The significance of DAF-16 and its role in the phenotypic covariance of longevity, immunity and stress resistance in the *Caenorhabditis* nematodes

by

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Abstract

Ageing, immunity and stress tolerance are inherent characteristics of all organisms. In animals, these traits are regulated, at least in part, by forkhead transcription factors in response to upstream signals from the Insulin/Insulin–like growth factor signalling (IIS) pathway. In the nematode *Caenorhabditis elegans*, these phenotypes are molecularly linked such that activation of the forkhead transcription factor DAF-16 both extends lifespan and simultaneously increases immunity and stress resistance. It is known that lifespan varies significantly among the *Caenorhabditis* species but, although DAF-16 signalling is highly conserved, it is unclear whether this phenotypic linkage occurs in other species. In this project we investigate this phenotypic covariance by comparing longevity, stress resistance and immunity in four *Caenorhabditis* species. We show, using phenotypic analysis of DAF-16 influenced phenotypes, that among four closely related *Caenorhabditis* nematodes, the gonochoristic species (*Caenorhabditis remanei* and *Caenorhabditis brenneri*) have diverged significantly with a longer lifespan, improved stress resistance and higher immunity than the hermaphroditic species (*Caenorhabditis elegans* and *Caenorhabditis briggsae*). Interestingly, we also observe significant differences in expression levels between the *daf-16* homologues in these species using Quantitative Real-Time PCR, which positively correlate with the observed phenotypes. We also provide additional evidence in support of a role for DAF-16 in regulating phenotypic coupling by using a combination of wildtype isolates, constitutively active *daf-16* mutants and bioinformatic analysis. Finally, we take a closer look at the *daf-16* gene and its isoforms in *C. elegans* and their role in driving specific responses to stress.

These findings impact upon our understanding of the diversification of the IIS pathway and the evolution of longevity in general, and illustrate how such differences could explain both inter and intra-species differences in ageing, immunity and stress response.

Dedicated to my dearest Mom, Josephine Joseph and my dad A.S Gandhi Das.

Thesis Overview

Ageing is a subject that generates much interest within both the scientific community and the wider population, in particular because of the potential applications of ageing research in treating age related disorders in the near future.

In this thesis I discuss the complex relationship between ageing, immunity and stress tolerance in the context of the transcription factor DAF-16, the final component of the IIS pathway. I look at how phenotypes driven by this signalling pathway and its molecules have evolved amongst the *Caenorhabditis* species and elicit responses that co-vary in a species-specific manner.

This thesis consists of seven chapters: an introduction (Chapter I), materials and methods (Chapter II), results (Chapter III – VI) and summary (Chapter VII). In this thesis I review recent finding in the realm of ageing research keeping in mind previous work and notions about ageing. I discuss ageing theories, model systems (especially *Caenorhabditis* species), key pathways and molecules that impact upon ageing and immunity, *Caenorhabditis* ecology, evolutionary impact and significance of DAF-16 and the covariance of life history traits (LHT). I then discuss my own findings, in which I show a co-variance of phenotypic characters of longevity, immunity and stress tolerance that correlate with expression levels of *daf-16* among the *Caenorhabditis* species. Finally I include a short summary about the entire project and potential future work.

Several sections of this thesis have been published. Parts of Chapter I have been published as a review in Current Ageing Science (Amrit & May, 2010) and Chapters III, IV and V have been published in PLoS One (Amrit, Boehnisch & May, 2010).

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Abbreviations

DNA	Deoxyribonucleic Acid
mtDNA	Mitochondrial DNA
cDNA	complimentary DNA
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
dsRNA	double-stranded RNA
ETC	Electron Transport Chain
ATP	Adenosine Triphosphate
AMP	Adenosine Monophosphate
IIS	Insulin/IGF (Insulin –like growth factor)- like signalling
PI3K	Phosphoinositide 3-kinase
ROS	Reactive oxygen species
CR	Caloric restriction
TOR	Target of Rapamycin pathway
MAPK	Mitogen activated protein kinase
Daf-c	Dauer constitutive
Daf-d	Dauer defective
TGF -β	Transforming growth factor beta
PCD	Programmed cell death
SOD	Super oxide dismutase
NGM	Nematode Growth Medium
LB	Luria Bertani Medium
BHI	Brain Heart Infusion
TBE	Tris/Borate/EDTA buffer
UV	Ultra Violet
NAD	Nicotinamide Adenine Dinucleotide
HNF	Hepatocyte Nuclear Factor
CML	Chronic Myeloid Leukaemia
JNK	Jun N-terminal Kinase
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time PCR
Ct	Threshold Cycle
DAVID	Database for Annotation, Visualization and Integrated Discovery

LHT	Life History Traits
DTC	Distal Tip Cells
WB	Worm Base
PIKK	Phosphatidylinositol Kinase-related Kinase
LIC	Leukaemia-Initiating Cells
cGMP	Cyclic guanosine monophosphate
RSAT	Regulatory Sequence Analysis Tools
EASE	Expression Analysis Systematic Explorer
PAMPS'	pathogen associated molecular patterns
MYA	Million years ago

Chapter I

Introduction

Francis R G Amrit and Robin C May, Younger for Longer: Insulin signalling, immunity and ageing, Current Ageing Science, In Press.

1.1 Ageing

The basis of ageing is an age-old question that dates back far in history. As a human race, we deal with ageing on a day-to-day basis with the notion of ageing always being related to frailty, disability, ailments and disease. Age is also pivotal to the decisions we make in a lifetime and is a topic of discussion across society from philosophers to scientists.

The human population is today on average older than ever before, a direct consequence of increase in life expectancy. Driven by modern day health care, World Bank figures to date show that average lifespan has increased worldwide with the western, developed countries having an expected lifespan of about 80 years and the developing countries having an expected lifespan in the range of about 60 – 70 years (Figure 1). These figures continue to rise with the socioeconomic implication of such an event being uncertain.

Ageing as a biological process is generally measured using lifespan. However, the term 'lifespan', which is just the length of life, is also used interchangeably with 'longevity', a term used when an unusually long lifespan is observed. 'Senescence' is another term that is more precisely used to describe a decline in physiological functioning with age but is generally restricted to the cellular level whilst 'ageing' encompasses cumulative changes in an organism, organ, tissue, or cell leading to a decrease in functional capacity.

The lifespan of any organism typically consists of maturation and reproduction followed by gradual, progressive and steady deterioration in fitness of the physical self. Accompanying this deterioration is the onset of various conditions and age related pathologies or diseases such as cancer,

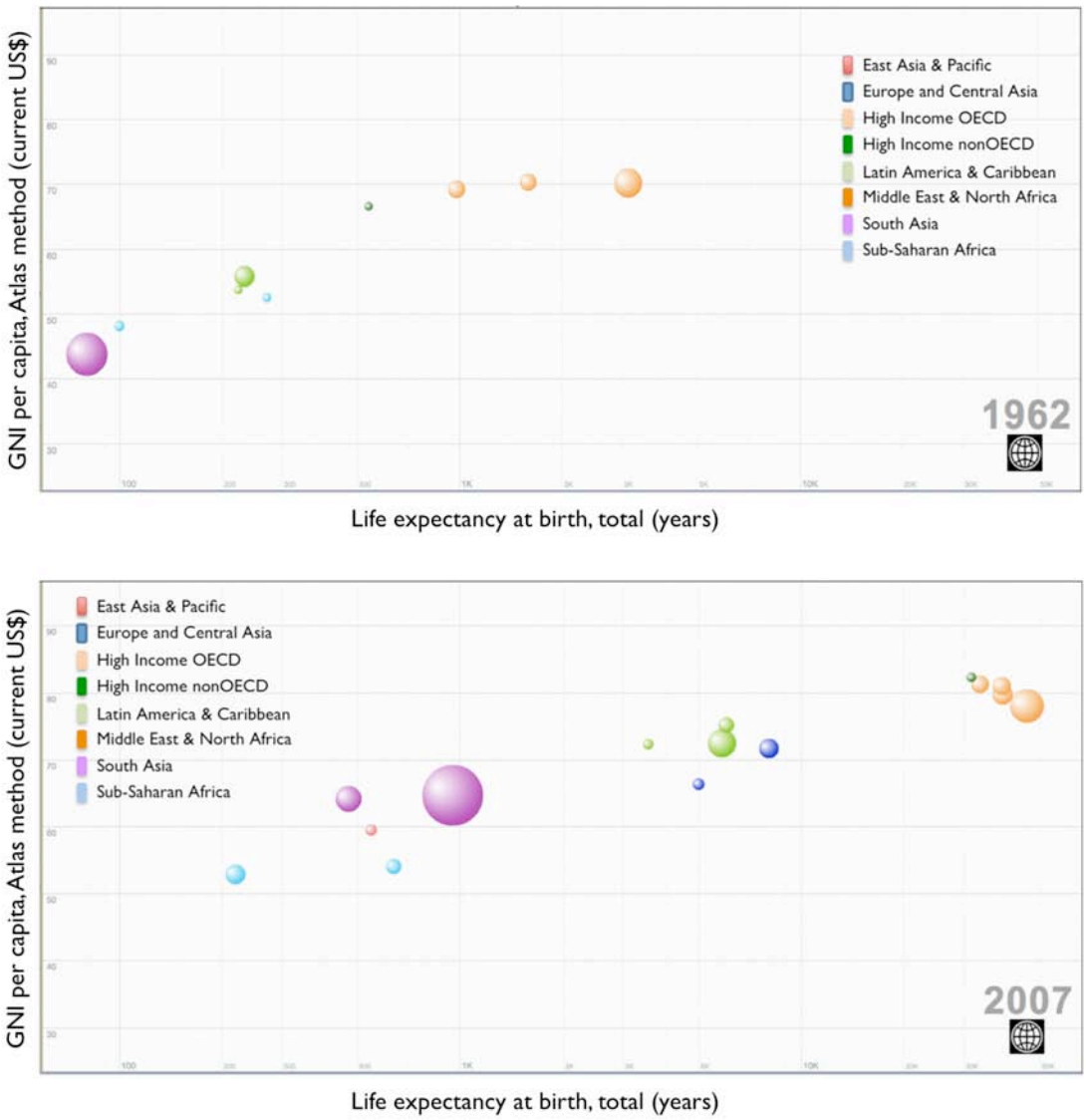

Figure 1 – World Bank indicators of the lifespan expectancy among countries in 1962 and 2007 shows an overall increase.

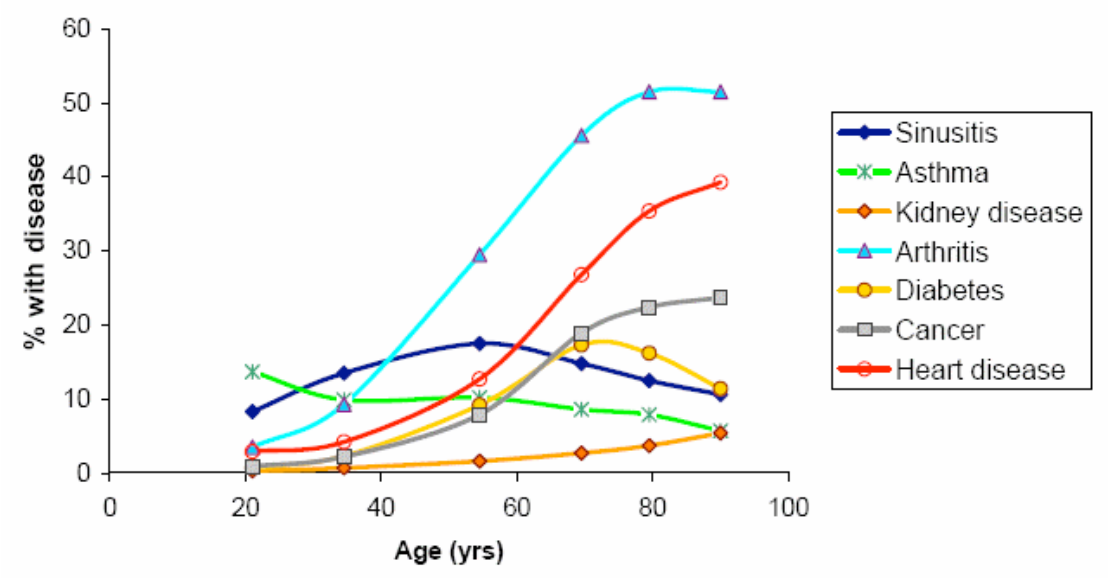

Figure 2 – Prevalence of number of pathologies expressed in percentages increase with age in US population (2002-2003 dataset). All forms of cancer and heart disease are featured. *

* Source: National Centre for Health Statistics, Data Warehouse on Trends in Health and Ageing.

diabetes, stroke and cardiovascular disease (Figure 2). In humans, ageing is also associated with degenerative changes in the skin, bones, blood vessels, lungs, nerves, and other organs and tissues. This damage has been speculated to occur as a result of a series of unintended biochemical side-effects of normal metabolism (Grey and Rae, 2007). These deleterious factors accumulate, eventually resulting in the loss of resilience and vitality coupled with the inability to maintain homeostasis and thus lead to death.

The phenomenon of ageing is a feature common to nearly all organisms that make up the tree of life. The same process that determines the three day lifespan in gastrotrichs also keeps the Quahog (*Mercenaria mercenaria*, the marine clam) going for over 200 years (Ropes and Murawski, 1983). Does this mean that a similar process molecularly drives this phenomenon of ageing? Also can the difference in this molecular function explain why ageing progresses at variable rates, resulting in the diverse lifespans we observe across different organisms in nature? And finally, are these determined lifespans a result of constant evolutionary chiselling and optimisation of the molecular processes in response to natural selection?

To answer such questions we now look to 'gerontology', a term used to describe the field of ageing research postulated by the Nobel prize-winning Russian scientist Ilya Metchnikoff in his 1908 book "The prolongation of life" (Metchnikoff and Mitchell, 1908). Through the last three decades we have witnessed tremendous research in this area and can finally say that ageing research has now come of age. The phenomenon of ageing, which was long considered as an inevitable process and looked upon as a programmed entity over which there was absolutely no control, is now being looked at from an entirely different perspective. Contrary to previous notions, gerontology dismisses the concept that ageing is a stochastic process but rather aims to understand the mechanics of ageing by approaching it as an environmentally, genetically and immunologically determined problem. Through these means we hope to unravel the mysteries of this universal process and perhaps in the near future come up with novel treatments to deal with age related disorders.

1.2 Theories Of Ageing

Widespread degenerative changes with increased incidence of various age related pathologies are characteristic features of growing old. Given the increase in average lifespan in today's modern world (Guttman, 2000) with better healthcare and an overall safer environment, the need to confront age related disorders is more imminent than ever before. An insight into the mechanisms driving ageing would then make it possible to come up with new and novel ways to tackle these problems (Kornfeld and Evason, 2006). Genetic studies in this area have already revealed molecular mechanisms and molecules that govern ageing and are evolutionarily conserved across taxa. An example for this is the evolutionarily conserved transcription factor DAF-16, which seems to have evolved to regulate key processes determining lifespan (Figure 3). The past twenty years have seen the identification of over 500 genes involved with ageing and/or longevity (de Magalhaes et al., 2009), and by genetically intervening in such pathways, it has been possible to dramatically increase the lifespan of model organisms like worms and flies (Kenyon et al., 1993, Clancy et al., 2001a). Although genetic modification of ageing is a recent phenomenon, the quest to investigate, understand and find treatments to prolong life has a very long, if not always illustrious, history (Figure 4).

1.2.1 Evolutionary Theories

Initial attempts to understand ageing was through postulation of theories that were based on the assumption that ageing is non adaptive in nature since the force of natural selection becomes weaker with age. Ageing brings with it vulnerability and ultimately leads to death. Therefore natural selection in this realm doesn't seem right as Darwin's evolutionary theory suggests that random and heritable variation of biological traits caused by mutations are selected for increased fitness. In view of this, evolutionary theories were postulated in a manner that biological evolution was linked with evolution of ageing, taking into consideration the interplay between mutation and

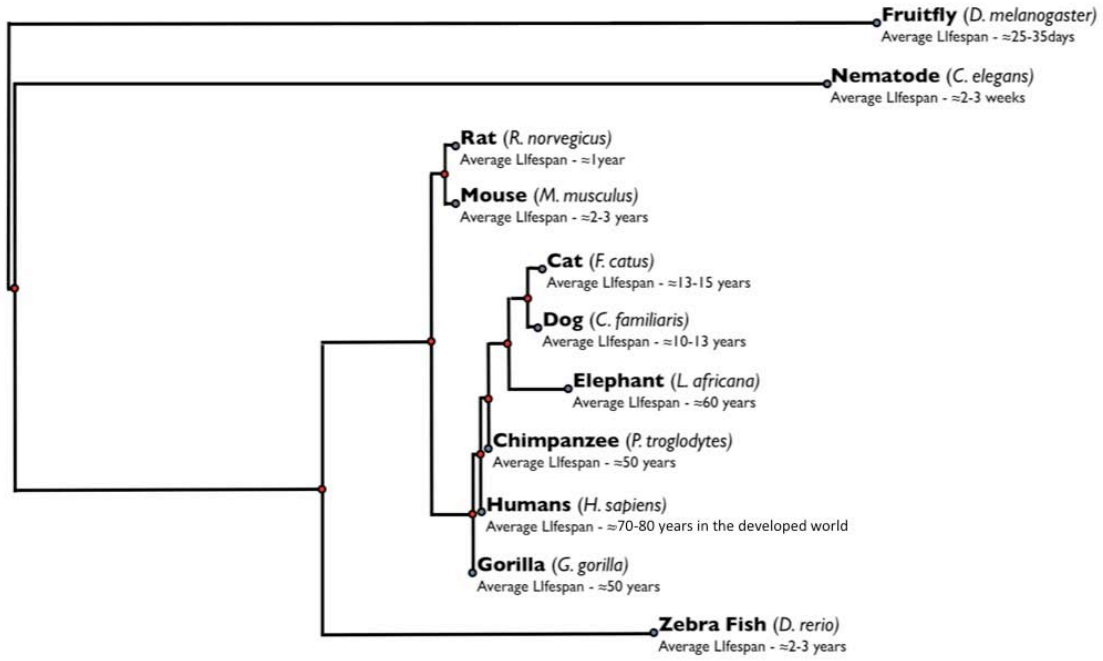

Figure 3 - Phylogenetic tree exemplifying the evolutionary relationship between various species based on the similarity between the longest FOXO/DAF-16 transcripts among them. The tree was constructed using the fast minimal evolution tree method employing a multiple sequence alignment tool, COBALT, that finds a collection of pairwise constraints derived from conserved domain database, protein motif database, and sequence similarity, using RPS-BLAST, BLASTP, and PHI-BLAST. The tree exemplifies the evolutionary relationship between various species based on the similarity between the longest FOXO/DAF-16 transcripts among them.

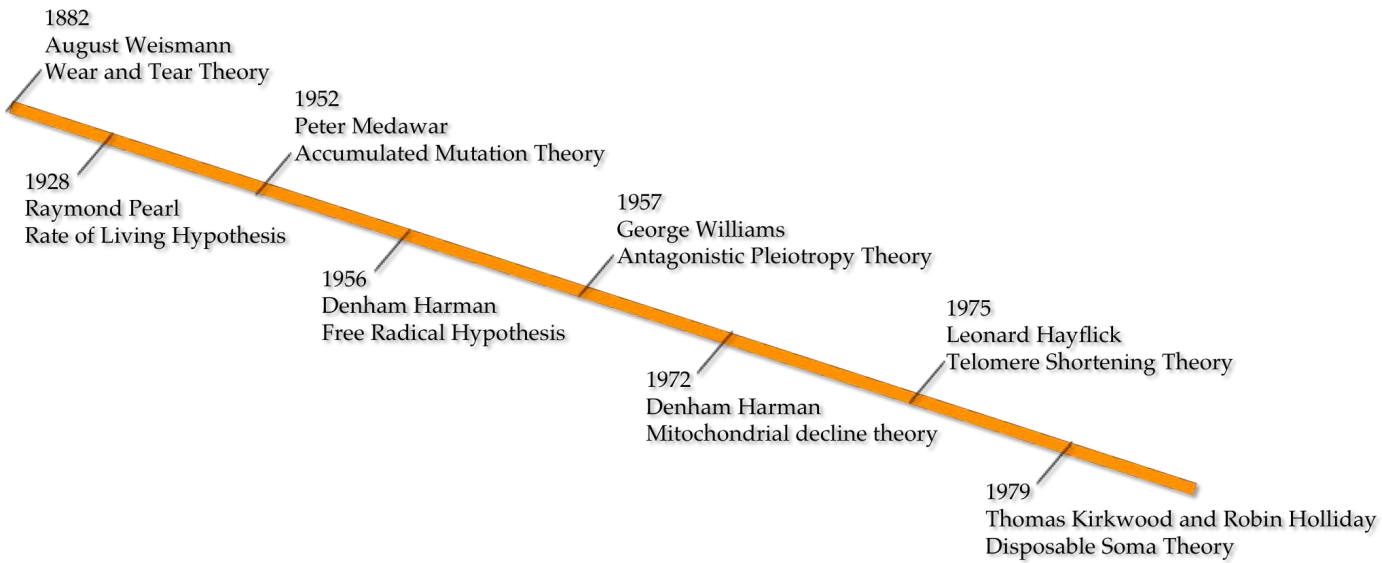

Figure 4 - Timeline showing the significant theories on ageing that have shaped modern models of ageing.

selection. Numerous evolutionary theories based on such concepts were postulated among which I discuss a few landmark theories.

1.2.1.1 Wear and Tear Theory – Germplasm Theory

Since the suggestion of biological evolution by Charles Darwin, the late 18th century saw the postulation of one of the first theories of ageing that kick-started the school of thought aimed at understanding this complicated process. August Weismann, a German biologist, in 1882 postulated the "Wear and Tear Theory" where he approached the problem by suggesting that ageing is mechanistic (Weismann, 1882). This was the first of the two concepts he proposed stating that over time there is gradual wear and tear of the body and cells due to overuse and abuse, which eventually leads to death. He suggested that ageing was a part of life's program and was included so that the old can make way for the next generation, reducing competition for the same resources. He found that the immortality of an animal would be a useless luxury without any advantage. According to Weismann ageing was beneficial for the long term health of the community but individualistically it would not make sense as to why an individual would acquire genes that would result in ageing and then death and also as to how individuals with these genes are better off then individuals without them. This was a general life history theory, which portrayed evolution as a force behind reproductive success and hence avoids extinction.

In order to explain the biological mechanism driving the theory based on the principle of programmed death, Weismann also suggested the concept of a specific limitation to number of divisions that a somatic cell may undergo in the course of an individual's life. He said that this limitation in a certain sense was under the control of the reproductive cells (Weismann, 1892). Employing this argument he went on to imply that the different life spans observed among animals would be due to the difference in the number of cell generations in which there is a specific norm for each different species. His postulation of a cell division limit was later observed experimentally in the 1960s by Hayflick and Moorhead as discussed later in this chapter.

1.2.1.2 Accumulated Mutation Theory

In the 1950s, the Nobel prize winner Peter Medawar theorized another evolutionary based idea that ageing occurs due to the accumulation of mutations (Medawar, 1952). His work was influenced by theories from Weismann and others, like Fisher and Haldane (Charlesworth, 2000). Terming it the "Accumulated Mutation Theory", he suggested that genes beneficial in early life are favoured by natural selection over genes beneficial late in life, since all organisms eventually die of diseases, predation, accidents etc. He hypothesised that the alleles that result in positive effects earlier in life (selected) could result in negative effects later on post reproduction due to physiological trade-offs. Also since the maximum likelihood of an individual to reproduce is in the young adult stage, he argued that deleterious mutations expressed at this stage are severely selected against because of their negative impact on fitness and hence reproduction. On the other hand, the selective pressure to select against deleterious mutations later on in life is weak since the genes have already been passed on to the next generation, ensuring propagation of traits that will in turn make certain the survival of the species as a whole.

Since the power of natural selection fades with age, deleterious mutations after the end of the reproductive period accumulate (as they are not selected against) leading to ageing and eventually death. Medawar portrayed ageing as a by-product of natural selection earlier in life.

1.2.1.3 Antagonistic Pleiotropy Theory

The Accumulated Mutation Theory was developed into the so-called "Antagonistic Pleiotropy Theory" formulated in 1957 by the evolutionary biologist George Williams (Williams, 2001). As the name suggests this theory was based on a couple of assumptions. The first assumption was that a particular gene may have an effect on not only one trait but can contribute to several traits of an organism (hence, pleiotropy) with the second assumption being that these pleiotropic effects may affect individual fitness in an

antagonistic manner. He suggested that evolution favours mutations that enhance fitness in younger fertile organisms, even if it comes at a cost of reduced fitness later on in life.

This theory seems rather robust with numerous examples supporting it. For instance, a study involving *Caenorhabditis elegans* displayed that a mutation which greatly increased lifespan was detrimental and did indeed exhibit a fitness cost (Walker et al., 2000). This was shown by subjecting cultures of wild-type and mutant worms to starvation cycles mimicking cyclic field conditions in nature, which ultimately resulted in the wild-type worms out competing (outnumbering) the mutants. In further support another example, which also can be explained to a certain extent by the honest signal theory comes from birds. Costly sexual ornaments of male birds that are crucial to attract females are vital in order to ensure mating and passing on of genes to the next generation. However, despite the reproductive advantage of possessing such ornaments, these extravagant features (as in peacocks) prove to be a handicap that reduces the bird's ability to escape predators later on in life, exemplifying the antagonistic pleiotropic effect of these genes (Zahavi, 1975).

Currently, both the antagonistic pleiotropy theory and the accumulated mutation theory are widely accepted.

1.2.1.4 Disposable Soma Theory

Another theory following up the theories by Medawar and Williams was the "Disposable Soma Theory" postulated by Thomas Kirkwood and Robin Holliday in 1979 (Kirkwood and Holliday, 1979). They suggested that organisms have an allocated amount of energy that they need to split between reproductive activities and the maintenance of the non-reproductive aspects of the organism (soma). Investment of energy into reproduction ensured the success of the species but at the same time meant that the lesser investment in bodily maintenance will result in natural degradation that leads to

accumulation of damage and therefore ageing. This was thus a special class of antagonistic pleiotropism in which hypothetical mutations that save energy for reproduction partly disable mechanisms that maintain somatic cellular homeostasis and vice versa. Therefore this theory highlights the fact that a fine balance between somatic maintenance and reproduction needs to be maintained and this balance could perhaps explain the variations in lifespan seen in nature.

1.2.2 Cellular and Molecular Theories

Recent evolutionary explanations of ageing and limited longevity of biological species are based on two major evolutionary theories: the mutation accumulation theory and the antagonistic pleiotropy theory. Despite the postulation of several such ageing theories over the years there has been no real consensus, leaving the experimental approach as the other alternative way to understand ageing.

1.2.2.1 Free Radical Hypothesis

A number of cellular/molecular theories of ageing were developed during the latter half of the twentieth century, amongst which the " Free Radical Hypothesis", proposed by Denham Harman in 1956, was highly influential (Harman, 1956). According to Harman, ageing occurred due to an accumulation of damage at the cellular level induced by reactive oxygen species (ROS) that are produced as by products of aerobic reactions. The superoxide radical (O_2^-) produced by aerobic reactions are metabolised by superoxide dismutase (SOD) into H_2O_2 and O_2. The H_2O_2 is further metabolised into the highly reactive hydroxyl radical HO^-. These species of molecules have been shown to damage proteins, membranes and DNA with recent studies showing that free radicals can result in the replacement of 10^4 to 10^5 DNA bases per cell per day (Wolff et al., 1986, Davies, 1987, Meneghini, 1988, Rao and Loeb, 1992, Pryor, 1973).

Therefore according to Harman, in order to retard the process of ageing, you need to minimize the damage caused by ROS.

1.2.2.2 Mitochondrial decline theory

The major site of production of ROS is the mitochondria, which are also the major site of production of energy in the form of adenosine triphosphate (ATP) in virtually all eukaryotic cells. Denham Harman in 1972 followed up his previous theory with the "Mitochondrial decline theory" stating that slow accumulation of impaired mitochondria is the driving force behind ageing. Mitochondria, also referred to as the powerhouse of the cell, contain a circular DNA genome (mtDNA), which is very small in comparison to DNA in the nucleus and codes for only a handful of polypeptides in humans (Anderson et al., 1981). This mtDNA has no histone protection or significant enzyme repair systems to offer free radical protection like that seen in the case of nuclear DNA, making the former highly vulnerable to oxidative stress (Richter, 1995). The location of the mtDNA, close to the inner mitochondrial membrane where most radicals are generated, further aggravates the damage caused. Therefore, this results in the accumulation of mitochondrial damage over a lifetime of an individual, which goes on to affect the Electron Transport Chain (ETC) complex that decreases the production of ATP leading to a cellular energy crisis. These mtDNA mutations are thought to impair cellular oxidative phosphorylations resulting in enhanced production of ROS that leads to further damage of mtDNA. Harman suggested that accumulation of such factors leads to ageing and death.

1.2.2.3 Oxidative damage theory

More recently, the ideas of Harman have developed into the "Oxidative damage theory", since it is now known that certain reactive oxygen species that do not belong to the class of free radicals can also cause damage at the molecular level (Beckman and Ames, 1998).

1.2.2.4 Rate of Living Hypothesis

Interestingly, the Free Radical Hypothesis provides a mechanistic explanation for a much earlier theory of ageing, the so-called "Rate of Living Hypothesis" proposed by Raymond Pearl in 1928. Pearl suggested that lifespan is inversely proportional to metabolic activity (Pearl, 1928), such that slowing down metabolism (whose by product is ROS) results in an increase in lifespan. Within this context, the shorter lifespan seen in ectotherms such as the nematode *Caenorhabditis elegans* when raised at higher temperature and the extended lifespan shown by most animals when provided with a minimal caloric diet both provide strong evidence for a link between metabolic turnover and ageing (Klass, 1977). However, several recent studies pertaining to this are suggesting otherwise. One such study looking at the effects of the deletion of *sod* genes (genes that constitute a family of antioxidant enzymes that detoxify superoxides, thus working against the negative effects of ROS) on lifespan of *C. elegans* documented an increase in lifespan contrary to what was observed in other model organisms such as yeast, flies and mice (Van Raamsdonk and Hekimi, 2009, Wawryn et al., 1999, Kirby et al., 2002, Phillips et al., 1989, Lebovitz et al., 1996, Li et al., 1995). Although an increased susceptibility to oxidative stress was seen in these *sod* mutants, mutants for single or multiple *sod* genes were shown to have a longer lifespan or a lifespan at least similar to that of wildtype worms (Van Raamsdonk and Hekimi, 2009). This increased lifespan in *sod* mutants has been suggested to result from an interaction with genes that affect mitochondrial function. Nevertheless, the increased oxidative stress resulting from *sod* mutations does not decrease lifespan in *C. elegans* and numerous studies using nematode worms have questioned the effect of ROS on lifespan (reviewed in (Gems and Doonan, 2009)).

1.2.2.5 Telomere shortening theory

The effects of ROS have also been implicated in another avenue of longevity research, that looking at the role of telomeres in ageing. The telomere region present at the ends of chromosomes consist of non-coding, repetitive DNA-

protein complex, which plays a key role in cell growth and maintaining proper segregation of chromosomes to daughter cells. Cellular senescence has been associated with a progressive reduction of these telomeric regions at every cell division (Morin, 1997). The role of these structures in ageing was first noted by Leonard Hayflick in 1975 when he discovered that cultured normal human cells had a limited capacity to divide, after which they become senescent, a phenomenon referred to as the 'Hayflick limit' (Hayflick, 1965). Oxidative stress has been shown to accelerate the shortening of telomeres significantly by inducing accumulation of single-strand breaks. This build-up was shown to result in the loss of distal fragments of telomeres during replication (Sitte et al., 1998). However, recent *in vivo* studies have argued against this based on their inability to exacerbate ageing phenotypes (by screening for oxidative nuclear DNA damage, an accumulation of nuclear DNA breaks, or an increased rate of telomere shortening) in telomere dysfunctional mice that have a heterozygous deletion of the superoxide dismutase gene (hence increased oxidative stress) (Guachalla et al., 2009). Nevertheless the "Telomere shortening theory" has been supported by findings such as the extension of replicative senescence among normal human cells (retinal pigment epithelial cells and foreskin fibroblasts) upon over expression of telomerase (Bodnar et al., 1998). Other studies using *C. elegans* have demonstrated that producing longer telomeres by over-expressing HRP-1, a telomere-binding protein, result in increased lifespan in a *daf-16* dependant fashion (Joeng et al., 2004).

1.2.2.6 Other theories of Ageing

There have been numerous theories postulated to explain and understand the phenomenon of ageing. This perhaps is an indication of the amount of interest this particular field attracts. In addition to the major theories discussed above, there exist numerous alternative hypotheses such as the Codon Restriction Theory (Strehler et al., 1971), Error Catastrophe Theory (Orgel, 1970, Orgel, 1963, Orgel, 1973), Somatic Mutation Theory (Failla, 1958, Szilard, 1959, Vijg, 2000), Dysdifferentiation theory (Cutler, 1985), Gene

Regulation theory (Kanungo, 1975) and Neuroendocrine Theory (Dilman and Dean, 1954).

Despite the postulation of such a vast number of theories and the existence of experimental evidence for a few, there is no single exclusive theory that can completely explain the process of ageing.

1.3 Relationship between Ageing, Immunity and Stress Tolerance

The modern view of ageing has made us treat ageing as a disease which can be defined as an accumulation of deleterious physical conditions through time which leads to widespread degenerative changes and an increase in incidence of age related pathologies or diseases. This damage to the physical self at the cellular and molecular level is a process that happens throughout life but is more evident in older individuals due to constantly weakening immune system and wearing down of repair mechanisms.

Ageing and the innate immune response are features that are shared across all living organisms. Invasion by pathogens triggers the immune system of the host to recognise and mount a counter offensive to try and eliminate the invading pathogens. In vertebrates there are two lines of defence apart from the physical barrier (cuticle/skin). These are the innate immune system, which responds immediately, and the acquired immune system, which responds slowly but is highly specific and long lasting. The innate immune system also contributes towards the initiation of the acquired immune system and appears to play the major role in immunity as a whole (Haag, Chamberlin et al. 2007). All organisms apart from vertebrates possess only an innate immune system, which has highly similar features across all organisms suggesting a common origin and conservation over millions of years of

evolution. So, based on the observed relationship between ageing, immunity and stress resistance, can the presence of a robust immune system translate into a longer lifespan as a knock on effect? If so, this can also address the problem of post reproductive ageing by selecting for enhanced immunity at an early age that then increases lifespan even after the animal has reproduced.

Such a link raises many questions. Does old age give rise to a weaker immune system or is it the other way around? Also, does a weaker immune system mean a weaker response to stress? If so, are they both governed at the molecular level through similar pathways? Are these pathways evolutionarily conserved and is differential functioning of this pathway the answer to the variable life spans we observe among nature?

1.4 Methods of Studying and Tackling Ageing

Until now two general methods have been used to study ageing. The older of these methods employed physiological means such as dietary restriction (DR) or calorie restriction (CR). The first of these experiments dates back to 1934 when Clive McCay and Mary Crowell of Cornell University delayed the onset of senescence in rats by reducing the calories in the diet by about 40%, while keeping the different nutrients at normal levels (McCay et al., 1935). This resulted in healthy and long-lived rats and mice. Ever since this observation, CR has been tried on a multitude of other organisms including *Drosophila melanogaster* (fly), *Caenorhabditis elegans* (worm) and recently macaque monkeys, with results pointing in the direction of an increased lifespan being a direct effect of calorie restriction (Klass, 1977, Ramsey et al., 2000, Chapman and Partridge, 1996).

In mammals calorie restriction has been shown to slow the progression or even entirely prevent the onset of a range of age-related pathologies such as cancer, cardiovascular disease, neurodegenerative disorders and diabetes

(Anson et al., 2003, Maswood et al., 2004, Mattson and Wan, 2005, Klebanov, 2007, Park and Prolla, 2005). This dietary restriction induced change in physiology however has limited application in the development of new medical interventions for diseases of ageing as compared to those that can be developed by understanding the genetic mechanisms that underlie these processes.

This has led to the modern and more recent method of studying ageing with the use of genetics and molecular biology. Until now, several age related genes (gerentogenes) have been identified whose activity, if altered, leads to increased longevity with better stress resistance or accelerated ageing (de Magalhaes et al., 2009). By studying the various pathways that are related to these gerentogenes, it then becomes a possibility to tap into the latter pharmacologically to deal with age-related problems.

1.5 *Caenorhabditis elegans* as a model system to study ageing and immunity

To develop an understanding of the general mechanisms determining the ageing process, the field of biological gerontology has been using model organisms and one such model is the nematode *Caenorhabditis elegans* which has proven to be a very potent and powerful tool for studying the genetics of longevity and stress resistance (Braeckman and Vanfleteren, 2007). Recent studies and genomic information on other *Caenorhabditis* species such as *Caenorhabditis remanei, Caenorhabditis brenneri* and *Caenorhabditis briggsae* has also opened up comparative genomic possibilities (Schulenburg, Leopold Kurz et al. 2004; Davies and Allen I. Laskin 2005; Ewbank 2006).

The free-living bacteriovorous nematode *Caenorhabditis elegans* (*Caeno*, recent; *rhabditis*, rod; *elegans*, elegant) has emerged recently as a powerful model to

study complex processes such as ageing and immunity. Ever since it was introduced by Sydney Brenner, this simple multicellular eukaryote has been studied intensively with exhaustive genotypic and phenotypic information now available. Features such as a short lifespan (~3 weeks), ease of maintenance, reduced cost, a small genome (one half that of *Drosophila melanogaster*), transparency, small size (1mm), rapid generation time (egg to egg in 3 days), ability to be stored for long periods by freezing and the fact that it is a simple and genetically tractable model have made this nematode species an ideal model to study longevity and the process of ageing (Ewbank, 2002, Houthoofd et al., 2003, Kurz et al., 2007).

The *Caenorhabditis* clade consists of both hermaphroditic and gonochoristic (male/female) species. The oocyte and sperm-producing hermaphrodites cannot fertilize each other and differ in appearance to males (Figure 5). However, both sexes posses the same general anatomy (Sulston et al., 1983, Sulston and Horvitz, 1977) with the males being slightly shorter and thinner than the hermaphrodites.

Hermaphroditism is considered to have evolved from male/female species, with a recent study showing that it is possible to produce a self-fertile XX hermaphroditic animal from a gonochoristic animal by lowering the expression of *tra-2* and *swm-1* (genes involved in sex determination and sperm activation in *C. elegans*) (Baldi et al., 2009). Self-fertilizing hermaphrodites (XX) can give rise to spontaneous males (XO) due to meiotic non-disjunction at a very low frequency. Males can be crossed with hermaphrodites to produce a 50% male population in the consequent generation. This attribute of this species offers conveniences previously only enjoyed in plant genetic systems. (Wood, 1988, 1997, Braeckman and Vanfleteren, 2007).

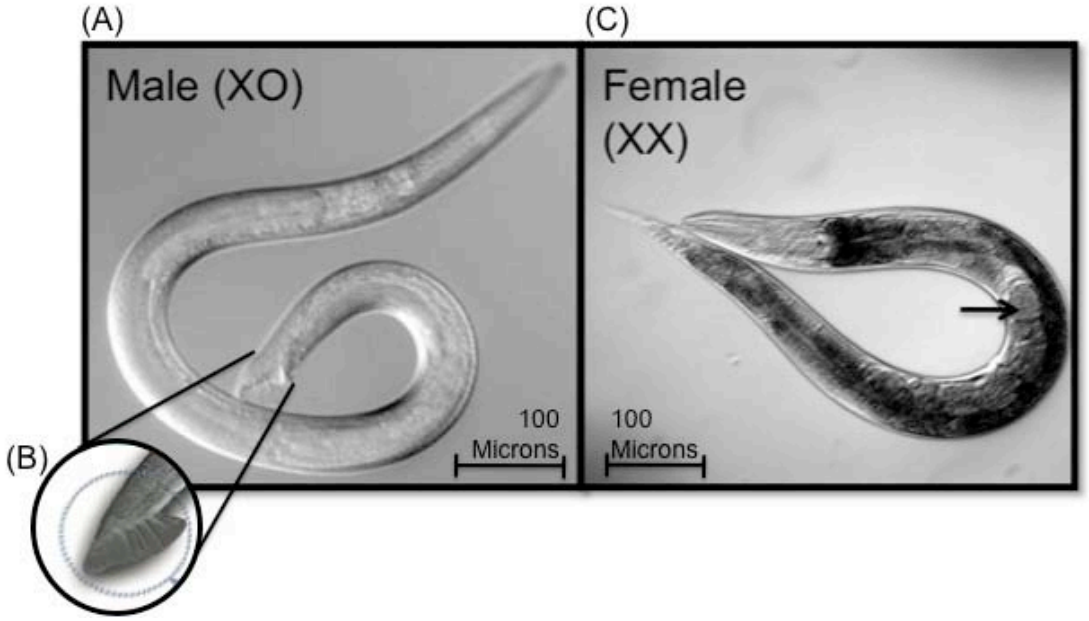

Figure 5 – Images of an adult male (A) and adult female (C) *C. elegans* nematode with the characteristic tail (B) of the adult male and the eggs in the adult female pointed out[*]. The fan shaped tail of the males distinguishes them from hermaphrodites and females across all the *Caenorhabditis* species.

Figure 6 – Image of wild type *C. elegans* infected with the fungus *Drechmeria coniospora* 24hrs after infection (Couillault et al., 2004). Scale bar in image represents a size of 100 microns.

[*] Images sourced from www.nematodes.org and http://lifecenter.sgst.cn/detoxiprot/pic/C_elegans.jpg

In addition, the evolutionarily ancient origin of nematodes means that they share many essential biological features present in higher vertebrates and hence provide a powerful model with which to investigate the complexity of biological processes such as ageing and immunity in higher animals.

1.5.1 Physiology

Physiologically these species belonging to the *Caenorhabditis* genus grow up to 1mm in length, 50-70 µm wide, are transparent, with six chromosomes (five pairs of autosomes and one pair of sex chromosomes), unsegmented with a long cylindrical body shape tapered at the ends. The genome size of *C. elegans* is about 100.2 Mega-bases (Mb). The nematode shape is maintained by internal hydrostatic pressure and the body plan consists of two concentric tubes separated by a fluid filled space, the pseudocoelom. The exterior collagenous cuticle secreted by the underlying hypodermis (Kramer, 1997) is impermeable and extremely tough and acts as a very effective physical barrier to invasion by harmful entities although a few specialised parasites such as fungus *Drechmeria coniospora* (Figure 6) (Pujol et al., 2008) and the bacterium *Pasteuria penetrans* (Bird et al., 2003) can break through this.

Sinusoidal locomotion seen in this clade of nematodes is achieved by synchronised contraction and relaxation of the four longitudinal muscle strips attached to the cuticle through a thin layer of the hypodermis (Moerman and Fire, 1997). The mouth is at the tip of the head (Figure 7A), which leads into the inner tube that consists of gut. The gut is made up of a pharynx and an intestine and runs straight from head to tail. The pharynx is bilobed and is responsible for ingestion of bacteria, crushing the bacteria in the terminal bulb and passing the food onto the intestine through the pharyngeal – intestinal valve. The intestine is a single cell thick tube that consists of 20 cells organized in 9 rings with all but the most anterior arranged in pairs. These cells surround a central lumen that connects to the anus near the tail. They have a simple nervous system which was a criteria for initially selecting this model system to study animal behaviour (Hope, 1999).

The reproductive system in a hermaphrodite consists of two functionally independent anterior and posterior arms. These arms both consist of an ovary distal to the vulva (Figure 7C), a more proximal oviduct, leading into the spermatheca, which then enters into the common uterus centred around the vulva (Figure 7B). Sperm is first produced and stored in the spermatheca and then oocytes are produced. Once fertilised oocytes squeeze through the spermatheca they enter the uterus as early embryos. Here gastrulation begins and they undergo development briefly whilst an eggshell is added and are laid through the vulva at about the 40-cell stage of development.

Wild type hermaphrodites hatch with 556 somatic cells and through development thereafter 1090 cells are generated. Of these precisely 131 cells are eliminated by programmed cell death, which results in an adult animal with 959 cells. This adult stage is reached at about 45–50 hours post hatching (the position and quantity of these cells remains constant throughout life). Cell lineage, depicting which cells are derived from which, was completely mapped in the 1970's for every cell by John Sulston and Bob Horvitz (Sulston and Horvitz, 1977). This discovery went on to fuel the use of *C. elegans* to perform studies at a single cell resolution in the context of a multi-cellular organism.

The *Caenorhabditis* species include free living and parasitic forms and the average worm can give rise to about 300-350 progeny. These species are predominantly found in the soil (especially rotting vegetables and feed on microbes such as bacteria) but are also known to inhabit aquatic environments (Kiontke and Sudhaus, 2006).

1.5.2 Life Cycle

The *Caenorhabditis* species have a short, temperature dependent life cycle that consists of embryogenesis (development from fertilization to hatching) and post-embryonic development that has four larval stages separated by molts

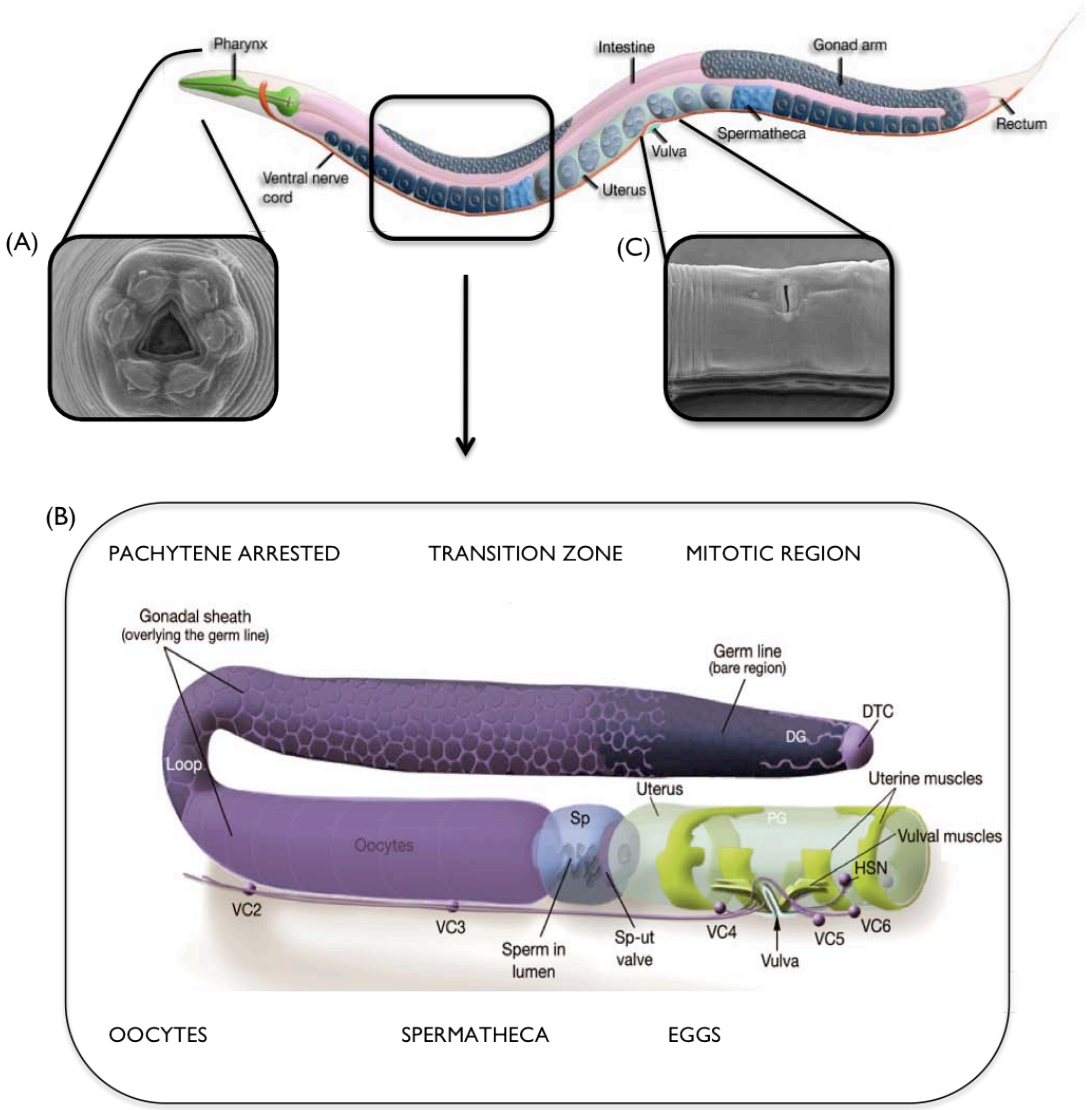

Figure 7 – Figure of an adult *C. elegans* hermaphrodite with scanning electron microscope images of the mouth[*] (A) and the vulva (C). Panel (B) illustrates one of the two arms of the hermaphroditic reproductive system where germ cells develop in an assembly-line fashion starting off with mitotic divisions at the distal end in response to a signal from the Distal Tip Cell (DTC) followed by the nuclei initiating meiosis and arresting at the at the pachytene stage of meiosis I as they move further away from the DTC. Once the nuclei reach the bend in the gonadal arm oogenesis is initiated, germ cells exit pachytene and go through diakinesis and proceed through the spermatheca where they are fertilised and then become eggs eventually (Lints and Hall, 2009).

[*] Scanning electron microscopy images obtained and modified from http://www.einstein.yu.edu/iag/samples/files/SEMworm.html

followed by the adult stage (Figure 8). *C. elegans* increases in size by about one-third during each larval stage and again as an adult after the final molt. Also, at every larval stage a new cuticle of stage-specific composition is secreted and the older one is shed. In L1 larvae, the nervous system, the reproductive system, and the digestive tract begin to develop, and this is completed by the L4 stage.

Sometimes these nematodes can adopt another non developmental stage known as the Dauer stage instead of the normal third Larval stage (Cassada and Russell, 1975). Entry into dauer is induced by stress like high temperature, starvation or overcrowding at the second moult.

1.5.2.1 Dauer Stage

Entering the dauer stage has been considered a strategy employed by the *Caenorhabditis* nematodes to survive harsh conditions and ensure survival and dispersion when suitable conditions return (Fodor et al., 1983, Cassada and Russell, 1975, Inoue et al., 2007).

Through development the worms make a choice between development into non-dauer L3's and dauer larvae. This reversible arrest of development seen in dauer larvae is modulated by food availability, dauer inducing pheromone and temperature. When the ratio between the dauer pheromone (that is constitutively produced by all worms) and the available food increases, this change in ratio or increase in pheromone relative to food availability is detected by the sensory neurons that signals through complex neurosecretory pathways to target tissues such as the germ line, pharynx and intestine, which in turn get remodelled and are metabolically shifted (Riddle and Albert, 1997). The dauer pheromone consists of ascaroside derivatives with short, fatty acid-like side chains. Although the dauer pheromone has been studied for 25 years, its biosynthesis is completely uncharacterized with the *daf-22* mutant being the only known mutant defective in dauer pheromone production (Golden and Riddle, 1985). Recently, three distinct

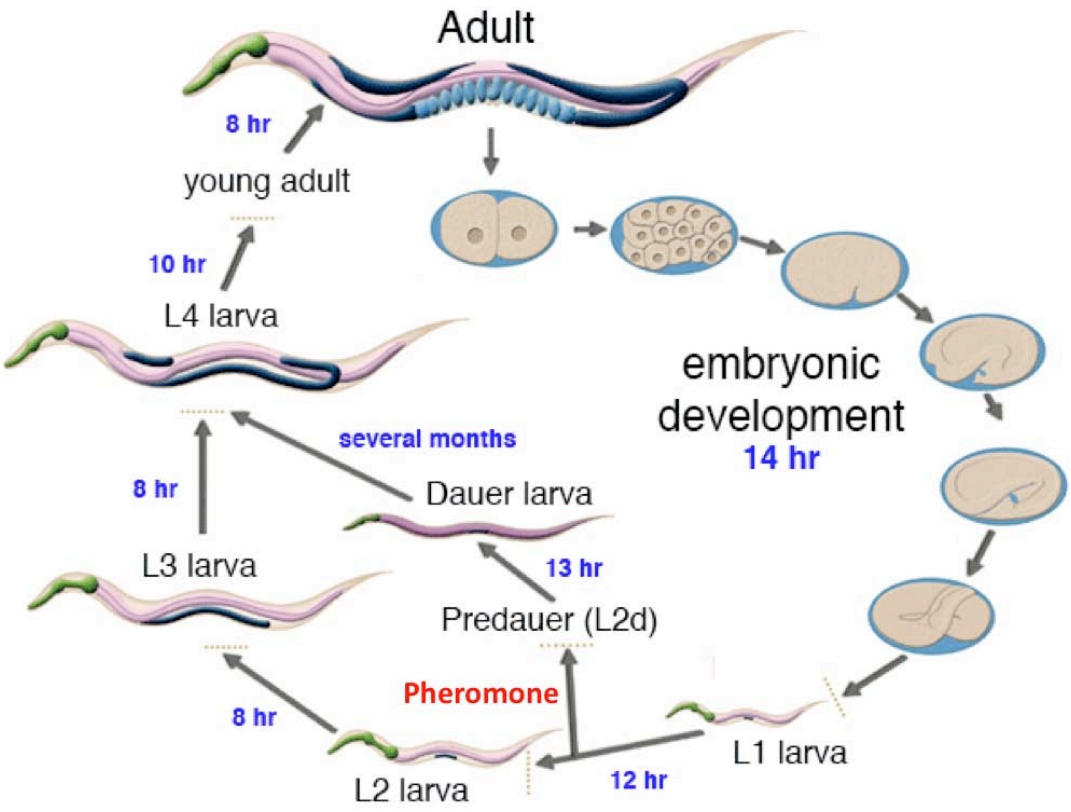

Figure 8 – The life cycle of *C. elegans* consists of four larval stages. At the first larval stage this life cycle can be interrupted by the dauer stage. This dauer stage is triggered by increased pheromone levels that result due to drop in food availability, over crowding and temperature. [*]

[*] Image sourced from ALTUN, Z. F. & HALL, D. H. (2005) Handbook of *C. elegans* Anatomy. *WormAtlas*.

ascarosides in *C. elegans* extracts with dauer pheromone activity have been identified (Butcher et al., 2007).

Dauer larvae are non-feeding and accumulate large amounts of fat in the intestines and hypodermis. They spend down these reserves, converting fat to glucose through the glyoxylate cycle (Riddle and Albert, 1997). Dauer larvae appear non-ageing, long lived (a mean lifespan of 60 days), stress resistant and are able to resume development into the L4 stage when they encounter a food source. In addition, they tend to live for a longer duration than worms that follow the normal life cycle (Klass and Hirsh, 1976, Finch and Ruvkun, 2001, Ogg et al., 1997).

The simplicity of the *Caenorhabditis* species is apparent from the anatomical and developmental description. In the wild these nematodes are thought to feed on bacteria and other microbes along with micronutrients found in the soil. However, in the laboratory the nematodes are cultivated on agar plates containing a particular strain of *Escherichia coli* (OP50) and, in general, most aspects of *C. elegans* biology have yet to be studied in wild populations.

1.5.3 OP50

In laboratories the *Caenorhabditis species* are usually grown monoxenically on lawns of *E. coli* strain OP50 as a food source (Brenner, 1974). This strain of bacteria is a uracil auxotroph that is more fragile and smaller than other *E. coli* strains. Although OP50 has been used universally to propagate *Caenorhabditis* cultures, it has been shown that when worms were grown on OP50 cells on Brain Heart Infusion (BHI) agar, they lived for significantly shorter times than when cultivated on OP50 cells on Nematode Growth Medium (NGM) agar (Garsin et al., 2001). This presumably owes to the fact that rich BHI medium is capable of inducing more rapid growth and/or expression of additional *E. coli* factors that accelerate the rate of *C. elegans* death. Nevertheless, the combination of OP50 and NGM agar is the choice of the *Caenorhabditis*

community with a considerable amount of research being done in this avenue with its genome being sequenced and published recently (May et al., 2009).

1.6 Molecules of Significance – Their role in Ageing, Immunity and Stress tolerance

A major advance in the study of ageing was the identification of the first age-altering gene by Klass and Johnson working in the *C. elegans* system in the 1980s. Mutations in this gene, called *age-1* in *C. elegans,* increased lifespan by approximately 50% (Klass, 1983, Friedman and Johnson, 1988). This discovery transformed the field, since it suggested that ageing is not a haphazard process but rather a process that is regulated at a cellular level. Since then, numerous genes and pathways/mechanism associated with ageing and stress resistance have been identified. *age-1* was shown to be a part of the IIS [Insulin/IGF (Insulin –like growth factor)- like signalling] pathway with other genes in the pathway such as *pdk-1, akt-1, sgk-1* and *daf-16* shown to have similar phenotypic effects (Kenyon et al., 1993, Paradis et al., 1999, Hamilton et al., 2005, Hertweck et al., 2004). The discovery of the tremendous impact of the components of the IIS pathway on ageing immediately stimulated this area of research, leading to the identification of numerous other molecules that influence IIS and thus ageing.

1.6.1 Sirtuins and Calorie Restriction

One such molecule was Sirtuin, a member of the silent information regulator 2 (*Sir2*) family of NAD+ dependent Class III histone/protein deacetylases (HDACs) (Tissenbaum and Guarente, 2001). Initially shown to mediate gene silencing in *S. cerevisiae*, this family includes five homologues in yeast (*Hst1-4* and *Sir2*), one each in *C. elegans* (*Sir2.1*) and *D. melanogaster* (*dSir2*) and seven mammalian counterparts (*Sirt 1-7*) (Allard et al., 2009, Rine and Herskowitz, 1987). Increased dosage of the *Sir-2.1* gene in yeast, worms and flies has been

confirmed to be sufficient to increase lifespan (Tissenbaum and Guarente, 2001, Kaeberlein et al., 1999, Rogina and Helfand, 2004). Intriguingly in *C. elegans*, longevity requires *daf-16* and recent data suggest that *Sir-2.1* works in tandem with 14-3-3 proteins (highly conserved small and acidic cytoplasmic proteins functioning as hetero or homo-dimers) to modulate DAF-16 activity (Berdichevsky et al., 2006). Also, research exploring the role of sirtuins in mammals has revealed that adult *sirt1*-null mice accumulated deposits of immune complexes in the liver and kidney leading to a mild autoimmune condition. These animals produced high titer anti-nuclear antibodies. Another observation of this study was that most of these mutant mice developed a disease resembling diabetes insipidus when they approached 2 years of age. Despite the lack of a direct link between SIRT1 and its role in autoimmunity, this study speculated SIRT1 to be playing a role in sustaining normal immune function that could therefore delay the onset of autoimmune disease (Sequeira et al., 2008).

Sir-2.1 catalyses a deacetylation reaction, resulting in the cleavage of NAD+ into nicotinamide (NAM) and 1-O-acetyl-ADP-ribose (Tanner et al., 2000). This relationship between *Sir-2.1* and NAD, which functions as a co-factor in various metabolic reactions, has led to the proposal that *Sir2.1* acts as a metabolic sensor, reporting upon the nutritional state of the organism (Tanner et al., 2000, Guarente, 2000). Altering metabolic state via calorie restriction (CR) increases lifespan and increases sirtuin mRNA levels in *D. melanogaster* (Rogina et al., 2002), providing a potential molecular explanation for the role of CR in ageing, which dates back to experiments conducted in the 1930's, by Clive McCay and Mary Crowell of Cornell University (McCay et al., 1935) discussed earlier in this chapter. In *C. elegans* and *D. melanogaster,* lifespan extension induced by CR has been shown to be dependant on *Sir-2.1* (Wang and Tissenbaum, 2006) since neither CR nor resveratrol (*Sir-2.1* activator) are capable of extending the lifespan of *dSir-2* mutant fruit flies and *Sir-2.1* mutant worms (Wood et al., 2004, Rogina and Helfand, 2004). CR has also been recently reported to improve T-cell function in humans. This study looking at Delayed-type hypersensitivity (DTH), T-cell proliferation (TP), and prostaglandin E(2) (PGE(2)) production in over-weight but not obese

participants aged 20-42 years assigned to 30% CR for 6 months showed a significant increase in DTH and TP along with a significant decrease in PGE(2) production (Ahmed et al., 2009). The fact that CR increases lifespan in a variety of organisms including yeast, flies, worms, spiders, fish, rotifers, mice, rats and macaque monkeys suggests that similar pathways are likely to occur in all animals (Koubova and Guarente, 2003, Klass and Hirsh, 1976, Bishop and Guarente, 2007). In addition, the recent finding that dietary glucose also can affect lifespan in *C. elegans* via inhibiting the *daf-16* pathway (Lee et al., 2009). This study showed that glucose completely prevented *daf-2* mutations from extending life span by altering *aqp-1* (aquaporin glycerol channel) expression. *aqp-1* function as a cell nonautonomous feedback regulator of the IIS pathway suggests that organisms may have multiple mechanisms for coupling nutritional status to ageing and immunity.

1.6.2 Target of Rapamycin Pathway and Autophagy

Target of Rapamycin pathway (TOR) proteins are members of the phosphatidylinositol kinase-related kinase (PIKK) family and are highly conserved from yeast to mammals. TOR has been shown to promote growth by phosphorylating ribosomal S6 kinase and translational initiation factor 4E binding protein 1 in response to nutrients (Inoki et al., 2005). Studies have shown extension in lifespan among worms and flies with mutations that decrease TOR activity (Jia et al., 2004, Kapahi et al., 2004). These mutations seem to activate a pathway that is distinct from the IIS pathway, as it extends lifespan independently of DAF-16 (Hansen et al., 2007, Vellai et al., 2003).

Caloric restriction in worms and flies has been demonstrated to down regulate pathways involved in growth and metabolic processing such as TOR, but lifespan extension produced by TOR inhibition (through other means) is not further increased by caloric restriction (Bjedov et al., Hansen et al., 2007, Kapahi et al., 2004). Down-regulation of TOR is also capable of influencing an increase in autophagy, resulting in extended lifespan (Figure 9) (Vellai et al., 2003, Kapahi et al., 2004). Autophagy, which is considered to be a host

defence mechanism triggered by adverse conditions, has also been shown to be negatively regulated by *daf-2*, with increased autophagy seen in *daf-2* mutants being coupled with lifespan extension (Melendez et al., 2003). Further insights into this have recently suggested the autophagy genes to be working in concert with the IIS pathway and *daf-16* (Jia et al., 2009), conferring immunity. In worms where the *atg* (autophagy) genes *bec-1* and *lgg-1* of the autophagy pathway were knocked down, there was an increase in bacterial intracellular replication. This made the worms more susceptible to the human pathogen *S. enterica* Typhimurium resulting in decreased lifespan and apoptotic-independent cell death. The study further showed that knockdown of autophagy genes nullified the pathogen resistance phenotype associated with the loss-of-function mutation, *daf-2(e1370)*, in the insulin-like tyrosine kinase receptor or by over-expression of the DAF-16 FOXO transcription factor. Thus, autophagy genes seem to play an integral part of the host defence mechanism in vivo against intracellular bacterial pathogens (biotic stress) and mediate pathogen resistance in long-lived IIS mutant nematodes (Jia et al., 2009). Similar observations have also been documented in *Drosophila* and mammals (Juhasz et al., 2007, Mammucari et al., 2007, Zhao et al., 2007). Interestingly, a very recent study has revealed that *Sir-2.1* mediates the effect of CR on autophagy (Eugenia Morselli, 2010). This study showed that autophagy under CR was completely abolished in a *Sir-2.1* mutant background (but not the autophagic response to rapamycin or tunicamycin).

1.6.3 AMP Kinase

Caloric restriction leads to a limited supply of energy. When such conditions persist it is vital that this message is translated into a phenotypic response to

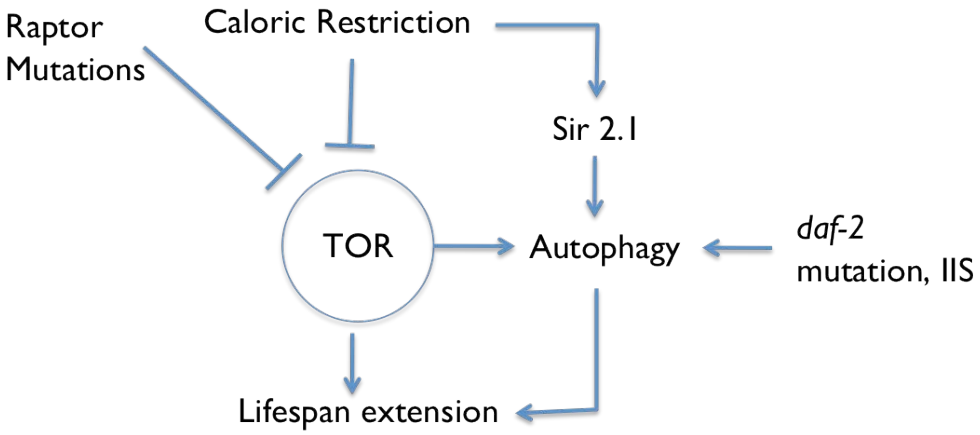

Figure 9 – Mutations in the *C.elegans* raptor (orthologoues regulatory associated protein of mTOR which transduce nutrient signals to the downstream translation machinery in mammals) homologue *daf-15* (Jia et al., 2004) and caloric restriction influence the Target of Rapamycin (TOR) pathway leading to lifespan extension.

survive. AMP kinase is one such sensor of nutrient and energy availability. It responds to the AMP:ATP ratio in cells and activates catabolic pathways whilst at the same time repressing anabolic pathways upon detecting high levels of the latter (AMP:ATP ratio). In *C. elegans*, overexpression of AMP kinase has been shown to extend lifespan, with it also being required for lifespan extensions through IIS mutations (Apfeld et al., 2004).

The complex network of various molecules determining lifespan and immunity towards biotic stresses such as pathogen invasion seem to always function with a relationship to the IIS pathway (discussed below). This significant role of the IIS pathway and *daf-16* in regulating innate immunity and determining lifespan suggests this is one of the most important molecular pathways in regulating ageing.

1.6.4 IIS [Insulin/IGF (Insulin –like growth factor)- like signalling] pathway in Ageing and Immunity

Insulin and Insulin like growth factors in mammals work through an evolutionarily conserved pathway to regulate several cellular processes that together contribute towards phenotypes such as stress resistance against abiotic factors, immunity against biotic factors and ageing (Rincon et al., 2005, Heemskerk et al., 1999, Holzenberger et al., 2003). Initially the impact of this pathway was identified and studied using the invertebrate model *C elegans*, but such studies have diverged more recently into non-model organisms such as mosquitoes (Arik et al., 2009). In *C. elegans*, harsh and unfavourable growth conditions (such as food deprivation and over-crowding) triggers an elevation in a set of secreted pheromones in all worms (Butcher et al., 2007). When such a cue is detected by early larval stage (L1) worms, the worms are driven into an arrested stage referred to as the dauer (1997). Worms in this adaptive, alternative developmental mode are non-feeding and highly stress resistant. Entry into this dauer stage is considered to be a survival strategy employed by the organism to outlive harsh conditions until favourable conditions return.

Studies based on observing dauer constitutive (Daf-c) and dauer defective (Daf-d) mutants eventually led to the discovery of several pathways that regulate dauer entry (Vowels and Thomas, 1992, Gottlieb and Ruvkun, 1994, Riddle et al., 1981, Thomas et al., 1993). The subsequent discovery of increased lifespan and stress resistance phenotypes as a result of hypomorphic mutations (mutations that result in reduced level of activity of altered gene product, or in which expression of wildtype gene product is reduced) in dauer formation genes such as *age-1* and *daf-2* (Morris et al., 1996, Kenyon et al., 1993, Kimura et al., 1997) immediately raised the question as to how their homologues in other organisms would function, given the evolutionary conservation of the IIS pathway. As a consequence of this, several targeted studies of homologous pathways and their functions in organisms ranging from yeast to invertebrates such as *C. elegans*, *D. melanogaster* and mammals such as *Mus musculus* have revealed a highly similar IIS pathway with overlapping functions (Clancy et al., 2001a, Holzenberger et al., 2003, Tatar et al., 2003, Longo, 2003). Intriguingly, the human orthologues of these genes were recently shown to change in expression level with age in muscle (Bell et al., 2009) and, correspondingly, additional genes implicated in human ageing via microarray analysis extend lifespan when knocked down in nematodes (Bell et al., 2009).

The significant role of the IIS pathway and *daf-16* in regulating innate immunity that combats biotic stresses such as an microbial infection also makes this a target to invading pathogens that have evolved mechanisms to combat host defences. *C. elegans* has been intensively used as a model system to study such interactions (Garsin et al., 2003, Evans et al., 2008). Recent data indicate that some pathogens, such as the Gram-negative bacterium *Pseudomonas aeruginosa,* may be able to colonise and infect *C. elegans* by activating the IIS pathway, thus inhibiting DAF-16 by cytoplasmic sequestration (Evans et al., 2008).

Thus far several other signalling cascades have been shown to contribute towards immunity (biotic stress) and abiotic stress resistance amongst nematodes, including the p38 MAP kinase (Kim et al., 2002, Kim et al., 2004),

ERK MAP kinase (Nicholas and Hodgkin, 2004), JNK-like MAP kinase (Huffman et al., 2004), TGF-β (DBL-1) (Mallo et al., 2002), programmed cell death (PCD) (Aballay and Ausubel, 2001), Toll-like receptor (Tenor and Aballay, 2008) and Wnt/Hox signalling pathways (Irazoqui et al., 2008, Nicholas and Hodgkin, 2009). There is substantial evidence that most of these pathways work in concert with the IIS pathway. For instance, upon oxidative stress the defence response mediated by translocation of DAF-16 into the nucleus was blocked in a p38-related *sek-1* (MAPKK) mutant and DAF-16 instead remained cytoplasmic (Kondo et al., 2005). Similarly, genetic analysis by Oh et al., discovered that the JNK pathway acted in parallel to the insulin-like signalling pathway to regulate lifespan and both pathways ultimately interacted with and phosphorylated DAF-16 (Kondo et al., 2005).

Thus, despite the presence of numerous pathways involved in immunity and stress responds, the IIS pathway seems to be the major influence on life history traits of organisms.

1.7 IIS pathway in *C. elegans*

Larval development, stress resistance and adult lifespan (among other phenotypes) of the *Caenorhabditis* species is regulated by the IIS pathway which is a part of the endocrine system that is remarkably conserved across organisms (Tatar et al., 2003, Russell and Kahn, 2007, Holzenberger et al., 2003, Murphy, 2006). In *C. elegans*, the IIS signalling cascade consists of proteins encoded by the genes *daf-2*, *age-1*, *akt-1*, *akt-2*, *daf-16* and *daf-18*. Upon environmental cues such as food availability, a subset of the 37 predicted insulin like genes (ins1-37) (Pierce et al., 2001, Finch and Ruvkun, 2001) such as *ins-7* get activated and in turn produce insulin like peptides which then bind to the single insulin/insulin-like growth factor (IGF)-1 transmembrane receptor, DAF-2. Upon stimulation, this receptor activates the

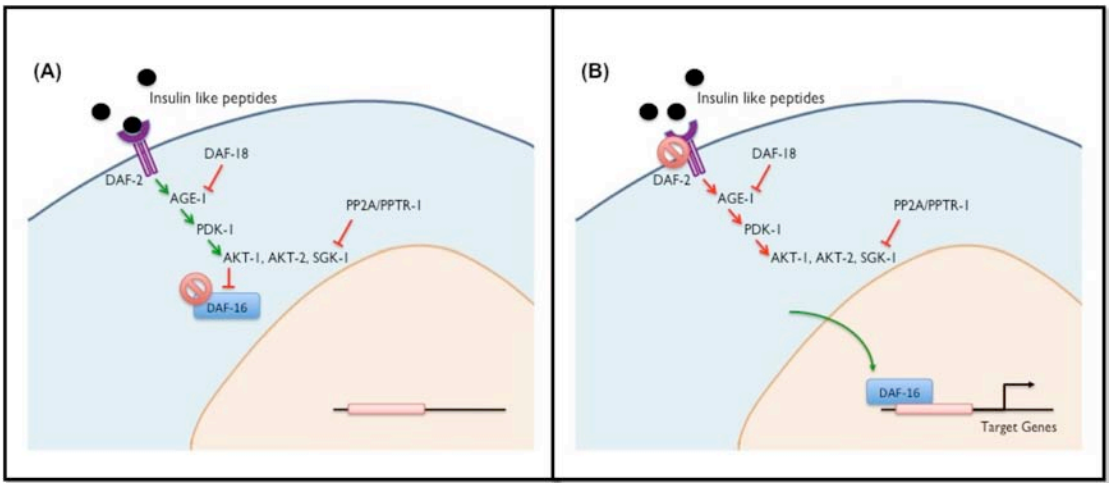

Figure 10 - The role of DAF-16 in regulating key processes such as lifespan, immune response and stress resistance in C. elegans. (A) In the presence of insulin-like molecules the receptor (DAF-2) on the cell membrane is active and, in turn, activates a series of protein kinase enzymes that result in the phosphorylation of DAF-16, keeping it in the cytoplasm. (B) Mutations in the IIS pathway upstream of DAF-16 (such as in DAF-2) lead to the opposite effect, with DAF-16 being dephosphorylated. This results in the movement of DAF-16 into the nucleus where it goes on to regulate a plethora of genes such as genes involved in overall metabolism or adaptation to stress.

phosphatidylinositol 3-kinase (PI 3-kinase) AGE 1 that consists of a p55-like regulatory subunit (Iser et al., 2007) and a p110 catalytic subunit (Morris et al., 1996) that are antagonized by DAF-18 (Human PTEN homolog). The PIP3 signal activates the AKT/PKB kinase homolog PDK-1, which then phosphorylates AKT-1, AKT-2 and SGK-1. The AKT proteins are involved in dauer formation and are also reported to be involved in lifespan extension while SGK-1 controls development, stress response and longevity. These kinases then phosphorylate the transcription factor DAF-16 and inactivate it by cytoplasmic localization (Figure 10A) (Brunet et al., 1999, Ewbank, 2006, Chavez et al., 2007, Ogg and Ruvkun, 1998, Finch and Ruvkun, 2001, Lin et al., 2001, Braeckman and Vanfleteren, 2007, Iser et al., 2007). This sub-cellular localization of DAF-16 was revealed to be regulated by the formation of a protein complex between 14-3-3 FTT-2 and DAF-16, a mechanism that appears to be conserved in mammals (Li et al., 2007). Mutations in *daf-2* or other upstream components such as *age-1* that result in a reduction in IGF-I signalling triggers dephosphorylation of DAF-16 and its subsequent translocation into the nucleus, where it regulates a plethora of target genes that have been identified to be involved in metabolism, development, stress response, detoxification and signalling among others (Figure 10B) (Murphy et al., 2003, Halaschek-Wiener et al., 2005b, Dong et al., 2007, Lee et al., 2003, McElwee et al., 2007, Oh et al., 2006). Expression changes in these genes result in phenotypes which include slower metabolism, increased pathogen resistance, increased dauer formation and increased longevity (Chavez et al., 2007, Ogg and Ruvkun, 1998, Dorman et al., 1995, Lee et al., 2003, Finch and Ruvkun, 2001, Lin et al., 2001, Lee et al., 2001, Ookuma et al., 2003, Schaffitzel and Hertweck, 2006). Also, these mutants are capable of staying younger for long after normal worms look old (Garigan et al., 2002, Herndon et al., 2002). However it is worth noting that not all mutations that lead to dauer arrest also result in an increased lifespan (Kenyon et al., 1993).

Among *daf-2* mutants there is interestingly a soma to germline transformation of gene expression. Germline in the wildtype (immortal line of cells) animals exclusively express *pie-1* and *pgl-1* genes (Kawasaki et al., 1998, Mello et al., 1992). However, in the background of a *daf-2* mutation these genes are also

expressed in the somatic cells and in turn contribute towards longevity (Curran et al., 2009).

1.8 IIS pathway in *Drosophila melanogaster* and *Mus musculus*

Endocrine signalling in *Drosophila melanogaster* and mice has also been shown to influence regulation of key processes such as ageing, immunity and stress resistance. Using *D. melanogaster* provides a useful intermediate platform for extending studies from *C. elegans* across to mammals, due to its closer tissue homology with mammals than *C. elegans* (Toivonen and Partridge, 2009). In *D. melanogaster*, the *daf-2* homologue insulin receptor (dINR) is activated by the seven insulin like peptides (DIPL1-7) that go onto function via the insulin receptor substrate Chico, the PI 3-kinase Dp110/p60, PKB (Akt1) and the dFOXO transcription factor regulating a repertoire of phenotypes such as growth, metabolism, size and lifespan (Figure 11) (Weinkove and Leevers, 2000, Clancy et al., 2001b, Bohni et al., 1999, Leevers et al., 1996, Verdu et al., 1999). Null mutations of the Chico gene have been shown to increase lifespan by 48% and 13% in homozygous female and heterozygous male fruit flies respectively (Clancy et al., 2001a).

The activity/regulation of dFOXO's mammalian counterparts, the FOXO family of transcription factors (FOXO1, FOXO3a and FOXO4) (Ogg et al., 1997) are also controlled by signalling through PI3K/Akt upon insulin growth factor and insulin stimulation (Brunet et al., 1999). This FOXO activity in mammals has, in turn, been shown to drive key processes such as cell cycle progression, apoptosis, growth, differentiation, stress resistance, reproduction and expression of several metabolic genes (Tatar et al., 2003, Alvarez et al., 2001, Kops et al., 2002, Tran et al., 2002, Saltiel and Kahn, 2001, Brunet et al., 1999, Holzenberger et al., 2003, Navarro et al., 1999). Interestingly, FOXO also plays an essential role in inducing resistance to oxidative stress, which has been speculated to have a knock on effect on lifespan extension (Tothova et al., 2007), in line with the "Oxidative damage theory". In addition, however, high FOXO3a levels have recently been shown to play an essential role in the

maintenance of Chronic Myeloid Leukaemia (CML) Leukaemia-Initiating Cells (LICs) (Naka et al., 2010), suggesting that the IIS pathway also operates at the cellular level to extend viability.

FOXO function is regulated by several post-translational modifications of which phosphorylation, acetylation and mono/polyubiquitination have been identified (Brunet et al., 1999, Kops et al., 1999, van der Horst et al., 2006, Matsuzaki et al., 2005). The predominant of these, phosphorylation controlled by signalling through PI3K/Akt, works at a more complex level in vertebrates than invertebrates, with multiple receptors for insulin (IR) and IGF-1 (IGF-1R) (Navarro et al., 1999) (Figure 11). The FOXO transcription factors are in a constant state of shuttling between the cytoplasm and the nucleus (Brownawell et al., 2001). When present in the nucleus during the absence of PI3K-Akt signalling, FOXO goes on to regulate numerous genes. However, upon activation of PI3K-Akt signalling, FOXO decouples from the DNA binding sites by phosphorylation. Phosphorylation is driven by the 14-3-3 protein after which FOXO is exported into the cytoplasm where it is degraded by proteosome dependant degradation (Brunet et al., 1999, Obsilova et al., 2005, Rinner et al., 2007, Brunet et al., 2002, Boura et al., 2007, Plas and Thompson, 2003).

A lot of data pertaining to the importance of the IIS pathways in various model systems is available. However, the translational potential of this evolutionarily conserved biological pathway with regards to its influence on ageing and longevity in humans has only recently started to be addressed. There have been studies where mutations known to impair IGF-1 function are overrepresented in a cohort of Ashkenazi Jewish centenarians (Suh et al., 2008). In addition, particular variants of the Insulin receptor gene have beenlinked to longevity in a Japanese cohort (Kojima et al., 2004). A 2008 study using a large, long-lived population of men with well-characterized ageing phenotypes demonstrated that individuals homozygous for the so-called “G” allele of FOXO3a showed both improved ageing and better insulin

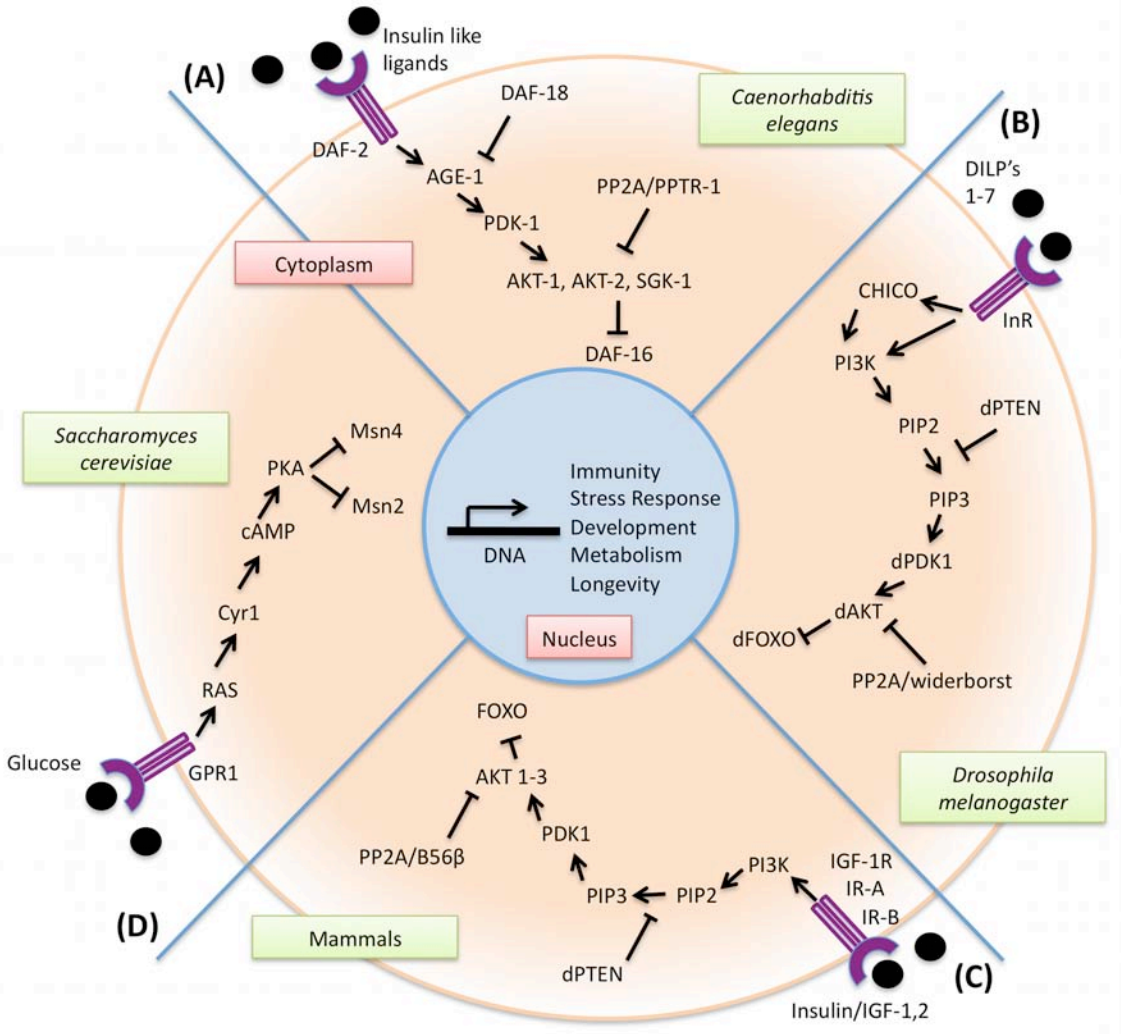

Figure 11 - Illustration of the functioning of the IIS pathway in various model systems. (A) The IIS pathway in *C. elegans* is activated through binding of Insulin like ligands to a transmembrane protein DAF-2 (Riddle et al., 1997). This then activates a cascade of intracellular kinases finally phosphorylating the transcription factor, DAF-16 (Riddle et al., 1981). (B) In the case of *D. melanogaster*, the IIS pathway is activated by DILP's (D. melanogaster Insulin like peptides) that in turn also activates a series of intracellular kinases that culminates in the phosphorylation of the DAF-16 homologue, dFOXO. (C) In mammals a very similar mechanism is in place, with the exception that there are multiple receptors for insulin (IR) and IGF-1 (IGF-1R) (Navarro et al., 1999). Phosphorylation of the mammalian homologue (FOXO) of DAF-16 nonetheless remains the final outcome of the series of intracellular kinases. (D) In yeast, glucose and other nutrients activate the Ras2/Cyr1/cAMP/PKA pathway via the G-protein coupled receptor Gpr1, which in turn negatively regulates the DAF-16 analogous transcription factors Msn2/Msn4. In all four cases, the phosphorylated, transcription factors are sequestered in the cytoplasm where they are inactive. However, when dephosphorylated by the inactivation of the IIS pathway, these transcription factor enter the nucleus regulating a myriad of genes involved in immunity towards biotic stress (such as microbial invasion), abiotic stress response (such as heat stress), development, longevity, metabolism.

sensitivity(Willcox et al., 2008). This association has been recently confirmed in a study involving German centenarians (Flachsbart et al., 2009).

Given the overlap of the importance of the IIS pathway and its downstream target DAF-16 in the various organisms, it is now clear that DAF-16 is a key molecule of the IIS pathway acting as a master regulator of a myriad of downstream targets which regulate phenotypes of stress resistance, immunity and lifespan.

1.9 DAF-16

DAF 16 encodes a member of the hepatocyte nuclear factor 3 (HNF-3)/ forkhead family of transcriptional regulators which is a downstream target of the IIS signalling pathway in *C. elegans* (Lin et al., 1997, Gerisch et al., 2007, Ogg et al., 1997). The *daf-16* gene codes for seven predicted isoforms, with the largest of them encoding an approximate 560aa polypeptide chain. This transcription factor binds as a monomer to DNA at the consensus binding site (TTG/ATTTAC) (Furuyama et al., 2000), although additional data suggest that binding at non-consensus sites also occurs (Mukhopadhyay et al., 2008).

DAF-16/FOXO molecules shuttle between the cytoplasm and nucleus, but under low PKB phosphorylation conditions DAF-16/FOXO becomes predominantly nuclear (Van Der Heide et al., 2004). The wide range of genes independently regulated by DAF-16 suggests the presence of additional mechanisms by which target specificity is achieved. In 2006, Wolff and colleagues demonstrated just such a role for *smk-1* (Wolff et al., 2006). They showed that DAF-16 in concert with molecules such as SMK-1 regulate the ageing process by providing specificity for the regulation of innate immunity, UV and oxidative stress, whereas SMK-1 is not required for the thermal stress resistance function of DAF 16. Additional specificity is conferred by the 14-3-3 proteins, highly conserved cytoplasmic proteins identified in all eukaryotic

organisms, which bind to DAF-16, inducing a conformational change and preventing DAF-16 from entering the nucleus by exposing its nuclear export signal (Li et al., 2007, Brunet et al., 2002). More recently, cGMP levels have been found to further modify *daf-16* phenotypes via transcriptional regulation of the *pde* genes (Hahm et al., 2009) and it is likely that many further pathways which moderate *daf-16* signalling remains to be identified.

DAF-16 activity shows considerable tissue specificity, with an increase in lifespan being more marked following increased DAF-16 activity in tissues such as the intestine and the nervous system than in other tissues (Libina et al., 2003). This is suggestive of secondary signals that coordinate phenotypes in the various other organs in the organism (Apfeld and Kenyon, 1998). In 2007 Murphy and colleagues showed that increasing the level of DAF-16 activity in just one tissue resulted in an increase of DAF-16 activity elsewhere through feedback regulation of insulin gene expression (Murphy et al., 2007). Interestingly, *daf-16* null mutations only reduce lifespan slightly, but suppress all life span extensions caused by other upstream IIS pathway mutations (Henderson and Johnson, 2001). Germline ablation also influences DAF-16 activity by instigating an upsurge in accumulation of DAF-16 in the nuclei (Figure 12), especially in the intestinal tissues (Lin et al., 2001), which provides a molecular explanation for the previous finding that ablation of the germ line extended the lifespan of *C. elegans* in a *daf-16* dependant manner (Hsin and Kenyon, 1999). This effect is not an indirect result of sterility, as removal of the entire gonad (germ cells and the somatic reproductive tissue) had no effect on lifespan. Interestingly, this relationship between the reproductive tissues and lifespan has also been noted in other organisms where transplantation of younger ovarian tissues into older mice have shown to increase lifespan along with similar phenotypes observed in *D. melanogaster* under certain conditions (Cargill et al., 2003, Flatt et al., 2008). Apart from influencing lifespan, the germline has recently been shown to control nematode resistance to the Gram-negative bacterial pathogens *Pseudomonas aeruginosa* and *Serratia marcescens* (Alper et al.). This aspect of pathogen resistance was found to be independent of DAF-16 activity, since *daf-16(mu86);glp-1* and *daf-16(mu86);mes-1* double mutants (*glp-1* and *mes-1* are

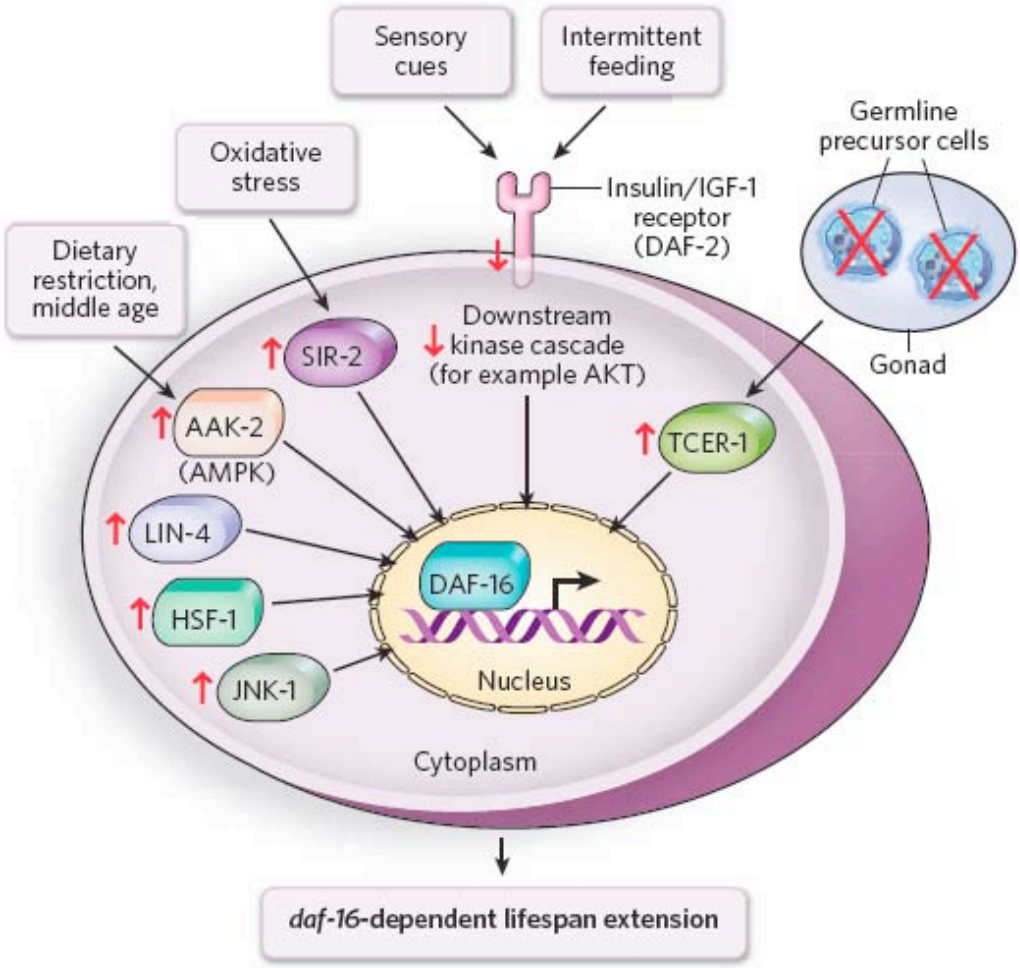

Figure 12 – Various inputs that are able to influence the activity of the *Caenorhabditis elegans* transcription factor DAF-16. Expression of proteins are depicted by the arrow to their left with overexpression of sirtuin SIR-2, the heat-shock transcription factor HSF-1, the developmental-timing microRNA LIN-4, AAK-2 (a subunit of AMP kinase), Jun kinase 1 (JNK-1) or the predicted transcription elongation factor TCER-1 extending lifespan. Also, inhibiting the DAF-2 Insulin/IGF-1 receptor or components of its downstream kinase cascade elicits a lifespan extension in a DAF-16 manner. Figure from (Kenyon, 2010).

genes required for germline proliferation) survive as long as the glp-1 or mes-1 single mutant in the presence of a pathogen and when grown on standard nematode culture conditions. These findings suggest that the germline acts in parallel, at least in part, to the p38 MAPK pathway to impart an effect on innate immunity. On the other hand, changes in lifespan induced by germline signalling have been shown to require *daf-16* (Arantes-Oliveira et al., 2002, Hsin and Kenyon, 1999).

Overall, DAF-16 seems to be a key molecule having a tremendous impact on life history traits. Therefore, from studies so far it is probably safe to suggest that this molecule is perhaps the 'molecular link' through which numerous problems such as post reproductive ageing can be explained.

1.10 *Caenorhabditis* species and their Ecology

We know very little about the natural ecology of the *Caenorhabditis* nematodes (Kiontke and Sudhaus, 2006). Although the species in the *Caenorhabditis* genus are morphologically very similar, their habitats and niches are variable and they are found nearly everywhere on the planet (Table 1). Despite the label of *Caenorhabditis* species as soil nematodes, in reality they are colonizers of nutrient and microorganism-rich organic material that is found predominantly in the soil. Their inability to propagate in soil is highlighted by the fact that dauer juveniles are unlikely to develop here without the addition of organic matter. A few *Caenorhabditis* species are also found to exist in a relationship with other organisms as discussed below.

Necromenic relationships where the worm ascends on a carrier host, waits for the host to die and lives on the bacteria which thrive in the dead animal are seen with certain species such as *C. briggsae,* which diverged from *C.elegans* about a 100 million years ago (MYA) (Coghlan and Wolfe, 2002). The dauer larvae of these species have been found to attach onto snails and wait for the

carrier to die after which they resume development on the decomposing cadaver (Sudhaus and Schulte, 1989, Kiontke and Sudhaus, 2006). Phoretic relationships where the worm rides on the carrier host until it finds a favourable environment are also seen in few species such as *C. elegans* where the dauer larvae use an associated host purely as a carrier to move onto other regions, where they resume development (Richter, 1993, Kiontke and Sudhaus, 2006).

Caenorhabditis elegans, Caenorhabditis briggsae and *Caenorhabditis remanei* are among the species that are found in anthropogenic habitats like compost and garden soil. These species are known to co-occur or share ecological niches with two of the three species being isolated from the same sample sites: *C. briggsae* and *C. elegans* in compost in France (Barriere and Felix, 2005) and *C. briggsae* and *C. remanei* in woodland in Ohio (Kiontke and Sudhaus, 2006). Despite the limited data suggesting a common niche shared by these nematodes, other observational studies have pointed out that species such as *C. briggsae* can tolerate and survive in a much higher temperature in comparison to *C. elegans*.

1.11 Evolutionary Spread and significance of *daf-16*

The ability to defend oneself from invading pathogens is a key feature that defines the fate of an organism. In vertebrates this defence comes in the form of an innate immune system, which responds immediately to infection, and the acquired immune system, which responds slowly but is highly specific and long lasting. Apart from contributing towards the initiation of the acquired immune system the innate immune system also appears to play the major role in coordinating immunity as a whole (Schulenburg et al., 2004).

The innate immune systems of all animals share highly similar features; such as identification of pathogen associated molecular patterns (PAMP's) that

Species	Habitat	Associate	Type of association	Geographical distribution	Reproduction mode
C. elegans	Compost, Garden soil	Millipede, Isopods, Insects, snails, slugs	Phoretic (facultative necormenic ?)	N. America, N Africa, Madeira, Europe, Asia, Australia, Hawaii	Hermaphroditic
C. briggsae	Compost, Garden soil, Woodland	Snails	Necromenic ?	India, N America, Europe, Hawaii, Taiwan, Japan	Hermaphroditic
C. remanei	Compost, Woodland	Snails, Isopods	Phoretic (facultative necormenic ?)	N America, Germany, Japan	Gonochoristic
C. brenneri	Compost-like material	?	?	India, Costa Rica, Guadeloupe, Trinidad, Sumatra	Gonochoristic
C. japonica	Cadavers/nests of burrower bugs	Burrower bug	Facultative necromenic	Japan	Gonochoristic
C. bovis	Auditory canal of zebu cattle (and goat)	Flies, Zebu cattle	Phoretic (Ectoparasite ?)	East Africa	Gonochoristic
C. drosophilae	Rotting Saguaro cactus	*Drosophila nigrospiracula*	Phoretic	Arizona	Gonochoristic

Table 1 – List of a few *Caenorhabditis* species and their ecological information (Kiontke and Sudhaus, 2006).

induce production of antimicrobial peptides, which are in turn, are able to deal with invading pathogens by numerous mechanisms. The similarity in induction, function, anti-microbial molecules and effects hence suggests that the innate immune system has evolved from a common origin and has been conserved over millions of years of evolution. Interestingly, recent discoveries have suggested that innate immunity is directly influenced by the IIS pathway and that this, too, may be conserved across phyla. Thus ageing induces knock-on effects on the immune system due to the intertwining relationship between ageing and immunity/stress resistance.

Studies in *C. elegans* have shown that ageing and immunity towards biotic stress are both defined to a large extent by the IIS pathway. Thus key components of the IIS pathway, such as *daf-16*, impact on both phenotypes simultaneously via a complex network of downstream genes. In other organisms too, the IIS pathway appears to play a dual role as a major effector of immunity and ageing (Clancy et al., 2001a, Tatar et al., 2003, Longo, 2003, Holzenberger et al., 2003). Even in yeast, which lack the IIS pathway, the precursor Ras/Cyr1/PKA pathway down-regulates glycogen storage and genes involved in the switch to the hypometabolic stationary phase, such as *ras2* and *cyr1* mutations, are capable of increasing longevity and also enhancing stress resistance (Longo and Fabrizio, 2002).

Why, then, given that the homologues of *daf- 16* assert similar pleiotropic effects in various organisms (Holzenberger et al., 2003), do we observe vast differences in terms of lifespan in nature? One possibility is that natural selection acts to favour enhancement of one DAF-16 regulated phenotype (stress resistance, for instance) and thus co-selects for other DAF-16 regulated phenotypes (such as ageing). Previous work has suggested that lifespan and pathogen/stress resistance are both species specific and sex specific, but that these phenotypes co-vary (van den Berg et al., 2006). Recent comparative studies using closely related nematode species has demonstrated that the coupling of most DAF-16 regulated phenotypes is conserved between species, even though the magnitude of the phenotype (maximal lifespan, for instance) may vary significantly between species (Inoue et al., 2007, van den Berg et al., 2006, McCulloch and Gems, 2003). Since our understanding of the natural ecology of the *Caenorhabditis* nematode species is still limited (Kiontke and Sudhaus, 2006),

understanding the selective pressures that may influence the evolution of IIS is difficult. Nonetheless, it can be speculated that the differences in the niches inhabited by these species may have imposed variable extrinsic stresses, such as exposure to different pathogens or environmental stresses, which over time have selected for a different IIS pathway "optimum" and thus differences in the immunity and lifespan phenotypes downstream of DAF-16. In conclusion we suggest that the IIS pathway may provide a molecular machinery that is modified by natural selection to influence immunity, stress resistance and ageing in a coordinated manner.

1.12 Project Outline

Previous work in our group has shown that lifespan and pathogen resistance are both species specific and sex specific. Ageing, immunity and stress tolerance are inherent characteristics of all organisms that are regulated, at least in part, by forkhead transcription factors in response to upstream signals from the Insulin/Insulin–like growth factor signalling (IIS) pathway. In the nematode *Caenorhabditis elegans*, these phenotypes are molecularly linked such that activation of the forkhead transcription factor DAF-16 both extends lifespan and simultaneously increases immunity and stress resistance. It is known that lifespan varies significantly among the *Caenorhabditis* species but, although DAF-16 signalling is highly conserved, it is unclear whether this phenotypic linkage occurs in other species.

To address this, here we investigate this phenotypic covariance by comparing DAF-16 influenced phenotypes that include longevity, stress resistance (towards abiotic factors such as heath) and immunity (towards biotic factors such as pathogens) in four representative *Caenorhabditis* species that include both hermaphroditic (*Caenorhabditis. elegans* and *Caenorhabditis briggsae*) and gonochoristic species (*Caenorhabditis remanei* and *Caenorhabditis brenneri*) (Chapter III). We measure for

expression levels of the *daf-16* homologues in these species using Real-Time PCR, looking for positive correlation with the observed phenotypes (Chapter IV). To further support our studies we use mutants with increased *daf-16* activity and perform bioinformatic analysis on the genes in *C. elegans*, *C. briggsae* and *C. remanei* that have DAF-16 consensus binding sites (Chapter V). Finally we look at *daf-16* Isoforms to probe if there are any isoforms that are particularly key under conditions of stress (Chapter VI).

Chapter II

Materials and Methods

2.1 Maintenance of the *Caenorhabditis* species

2.1.1 Introduction

The species belonging to the *Caenorhabditis* clade are ideal for biological research due to their cost effective nature and ease of maintenance. Species of this clade can be obtained from the *Caenorhabditis* Genetics centre (CGC) based at Washington.

In the laboratory worm stocks are stored by freezing them down in freezing medium containing glycerol at -80℃ which upon requirement can be propagated into culture on nematode growth media (NGM) spread with a lawn of bacteria, which serve as a food source.

2.1.2 Growth Media

2.1.2.1 Nematode Growth Media (NGM)

The *Caenorhabditis* species were grown and maintained as a culture on nematode growth medium (NGM) in 9cm petri dishes. NGM was prepared by mixing 3g of sodium chloride (NaCl), 17g of agar and 2.5g of peptone in a 1 litre bottle. To this 1ml of cholesterol (5mg/ml in 100% EtOH) was added followed by 975ml of distilled water. The media was then sterilized by autoclaving for 20 minutes at 120℃ at 15 psi, which is 1.02 atmospheric pressure. After the autoclave cycle the bottle along with its contents was allowed to cool down to approximately 55℃, when 1ml each of 1M calcium chloride ($CaCl_2$) and 1M magnesium sulphate ($MgSO_4$) along with 25ml of 1M potassium phosphate (pH 6) was added. The media was then mixed thoroughly to ensure its homogeneity and then poured aseptically by a flame into petri dishes.

For experimental assays, medium sized (6cm) NGM plates were used and for experiments such as mating the small sized (3cm) NGM plates were used.

2.1.2.2 Luria Bertani Media (LB)

Bacterial strains were streaked onto Luria Bertani medium in petri dishes. For 1 liter of LB 5g of yeast extract, 10g of tryptone, 5g of sodium chloride (NaCl) and 10g of agar was mixed together in a 1 liter bottle. 800ml of distilled water was then added to this and the pH was adjusted to 7.5 with sodium hydroxide (NaOH). The volume was then made-up to 1 liter. This was then sterilized by autoclaving for 20 minutes at 120°c at 15 psi, which is 1.02 atmospheric pressure. After the autoclave cycle the bottle along with its contents was allowed to cool down to approximately 55°C, and then poured aseptically by a flame into petri dishes.

Also, LB broth was prepared following the exact same protocol as above with the exception of addition of agar.

2.1.2.3 Media for RNA interference experiments

Two types of media were made up for the RNAi experiments. LB media (containing 50μg/ml ampicillin and 10μg/ml tetracycline) and NGM media (containing 50μg/ml ampicillin and 1mM IPTG) were poured into plates a few days before use to ensure they are dry, as wet plates give weaker phenotypes.

2.1.2.4 K Medium

K medium was used as a buffer for the metallo-tolerance assay. This medium was prepared by dissolving 53mM NaCl and 32mM KCl in distilled water followed by autoclaving at 121°C for 15 mins (Barsyte et al., 2001, Dusenbery, 1990).

2.1.3 Bacterial strains

Escherichia coli OP50 that has a genetic lesion preventing the de novo synthesis of uracil (Brenner, 1974), HT115 (with transformed plasmid clone for RNAi), *Salmonella enterica* subsp. *enterica* ser. Typhimurium SL1344 (Aballay and Ausubel, 2001), *Pseudomonas aeruginosa* PA01 and *Staphylococcus aureus* NCTC8532 were used for this study.

2.1.3.1 Growth and cultivation of bacterial strains

Bacteria were streaked onto LB plates from frozen stocks and grown overnight at 37°C. Single colonies from these plates were then picked and grown in nutrient rich Luria-Bertani (LB) broth overnight with shaking (180-200rpm) at 37°C. As for the strain HT115, the single colonies were grown in LB broth containing 50μg/ml ampicillin for 6-8 hours.

From these bacterial cultures, OP50 (standard laboratory food source for the *Caenorhabditis* species) was then spread onto standard NGM (Brenner, 1974, Hope, 1999) plates, taking care not to spread right till the edges to try and keep the worms more towards the interior of the plate since they prefer staying on the bacterial lawn. This also prevents the worms from crawling up the sides of the plate, drying out and dying.

As for the other bacterial cultures that were used for assays and RNAi, a drop of approximately 20μl to 30μl of the culture was dropped in the middle of the assay plate and allowed to soak into the NGM.

All plates were incubated overnight at 37°C (~16hrs) followed by storage at 4°C. Plates were always equilibrated to room temperature by leaving them on the bench for a few hours before use.

2.1.4 Worm strains

Four species from the *Caenorhabditis* genus were used for our studies. These consist of *Caenorhabditis elegans* (N2, Var. Bristol), *Caenorhabditis briggsae* (AF16), *Caenorhabditis remanei* (EM464) and *Caenorhabditis brenneri* (CB5161) along with wild-type isolates from the same species as listed in Table 2. Also transgenic *C. briggsae* (JU1018. JU1076) and *C. remanei* (JU1184) strains, capable of undergoing RNAi by feeding, were used in this study. In addition, we made use of the following *C. elegans* mutants:

CB1370 - *C. elegans daf-2*(e1370)

This is a temperature sensitive dauer constitutive mutant that forms 100% dauers at 25℃ and is wild –type at 20°C.

PS5531 - *C. briggsae daf-2*(sy5445)

This is a *C. briggsae* mutant that has a missense mutation in the kinase domain of Cb-*daf-2* gene. The mutation has been shown to affect a residue conserved in *daf-2* and other insulin receptors, but not conserved in other receptor tyrosine kinases (Inoue et al., 2007).

BA17 - *C.elegans fem-1* (hc17)

This is a temperature sensitive, non-sperm producing feminizing recessive mutant.

2.1.4.1 Growth, cultivation and propagation of worm strains

Worms were grown on NGM plates seeded with a lawn of OP50 bacteria. Once the nematodes consumed most of their food source a chunk of agar (usually containing large numbers of worms) was cut out using a sterile scalpel and transferred onto a fresh plate. This process called "chunking" was ideal for transferring homozygous worm strains. Also, chunking agar with a relatively high number of worms ensured that a genetic bottleneck was avoided.

Species	Strain name	Isolated from	Genotype	Outcrossed
C. elegans	RC301	-	npr-1(g320) X [*C. elegans* wild type (Tc1 pattern HCF)]	x
	CB4856	Hawaii, USA	*C. elegans* wild type, CB subclone of HA-8 (Tc1 pattern IX)	x
C. briggsae	ED3033	Taipai, Taiwan	-	x
	ED3034	Taipai, Taiwan	-	x
C. remanei	JU1082	Okazaki, Japan	-	x
	JU1084	Kakegawa, Japan	-	x
C. brenneri	LKC28	Costa Rica	-	x
	SB129	Bohorok, Sumatra	-	x

Table 2 - List of wild type isolates of the *Caenorhabditis* species

All strains except BA17 (which was grown at 25°C to induce sterility prior to analysis) were grown at 20°C. Fourth larval stage hermaphrodites from the hermaphroditic species and females from the gonochoristic species were used for all assays performed unless stated otherwise.

2.1.4.2 Bleaching worm strains

To produce age synchronized populations of worms and to get rid of the occasional bacterial contamination in the nematode stock, gravid worms were "bleached" by treatment with sodium hydroxide and sodium hypochlorite that kill all the worms (but not eggs) and bacterial/yeast contaminants.

This process included washing the worms/eggs off the culture plate using 1-1.5ml of M9 buffer (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1ml 1M $MgSO_4$, H_2O to 1 liter and sterilized by autoclaving) into a 1.5ml eppendorf tube. This tube was then spun using a centrifuge at about 150 g for 30 seconds, followed by removal of most of the supernatant leaving behind the worm pellet in a total volume of 500μl of M9. 80μl of 5M NaOH and 160μl of 5% solution of sodium hypochlorite was then added to the eppendorf tube and shaken vigorously every 20 secs for two minutes to break open the worm bodies. The tube was then filled up with M9 buffer and spun again at 150 g for 30 seconds followed by removal of supernatant. The pellet was re-suspended again in M9, spun down at 150 g followed by removal of supernatant. This wash was repeated once more. In the final step of this wash, most of the supernatant was removed leaving behind 50-100μl. The eppendorf tube with the worm suspension was then rolled overnight using a roller. The following day, the worm suspension was dropped onto the edge of a fresh NGM/OP50 plate and allowed to dry.

This process kills larval and adult animals, but allows the eggs to hatch overnight producing L1 larvae, which were used to propagate a fresh, age synchronous and clean worm culture.

2.1.4.3 Freezing worm strains

Stocks of worm strains can be preserved for years on end at -80℃, which can be thawed instantly to produce fresh cultures when needed. Frozen stocks of worm strains were produced by washing off freshly starved L1-L2 animals using M9 buffer. To this worm suspension an equal amount of autoclaved 30% glycerol (v/v) was added. 1ml from this mixture was then aliquoted into 1.8ml cryovials labeled with strain name and date. This worm suspension in the cryovial was then insulated in a freezing blanket to ensure a gradual freezing and frozen at -80°C. An extra vial was always frozen as a tester which was thawed after 24 hours, put on NGM plates and left to incubate overnight at 20°C. The plate was then examined the following day using light microscopy to check for viability of the thawed stock. Once this was ascertained, the remaining tubes were then transferred from the freezing blanket into cryo boxes and stored at -80°C.

2.1.4.4 Checking worm strains by PCR/ mutants by sequencing

All the worm strains used in this experiment were checked for their species on a regular basis by PCR (this technique is explained later on in the molecular biology section of this chapter) using species-specific primers (Barriere and Felix, 2005) that are listed in Table 4 along with the PCR program. Mutant nematode strains were checked by PCR'ing across the region of the mutation using specific primers (listed in the table below) and then sequencing the resultant PCR product to look for the mutation.

Mutation	Species	Forward 5'	Reverse 3'
PS5531	*C. briggsae*	TTT CTA CAA CAT TGT GCC GC	CAT CAT TCT CTT TCC GGA CG

Table 3 – Primers used to PCR across the region of mutation in *C. briggsae* mutant PS5531

Table 4 – (A) List of species specific primers used to identify between the *Caenorhabditis* species. Primers for *C. elegans, C. briggsae* and *C. remanei* were originally described by Barriere and Felix, 2005 and primers for *C. brenneri* were designed used Primer 3 software. (B) PCR program used with the primers listed in 2(A) and 5.

(A)

Gene symbol	Wormbase gene ID	Species	Forward Primer 5′	Reverse Primer 3′
glp-1	WBGene00001609	*C. elegans*	CCGCTTGGATTCTATGGATTG	CTCTCCTTGCTCCGGGATTG
glp-1	WBGene00029022	*C. briggsae*	GAACCTGCGAGTGCATGTAC	CCGTCTGCAAACGAACGGGC
glp-1	WBGene00055276	*C. remanei*	CAACGGAGGTATCTGCTCAG	CCGCCGTCAAATTTGCATTC
daf-16	WBGene00146715	*C. brenneri*	CCTTAGTAGTGGCCTCAATGGTGT	CACAACCTATCACTTCACTCTCGC

(B)

Temp (°C)	Duration	No of Cycles
94 °C	2 min	1
94 °C	25 sec	35
60 °C	30 sec	35
72 °C	1 min	35
72 °C	7 min	1
15 °C	5 min	1

2.2 Microscopy

The ideal size of the *Caenorhabditis* species (adult 1mm) is suited perfectly for analysis using light microscopy. For all experiments a stereoscopic zoom microscope (Nikon SMZ800) was used. Worms were handled under the microscope using a platinum wire that was always pre heated on a flame for sterilization. Checking and counting of worms for phenotypic assays and other experiments were done using these means.

2.3 Production of male nematodes from the hermaphroditic species

2.3.1 Introduction

The *Caenorhabditis* clade consists of both hermaphroditic and gonochoristic (male/female) species. Despite the occurrence of spontaneous males (XO) in the self-fertilizing hermaphrodites (XX) due to meiotic non-disjunction, the frequency of occurrence is very low (0.2%). For this study we needed a few hundred males that we produced using the following method.

2.3.2 Methodology

C. elegans males were produced directly from a homozygous hermaphroditic stock of *C. elegans*. About 10-15 L4 hermaphrodites were transferred onto a small NGM plate with OP50. This plate was then subjected to a heat shock at 30°C for a period of 6 hours. Post heat shock treatment, the animals were left to self fertilize at 20°C. This resulted in progeny with about 4-5 males per plate. These males were then picked/transferred onto a fresh NGM plate with OP50 that already has L4 hermaphrodites on it. At this point it is essential to make sure that the

hermaphrodites are transferred onto this plate prior to introducing males, as this tends to keep the males from straying away. Also a ratio of 4 males to 1 hermaphrodite was maintained to ensure crossing that produced 50% males in the consequent generation. These males were then picked for experimentation.

2.4 Phenotypic Assays

2.4.1 Introduction

Phenotypes are consequences of gene function and are of paramount significance whilst typing or classifying organisms. In this study we looked for particular phenotypes among species of the *Caenorhabditis* clade under normal laboratory growth conditions and other varied conditions. For all these experiments fourth larval stage hermaphrodites from the hermaphroditic species and females from the gonochoristic species were used. Animals were considered dead when

- They failed to respond to prodding by a platinum wire
- Absence of spontaneous movement
- Cessation of pharyngeal pumping

The worms were transferred onto new plates every one to two days until they stopped egg laying, in order to prevent F1 progeny from interfering with the experiment.

2.4.2 Longevity Assay

The hermaphroditic and gonochoristic strains of worms were bleached (Hope, 1999) to produce age-synchronous L4 moult populations. Between 80 and 250 of these L4 worms were transferred onto NGM plates (~30 worms per plate, yielding up to 10

replicates) seeded with OP50 for longevity assays. These plates were incubated at 20°C and monitored for survival every 24hrs.

2.4.3 Biotic Stress Assay

We studied the extent to which *Caenorhabditis* species were able to tolerate various types of biotic stress, mimicked by replacing the nematodes' normal food source (OP50) with pathogenic bacteria such as *Pseudomonas aeruginosa*, *S. enterica* Typhimurium and *Staphylococcus aureus* previously shown to infect *C. elegans* by eliciting an infection leading to premature death (Gallagher and Manoil, 2001, Sifri et al., 2003, Labrousse et al., 2000). The negative effects of heat as an experimental determinant has been ruled by previous studies by others and us where worms grown at 25°C on OP50 have been shown to have a significantly longer lifespan than those grown on pathogenic bacteria such as *S. enterica* Typhimurium (Aballay et al., 2000).

2.4.3.1 S. enterica **Typhimurium** *(SL1344)*

Fourth larval stage animals were transferred onto pathogenic assay plates containing rod shaped, flagellated, aerobic, Gram-negative bacterium *S. enterica* Typhimurium, which was prepared as per protocol stated before in this chapter. These plates were incubated at 25°C and nematodes were monitored for survival every 24hrs.

2.4.3.2 Pseudomonas aeruginosa (PA01)

Fourth larval stage animals were transferred onto pathogenic assay plates containing Gram-negative, aerobic, rod-shaped bacterium with unipolar motility *Pseudomonas aeruginosa*, which were prepared as per protocol stated before in this chapter. These plates were incubated at 25°C and nematodes were monitored for survival every 24hrs.

2.4.3.3 Staphylococcus aureus (NCTC8532)

Fourth larval stage animals of the various species were also tested for tolerance against the facultatively anaerobic, Gram-positive cocci, *Staphylococcus aureus*. These plates were prepared as per protocol stated before in this chapter. These plates were incubated at 25°C and the nematodes were monitored for survival every 24hrs.

2.4.4 Abiotic Stress Assay

This study also involved the study of the extent of tolerance in the varied *Caenorhabditis* species to various types of abiotic stress factors.

2.4.4 .1 Heat Assay

L4 worms from the hermaphroditic species, and females in the case of gonochoristic species, were transferred onto NGM plates with OP50 that had been pre-warmed by placing them in a 37℃ incubator for a couple of hours prior to use. Post transfer these plates were then incubated at 37°C and the worms were scored for survival at hourly intervals. The control for this experiment was worms of the various species grown on NGM plates with OP50 at 20℃.

2.4.4 .2 Heavy Metal Stress Assay

Age synchronous L4 worms were transferred from NGM plates into 24-well tissue culture plates containing copper chloride (7mM) dissolved in K medium (53mM NaCl, 32mM KCl) (Barsyte et al., 2001, Dusenbery, 1990). The plate was incubated at 20°C and the worms were scored for survival every hour. The control for this experiment was a couple of wells with worms in only K medium.

2.5 Bioinformatic analysis*

2.5.1 Introduction

From previous studies involving *C. elegans* it has been shown that DAF-16 contributes towards the regulation of a plethora of genes (Murphy et al., 2003, Halaschek-Wiener et al., 2005b, Lee et al., 2003, Dong et al., 2007, Oh et al., 2006). Since no such studies have been conducted in other species we set out to identify genes regulated by homologues of the *daf-16* gene. We performed bioinformatic analysis on the genomes available for the representative *Caenorhabditis* species to try and establish an estimate of the gene groups (based on function of the gene) regulated.

2.5.2 Bioinformatic analysis of DAF-16 downstream targets

We screened through the genomes of *C. elegans* (20,189 genes), *C. briggsae* (21,976 genes) and *C. remanei* (31,614 genes) looking for the presence of two known canonical DAF-16 binding sites (ttatttac/gtaaataa, ttgtttac/gtaaacaa) (Furuyama et al., 2000). This involved the survey of a 3000bp upstream flanking region of every gene (upstream of the lead ATG) done using the package " Regulatory Sequence Analysis Tools" (RSAT; http://rsat.bigre.ulb.ac.be/rsat/; (Van Helden et al., 2000) by applying a perfect match approach to identify genes. From these identified genes, only those with upstream flanking region containing one or more perfect matches to the consensus sites were included in further analyses wherein we retrieved a subset of genes for each species that were orthologous either between *C. elegans* and *C. briggsae, C. elegans* and *C. remanei* or between all three species.

Using the functional annotation tools available from the non-commercial bioinformatic database DAVID (Database for Annotation, Visualization and

* Bioinformatic analysis was performed by Dr. Claudia Boehnisch

Integrated Discovery) (Huang et al., 2008, Dennis et al., 2003) we subsequently screened the derived gene subsets to identify functional groups that were significantly over-represented in the DAF-16 regulon (enriched functional gene groups), relative to the representation of that functional group in the genome as a whole .

We then went onto compare our *C.elegans* gene list results with the available datasets of Oh (Oh et al., 2006), Murphy (Murphy et al., 2003), Halaschek-Wiener (Halaschek-Wiener et al., 2005a), Lee (Lee et al., 2003), McElwee (McElwee et al., 2007) and Dong (Dong et al., 2007). Prior to the analysis these datasets were combined and duplicates removed.

The resultant compared gene list was then further compared to the gene lists obtained for *C. briggsae* and for *C. remanei* and the three resulting lists were analysed using DAVID. Finally, we went through the dataset of Oh and the datasets of Murphy, McElwee, Lee, Halaschek-Wiener and Dong to highlight any overlap.

We determined the number of genes that were enriched within the functional annotation category Gene Ontology GOTERM BP_ALL and especially enriched in GOTERM BP_2. The results were obtained by using the Functional Annotation Chart tool. The GOTERMS BP are available from the DAVID database. The p-value obtained in this analysis is equivalent to the EASE score, which uses a conservative adjustment of the Fisher's exact probability, and was applied to identify significantly enriched gene categories. DAVID provides several methods to correct for multiple testing which include Bonferroni adjustment of the p-value, and the Benjamini-Hochberg approach to control for family-wide false positive rate. The fold enrichment value measures the magnitude of enrichment and is considered significant if 1.5 or above (Huang et al., 2008) .

For all orthologs, the corresponding WormBase IDs of *C. elegans* genes were used as input files. Orthologs between *C. remanei* and *C. briggsae* but not occurring in *C. elegans* could not be addressed with this approach. All orthologous genes with a duplicate output in one of the species were counted only as one gene.

2.5.3 Adaptive sequence evolution

We probed for adaptive sequence evolution along the IIS pathway by aligning amino acid sequences and DNA sequences of the coding regions of homologous *daf-2*, *age-1*, *pdk-1*, *akt-1*, and *daf-16* genes of *C. elegans*, *C. briggsae* and *C. remanei*. (sequences obtained from WormBase WS197 - www.wormbase.org). This was done using ClustalX2 (Larkin MA, 2007) and for each gene of interest, the presence of adaptive sequence evolution (ratio between synonymous [K_S] and non-synonymous [K_A] substitutions) was calculated between a pair of sequences (*C. elegans* and *C. briggsae*; *C. elegans* and *C. remanei*; *C. briggsae* and *C. remanei*) using PAL2NAL (Suyama et al., 2006) that calculates K_S and K_A by the codeml program in PAML. Pairwise protein alignments in CLUSTAL format and the corresponding DNA sequence alignments in FASTA format were used as input files. The following option settings were used

- Codon table: "universal"
- Remove gaps and inframe stop codons: "Yes"
- Calculate K_S and K_A: "Yes"
- Remove mismatches: "No".

2.6 Molecular Biology

2.6.1 Worm Lysis

Worm extracts containing genomic DNA were obtained by lysing worms. 5µl of 20mg/ml proteinase K was added to 95µl of 1x PCR buffer (50 mM KCl, 10 mM Tris-

HCl - pH 8.3 and 1.3 mM $MgCl_2$). 5-10μl of this solution was then taken in a 0.1ml PCR tube and 2-3 worms were added to this. The tube was immediately spun down to ensure the worms were in solution at approximately 10,000 g in a tabletop centrifuge. The PCR tube was then transferred to the -80° C freezer and left in there for approx. 30 minutes. To obtain genomic DNA, this tube from the freezer was then heated to 65°C for 60-90 minutes followed by a further incubation step at 95°C for 15 minutes in a PCR machine. The resultant worm lysate if not used immediately was stored at -20° C.

2.6.2 RNA Isolation

RNA was isolated from 9cm plates of nematodes. The worms were washed off the plates using 1-2ml of M9 solution into eppendorf tubes. The tubes were then spun down at 150 g for 1 min. Most of the supernatant was removed leaving behind the worm pellet in approximately 0.2ml of M9 buffer. The tube was then refilled with M9 and the pellet resuspended, followed by another spin and supernatant removal. This whole process was repeated once more to try and remove most of the bacterial cells, finally resulting in a worm concentrated 0.2-0.4 ml solution of M9.

2.6.2.1 RNA extraction using the RNeasy kit by Qiagen (cat. no. 74104)

The RNeasy kit provided enrichment for extraction of mRNA since most RNA's smaller than 200 bases were selectively excluded. RNA extraction was performed following manufacturers instructions.

The worms were pipetted into a mortar followed immediately by addition of liquid nitrogen. The worms were then ground thoroughly using a pestle. As the liquid nitrogen evaporated and before the tissues thawed, buffer RLT containing highly denaturing guanidine thiocyanate and β-mercaptoethanol was added. This buffer also functioned as a RNase inhibitor thus ensuring purification of intact RNA.

The resultant extract was then run through a QIAshred column using a microspin that further helped in lysing and homogenising the tissue.

Ethanol was then added to the supernatant from the previous step to provide appropriate binding conditions. This sample was then applied to an RNeasy Mini spin column that consists of a silica membrane capable of binding up to 100μg of RNA longer than 200 bases facilitated by a specialized high-salt buffer system. With the RNA bound to the silica membrane, all the contaminants were efficiently washed away and RNA was finally eluted in 30μl of water. RNA was then stored at -80°C.

2.6.3 Nanodrop

RNA was quantified using a microvolume spectrophotometer called the nanodrop. We used 1μl of sample for measurement of concentration. A measurement cycle involved pipetting a droplet of sample onto the measurement pedestal followed by closing the apparatus that resulted in the sample arm slightly compressing the droplet and a liquid column being drawn. The surface tension alone is able to hold the sample in place for the spectral measurement. The spectral measurement is then made which consists of sample assessment at both 1-mm and 02.mm path providing a greater dynamic range and eliminating the need to perform dilutions and make assumptions regarding sample concentration prior to measurement (Gallagher and Desjardins, 2006). Finally once the reading was obtained the apparatus arm was opened and the sample was simply wiped from both the upper bushing and lower pedestal using filter paper.

2.6.4 Primer/Probe design

Primers and probes for all the experiments were designed taking into account factors such as GC content, size and melting temperature using the freely available software

online called Primer3 (http://frodo.wi.mit.edu/primer3/). All primers designed were also checked for their molecular properties using another online tool called the "oligonucleotide property calculator" (http://www.basic.northwestern.edu/biotools/oligocalc.htm).

2.6.5 Polymerase chain reaction (PCR)

Several primer pairs as listed in Table 4A (for identification of *Caenorhabditis* species) and Table 5 (to profile housekeeping genes *gpd-3* and 18s) were designed and used to amplify specific segments of DNA. The PCR reactions containing PCR buffers, DNA template, respective primers (10mM), dNTP's (2mM) and the heat stable enzyme Taq polymerase were cycled through various temperatures (as listed in Table 4B) in a Sensoquest thermocycler.

2.6.6 Sequencing

All the sequencing in the project was done in house at the genomics facility, School of Biosciences, University of Birmingham.

2.6.7 Agarose gel electrophoresis

A 1% (w/v) TBE agarose gel with 0.2μg/ml ethidium bromide was used for resolving DNA. The gel matrix was prepared by dissolving agarose (1g) in 1x TBE buffer (100ml) by boiling in a microwave followed by addition of ethidium bromide once the gel cooled down to approximately 55°C. DNA was mixed with 0.1 volumes of 6x DNA loading dye from promega (0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll 400, 10mM Tris-HCl - pH 7.5 and 50mM EDTA - pH 8.0) and subjected to electrophoresis at 60V for approximately 45min-1hr along with a marker/ladder (0.2μg 100bp ladder) as a reference for size and quantification. The gel was then viewed under UV light using Geneflow Syngene Bio Imaging for identification of bands.

2.6.8 DNase treatment

All the RNA samples were treated with RNAse-free DNase (Promega) to eliminate any DNA residues that may be present prior to use by incubating the RNA with appropriate buffer and DNase enzyme for 1hr at 30°C followed by addition of the stop solution (which killed the DNase activity) and another incubation for 10 mins at 75°C.

2.6.9 Reverse transcriptase PCR

cDNA (complementary DNA) was prepared from the isolated RNA (DNase treated) using a Superscript II reverse transcriptase (Invitrogen). A 20μl reaction was set up initially with random primers (200ηg), RNA template (1μg), dNTP mix (10mM each) and made upto 12μl with distilled water. This mix was incubated at 65°C for 5 mins, quick chilled on ice. A mixture of 5X First-Strand Buffer (250 mM Tris-HCl, pH 8.3 at room temperature; 375 mM KCl; 15 mM MgCl2), DTT (0.1M), RNase Inhibitor (40units/μl) and the reverse transcriptase enzyme superscript II (200units) was then added to the reaction mix (referred to now as reverse transcriptase +ve) and subjected to further incubations as illustrated below

Temperature (°C)	Time (mins)
25	12
42	90
70	15

Figure 13 - Figure illustrating the various steps to prepare cDNA

In addition, control reactions using distilled water instead of superscript II were performed in order to ensure the absence of any DNA contamination (referred to as reverse transcriptase –ve control). To confirm this both the reaction mixes (reverse

transcriptase +ve and –ve) were used as a template in a PCR against the housekeeping primer *gpd-3* (Table 5).

2.6.10 Real Time PCR

Real-time quantitative RT-PCR was performed (7300 Real Time PCR System; Applied Bio Systems) using the Quantace SensiMix dU kit to quantify gene expression of *daf-16* among the *Caenorhabditis* species (N2, N2 males, AF16, EM464 and CB5161) at various stages of development (L1/L2, L4 and adult) and expression of various *daf-16* isoforms (under normal conditions and conditions of stress) in *Caenorhabditis elegans*. This technique employed the Sybr Green methodology, which exploited the ability of SYBR Green (fluorescent dye) to bind to double stranded DNA with great specificity. Every reaction in the experiment was done in triplicate. The details of the candidates and primers are listed in Table 5 and Table 6.

2.6.11 Calculating gene expression

Real time results were analysed using the ΔCT method with the *gpd-3* and 18S RNA expression levels as controls for normalization and expressed as fold change compared to *C.elegans* (Livak and Schmittgen, 2001)

2.6.12 Calculating primer efficacy

All primers were tested for maximum efficiency with their respective cDNA prior to qRT-PCR. To assess the efficacy of the primers and the sensitivity of the qPCR assay, 2-fold dilution series of the template DNA for all the species tested were prepared and subjected to qPCR amplification. The results obtained were extrapolated to produce standard curves by linear regression analysis between threshold cycle (Ct) and sample dilution that gave coefficients of determination (r^2) that exceeded 0.95 for all template/primer combinations. Once amplification efficiencies of the target and

Table 5 - List of Real Time primers used to amplify *daf-16* in the various species and the reference genes *gpd-3* and 18s

Gene symbol	Wormbase gene ID	Species	Forward Primer 5′	Reverse Primer 3′
daf-16	WBGene00000912	*C. elegans*	GCGAATCGGTTCCAGCAATTCCAA	ATCCACGGACACTGTTCAACTCGT
daf-16	WBGene00033715	*C. briggsae*	AGAAGGCTACCACTAGAACCAACG	TCCATCCAGCGGAACTGTTCGAAT
daf-16	WBGene00056236	*C. remanei*	CGACGGCAATACTCATGTCAATGG	ACGGTTTGAAGTTGGTGCTTGGCA
daf-16	WBGene00146715	*C. brenneri*	CCTTAGTAGTGGCCTCAATGGTGT	CACAACCTATCACTTCACTCTCGC
gpd-3	-	All species	TGAAGGGAATTCTCGCTTACACC	GAGTATCCGAACTCGTTATCGTAC
18s	-	All species	TTCTTCCATGTCCGGGATAG	CCCCACTCTTCTCGAATCAG
act-4	-	All species	CCATCATGAAGTGCGACATTG	ATCCTCCGATCCAGACGGACTA

Table 6 - List of Real Time primers used to amplify the various *daf-16* isoforms

Gene symbol	Isoform ID	Species	Forward Primer 5′	Reverse Primer 3′
daf-16	>R13H8.1a	*C. elegans*	CCTATTCGGATATCATTGCC	TGACGGATCGAGTTCTTCCAT
daf-16	>R13H8.1b	*C. elegans*	ATTCCTGAAGAAGATGCTGACC	CGATTCGGACATTTCCGAA
daf-16	>R13H8.1c	*C. elegans*	CCTGAAGAAGATGCTGACCTAT	TTCGGACATTCTGAAATCCG
daf-16	>R13H8.1d	*C. elegans*	TTACATTGCTCGAAGTGCCG	CAGTGGAAAAACTCATTCCG
daf-16	>R13H8.1e.2	*C. elegans*	CGTGGGGAAAACTATGAA	CGCATGAAACGAGAATGA
daf-16	>R13H8.1f	*C. elegans*	TTTGAACCCGATTTTGTGCG	GTTCGAGATTGTGCAGTGTGA

the reference were determined to be approximately equal, RT PCRs were carried out for all the experimental conditions.

2.6.13 RNAi

RNA interference was used to try and target specific gene products for degradation by exploiting the fact that feeding or injecting double-stranded RNA possess the ability to nullify gene action by specifically inactivating endogenous genes with a corresponding sequence (Siomi and Siomi, 2009).

2.6.13.1 Production of RNAi clones

In the case of *C. briggsae,* DNA fragments for *daf-2* and *daf-16* were PCR amplified from genomic DNA. All PCR reactions were performed using Pfu DNA Polymerase (Promega). The fragments were 277bp for *daf-2* and 270 bp for *daf-16*. The following primers with recognition sites for either one of the restriction enzymes PstI, SacI or NheI were used for PCR amplification:

daf-2 – For -NheI (5'- TACATTGCTAGCAGTGTCGATGCCAAGCTTCT-3'),

daf-2- Rev - PstI (5'- ATATTACTGCAGTTCCCTGTGCCAGATAGACC-3'),

daf-16 – For - NheI (5'-TACATTGCTAGCAGAGTTCGTGGTGGGTCATC-3')

daf-16 – Rev - SacI (5'- ATATTAGAGCTCTGATTGCATCGAATCGTCAT-3')

All restriction enzymes were obtained from Fermentas. The set up for PCR reactions was the following: initial DNA - denaturation for 5min at 94°C. This step was followed by 35 cycles of target amplification comprising of denaturation for 30sec at 94°C, primer annealing at 55°C for 30sec and elongation for 2min at 72°C. This was followed by a final elongation step for 10 min at 72°C.

Target gene	Species	Strain	Plasmid	Insert Sequence
Control	*C. briggsae*	JU1018	HT115	-
(No insert – only	*C. briggsae*	JU1076		-
plasmid)	*C. remanei*	JU1184		-
	C. elegans	N2		-
lin-12	*C. briggsae*	JU1018	HT115	-
(Positive control)	*C. briggsae*	JU1076		-
	C. remanei	JU1184		-
	C. elegans	N2		✓
rol-6	*C. briggsae*	JU1018	HT115	-
(Positive control)	*C. briggsae*	JU1076		-
	C. remanei	JU1184		-
	C. elegans	N2		✓
daf-16	*C. briggsae*	JU1018	HT115	✓
	C. briggsae	JU1076		✓
	C. remanei	JU1184		
	C. elegans	N2		✓
daf-2	*C. briggsae*	JU1018	HT115	✓
	C. briggsae	JU1076		✓
	C. remanei	JU1184		
	C. elegans	N2		✓

Table 7 - List of RNAi clones. Ticks in the last column indicate that we were successfully able to produce an insert sequence and insert it into the respective plasmid.

The resultant PCR fragments were then cloned into the L4440 vector (Timmons and Fire, 1998) after double digestion with the corresponding restriction enzymes for 4h at 37°C. For cloning T4 DNA ligase from New England BioLabs were used. Ligation was performed overnight at 4°C. The resulting plasmids were firstly transformed into *E. coli* DH5alpha for amplification. Transformed bacteria were selected on LB agar containing ampicillin (50 μg/ml). Plasmids from one positive colony per plasmid construct were isolated with the Qiagen Miniprep Kit. (Qiagen Ltd.) and sequenced prior final transformation into *E. coli* HT115. Only clones whose insert matched perfectly to the published sequences of *C. briggsae daf-2* and *daf-16* were used for final transformation. Clones were selected on LB agar containing ampicillin (50 μg/ml) and tetracycline (12.5 μg/ml). Several single positive colonies from independent LB plates were then used to establish a frozen stock of *daf-2* and *daf-16* RNAi *E. coli* HT115.

As for *C. elegans* the *daf-2* and *daf-16* clones were used from Dillin et al. (Dillin et al., 2002). The controls *lin-12* and *rol-6* clones were kindly provided by Marie Anne Felix.

2.6.13.2 Production of competent cells

In order to obtain competent cells of both *E. coli* DH5alpha and *E. coli* HT115, bacteria were grown overnight at 37°C in liquid LB containing either ampicillin (DH5alpha) or tetracycline (HT115). The next day 25ml of liquid LB (and appropriate antibiotic) were inoculated with the overnight culture resulting in a 1:100 dilution of overnight culture. Cells were grown to OD595=0.4. Cells were centrifuged at 500 g for 10min at 4°C. Cells were gently resuspended in 0.5x original volume cold, sterile 50mM $CaCl_2$. Thereafter they were incubated on ice for 30min and subsequently centrifuged as before. The resulting pellet was resuspended in 0.1x original volume cold, sterile 50mM $CaCl_2$. 100μl of this was then immediately used for transformation.

2.6.13.3 Performing RNAi

We constructed a vector (L4440) containing a cloned genomic fragment of interest that we then transformed into HT115, *E. coli* bacterial strain. Using this as a food source, we then induced RNAi following methods described by Kamath et. al. (Kamath and Ahringer, 2003). *E. coli* RNAi clones (Table 7) were grown for 6-12 hours in LB broth containing 50μg/ml ampicillin (until a turbid culture was obtained) and then seeded on NGM RNAi plates (described previously in this chapter), let to dry and induce overnight. L3/L4 stage nematodes were then transferred to these plates and left for 72 hours for RNAi to take effect after which the gravid adult animals were bleached and the eggs transferred to a fresh RNAi plate (prepared the day before) and kept at 20^{o}C till the progeny reached L4 stage. These L4 nematodes were then subjected to various phenotypic assays. For all RNAi experiments carried out we included an *E. coli* RNAi clone carrying the HT115 (RNAi expressing) plasmid without insert as a control.

2.6.14 Northern Blotting

A Northern Max kit from Ambion was used for Northern Analysis. Following manufacturers guidelines, RNA was size fractionated in a denaturing gel followed by transfer and immobilization of the latter onto a membrane. This was performed in a manner that the relative positions of the RNA molecules were maintained. The resulting northern blot was then hybridized with a radioactively labelled probe (prepared using primers listed in Table 8) complimentary to the mRNA of interest. The signal produced by this bound radioactive probe was then used to determine the presence, size and abundance of the target mRNA. Along side a positive control (*gpd-3* mRNA) was run for all experiments.

3.3 Survival analysis of type *Caenorhabditis* species to abiotic stress

From numerous previous studies it has been established that lifespan in *C. elegans* is significantly influenced by the activity of the evolutionarily conserved transcription factor DAF-16, which is a key molecule in the IIS pathway (Kenyon et al., 1993). It has also been demonstrated in *C. elegans* that DAF-16 plays an important role in regulating the expression of genes conferring resistance to high temperature or heavy metals (Barsyte et al., 2001, Galbadage and Hartman, 2008). Given this link of DAF-16 with longevity, stress resistance and immunity, we probed further to see if the observed variance between lifespans among the *Caenorhabditis* species also extended correlatively to other DAF-16 dictated phenotypes such as tolerance towards abiotic stress factors.

To test this we exposed multiple isolates of all four species to prolonged high temperature of 37°C (Figure 16, Table 11 for p - values) or toxic heavy metals such as $CuCl_2$ (Figure 17, Table 12 for p - values). In general the gonochoristic species showed significantly higher survival than either hermaphroditic species with the exception of the difference in survival between *C. briggsae* and *C. brenneri* under toxic heavy metal stress.

Table 8 - List of primers used to produce probes for Northern Analysis

Gene symbol	Wormbase gene ID	Species	Forward Primer 5′	Reverse Primer 3′
daf-16	WBGene00000912	*C. elegans*	AATGAAGGAGCCGGAAAGAG	CGTTATCAAATGCTCCTTGC
daf-16	WBGene00033715	*C. briggsae*	GATTCGCCAGAAGATGGAAG	CCCTTATCTCTGAAGTATGG
daf-16	WBGene00056236	*C. remanei*	ACTCAATCCGCCACAATCTC	CGGAAAGTAGTCATGCTGTCG
daf-16	WBGene00146715	*C. brenneri*	GGGAATCGCTCTGTTTACCA	GAGAAGAGAGAAAGAGACCC

2.7 Construction of Phylogenetic trees

DNA sequences for the various species were obtained either from Ensemble (www.ensembl.org) or The National Centre for Biotechnology Information - NCBI (www.ncbi.nlm.nih.gov). Homologous sequences were fished out employing the blast tool in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For multiple sequence alignments and production of phylogenetic trees the online software Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and Cobalt from NCBI (http://www.ncbi.nlm.nih.gov/tools/cobalt/) were used respectively.

2.8 Statistical analysis

For all survival assays, survival curves were produced based on the Kaplan-Meier method using MS-Excel and the significance was calculated using the non-parametric log-rank method. Assays were then corrected for multiple testing using the Bonferroni correction. The details for these tests are as follows.

2.8.1 Kaplan-Meier Analysis

Kaplan and Meier, as a means to perform survival analysis proposed the Kaplan-Meier Analysis (Kaplan and Meier, 1958). This test is used extensively in clinical trials where the investigator is often interested in the time until participants in a study present a specific event or endpoint such as death. In such research there are always subjects who do not last the entire duration of the study and may prematurely leave. Such candidates cannot be eliminated from the study, as this would skew the results. When the first subject is censored, the part of the curve post this event is an estimation of the survival for the group rather than actual survival. The actual survival is not known since the subjects censored are still alive at the time of the analysis. Censoring a subject in effect reduces the sample size of subjects at risk after the time of censorship. Reducing sample size always reduces reliability, so

the more subjects are censored and the earlier they are censored the more unreliable the curve is. Because each censored subject reduces the reliability of the curve from that point forward, the end of the curve is most affected. This is unfortunate, since the end of the curve represents long term survival which is the ultimate goal. However the Kaplan-Meier test does take in to consideration the censored/missing individuals of such studies. The test records deaths as of [time] t as if they occurred slightly before t and censored/missing as of [time] t slightly after thereby calculating the survival probability estimate for each of the t time periods, except the first, as a compound conditional probability. Henceforth when a subject is censored, the number of patients "at risk" is reduced by one. So when a subject dies, the survival for the interval ending with his death is calculated according to the number remaining at risk at the time of death.

Since our studies were aimed at the assessing survival of the *Caenorhabditis* species and involves numerous worms missing from the survival assay plates (censored) the Kaplan-Meier analysis was ideal to analyse our data.

We also calculated Confidence intervals (CI) at key points along the Kaplan-Meier survival curve to give an indication of the reliability and robustness of the estimates at these points according to Peto's method (Peto, 1984, Parmar and Machin, 1995).

2.8.2 Log Rank Analysis

The logrank test, also referred to as the Mantel-Cox test, was used to test for significant difference between the cumulative probability of survival between the Kaplan-Meier survival curves of the various tested species.

2.8.3 Bonferroni Correction

The Bonferroni correction was used as a final step in our survival analysis to test for multiple-comparison correction since several statistical tests were being performed simultaneously per survival assay.

2.8.4 Other statistical tests

Statistical details and detailed description of the annotation methods used in DAVID for Bioinformatic analysis was sourced from references in the respective section of this chapter.

2.9 List of kits used

- RNA Isolation – Qiagen RNeasy (cat. No. 74106)

  Qiashred (cat. No. 79654)
- Real Time PCR – 2x SensiMix dU SYBR® Green Kit
- Northern Analysis – Ambion Northern Max kit (cat. No. AM1904)
- 6x loading dye – Promega (cat. No. G1881

Chapter III

Phenotyping of Species

AMRIT, F. R., BOEHNISCH, C. M. & MAY, R. C. (2010) Phenotypic covariance of longevity, immunity and stress resistance in the *Caenorhabditis* nematodes. PLoS One, 5, e9978.

3.1 Introduction

The presence of many similar phenotypes among organisms suggests a common origin of life. Thus tracing genes through evolutionary time could potentially explain why conserved phenotypes function to variable extents among species.

One such phenomenon shared by all living organisms but which varies hugely across species and between different sexes of the same species is longevity. Several recent findings with regards to this have now established genetic pathways to be the driving force behind them contrary to what was believed earlier (discussed in the introduction). Pioneering genetic studies using the roundworm *Caenorhabditis elegans* identified the first gene with a substantial role in determining lifespan (Klass, 1983, Friedman and Johnson, 1988, Kirkwood, 2005). This process has now been attributed to the functioning of the IIS pathway.

The IIS pathway, especially its key molecule *daf-16*, is highly conserved and dictates lifespan in organisms ranging from yeast to humans and, in many cases, appears to retain its dual role as a major effector of immunity and longevity (Clancy et al., 2001a, Holzenberger et al., 2003, Tatar et al., 2003, Longo, 2003). Studies in *C. elegans* have explored this coupling relationship between the *daf-16* determined phenotypes of longevity, immunity and stress tolerance to a great extent, but little is known about the corresponding phenotypes in other nematode species. Since *Caenorhabditis* species are found all across the globe they represent an excellent tool with which to assess how the IIS pathway varies during evolution.

In this study we undertook a comprehensive analysis of immunity, stress response and longevity phenotypes in several representative isolates of four nematode species within the *Caenorhabditis* genus. We compared hermaphrodites and females (from the gonochoristic species) in our assays. Females were the most appropriate to compare in comparison to the males as the males differ from hermaphrodites in their gross morphology, many aspects of their anatomy, expression of a number of behaviours

including regulation of defecation (Reiner and Thomas, 1995), response to media conditioned by the same- versus opposite sex (Simon and Sternberg, 2002) and mate-searching (Emmons and Lipton, 2003). Numerous worms were tested for survival per phenotypic assay. These worms were all not monitored on the same plate but were on replicative plates with a maximum of 15 worms per plate. Once the assay was completed the replicates were tested for significance between themselves and the data was pooled. Replicative plates that were significant outliers were discarded. Also plates with bacterial contamination were eliminated from the final pooled data set per assay.

Furthermore, in our studies we investigated conservation in the DAF-16 downstream regulon (target genes) by surveying the three available *Caenorhabditis* genomes (*C. elegans, C. briggsae* and *C. remanei*) for genes containing the known consensus sites for DAF-16. Based on orthologous sets of genes containing the consensus sites, we asked whether certain biological processes are more prevalent in one species than in others (divergent targets) and which processes are shared between all three species. We also tested for adaptive sequence evolution along the IIS pathway in these species.

3.2 Lifespan assay of representative *Caenorhabditis* species

Much work has gone into exploring the life-history traits (LHTs) of *C. elegans*. Among these reports the majority include studies on ageing, looking at traits such as age-specific fecundity, total reproduction, generation time, age-specific mortality, mean or maximum lifespan, and growth rate. A few studies have also reported findings in other *Caenorhabditis* species, noting significant differences from the workhorse of nematode/ageing community, *C. elegans* (Berg et al., 2006, McCulloch and Gems, 2003).

In this study we examined differences in lifespan among four *Caenorhabditis* species that included *C. elegans* (N2), *C. briggsae* (AF16), *C. remanei* (EM464) and *C. brenneri* (CB5161). These species represented both hermaphroditic (self-fertilizing) and gonochoristic (male-female) species of the *Caenorhabditis* clade. Experimentally, since different laboratory isolates of the same strain are known to exhibit variation in lifespan (Patel et al., 2002), we conducted parallel longevity assays on our isolates on NGM plates with OP50 as a food source.

From our results (Figure 14) it was striking that the hermaphroditic and the gonochoristic species fell into two groups. We observed, as previously reported (McCulloch and Gems, 2003), that the two gonochoristic species (*C. remanei* and *C. brenneri*) exhibited a significantly ($p<0.0001$; see Table 9 for all p-values) longer lifespan than both the hermaphroditic species (*C. elegans* and *C. briggsae*).

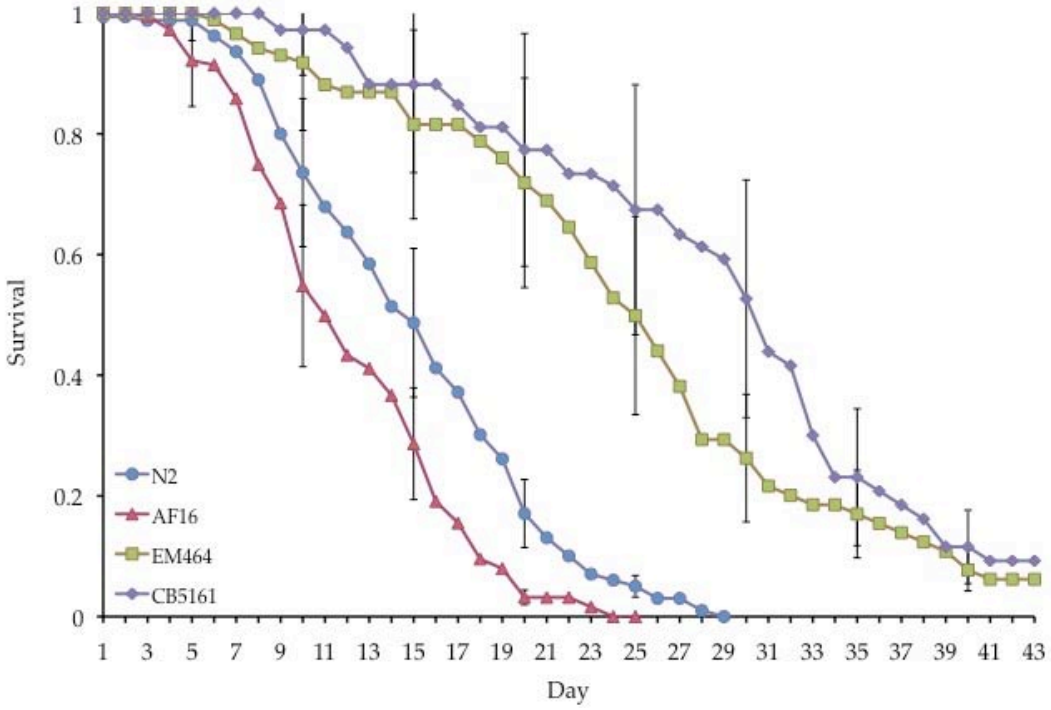

Figure 14 – Lifespan analysis of hermaphrodite (N2, AF16) or female (EM464, CB5161) animals at the fourth larval stage (L4) monitored for survival over fifty days. Whilst hermaphrodite animals showed 100% lethality over this period, survival is significantly higher for both gonochoristic species ($p < 0.0001$), with more than 50% of animals surviving longer than twenty days. Bars every 5 days indicate 95% confidence intervals.

Species compared	Lifespan - p value
N2/AF16	<0.0001
N2/EM464	<0.0001
N2/CB5161	<0.0001
AF16/EM464	<0.0001
AF16/CB5161	<0.0001
EM464/CB5161	<0.1

Table 9 - Table of p values comparing all of the type strains for the lifespan assay. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

3.2.1 Lifespan assay of *Caenorhabditis* Wild type isolates

Our lifespan analysis of the representative species clearly demonstrated variable lifespans among the closely related species in the same *Caenorhabditis* clade. However, to rule out the possibility that this observed phenotype was not a one off event among these species, we tested for lifespan among several wildtype isolates (as listed in Table 2) for each of the four type species.

This additional testing confirmed that this trend was highly conserved across multiple wild-type isolates of each species (Figure 15, Table 10 for p-values). The testing of several hermaphroditic wild-type isolates also ruled out the possibility that our observations were due to the fixation of novel mutations under the force of genetic drift in our laboratory *Caenorhabditis* lines.

Species Compared	Lifespan
N2/N2	-
N2/RC301	>0.2
N2/CB4856	>0.2
N2/ED3033	>0.2
N2/ED3034	>0.2
N2/JU1082	<0.0001
N2/JU1084	<0.0001
N2/SB129	<0.0001
N2/LKC28	<0.0001

Table 10 - Table of p values comparing all of the wild type isolates for the lifespan assay. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

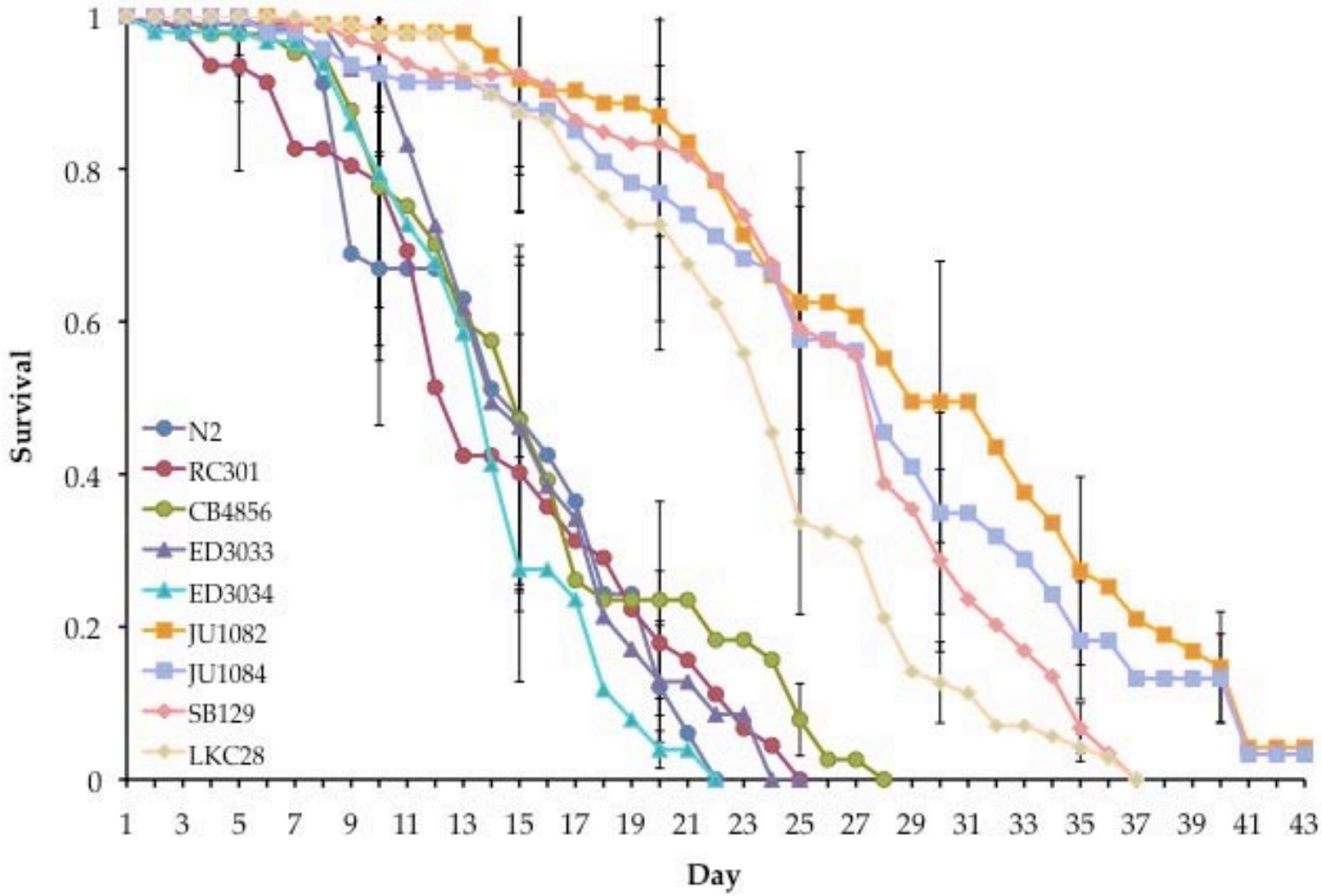

Figure 15 - Lifespan analysis of two wild isolates for each of the type strains of the *Caenorhabditis* species (listed in table 2). The observed trend is similar to that of the type strains with the hermaphroditic animals showing 100% lethality in 28 days. Bars every 5 days indicate 95% confidence intervals.

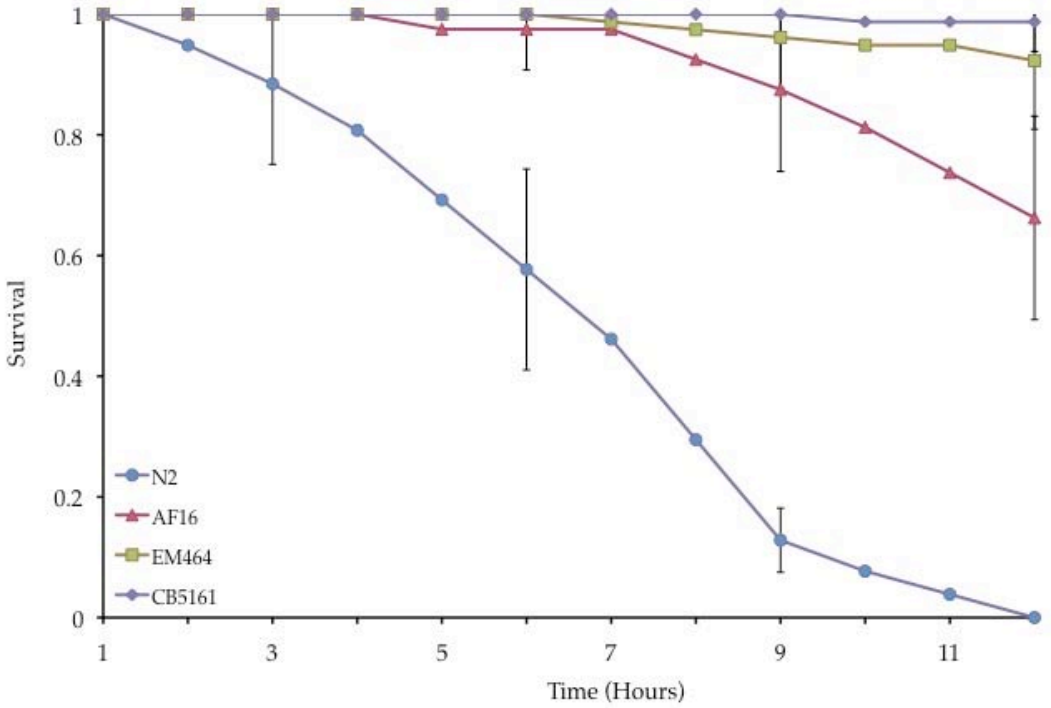

Figure 16 - Monitoring of survival of hermaphrodite (N2, AF16) or female (EM464, CB5161) animals at the fourth larval stage (L4) under conditions of heat stress (37°C). Gonochoristic species show improved survival relative to *C. briggsae* and, in particular, *C. elegans*. Bars every 3 hours indicate 95% confidence intervals.

Species Compared	Thermotolerance
N2/AF16	<0.0001
N2/EM464	<0.0001
N2/CB5161	<0.0001
AF16/EM464	<0.0001
AF16/CB5161	<0.0001
EM464/CB5161	<0.1

Table 11 - Table of p values comparing all of the type strains for the heat stress (37°C) assay. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

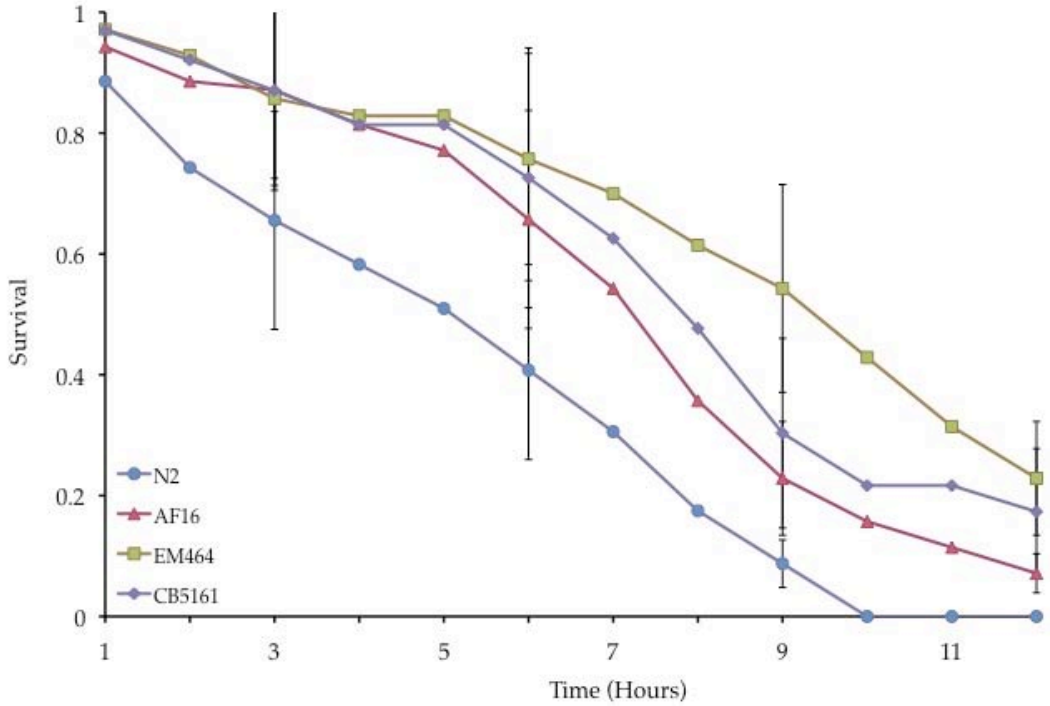

Figure 17 – Monitoring of survival of hermaphrodite (N2, AF16) or female (EM464, CB5161) animals at the fourth larval stage (L4) when exposed to heavy metal (7mM $CuCl_2$). A similar survival trend to that seen when exposed to thermal stress was observed. Bars every 3 hours indicate 95% confidence intervals.

Species Compared	Metallotolerance
N2/AF16	<0.001
N2/EM464	<0.0001
N2/CB5161	<0.0001
AF16/EM464	<0.01
AF16/CB5161	>0.2
EM464/CB5161	>0.2

Table 12 - Table of p values comparing all of the type strains for resistance against heavy metal (7mM $CuCl_2$) stress. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

3.3.1 Survival analysis of wild type *Caenorhabditis* isolates to abiotic stress

To further confirm our observations we also tested for resistance to abiotic stress among wildtype isolates for the species. We subjected the nematodes to heavy metal stress and monitored survival on an hourly basis. Our results (Figure 18, Table 13 for p – values) recapitulated the trend observed with the type isolates, although it should be noted that the smaller sample sizes (number of worms per assay) and time constraints meant that not all comparisons show statistical significance as seen for the other assays with the exception of the survival of CB4856, JU1082 and JU1084.

Species compared	Metallotolerance
N2/N2	-
N2/RC301	>0.2
N2/CB4856	<0.0001
N2/ED3033	>0.2
N2/ED3034	>0.2
N2/JU1082	<0.01
N2/JU1084	<0.0001
N2/SB129	>0.1
N2/LKC28	>0.2

Table 13 - Table of p values comparing all of the wildtype strains for resistance against heavy metal (7mM $CuCl_2$) stress . Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

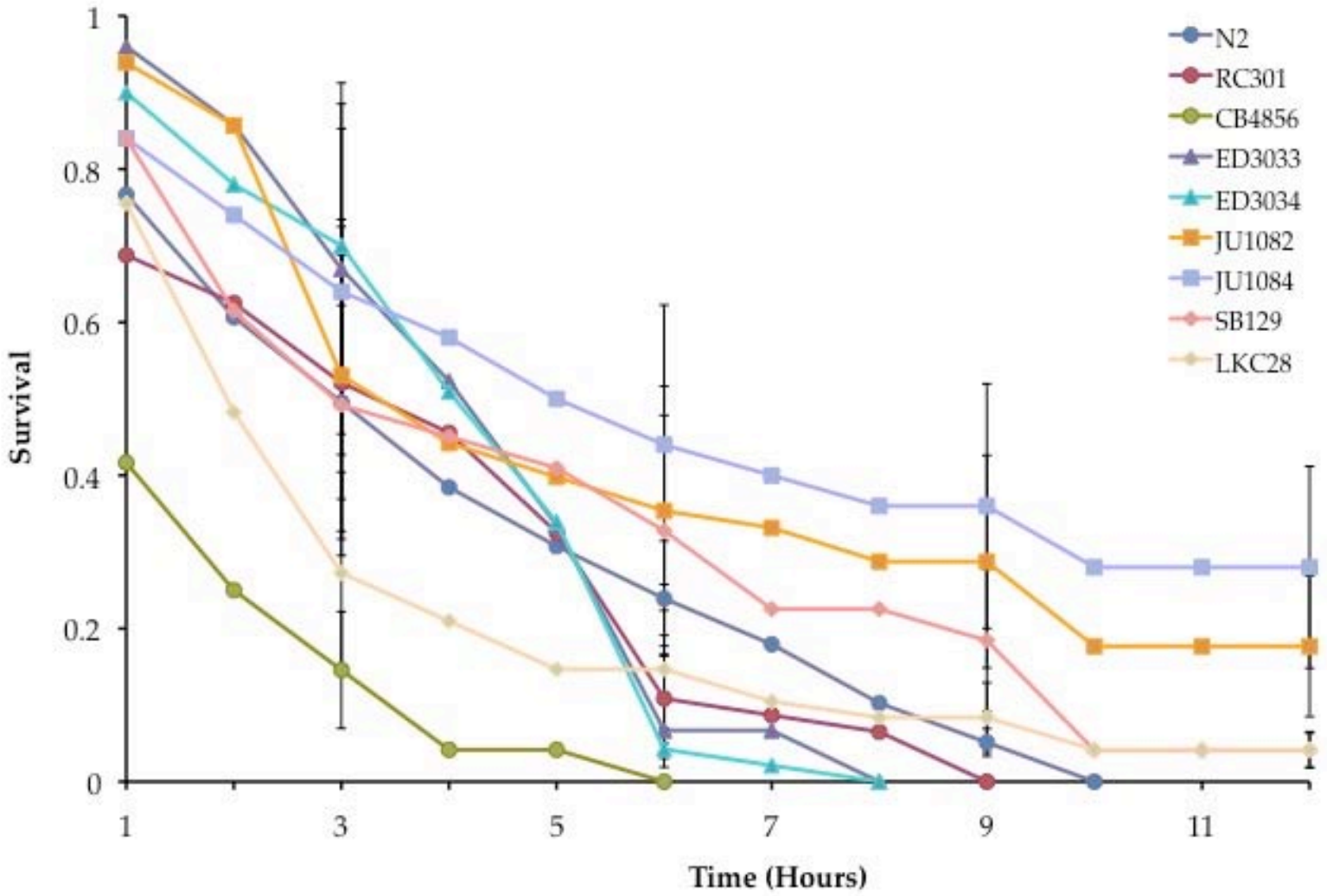

Figure 18 – Survival plot of two wild isolates for each of the tested *Caenorhabditis* species (listed in table 2) when exposed to heavy metal stress. The observed trend is similar to that shown by the type strains, with the hermaphroditic animals showing 100% lethality. Bars every 3 hours indicate 95% confidence intervals.

3.4 Survival analysis of type *Caenorhabditis* species to biotic stress

C. elegans has been used extensively as a model to study pathogenesis of numerous human pathogens (Gallagher and Manoil, 2001, Joshua et al., 2003, Kurz et al., 2003, Sifri et al., 2003, Aballay et al., 2000, Labrousse et al., 2000, Mylonakis et al., 2004).

Studies by Garsin et al., in 2003 highlighted the protective role of DAF-16 in activating host defence genes that confer enhanced stress resistance during a pathological infection (Garsin et al., 2003). Based on this observation and our own findings, we predicted the existence of a correlative DAF-16 influenced phenotypic pattern across species. To test this, we assessed survival of the four *Caenorhabditis* species following exposure to a range of pathogens. All these assays were carried out at 25℃. The effect of change in temperature as a determinant of phenotypic response can be ruled out as we and others have previously shown that worms on OP50 had a longer lifespan than what we observed when grown on pathogenic bacteria (Aballay et al., 2000).

Our results demonstrated that the type strains of the two long-lived, gonochoristic species (EM464 and CB5161) showed significantly higher resistance to the Gram-negative bacterium *Pseudomonas aeruginosa* (Table 14 for p – values) and the Gram-positive bacterium *Staphylococcus aureus* (Figure 20, Table 15 for p – values) than type strains of the two hermaphroditic species (AF16 and N2). This finding was in accordance with previous observations reported using the fungus *Cryptococcus neoformans* (Berg et al., 2006) .

However, although significant, the magnitude of the difference between all four species was smaller when tested for sensitivity to the Gram-negative pathogen *S. enterica* Typhimurium (Figure 21, Table 16 for p – values). This result may be due to the persistent infection caused by this bacterium (in contrast to the transient infection caused by *P. aeruginosa* and *S. aureus*), suggesting that DAF-16 coupled responses

may be important for dealing with transient infections but not very efficient towards mediating resistance to colonizing pathogens.

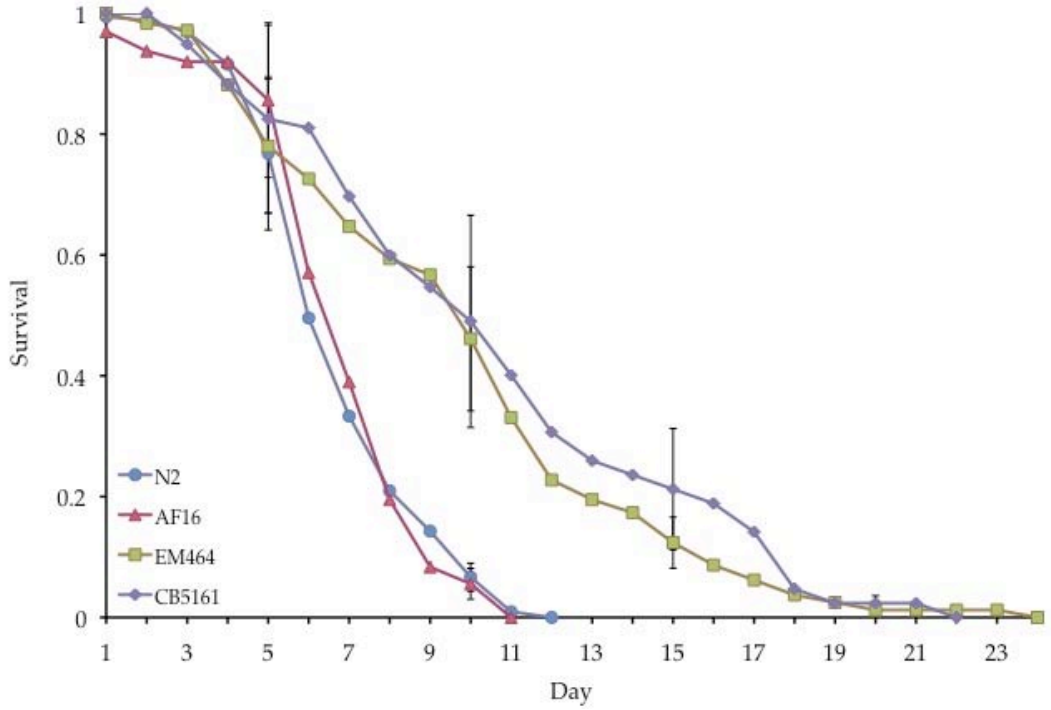

Figure 19 - Monitoring of survival of hermaphrodite (N2, AF16) or female (EM464, CB5161) animals at the fourth larval stage (L4) when grown on a diet of pathogenic bacterium, *Pseudomonas aeruginosa*. *C. remanei* and *C. brenneri* exhibit significantly higher resistance to the pathogen. Bars every 5 days indicate 95% confidence intervals.

Species compared	PA01
N2/AF16	>0.2
N2/EM464	<0.0001
N2/CB5161	<0.0001
AF16/EM464	<0.0001
AF16/CB5161	<0.0001
EM464/CB5161	>0.2

Table 14 - Table of p values comparing all of the type strains for resistance to the ubiquitous Gram-negative bacterium *Pseudomonas aeruginosa*. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

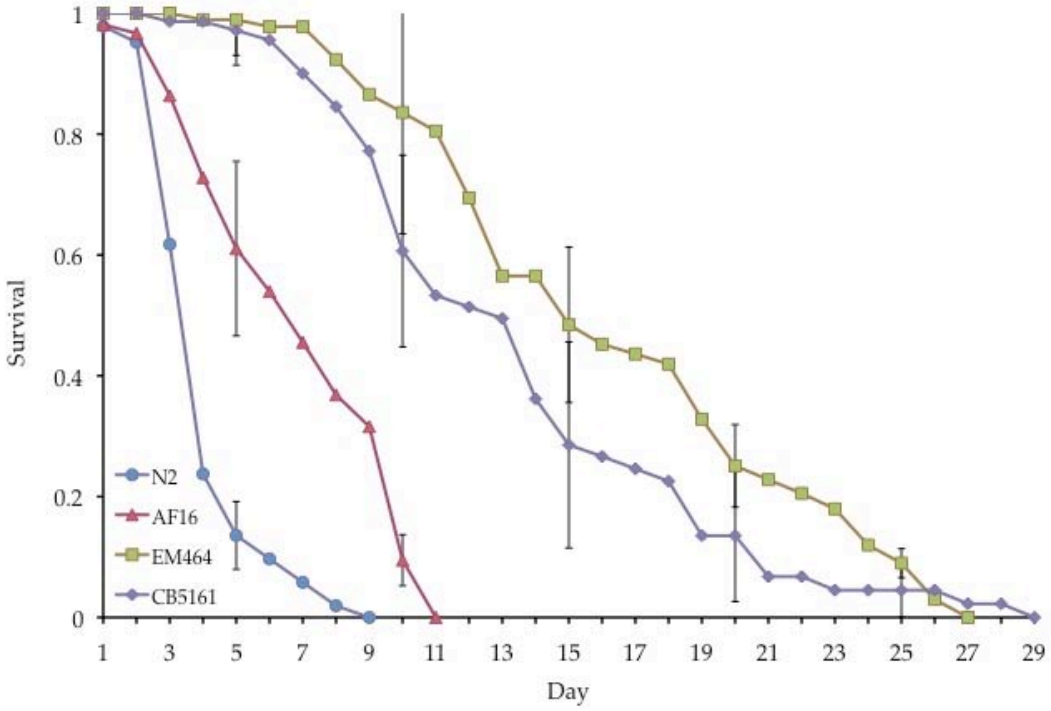

Figure 20 - Monitoring of survival of hermaphrodite (N2, AF16) or female (EM464, CB5161) animals at the fourth larval stage (L4) when exposed to the Gram positive pathogen *Staphylococcus aureus*. *C. remanei* and *C. brenneri* exhibit significantly higher resistance to the pathogen. Bars every 5 days indicate 95% confidence intervals.

Species compared	NCTC8532
N2/AF16	<0.0001
N2/EM464	<0.0001
N2/CB5161	<0.0001
AF16/EM464	<0.0001
AF16/CB5161	<0.0001
EM464/CB5161	<0.05

Table 15 - Table of p values comparing all of the type strains for resistance to the Gram-positive bacterium *Staphylococcus aureus*. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

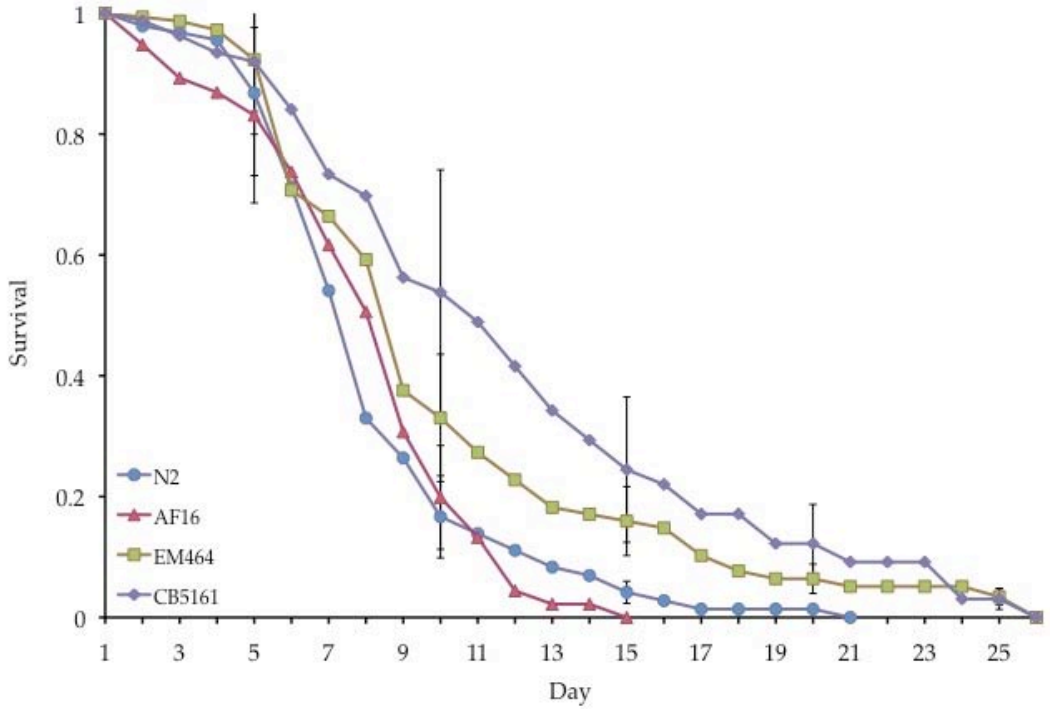

Figure 21 - Monitoring of survival of hermaphrodite (N2, AF16) or female (EM464, CB5161) animals at the fourth larval stage (L4) when grown on a diet of pathogenic bacterium, *S. enterica* Typhimurium. *C. remanei* and *C. brenneri* exhibit significantly higher resistance to the pathogen.

Species compared	SL1344
N2/AF16	>0.2
N2/EM464	<0.01
N2/CB5161	<0.001
AF16/EM464	<0.02
AF16/CB5161	<0.001
EM464/CB5161	>0.1

Table 16 - Table of p values comparing all of the type strains for resistance to ubiquitous Gram-negative bacterium *S. enterica* Typhimurium. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

3.4.1 Survival analysis of wild type *Caenorhabditis* isolates to biotic stress

To ensure that these differences were not isolate dependent, we tested multiple additional isolates of each species for resistance to *Staphylococcus aureus*. In all cases, gonochoristic isolates exhibit substantially higher resistance to killing by this pathogen some, but not all, pathogens can be attributed to higher DAF-16 levels.

Species compared	NCTC8532
N2/N2	-
N2/RC301	>0.2
N2/CB4856	<0.001
N2/ED3033	<0.0001
N2/ED3034	<0.0001
N2/JU1082	<0.0001
N2/JU1084	<0.0001
N2/SB129	<0.0001
N2/LKC28	<0.0001

Table 17 - Table of p values comparing all of the wildtype strains for resistance against Gram-positive bacterium *Staphylococcus aureus*. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction

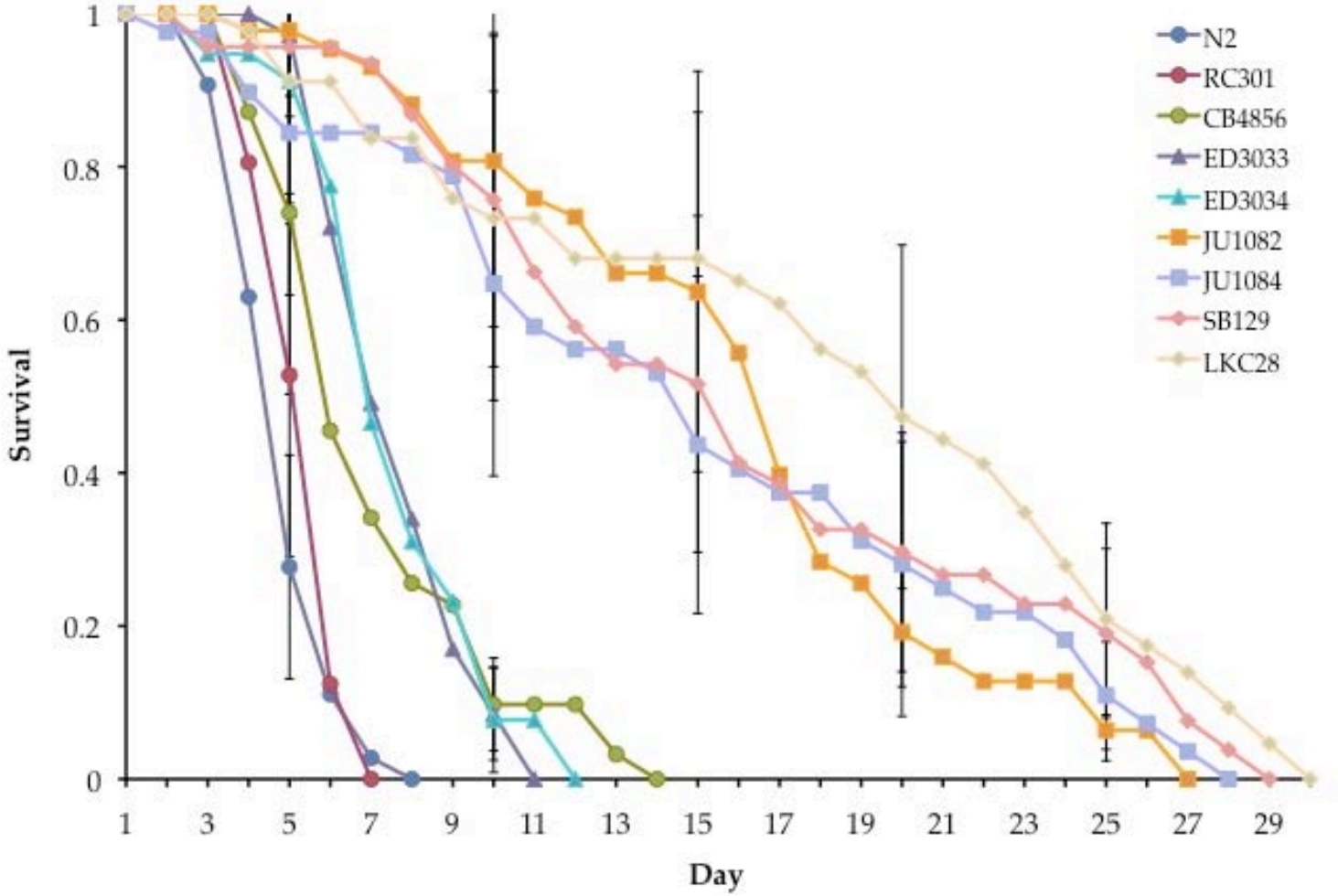

Figure 22 - Survival plot of two wild isolates for each of the tested *Caenorhabditis* species (listed in table 2) when grown on Gram positive bacterium, *Staphylococcus aureus.* The observed trend is similar to the type strains with the hermaphroditic animals showing 100% lethality before day 15. Bars every 5 days indicate 95% confidence intervals.

3.5 Survival analysis of *C.elegans* feminizing mutant under biotic stress

Since progeny production and the consequent risk of matricidal killing by bagging (a phenomenon where the eggs hatch internally with subsequent emergence of active larvae from the parent, resulting in the parents death) has previously been shown to shorten *C. elegans* lifespan, particularly when exposed to pathogens, (Sifri et al., 2003, O'Quinn et al., 2001, Aballay et al., 2000) we considered the possibility that the enhanced survival of gonochoristic species may result from the absence of matricidal killing. To test this, we exposed *C. elegans* fem-1(hc17) BA17 animals which are XX female worms (which if virgin were not fertile) to the pathogenic bacteria *Staphylococcus aureus.* As previously reported, feminised *C. elegans* exhibited improved survival under pathogenic conditions (Figure 23), but this increase is far smaller than that seen in the higher *daf-16* producing gonochoristic species on *Staphylococcus aureus.* Thus the enhanced survival of gonochoristic species is not attributable to the lack of progeny production.

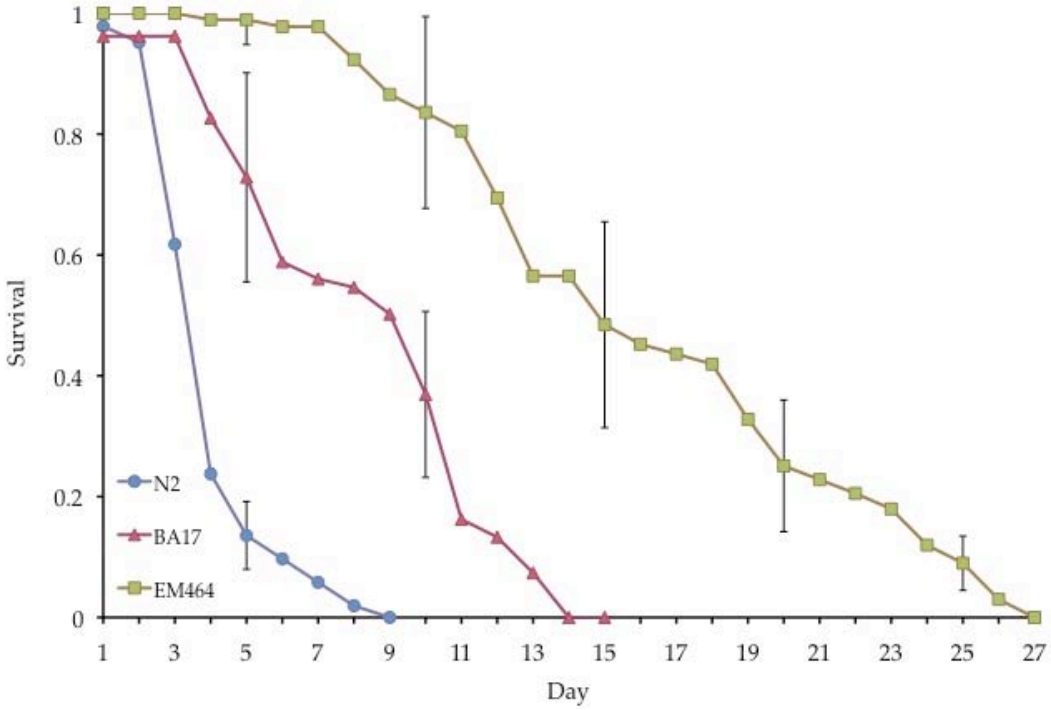

Figure 23 - *C.elegans* feminised mutant (BA17) animals and the gonochoristic (EM464) animals are significantly (<0.0001) longer lived than the hermaphrodites (N2). However the significance is greater in the case of the gonochoristic (EM464) species. Bars every 5 days indicate 95% confidence intervals.

3.6 Comparative analysis of the DAF-16 regulon in *C. elegans, C. briggsae* and *C. remanei* [*]

The IIS pathway is highly conserved with its key molecule, DAF-16 driving a plethora of phenotypes. Since our phenotypic analysis among closely related *Caenorhabditis* nematodes revealed variances in DAF-16 determined phenotypes, we considered the possibility that the DAF-16 pathway itself may have become modified during the diversification of the *Caenorhabditis* nematodes. Calculating Ka/Ks ratios for all of the components in the IIS signalling pathway (*daf-2*, *age-1*, *pdk-1*, *akt-1*, and *daf-16*) between *C. elegans*, *C. briggsae* and *C. remanei* we looked for evidence of positive selection in any of the genes, but found none (Table 18).

The presence of positive selection is defined by a Ka/Ks ratio >1. Purifying selection is indicated by Ka/Ks ratio values ≈ 0 and neutral selection by Ka/Ks ratios =1. In general all Ka/Ks rate ratios are ≈ 0, which indicates purifying selection. These findings agree very well with the high evolutionary conservation of all factors studied across different species. For all members of the *daf-2*/*daf-16* cascade, amino acid changes are disfavoured by selection.

This seems to be particularly relevant for the upstream receptor *daf-2*. The very low rate observed in all three species indicates the importance of a functional *daf-2* gene. Since *daf-2* is involved in many other biological processes any mutational changes are very likely to be detrimental for the mutation-carrying organism. As for the evolution of *daf-16*, it is characterized by less strict purifying selection because of the general higher Ka/Ks values. However, since the value is still clearly below 1, it may reflect purifying selection as well.

[*] Work in this section was carried out by Dr Claudia Boehnisch

Our studies have given us a very interesting insight about the rates of evolution of the IIS pathway between the species studied or within the IIS pathway itself (Table 18). Could these differences reflect upon the different selection pressures, varied life styles, and hence some of the differences in longevity and stress resistance? In order to answer these questions a different phylogenetic approach has to be chosen which was not the purpose of this study.

Since the IIS pathway did not seem to have undergone any major modification through evolutionary time in these species, we went on to investigate whether the downstream targets of DAF-16 differed between the three sequenced nematode species (*C. elegans*, *C. briggsae* and *C. remanei*).

Our analysis, looking for the presence of perfectly matched DAF-16 canonical consensus sites (ttatttac/gtaaataa, ttgtttac/gtaaacaa) in the 3kb upstream of every predicted gene in *C. elegans, C. briggsae* and *C. remanei,* revealed the following:

- In *C. elegans* our approach yielded 6,293 genes (31.2% of the genome) containing either one or both of the known sites in their 3kb upstream region
- In *C. briggsae* we identified 5,150 genes (23.4% of the genome) containing at least one of the consensus sites
- As for *C. remanei,* there were 8,456 genes (26.7%) containing at least one of the consensus sites

We analyzed our data splitting up our results on the following basis:

- A species-specific DAF-16-regulon, consisting of orthologs that contain the consensus site in only a single species.
- A species-shared DAF-16 regulon, consisting of orthologous genes that contain the consensus site in two of the species.

Genes	*C. briggsae* vs. *C. remanei* KA/KS	*C. elegans* vs. *C. briggsae* KA/KS	*C. elegans* vs. *C. remanei* KA/KS
age-1	0.0955	0.0717	0.0661
akt-1	0.0636	0.0437	0.0459
daf-2	0.0926	0.0081	0.0066
daf-16	0.2391	0.1613	0.1616
pdk-1	0.0829	0.0829	0.0472

KA non-synonymous substitutions

KS synonymous substitutions

Table 18 – Adaptive sequence evolution. Ka/Ks ratios for components of the IIS pathway among type *Caenorhabditis* species

- A core-DAF-16 regulon, consisting of orthologs that contain the consensus site in all three species.

Clearly the short length and relative variability of the DAF-16 consensus sequence means that this approach inherently overestimates the number of DAF-16 binding sites in the genome. However, given the absence of experimental techniques (such as chromatin immunoprecipitation) in the non- *C. elegans* species, such a bioinformatic approach is, at present, the only way of obtaining an approximate estimate of genome-wide differences in the IIS pathway within this group of organisms.

Based on this analysis, the number of orthologous genes that contain perfect matches to the DAF-16 consensus binding sites appeared similar between *C. elegans* and *C. briggsae* (1900 genes), *C. elegans* and *C. remanei* (2165 genes) and *C. briggsae* and *C. remanei* (2111 genes). However, although *C. elegans, C. briggsae* and *C. remanei* have 13,015 genes in common (64.4% of the *C. elegans* genome) only 913 of these contain the DAF-16 binding elements in all three species, a group that we define as the core DAF-16 regulon (Figure 24).

Next, based on our gene sets we sought to ask whether the core DAF-16 regulon and the species-specific DAF-16 regulons differ in the type of genes they contain by testing whether particular gene ontology (GO) terms (using GOTERM BP_ALL and GOTERM BP_2) are overrepresented (Appendix file 1 and 2). For this we used the online tool DAVID. Using DAVID analysis we ran our perfect match gene lists (input list) against the background gene list, which was in this case the *C. elegans* genome. This genome has been annotated and its genes have been put into different categories based on numerous criteria such as biological function, molecular function, chromosome etc. The biological functions are contained in the category GOTERM_BP_ALL. GOTERM BP_ALL consists of subcategories such as GOTERM_BP_2, which in turn contains categories such as immunity, aging, longevity etc. It is known how many genes are contained in which category. The enrichment analysis uses the Fisher Exact test to compares the likelihood of the presence of genes in each category or subcategory by random chances of a given

gene list. It calculates whether the number of genes of the input list for each category investigated is more than a random chance when compared to the number of genes per category of the background list. In our case we were interested whether genes which were identified as containing the perfect match for the DAF-16 binding motif were significantly enriched in categories such as immunity, stress resistance, longevity etc., or in other words in the categories which are known to contain DAF-16 mediated biological processes.

Therefore the null hypothesis in our case would be that there is no difference between the proportional distributions of GO terms in our list verses the distribution in the genome. This hypothesis is the same for all the three species and can be rejected since our studies show an over representation of gene groups.

Amongst others, genes involved in lifespan regulation, immune response and responses to chemical stimuli (including detoxification and stress response) were enriched in the DAF-16 core regulon (Appendix 1 and 2). For the species-specific regulons we observed that:

- *C. elegans*-specific DAF-16 regulon showed overrepresentation of genes involved in immunity (11 genes) and stress responses (38 genes)
- *C. remanei*-specific DAF-16 regulon showed overrepresentation of genes involved in immunity (13 genes) and stress responses (14 genes)
- Intriguingly, these groups were not overrepresented in the *C. briggsae*-specific DAF-16 regulon

We did not look for any significant differences between the species, but we were just generally interested in the gene overlap between the species. We again point out, that this bioinformatic analysis should not be over-interpreted. It just represents a starting point for further investigations.

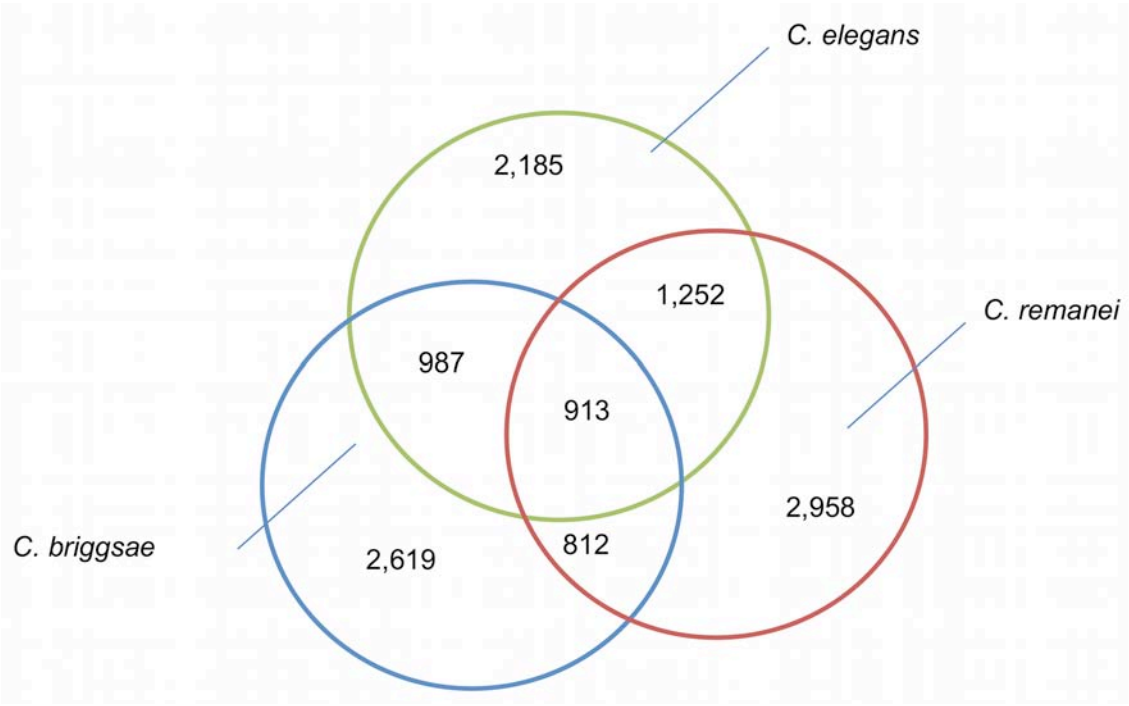

Figure 24 – Venn diagram illustrating the number of orthologous genes that contain perfect matches to the DAF-16 consensus binding sites between species.

Since false positives are a liability in such studies, we compared our *C. elegans* dataset to a gene list containing all putative DAF-16 targets recently identified in *C. elegans* via a range of other approaches by Oh (Oh et al., 2006), Murphy (Murphy et al., 2003), Halaschek-Wiener (Halaschek-Wiener et al., 2005a), Lee (Lee et al., 2003), McElwee (McElwee et al., 2007) and Dong (Dong et al., 2007). A total of 1,746 genes were identified as putative DAF-16 targets in at least one of these studies and 678 genes of these matched those identified by our approach, a group we refer to as the adjusted dataset. Of the 678 potential *C. elegans* DAF-16 target genes:

- 283 overlapped with the *C. brigssae* dataset
- 274 genes were found in common with the *C. remanei* list.
- Overall, the adjusted DAF-16 core regulon (genes found in all three species) contained 145 genes (Figure 25).

Partitioning the adjusted DAF-16 core regulon using the GOTERM BP_ALL and GOTERM BP_2 gene categories revealed significant enrichment for genes involved in the regulation of lifespan, stress response, transport, localization and metabolism (Figure 26 and Appendix 3). As expected the outcomes of the analyses of the unadjusted and the adjusted datasets differ slightly. However, the overall pattern is the same between the two approaches for both the core regulon as well as the species-specific regulon.

Finally, we compared the list of putative *C. elegans* DAF-16 targets identified by Oh and colleagues via a direct, chromatin immunoprecipitation (ChIP) approach (Oh et al., 2006) with those identified via microarray or bioinformatic approaches in the other studies (Murphy (Murphy et al., 2003), Halaschek-Wiener (Halaschek-Wiener et al., 2005a), Lee (Lee et al., 2003), McElwee (McElwee et al., 2007) and Dong (Dong et al., 2007)) or our own dataset (Table 19). Interestingly, there is very little

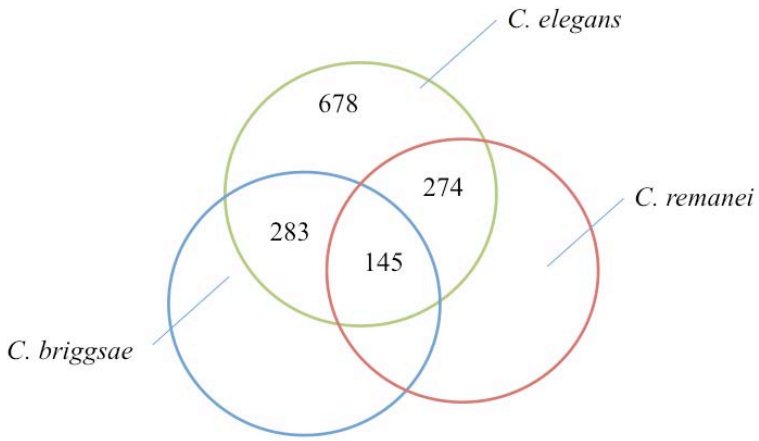

Figure 25 – Venn diagram representing fresh gene lists obtained comparing our data set with those previously published.

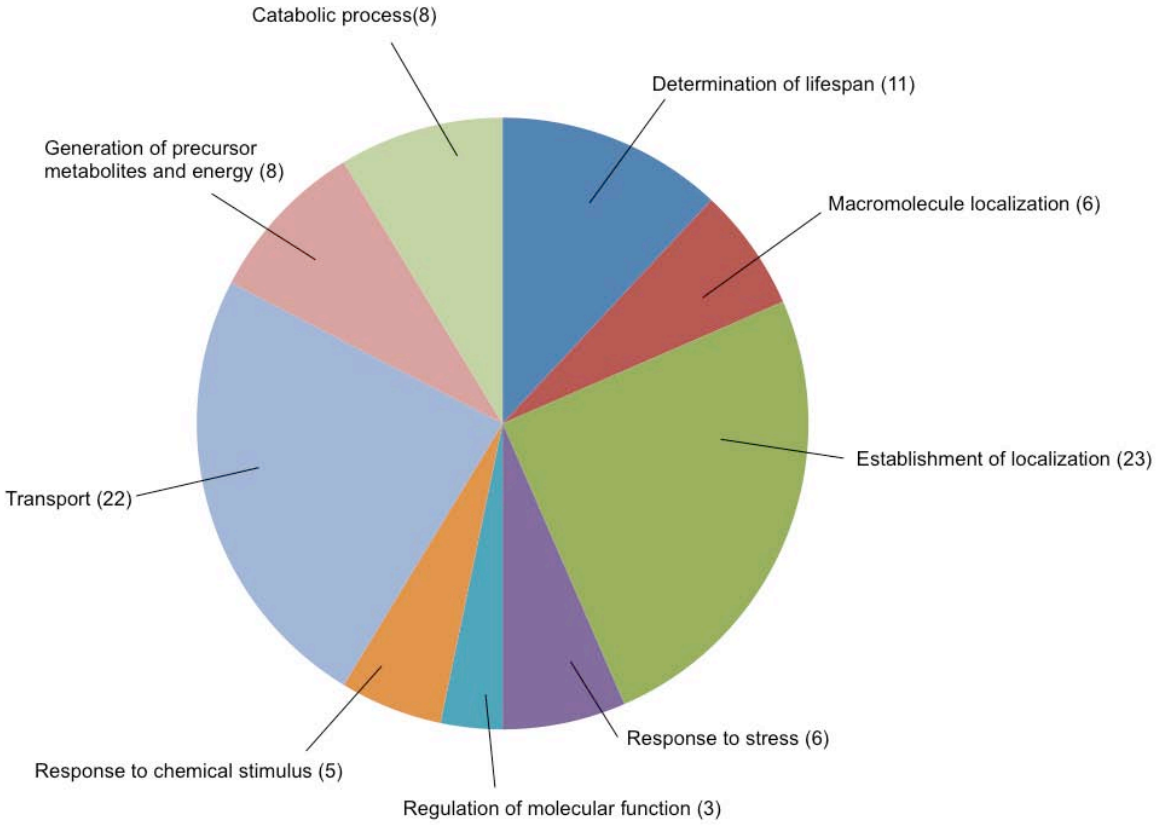

Figure 26 – Venn diagram representing the core DAF-16 regulon shared between all the *Caenorhabditis* species tested.

overlap between DAF-16 targets identified by ChIP and those inferred from microarray or bioinformatic analysis, with the exception of 11 genes shared between Oh et al and McElwee et al and 30 genes shared between Oh et al and our dataset. Thus there is likely to be considerable benefit in combining a range of experimental approaches in order to narrow down the list of true DAF-16 target genes.

	Lee (2003)	Murphy (2003)	Halaschek-Wiener (2005)	McElwee (2007)	Dong (2007)	This study
Number of genes overlapping with Oh (2005) 103 genes	1/81	2/473	1/317	11/953	1/93	30/6293

Table 19 - This table shows the overlap between the reference dataset of Oh and other datasets obtained by Murphy, Halaschek-Wiener, Lee, McElwee and Dong and the *C. elegans* gene list of this study. The first number gives the number of genes that are shared between Oh and the dataset of comparison. The second number stands for the total number of genes identified as potential downstream targets of DAF-16 in the corresponding study.

3.7 Discussion

Based on phenotypic analysis of traits pertaining to longevity, stress response and immune response among the *Caenorhabditis* species we are able to classify the clade (among species studied) into two distinguishable groups. Hermaphrodites, which are considered to have evolved from the gonochoristic species, were observed to group together displaying co-varied phenotypic traits such as a shorter lifespan, lesser stress and tolerance to infection contrary to that observed among the gonochoristic species.

The life history traits studied in this chapter have been previously probed to great detail in *C. elegans* and the role of DAF-16, a critical gene regulator that controls the transcription of hundreds of genes, has been proven to be contributing towards this (Murphy, 2006). Since this transcription factor is highly conserved and its homologues in other organisms have been shown to perform similar functions (Holzenberger et al., 2003), it is very likely that similar phenotypes can be expected at least among the sister *Caenorhabditis* species. However, it is very interesting that we observed significant variance of potential DAF-16 determined fates among the tested *Caenorhabditis* species despite their close evolutionary relationship. This raises the question of how such DAF-16 mediated phenotypes have changed through evolutionary time.

There are extrinsic and intrinsic factors that drive and chisel evolution at an optimum rate to maximize reproductive success. Here we show covariance of three DAF-16 mediated phenotypes, longevity, immunity and stress response, across the *Caenorhabditis* genus. The fact that this pattern is seen in multiple isolates of each species suggests that this co-variance may reflect their need to search for a partner to mate. This lifestyle means that for the gonochoristic species in their endeavour to find partners, the chance of encountering stressful conditions (eg. pathogens, high temperature) increases and is likely to favour the evolution of a longer lifespan in order to increase mating opportunities. In addition, since we know very little about the natural ecology of the *Caenorhabditis* nematodes (Kiontke and Sudhaus, 2006), it is

possible that differences in the niches inhabited by these species may impose extrinsic stresses that have led to the evolution of improved stress tolerance via the over-expression of DAF-16.

It is interesting to note that susceptibility to several pathogens correlates with other DAF-16 mediated effects, with the exception of the Gram-negative bacterium *S. enterica* Typhimurium, which shows similar lethality in all four species and the two *daf-2* mutants. Interestingly, *S. enterica* Typhimurium is one of the few human pathogens thus far shown to establish a truly persistent infection in the worm due to its resistance towards antimicrobial peptides (Alegado and Tan, 2008, Aballay et al., 2000). Thus, this is suggestive of the fact that the (*daf-16* mediated) genes driving resistance to infection do not work effectively in dealing with gut-colonising pathogens.

The insulin-like signalling pathway contributes to both innate immune responses and stress responses in *C. elegans*. Our data suggests that this may also hold true in closely related nematode species as well. In line with this, we show that the components of this pathway do not show evidence of adaptive sequence evolution during the diversification of these species. In contrast, however, the set of putative downstream targets controlled by DAF-16 show changes between these species. All three sequenced species share a core DAF-16 regulon comprised of genes functioning in longevity, stress response and other biological processes. However, whilst *C. elegans* and *C. remanei* contain a similar set of target types in their species-specific DAF-16 regulons, the species-specific DAF-16 regulon of *C. briggsae* comparatively lacks genes involved in immunity and stress response. Interestingly, in line with this finding, we observed that a *daf-2* mutant in *C. briggsae* is long-lived and resistant to abiotic stress, but only moderately resistant to killing by a range of pathogens.

The majority of enriched genes identified by our approach are associated with other biological processes such as metabolism, transport and other functions, in line with previous studies that have identified downstream targets of DAF-16 in *C. elegans* (Halaschek-Wiener et al., 2005a, McElwee et al., 2003, Murphy et al., 2003, McElwee

et al., 2007, McElwee et al., 2004, Oh et al., 2006). We note, however, that such bioinformatic analyses are susceptible to false positive (due to the chance occurrence of DAF-16 consensus sequences) and false negative (due to its reliance on perfect-match sequence motifs) errors. Indeed, depending on the approach used, others have estimated that up to 78% of *C. elegans* genes might be potential DAF-16 downstream targets (Murphy, 2006). As such, we would emphasize that our bioinformatic analysis is intended only as a guide for future experimental analyses once tools become available.

In conclusion, we demonstrate covariance of DAF-16 mediated phenotypes in the four most well-characterized species (both type and wild type isolates) of the *Caenorhabditis* clade.

Chapter IV

Real time analysis of *daf-16* expression

AMRIT, F. R., BOEHNISCH, C. M. & MAY, R. C. (2010) Phenotypic covariance of longevity, immunity and stress resistance in the *Caenorhabditis* nematodes. PLoS One, 5, e9978.

4.1 Introduction

The translation of a robust immune/stress defence system into a strategy for a longer lifespan is key to an organism's survival and consequently mechanisms that allow animals to efficiently fend off infections and handle stress are evolutionarily selected.

The innate immune system is one such evolutionarily conserved mechanism. Shared across phyla, this system mounts an immediate response to invading pathogens and plays a major role in coordinating immunity as a whole (Schulenburg et al., 2004).

This evolutionary conservation of the innate immune system has been exploited by science, allowing model systems, such as invertebrates, to provide parallels with higher vertebrates such as humans. Such studies using the nematode worm *C. elegans* have revealed that immunity to a large extent is under the transcriptional control of the evolutionarily conserved transcription factor known as DAF-16 (Gravato-Nobre and Hodgkin, 2005). This transcription factor has been shown to contribute towards stress resistance by regulating a myriad of genes (Murphy et al., 2003, Halaschek-Wiener et al., 2005b, Dong et al., 2007, Lee et al., 2003, Oh et al., 2006). In addition, *daf-16* is the closest candidate for a genuine 'ageing' gene, since studies pertaining to DAF-16 homologs in flies (dFOXO) and mammals (FOXO1, FOXO3a, FOXO4 and FOXO6) have exemplified the role of these transcription factors in determining several life history traits such as longevity, biotic and abiotic stress resistance (Hwangbo et al., 2004, Brunet et al., 2004, Clancy et al., 2001b).

Our phenotypic analyses, described in the previous chapter, have highlighted the covariance of phenotypes that include longevity, biotic and abiotic stress resistance among the species of the *Caenorhabditis* clade. Given this, the close relatedness of the *Caenorhabditis* species tested and the conserved function of *daf-16*, we wondered if the covariance we observed were driven by variable levels of expression of the homologues of this transcription factor.

In order to probe this we measured *daf-16* expression levels among the four *Caenorhabditis* type species at various stages of development looking for correlative expression.

4.2 *daf-16* expression levels among the mixed populations of the *Caenorhabditis* species

Given the evolutionary conservation and critical role played by DAF-16 in regulating lifespan and resistance to stress (biotic and abiotic) in *C. elegans*, we quantified *daf-16* mRNA levels in mixed populations (nematodes at various stages of development) of all four type *Caenorhabditis* species. *daf-16* expression levels were normalized to the expression of the commonly used reference gene *gpd-3* (Higashibata et al., 2006, Longman et al., 2007, Welker et al., 2007), encoding the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. This gene has been shown not to be one of the downstream targets of *daf-16* (Murphy et al., 2003) and hence can be used as a reference to study variation in *daf-16* expression. To further ensure that the control gene *gpd-3* is true to its expression and can be used a reference we tested another control gene β-actin and compared the relative *daf-16* expression levels of *C. elegans* and *C. briggsae* to these endogenous genes. In both cases (*gpd-3* and β-actin) the relative *daf-16* expression levels are identical (Figure 27) thereby justifying the use of *gpd-3* as an endogenous gene for all our assays. Initially prior to experimentation we optimized all the primers used in this study and ensured the efficiencies of our target genes and reference genes were approximately equal. To assess the efficacy of the primers and the sensitivity of the qPCR assay, 2-fold dilution series of the template DNA for all the species tested were prepared and subjected to qPCR amplification. The results obtained were extrapolated to produce standard curves by linear regression analysis between threshold cycle (Ct) and sample dilution that gave coefficients of determination (r^2) that exceeded 0.95 for all template/primer combinations (Table 20). Once amplification efficiencies of the target and the reference were determined to be approximately equal, RT PCRs were carried out for all the experimental conditions.

In our study looking at daf-16 expression levels in mixed populations of the four type *Caenorhabditis* species, *C. briggsae* showed *daf-16* levels similar to those in *C. elegans*. *daf-16* expression in the two gonochoristic species was between seven (*C.*

brenneri) and twelve (*C. remanei*) fold higher than *C. elegans* in the mixed populations (Figure 28).

Thus there appears to be a general trend, with lower *daf-16* expression in the hermaphroditic species (exhibiting weaker immune and stress responses and a shorter lifespan) and higher *daf-16* expression in gonochoristic species (exhibiting more robust immune and stress responses coupled with a longer lifespan).

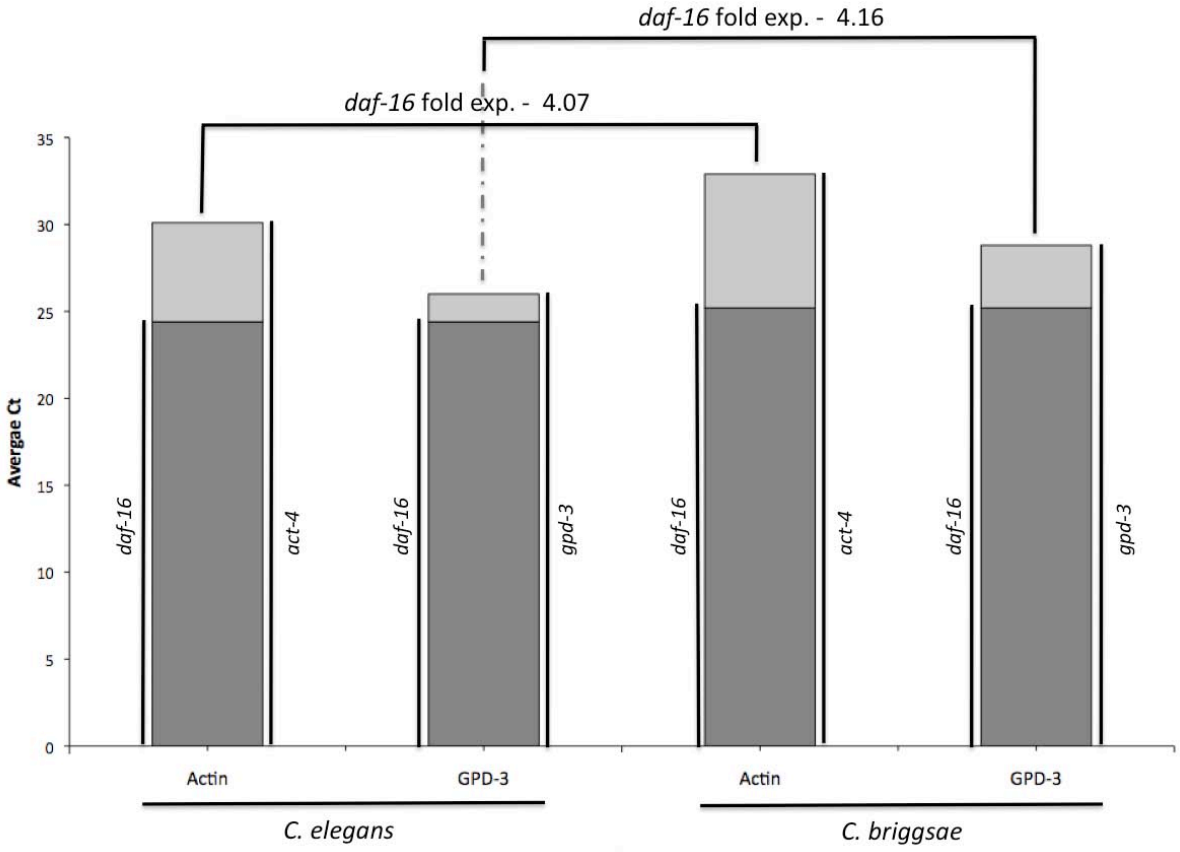

Figure 27 - Expression levels of *daf-16* (normalized to the reference gene *gpd-3 and* β-actin) among mixed populations (nematodes at various stages of development) of *C. elegans* and *C. briggsae.* Although the absolute value of average threshold cycle (Ct) between the species and genes vary the relative expression levels of *daf-16* relative to *gpd-3* and *act-4* is very similar. ·.

· Expression levels represent pooled data from technical triplicates but since this was to confirm the validity of the endogenous gene *gpd-3*, only a single biological replicate.

R2 Value	*daf-16*	*gpd-3*	18s RNA
C. elegans hermaphrodites	0.99036	0.99542	0.99565
C. elegans males	0.99952	0.99584	0.99501
C. briggsae hermaphrodites	0.93219	0.99008	-
C. brenneri males and hermaphrodites	0.99927	0.98738	-
C. remanei males and hermaphrodites	0.99813	0.98323	-

Table 20 - Table represents the coefficients of determination (r2) for all template/primer combinations. Once amplification efficiencies of the target and the reference genes were determined to be approximately equal, RT PCRs were carried out for all the experimental conditions.

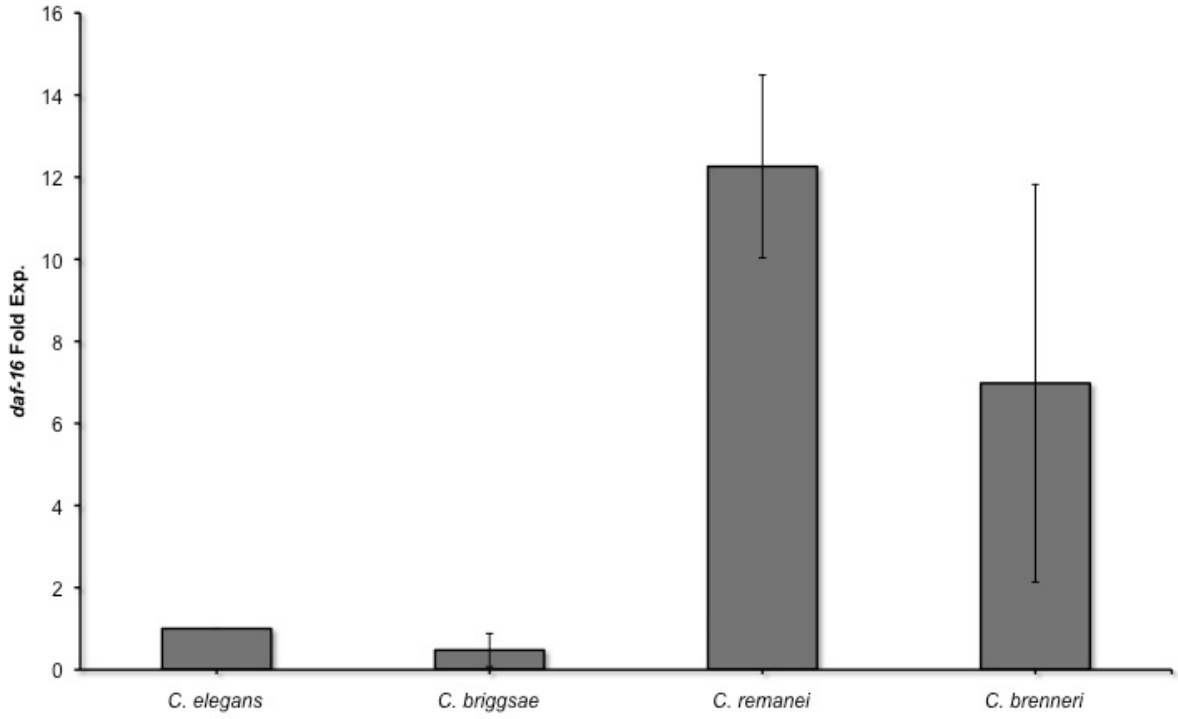

Figure 28 - Expression levels of *daf-16* (normalized to the reference gene *gpd-3*) among mixed populations (nematodes at various stages of development) of the type *Caenorhabditis* species. Data represent the mean of six experiments (biological replicates), error bars show standard deviation.

4.3 *daf-16* expression levels among the staged populations of the *Caenorhabditis* species

To establish whether our *daf-16* expression data shown before was dependent on gene expression at a particular life stage, we assessed *daf-16* expression levels between species of all strains of the four *Caenorhabditis* species when matching for developmental stage using synchronised populations (L2-L3, L4 and adult stages).

From our results we observed that a similar trend to that observed with mixed populations was maintained at the various stages of development. Higher daf-16 expression levels among the gonochoristic species was observed throughout development in the staged populations (Figure 29, Figure 30 and Figure 31) with the difference being most prominent in the L4 stage (Figure 30).

Thus higher levels of *daf-16* expression seem to positively correlate with our phenotypic analysis of stronger stress resistance (biotic and abiotic) and longer lifespan at all life stages.

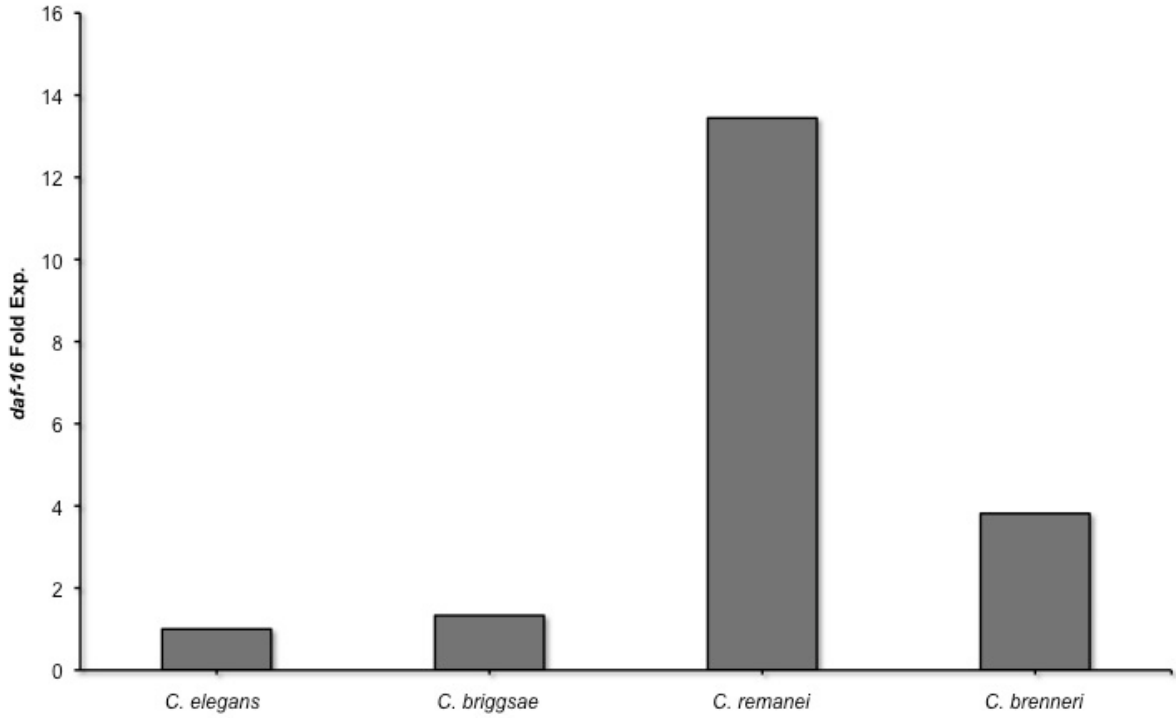

Figure 29 - Expression levels of *daf-16* (normalized to the reference gene *gpd-3*) among staged populations (nematodes from L2 and L3 stages of development) of the type *Caenorhabditis* species [*]

[*] Expression levels represent pooled data from technical triplicates but due to constraints of time, only a single biological replicate.

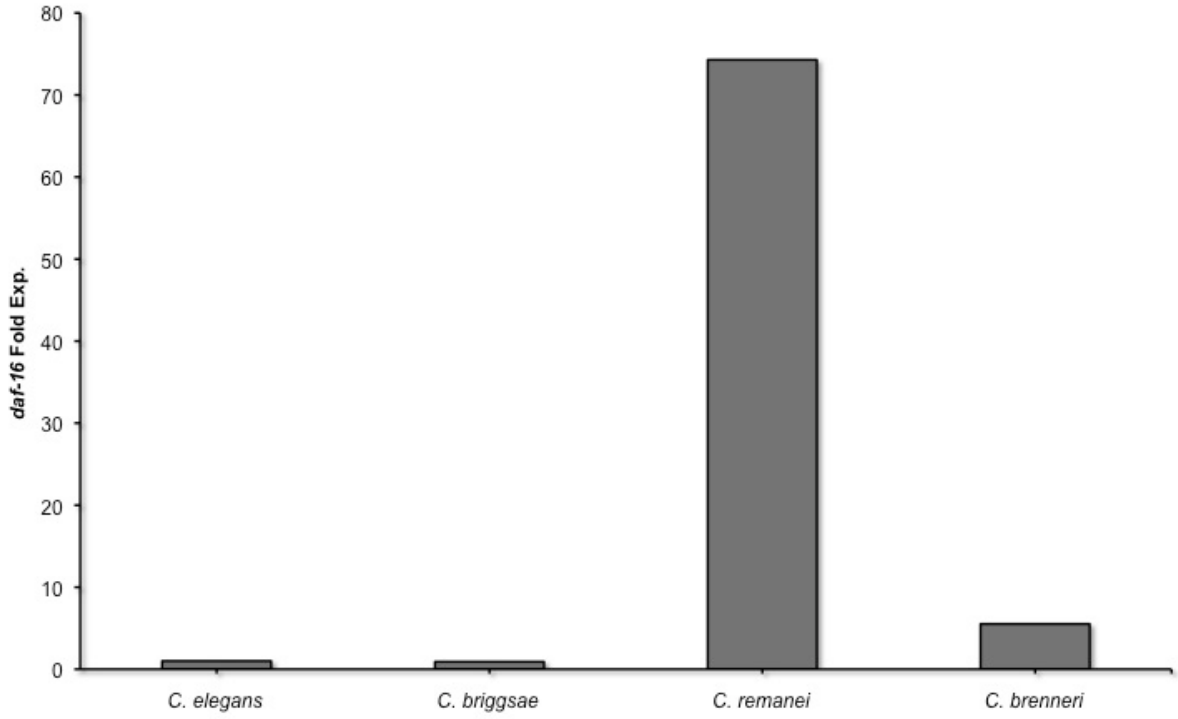

Figure 30 - Expression levels of *daf-16* (normalized to the reference gene *gpd-3*) *among* staged populations (nematodes at L4 stage of development) of the type *Caenorhabditis* species[*].

[*] Expression levels represent pooled data from technical triplicates but due to constraints of time, only a single biological replicate

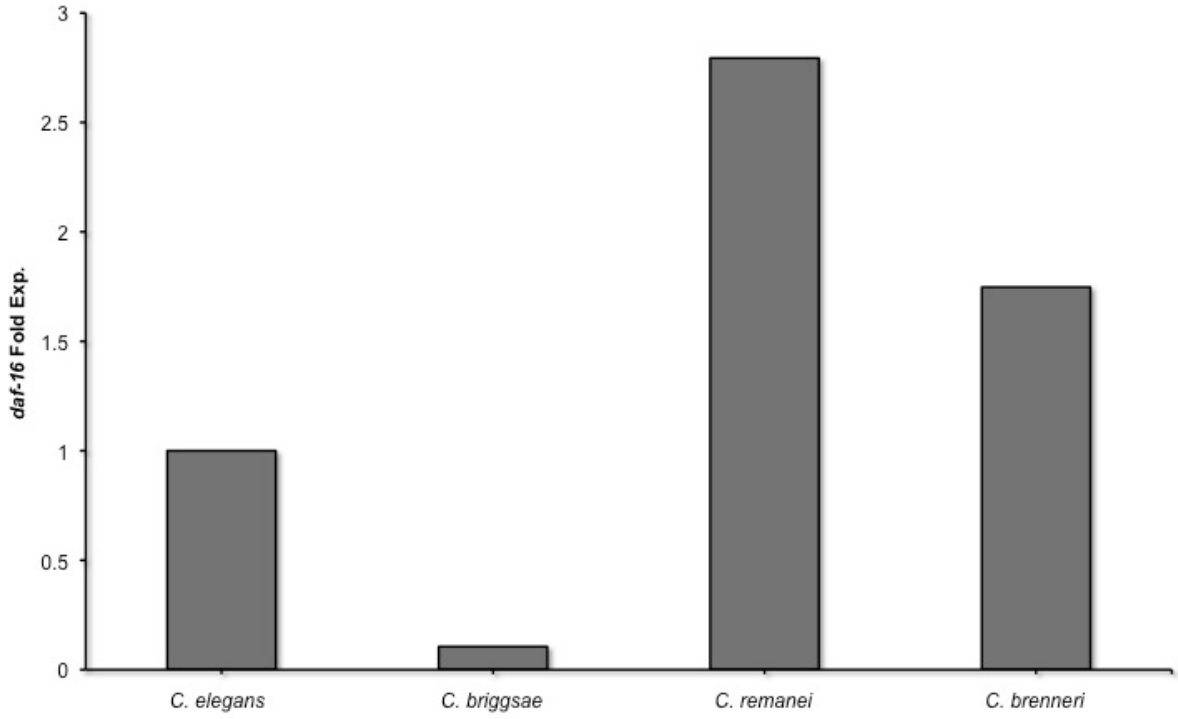

Figure 31 - Expression levels of *daf-16* (normalized to the reference gene *gpd-3*) *among* staged populations (nematodes at the adult stage of development) of the type *Caenorhabditis* species[*].

[*] Expression levels represent pooled data from technical triplicates but due to constraints of time, only a single biological replicate

4.4 *daf-16* expression levels among males from *Caenorhabditis elegans*

Lifespan of *C. elegans* has been shown to reduce by mating but not by gamete production (Gems and Riddle, 1996). Contrary to this our studies have demonstrated that species with obligate mating strategies (*C. remanei* and *C. brenneri*) have increased lifespan and *daf-16* expression relative to the hermaphroditic species (*C. elegans* and *C. briggsae*).

To confirm that our observations were not biased by the absence of males in the hermaphroditic stocks, we tested expression levels of *daf-16* in *C.elegans* males using two independent reference genes (*gpd-3*, as described above, and 18S rRNA, a component of the eukaryotic ribosome). From our results we found no significant transcriptional difference among *C.elegans* males in comparison to *C.elegans* hermaphrodites, indicating that the absence or presence of males in a population has no effect with regards to *daf-16* expression levels (Table 21). However, we do note that the increased *daf-16* expression that we observe in the gonochoristic species could be attributed to one or the other gender. But since earlier studies have demonstrated that both male and female animals in the gonochoristic species are longer lived than either gender of *C. elegans* or *C. briggsae* (van den Berg et al., 2006) it seems more likely that *daf-16* is highly expressed in both genders of the gonochoristic species.

Species	*daf-16* Expression level	Reference gene used
C. elegans hermaphrodites	1	*gpd-3*
C. elegans males	0.318147274	*gpd-3*
C. elegans hermaphrodites	1	18s
C. elegans males	0.909917931	18s
C. briggsae hermaphrodites	0.478474293	*gpd-3*
C. brenneri males and hermaphrodites	6.978255528	*gpd-3*
C. remanei males and hermaphrodites	12.25907791	*gpd-3*

Table 21 - Expression levels of *daf-16* in *C.elegans* males using two independent reference genes in comparison to all other species/genders.

4.5 Discussion

Increased *daf-16* dosage in *C. elegans* has been shown to have very minimal effects on lifespan (Lin et al., 2001). Despite the abundance of DAF-16 in the cells of these animals, DAF-16 activity is usually inhibited by retention of the transcription factor in the cytoplasm due to constitutive signalling through the IIS pathway (*daf-2)*. Why, then, does *daf-16* expression level appear to correlate with longevity at the species level? One possibility is that a higher *daf-16* expression could mean that under conditions of stress the IIS pathway has a bigger reservoir of proteins to activate, hence mounting a stronger response. Also, perhaps the existence of a higher expression of *daf-16* through life may also contribute to a higher overall lifespan, taking into account that through a lifespan numerous stress factors are encountered which will activate an increased stress response whose knock on accumulated effect could be a longer lifespan. Finally, it is possible that, in non - *C. elegans* species, DAF-16 nuclear localization is not so strictly controlled and thus that available DAF-16 is a predictor of DAF-16 mediated phenotypes even under non-stress conditions.

Our phenotypic and *daf-16* expression data are correlative and, as yet, we cannot prove a causative influence of *daf-16* expression level on the observed phenotypes in the gonochoristic species. Unfortunately, current technology in non - *C. elegans* species is limited, which makes demonstrating a direct role for DAF-16 in phenotypic covariance in the gonochoristic nematodes not feasible. Very few genetic mutants have been made in these species, RNA interference is of low efficiency and no antibodies exist for chromatin immunoprecipitation approaches. However, many groups are currently attempting to develop such tools for these species and, as such, we hope that a full mechanistic investigation of the IIS pathway in non - *C. elegans* species will be feasible in the near future.

Chapter V

Manipulation of the IIS pathway

AMRIT, F. R., BOEHNISCH, C. M. & MAY, R. C. (2010) Phenotypic covariance of longevity, immunity and stress resistance in the caenorhabditis nematodes. *PLoS One,* 5**,** e9978.

5.1 Introduction

The IIS pathway in *C.elegans* plays a vital role in deciding fates such as developmental decisions (reproductive growth or arrest at the dauer stage), fat storage, metabolism, stress resistance, fertility and life span (Kenyon et al., 1993, Lee et al., 2003, Ashrafi et al., 2003, Riddle and Albert, 1997).

Consisting of a transmembrane protein DAF-2 (Riddle et al., 1997), several intracellular kinases and the DAF-16 transcription factor (Riddle et al., 1981), inactivation of the IIS pathway not only extends lifespan but also increases resistance to pathogens and abiotic stresses (Barsyte et al., 2001, Murakami and Johnson, 1996, Evans et al., 2008). This observation of DAF-16 mediated pleiotropic effects can be triggered by one of several upstream inhibitory mutations in genes such as *age-1* (a homologue of the mammalian phosphatidylinositol 3-OH kinase) or *daf-2* (a homologue of the mammalian insulin receptor). The consequence of these mutations is the relocalization of the transcription factor DAF-16 into the nucleus where it regulates a plethora of downstream genes (Kenyon et al., 1993, Murphy et al., 2003, Friedman and Johnson, 1988, Kimura et al., 1997, Lee et al., 2003).

Our observations and data from chapters III and IV display a correlative trend between phenotypic co-variances and *daf-16* expression levels among the type *Caenorhabditis* species and suggest the hypothesis that *daf-16* is the molecule that could perhaps be determining the molecular basis of stress tolerance (to biotic and abiotic stress factors) and ageing.

To further quantify the effect of DAF-16 and to get a glimpse into the molecular functioning/significance of this molecule we looked into the possibility of manipulating the IIS pathway among our type *Caenorhabditis* species. Through these means we hoped to alter DAF-16 activity to see if we could direct a species towards particular phenotypes based on their DAF-16 activity.

5.2 RNA interference

Currently, there are no available genetic mutants for the gonochoristic species (*C. remanei* and *C.brenneri*), since the dioecious nature of the gonochoristic species makes it particularly hard to produce and maintain stable mutant lines. We therefore sought to exploit RNAi interference in these species, since RNAi has a proven track record for use in the sister species *C. elegans*.

In *C. elegans* RNAi can be induced by soaking animals in a solution containing double-stranded RNA (dsRNA) or by feeding on bacteria expressing dsRNA, or by injecting dsRNA directly into the animal (Tabara et al., 1998, Timmons and Fire, 1998). Once taken up by cells, the dsRNA leads to post- transcriptional degradation of homologous mRNA sequence, thus nullifying the phenotypic consequence of gene expression. Environmental RNAi in *C. elegans* requires the presence of an intestinal luminal transmembrane protein called SID-2 (Winston et al., 2007) that facilitates initial import of dsRNA from the gut lumen into the animal. This function of SID-2 is strangely not conserved in *C. briggsae,* preventing RNAi by feeding/soaking in this species. Genomic comparison suggests that, as with *C. briggsae*, *C. remanei* also seems to lack the conserved function of SID-2.

Therefore for our experiments we used mutant lines for these species that express *C. elegans* SID-2 to make them susceptible to RNAi by feeding (kindly provided by Marie Anne Felix). We performed initial experiments to optimize and confirm the function/effect of RNAi using species specific RNAi clones in these SID-2 mutant type species prior to moving onto phenotypic studies.

We report that penetration of RNAi among species other than *C. elegans* is minimal. Despite the expression of SID-2 in these strains (JU1018 and JU1076), we did not observe any significant RNAi induced changes among our controls *rol-6* and *lin-12* with approximately 5% of the F1 progeny showing a roller or lineage phenotype.

Numerous attempts with varying parameters also proved futile. We grew RNAi bacteria (HT115) for 6-8 hours under varied conditions in parallel as follows:

- Ampicillin (50µg/ml) containing LB medium with and without IPTG (1mM)
- Ampicillin (50µg/ml) and tetracycline (10µg/ml) containing LB medium with and without IPTG.
- We also cultivated the RNAi bacteria for 4 hours in ampicillin (50µg/ml) containing LB medium and added 0.4mM IPTG during the 5th hour of growth.

After worms were transferred to the RNAi plates according to the culture scheme we chose two temperatures in order to induce RNAi. Worms were exposed to either 20°C or 25°C. After 40 hours adult worms were transferred to fresh RNAi plates and incubated for another 24 hours at either 20°C or 25°C.

Although the transmission rate of RNAi was not as expected we did carry out some experiments that included looking for survival rates (lifespan assay, heat stress assay at 37°C, pathogenic assay on *S. aureus* (NCTC 8532) at 25°C and metallo-tolerance assay). For these experiments we used the F2 generation of RNAi fed worms. We also used the varying parameters regime as described above. We included the two positive RNAi controls, negative controls and the experimental run (RNAi silencing of *daf-16* and *daf-2*) in both species. Again, we only observed RNAi in 5% among the positive controls.

Since lifespan or pathogen sensitivity analyses rely on a large population of animals, the poor penetrance of RNAi in these species precluded us from pursuing this experimental route. We therefore decided instead to use the available *daf-2* mutants (with high DAF-16 activity) for *C.elegans* (CB1370) and a recently produced *daf-2* mutant in *C. briggsae* (PS5531) to probe for the role of DAF-16 in stress resistance (to biotic and abiotic stress factors) and ageing.

5.3 Lifespan assay of *daf*-2 mutants

In *C. elegans*, loss-of-function mutations in the upstream insulin-like growth factor receptor DAF-2 have the ability to dramatically enhance DAF-16 activity (Lee et al., 2001, Henderson and Johnson, 2001). This increased activity influences the DAF-16 mediated pleiotropic effects in a positive manner enhancing stress resistance and increasing lifespan.

Among our type species, *C. briggsae* is a hermaphroditic nematode species that is most closely related to the model organism *C. elegans* (Gupta et al., 2007). *C. briggsae* has a completely sequenced and annotated genome along with a repertoire of molecular, genetic, and genomic resources (Stein et al., 2003), making it an ideal target of comparative studies with *C. elegans*. To decipher the evolution of dauer formation in the genus *Caenorhabditis* at the molecular level, Inoue et. al., isolated dauer-formation mutations in *C. briggsae*. Their studies identified orthologs of *C. elegans* genes *daf-2* (insulin receptor), *daf-3* (Smad), and *daf-4* (TGF-ß type 2 receptor) and genes required for formation of sensory cilia. They also observed that the functions of these genes were conserved between *C. elegans* and *C. briggsae*.

Since *daf-16* is also an evolutionarily conserved gene, we investigated whether the function of increased activity of DAF-16 is also conserved in *C. briggsae*, which, like *C. elegans*, has low basal levels of DAF-16 (Figure 28), by comparing daf-2 loss-of-function mutants in both species. We examined for differences in lifespan among *C. elegans* (N2), *C. briggsae* (AF16) and *daf-2* loss-of-function mutants for both of these species (CB1370 and PS5531 respectively).

As previously reported (Kenyon et al., 1993, Inoue et al., 2007) the *daf-2* loss-of-function mutants (CB1370 and PS5531) performed significantly better with a longer lifespan than their wildtype counterparts (Figure 32, Table 22 for p – values). Loss of *daf-2* almost doubled the lifespan in *C.elegans* (CB1370) and had a significant (<0.0001), though smaller, effect in *C. briggsae*.

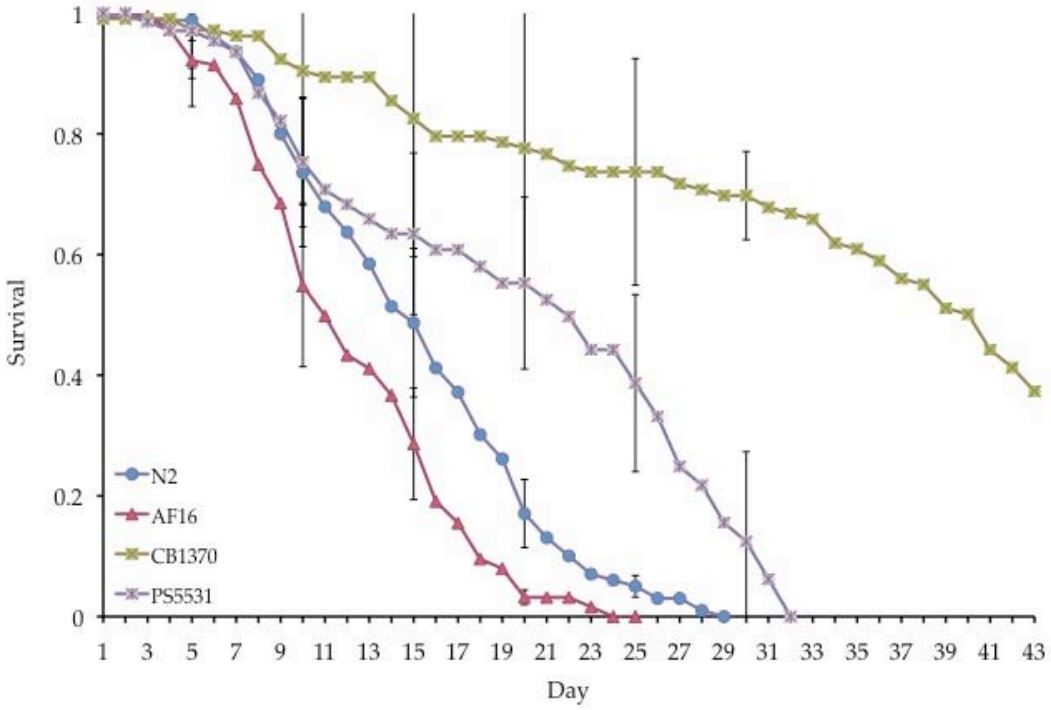

Figure 32 - *daf-2* mutations in *C.elegans* (CB1370) and *C.briggsae* (PS5531) result in enhanced lifespan in comparison to their wildtype counterparts (N2 and AF16) that were monitored for survival over fifty days. Whilst the magnitude of increase in lifespan of PS5531 is relatively small in comparison to CB1370, the increase is still significant ($p < 0.001$).

Species compared	Lifespan
N2/N2	-
N2/CB1370	<0.0001
N2/PS5531	<0.001
AF16/CB1370	<0.0001
AF16/PS5531	<0.0001
CB1370/PS5531	<0.0001

Table 22 - Table of p values comparing all of the experimental strains for the lifespan assay. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

5.4 Survival analysis of *daf*-2 mutants to abiotic stress

Organisms have evolved to develop survival strategies to evade or handle stress resultant from an abiotic source such as heat. Since DAF-16 is involved in handling stress through activation of numerous downstream genes (Barsyte et al., 2001, G J Lithgow, 1995), we tested our *daf-2* hermaphroditic mutants (CB1370 and PS5531) to see what effect an increased DAF-16 activity had upon stress from abiotic factors.

To test this we exposed multiple isolates of all four species to prolonged high temperature of 37°C or toxic heavy metals such as $CuCl_2$. In both cases, inactivation of *daf-2* enhanced resistance of the *C.elegans* mutant CB1370 and the *C.briggsae* mutant PS5531 (whose wild type is inherently more resistant to high temperatures) to high temperature (Figure 33, Table 23 for p - values) and heavy metal toxicity (Figure 34, Table 24 for p - values).

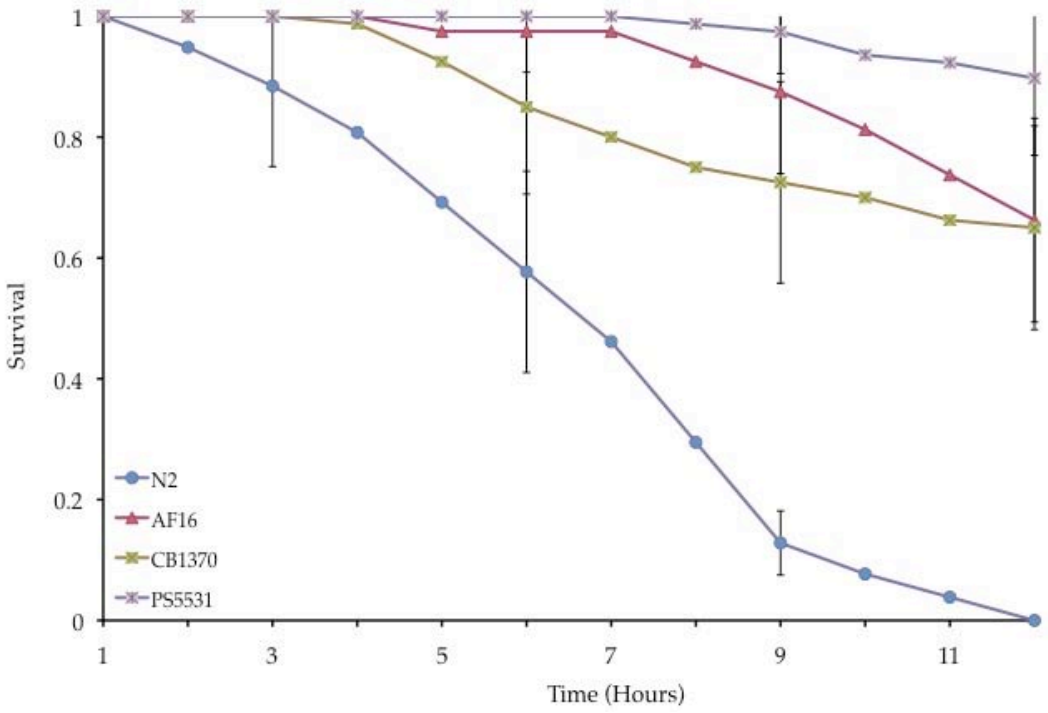

Figure 33 - *daf-2* mutations in *C.elegans* (CB1370) and *C.briggsae* (PS5531) result in enhanced survival to high temperature (37°C) in comparison to their wildtype counterparts (N2 and AF16). Bars every 3 hours indicate 95% confidence intervals.

Species compared	Thermotolerance
N2/N2	
N2/CB1370	<0.0001
N2/PS5531	<0.0001
AF16/CB1370	>0.2
AF16/PS5531	<0.001
CB1370/PS5531	<0.001

Table 23 - Table of p values comparing all of the experimental strains against high temperature (37°C) stress. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

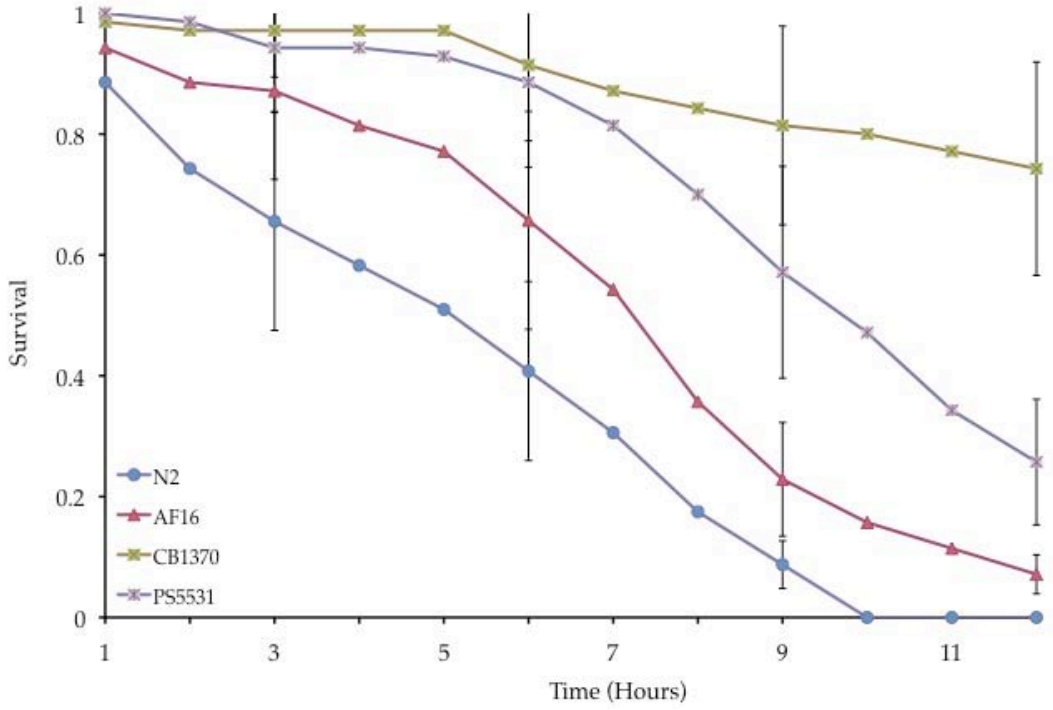

Figure 34 - *daf-2* mutations in *C.elegans* (CB1370) and *C.briggsae* (PS5531) result in enhanced survival to heavy metal stress (7mM $CuCl_2$) in comparison to their wildtype counterparts (N2 and AF16). Bars every 3 hours indicate 95% confidence intervals.

Species compared	Metallotolerance
N2/N2	-
N2/CB1370	<0.0001
N2/PS5531	<0.0001
AF16/CB1370	<0.0001
AF16/PS5531	<0.0001
CB1370/PS5531	<0.0001

Table 24 - Table of p values comparing all of the experimental strains against heavy metal ($CuCl_2$) stress. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

5.5 Survival analysis of *daf*-2 mutants to biotic stress

C. elegans has been used extensively as a model to study pathogenesis and responds to infection by upregulating the expression of several genes encoding antimicrobial proteins (Troemel et al., 2006, Wong et al., 2007) by activating pathways on identification of pathogen associated molecular patterns (PAMPS'). Thus far numerous signalling cascades have been shown to contribute towards immunity and stress resistance among nematodes, including the p38 MAP kinase (Kim et al., 2002, Kim et al., 2004), ERK MAP kinase (Nicholas and Hodgkin, 2004), JNK-like MAP kinase (Huffman et al., 2004), TGF-β (DBL-1) (Mallo et al., 2002), programmed cell death (PCD) (Aballay and Ausubel, 2001), Toll-like receptor (Tenor and Aballay, 2008) and Wnt/Hox signalling pathways (Irazoqui et al., 2008, Nicholas and Hodgkin, 2009). Most of these pathways have been speculated to be working in concert with the IIS pathway(Kondo et al., 2005).

Our studies and work from others has implicated DAF-16 in conferring resistance to biotic stress by activating host defence genes during a pathological infection (Garsin et al., 2003). We therefore tested the *C. elegans* and *C. briggsae daf*-2 mutants for pathogen sensitivity following infection by both Gram-positive (*S. aureus*) and Gram-negative bacteria (*P. aeruginosa*, *S.* typhimurium).

Interestingly, *C. briggsae daf*-2 (PS5531) mutants showed enhanced resistance towards *S. aureus* (Figure 35, Table 25 for p-values) and *P. aeruginosa* (Figure 36, Table 26 for p-values), but the magnitude of the increase was substantially smaller than that for *C. elegans daf*-2 mutants (Figure 35 and Figure 36). Intriguingly, loss of *daf*-2 in *C. elegans* or *C. briggsae* did not elicit the same extent of resistance to S. *typhimuri*um as observed with the other pathogens. But nevertheless the response was significantly higher than that exhibited by wild types (Figure 37, Table 27 for p-values).

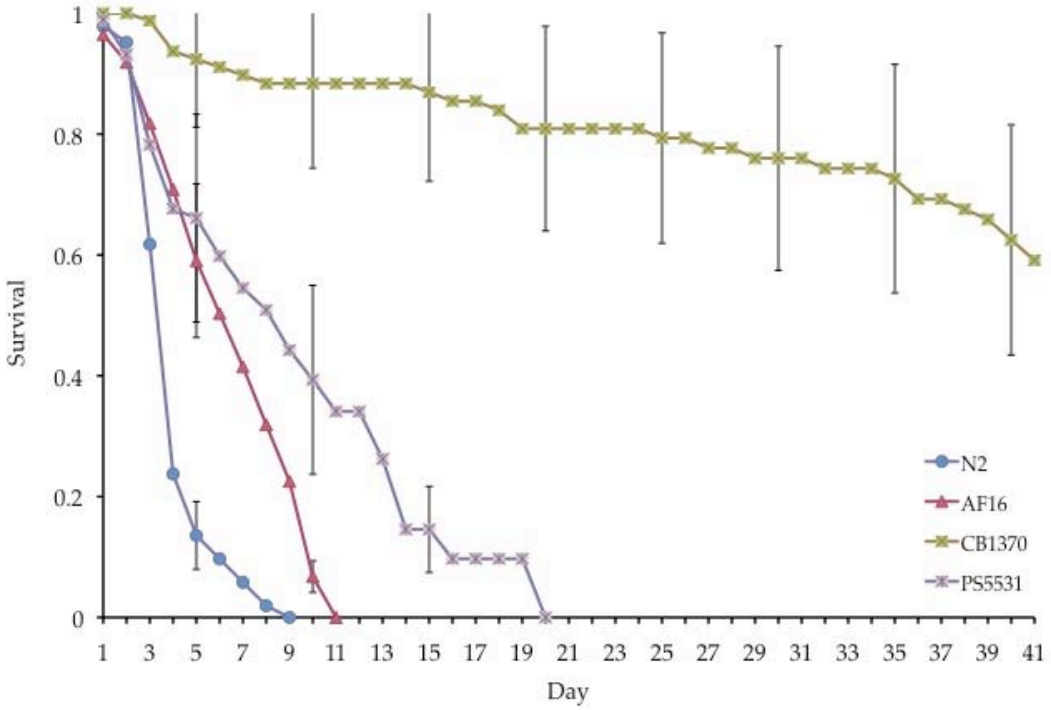

Figure 35 – *C. briggsae daf-2* (PS5531) mutants and *C.elegans daf-2* (CB1370) mutants display enhanced resistance against Gram-positive bacterium *Staphylococcus aureus*. Bars every 5 days indicate 95% confidence intervals.

Species compared	NCTC8532
N2/N2	-
N2/CB1370	<0.0001
N2/PS5531	<0.0001
AF16/CB1370	<0.0001
AF16/PS5531	<0.02
CB1370/PS5531	<0.0001

Table 25 - Table of p values comparing all the experimental strains for resistance against Gram-positive bacterium *Staphylococcus aureus* . Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

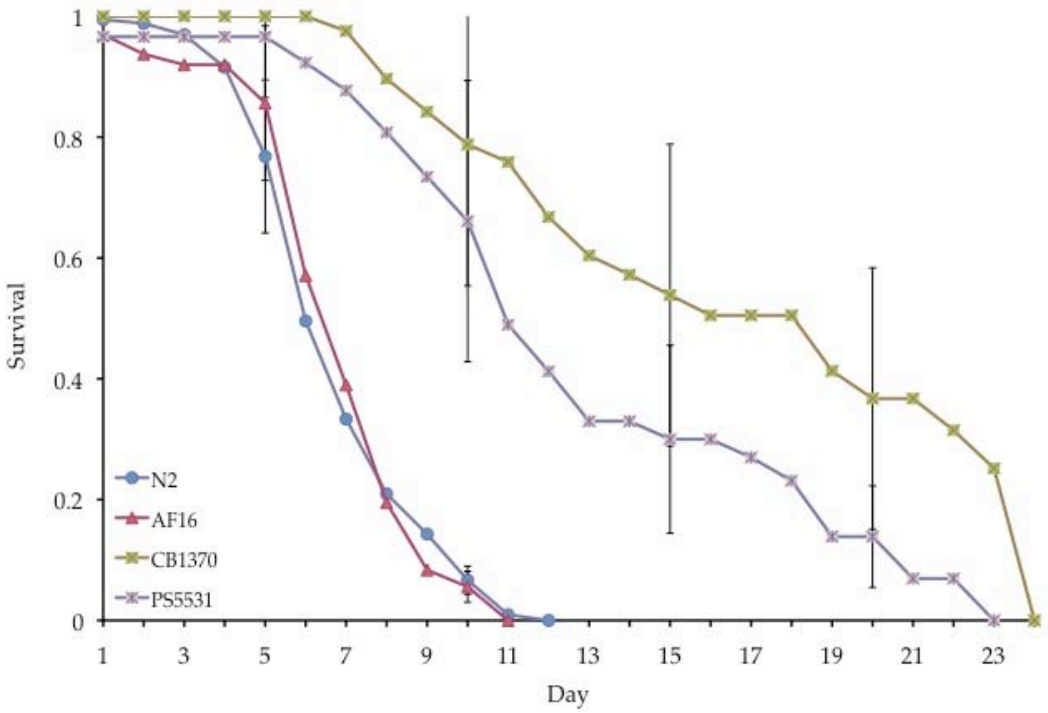

Figure 36 – *C. briggsae daf-2* (PS5531) mutants and *C.elegans daf-2* (CB1370) mutants display enhanced resistance against Gram-negative bacterium *Pseudomonas aeruginosa*. Bars every 5 days indicate 95% confidence intervals.

Species compared	PA01
N2/N2	-
N2/CB1370	<0.0001
N2/PS5531	<0.0001
AF16/CB1370	<0.0001
AF16/PS5531	<0.0001
CB1370/PS5531	<0.01

Table 26 - Table of p values comparing all the experimental strains for resistance against Gram-negative bacterium *Pseudomonas aeruginosa* . Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

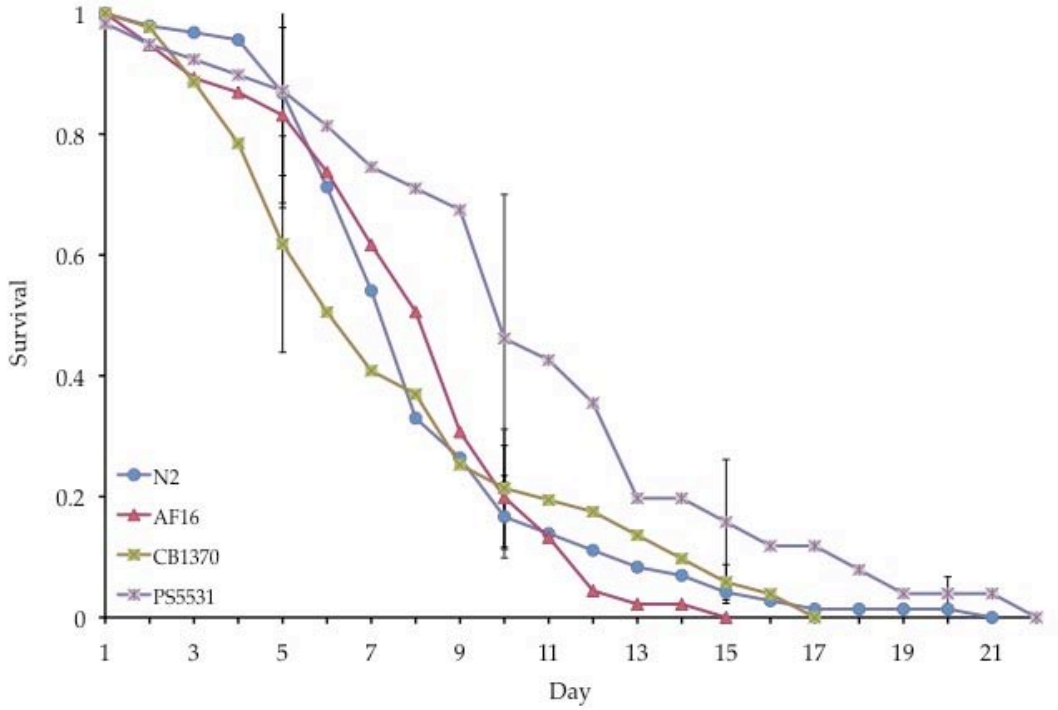

Figure 37 – *C. briggsae daf-2* (PS5531) mutants and *C.elegans daf-2* (CB1370) mutants did not show enhanced resistance against the Gram-negative bacterium *S.* typhimurium. Bars every 5 days indicate 95% confidence intervals.

Species compared	SI1344
N2/N2	-
N2/CB1370	>0.02
N2/PS5531	<0.01
AF16/CB1370	>0.02
AF16/PS5531	<0.01
CB1370/PS5531	<0.01

Table 27 - Table of p values comparing all the experimental strains for resistance against Gram-negative bacterium *S.* typhimurium. Results were corrected for multiple testing using the Bonferroni correction. P values in bold in the table represent significance after correction.

5.6 Discussion

The data presented in this chapter demonstrate that activation of DAF-16 via upstream mutation of *daf-2* enhances both lifespan and resistance to stressful conditions, both biotic and abiotic, in both *C. elegans* and *C. briggsae*. Interestingly, the *daf-2* mutants showed very limited resistance to infection by *S. enterica* Typhimurium. Even though we did observe enhanced resistance in these mutants, the extent of this resistance was not as dramatic as that seen when these mutants were subjected to other kinds of pathogens. One possibility is that this difference is due to the persistent nature of the infection that *Salmonella* has been shown to cause in *C. elegans* (Aballay et al., 2000) that has been attributed to their resistance to anti-microbial peptides (Alegado and Tan, 2008).

Interestingly, in all our phenotypic analysis of the *daf-2* mutants, the *C. briggsae* mutant (PS5531) showed a weaker phenotype than the corresponding *C. elegans* mutant. The *C. briggsae daf-2*(sy5445) mutant has not been fully characterised and thus this difference may result from the sy5445 mutation being more weakly hypomorphic than the e1370 mutation in *C. elegans*.

Regrettably, genetic mutants in *daf-2* or *daf-16* are not available for either gonochoristic species, nor is RNA interference efficient enough in these species to allow direct manipulation of the IIS pathway in a similar manner. However, should such studies become feasible in the future, then our data would predict that loss of *daf-2* would likely have only a minimal effect on lifespan and stress resistance in the gonochoristic species.

From this chapter we can conclude that DAF-16 activity plays a role in determining survival among the tested *Caenorhabditis* species, a trait that can be extrapolated to other species. Our analysis using mutants suggests that by increasing DAF-16 activity among hermaphrodites we can induce pleiotropic effects similar to that seen among the wildtype gonochoristic species. As for *daf-2* mutations in the

gonochoristic species we can as of now only speculate that a similar effect may be seen.

Chapter VI

daf-16 and its splice variants

6.1 Introduction

Eukaryotic genes are composed of alternating exonic and intronic regions. Upon gene expression the introns are spliced out and the adjacent exon sequences come together in a reaction catalyzed by a large complex of RNAs and proteins called the spliceosome. The final spliced RNA is then translated into protein.

This process, termed alternative splicing (intron removal), is an ancient mechanism in eukaryotes (Johnson, 2002) that results in various forms of the same protein. This increases the proteome of an organism without necessarily increasing the genome and, in some cases, also functions as a mechanism to turn off gene expression after gene transcription. Splicing is an immensely important process that may potentially have numerous evolutionary implications, taking into consideration recent discoveries showing 95-100% of human pre-mRNAs that have more than one exon are processed to yield multiple mRNAs (Pan et al., 2008, Wang et al., 2008). Changes in the primary amino acid sequence of the protein are often a consequence of alternative splicing, and can lead to either subtle or dramatic end results.

In *C. elegans,* intron and exon structure are conserved and are similar to that found in other higher eukaryotes (Blumenthal and Steward, 1997). Initial screening identified 844 alternatively spliced *C. elegans* genes by comparing cDNA and genomic sequences (Kent and Zahler, 2000). An updated list of these spliced genes can be found at http://genome-test.cse.ucsc.edu/IntronWS120/.

One such *Caenorhabditis* gene that is spliced to produce numerous transcripts is the *daf-16* gene. The importance of the role played by this gene has echoed through our study with results indicating that among the type *Caenorhabditis* clade, this evolutionarily conserved gene functions at differential levels (Chapter IV) leading to phenotypic covariance (Chapter III) between species. This could very well mean that DAF-16 function dictates the molecular basis of immunity and ageing in the *Caenorhabditis* clade and, potentially, in more distantly related species.

This key role of *daf-16* led us to investigate the molecular nature of DAF-16 by looking at alternative splicing of this gene. We hypothesized that various isoforms of *daf-16* could have evolved as a specialized defence mechanism to particular types of stress. In other words the worm has stress specific *daf-16* isoformic response. We also hoped to see if we could find a key isoform that played an important housekeeping role.

6.2 Northern Blotting

To probe alternative splicing of *daf-16*, we initially attempted to identify *daf-16* isoforms in all the type species of the *Caenorhabditis* genus we studied using Northern blotting. Within the genus, two species have fully sequenced and annotated genomes (*C. elegans* N2 and *C. briggsae* AF16) whilst *C. brenneri* (CB5161) and *C. remanei* (EM464) have been sequenced but not annotated. From this genomic information we designed (species specific) *daf-16* probes for all four species and ran northern blots to identify isoforms.

From our studies we were able to identify several isoforms for the type species *C. elegans,* but unfortunately, despite several attempts varying numerous experimental parameters such as increasing probe concentration, optimising hybridisation, washing and exposure times (as explained in the Ambion Northern Max kit) we were unable to identify *daf-16* isoforms in the other type species.

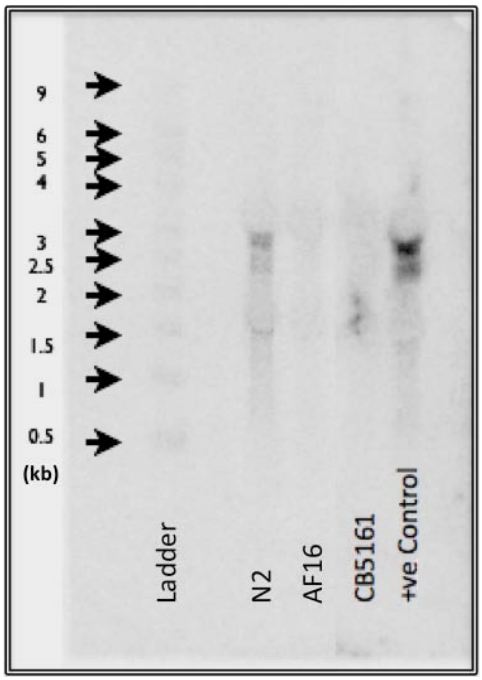

Figure 38 – Northern blot to identify *daf-16* isoforms among the *Caenorhabditis* species. Positive control used was mouse GAPDH.

6.3 *daf-16* Isoforms in *C. elegans*

After our attempt to identify *daf-16* isoforms in the various *Caenorhabditis* species, we decided to concentrate more on the molecular diversity of *daf-16* in *C. elegans*. The *C. elegans* reference site (www.wormbase.org) describes seven putative *daf-16* isoforms in *C. elegans* (Figure 39, Table 28). Our goal was quantitate the expression levels of each isoform to determine whether there may be isoform dependence for specific phenotypes under conditions of stress.

Name	Length (bp)	Length (aa)	Biotype
R13H8.1a	2575	530	Protein coding
R13H8.1b	3021	508	Protein coding
R13H8.1c	3035	510	Protein coding
R13H8.1d	1616	487	Protein coding
R13H8.1e.1	1128	303	Protein coding
R13H8.1e.2	1027	303	Protein coding
R13H8.1f	2852	568	Protein coding

Table 28 – List of the properties of all the putative splice variants of the *daf-16* gene. Based on the EST information from Wormbase we can determine if the isoform is protein coding (Biotype) or not.

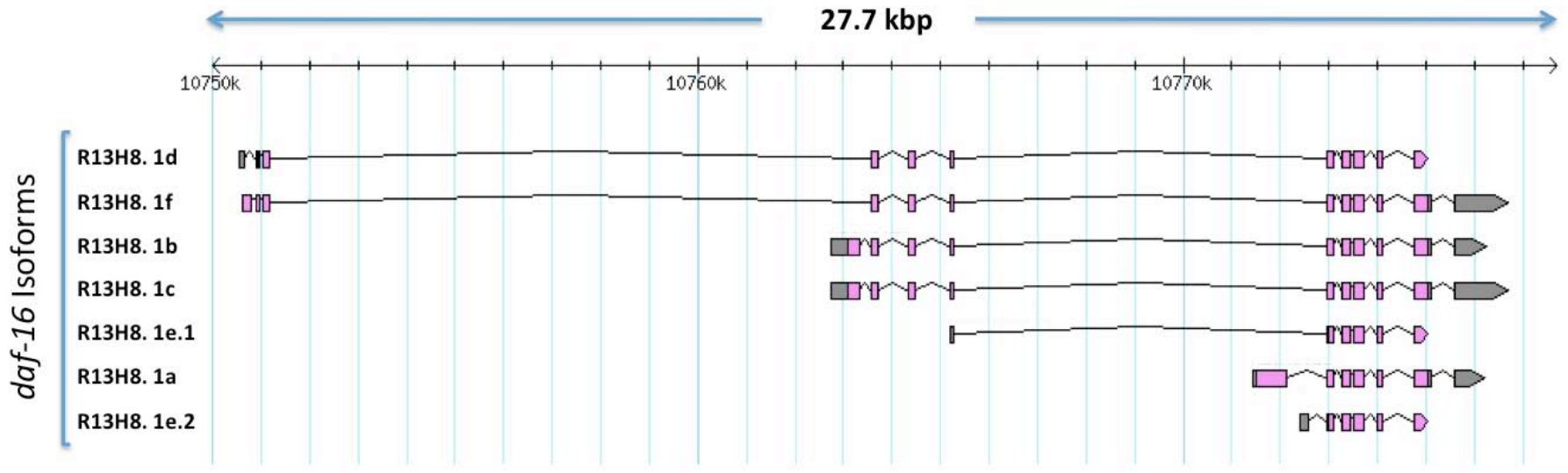

Figure 39 - Illustration depicting the Isoforms of the *daf-16* gene in *C. elegans*. Image obtained from Wormbase (http://www.wormbase.org)

6.3.1 Identification of error in Wormbase

We first designed primers for isoform specific regions for each of the seven *daf-16* isoforms. We were successfully able to design primers for six of the seven isoforms (Table 6). The inability to find an isoform exclusive region for the seventh isoform was because isoform R13H8.1e.1 is an exact splice variant of R13H8.1e.2 with the exception of having one less exon and therefore has no unique sequence within it.

Pre testing/optimisation of these primers revealed that all our primer pairs gave us the expected products (using cDNA as the template) apart from R13H8.1e.2 and R13H8.1f (Figure 40). R13H8.1e.2 gave us a product of approximately 500bp contrary to the 147bp as predicted by wormbase (Figure 41). In contrast, we were unable to generate any product for R13H8.1f, despite the Wormbase prediction of a 137bp product (Figure 42). These two discrepancies suggested that the Wormbase annotation may be incorrect and thus to confirm this we designed another set of experiments to check and verify these isoforms.

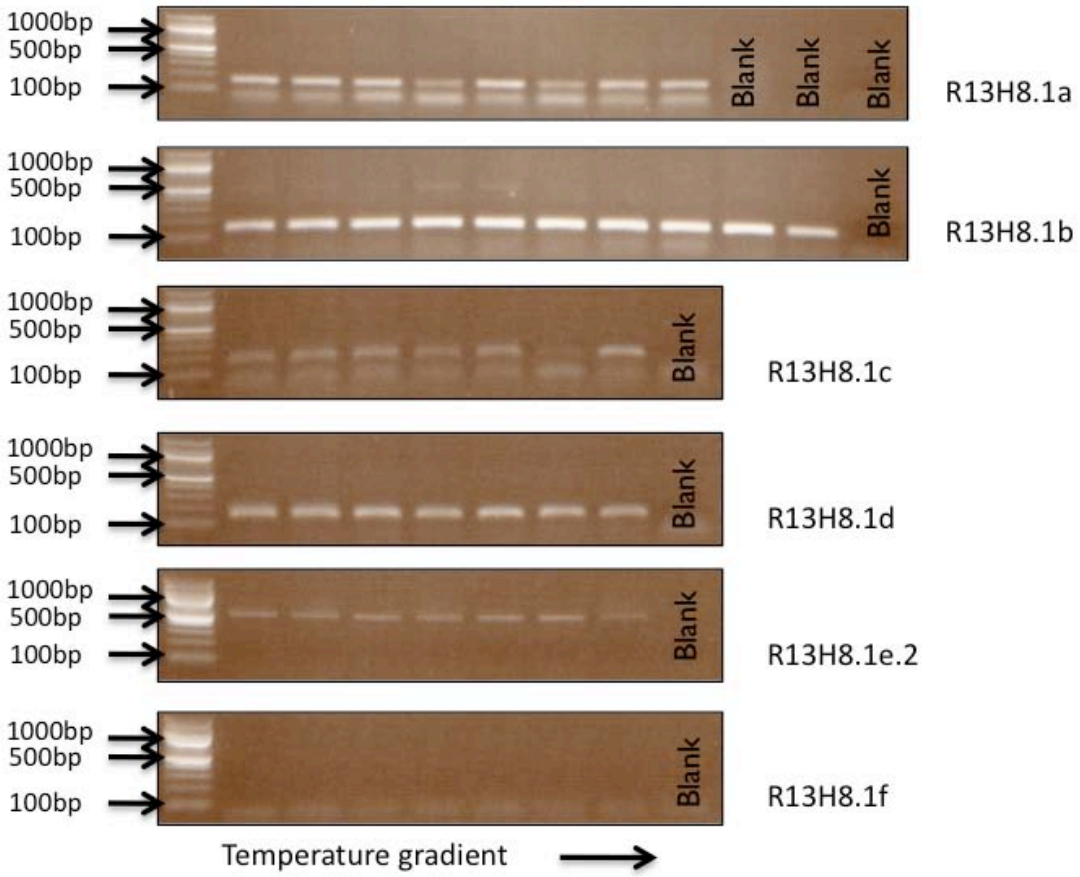

Figure 40 – Results of *daf-16* primer optimisation studies. A temperature gradient PCR was run for all the primer pairs with *C. elegans* cDNA as a template. The resultant products were run on an agarose gel with a 100bp ladder as a reference. Blank lanes consist of only mastermix and no template (negative control).

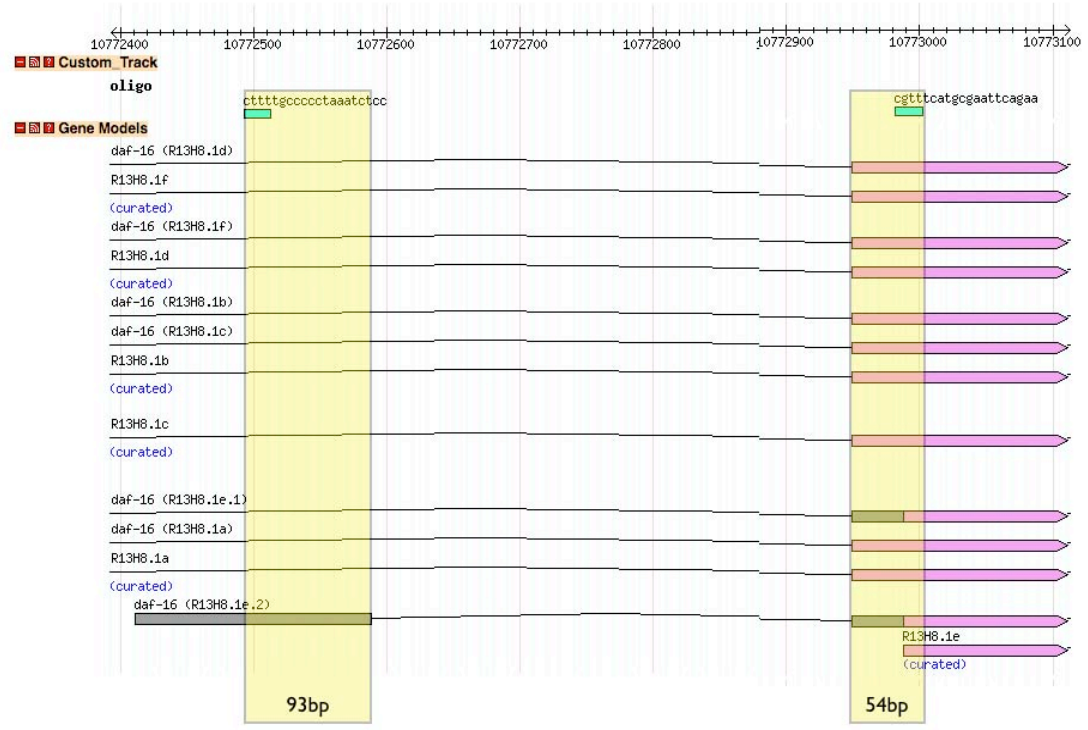

Figure 41 – Position of designed primer (R13H8.1e.2) binding sites in the *C. elegans* genome as according to Wormbase that will result in a 147bp product. *

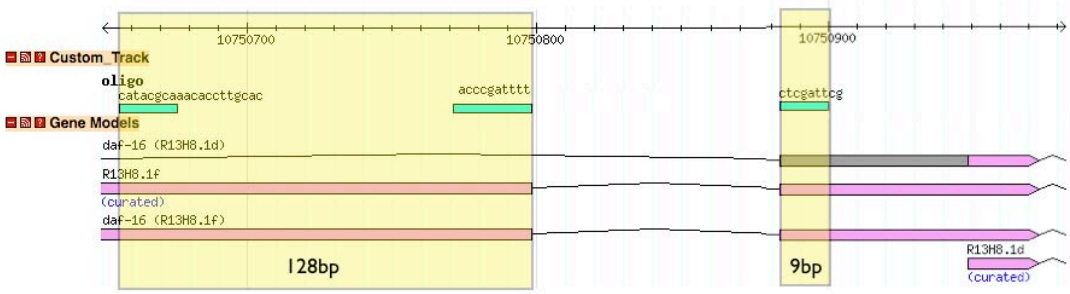

Figure 42 - Position of designed primer (R13H8.1f) binding sites in the *C. elegans* genome as according to Wormbase that will result in a 137bp product. *

* The primers in these figures were the initial set used that didn't give us a product. However based on this observation we were able to design another set of primers (Table 6) that highlighted the mistakes in wormbase.

6.3.2 Rectification of error in Wormbase

To confirm and rectify the potential error on wormbase we designed a new set of primers for isoforms R13H8.1e.2 and R13H8.1f (Table 6) in such a way that the forward and reverse primers were on two adjacent exons positioned on both sides of the region of uncertainness. A PCR was run with these new primers with cDNA as a template (4 samples – biological replicates) that gave us a product of the expected size (150bp, Figure 43). The PCR products were then sequenced to confirm these results. Sequencing revealed that the intron suggested/predicted by wormbase to be spliced out is actually retained within the spliced mRNA (Figure 44 and Figure 45).

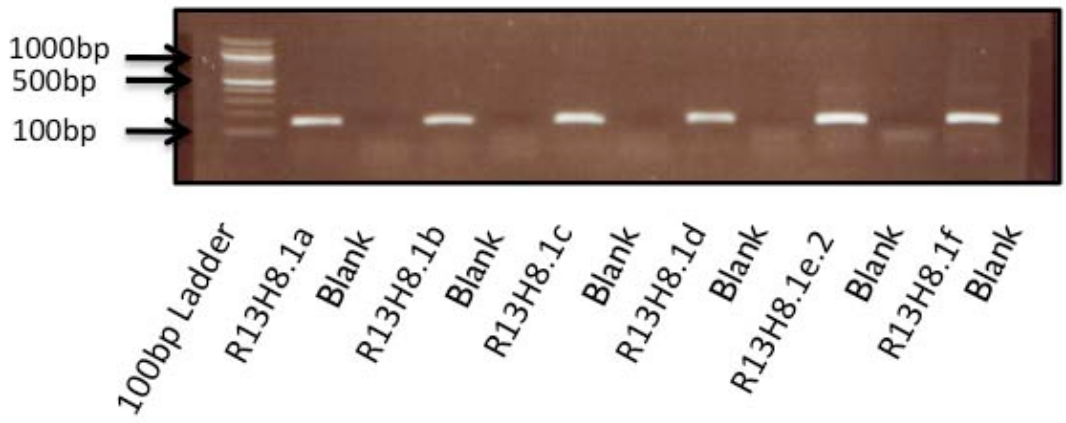

Figure 43 – Agarose Gel showing PCR products of expected size for all of the *daf-16* isoform primer pairs with *C. elegans* cDNA as a template. Blank lanes consist of only mastermix and no template (negative control).

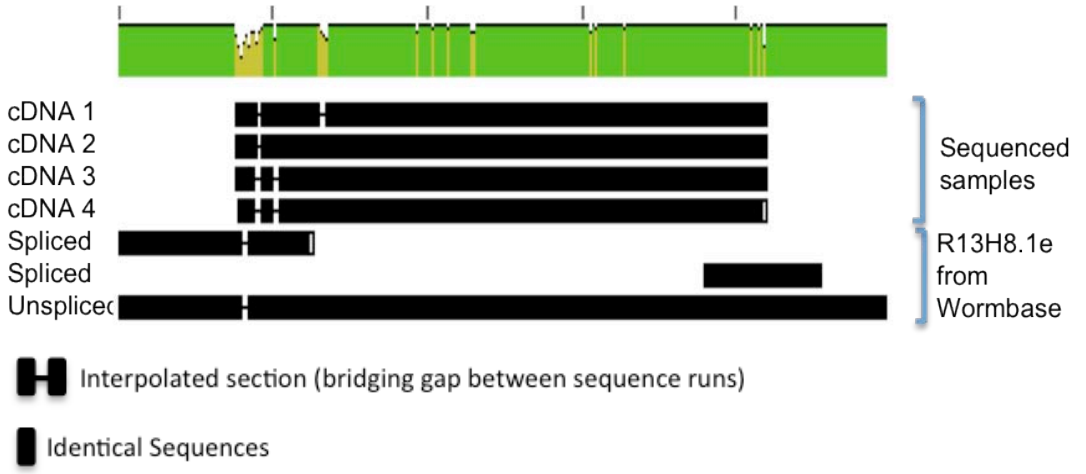

Figure 44 – Sequenced PCR product of cDNA corresponding to R13H8.1e.2 *daf-16* isoform aligned with predicted sequence available on Wormbase·. *

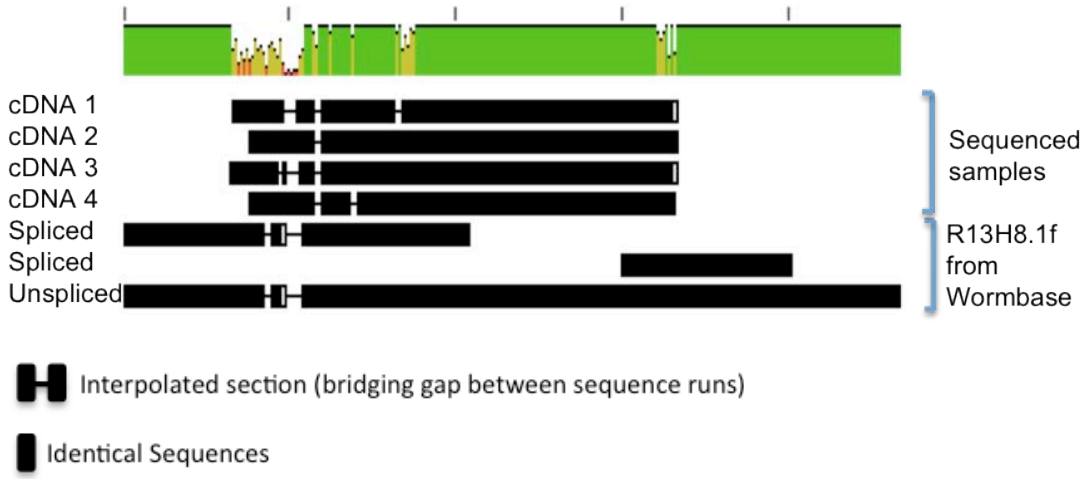

Figure 45 - Sequenced PCR product of cDNA corresponding to R13H8.1f *daf-16* isoform aligned with predicted sequence available on Wormbase. *

· Thickness and intensity of the coloured bar indicates level of sequence conservation across all of the sequencing runs (Low depth, yellow colour = Poor conservation; High depth, dark green colour = 100% identity)

6.4 Real time analysis of *daf-16* isoform expression

After verification of primers and their products we checked for expression of *daf-16* isoforms under varied conditions. *daf-16* is a vital gene whose importance in determining life history traits have been highlighted in this study. Through this experiment we wanted to see if there is a stress specific isoform response. Since DAF-16 regulates a huge variety of phenotypes it is very likely that such a response could exist with certain isoforms specialised to handle certain conditions.

To test this we extracted RNA from animals (mixed population) exposed to various kinds of stress and measured for their *daf-16* isoform expression levels against a control sample of RNA extracted from a mixed population of nematodes grown at 20°C on OP50.

Overall, from our results we were able to see a pattern emerging with regards to *daf-16* isoform expression levels in worms exposed to heat stress (37°C for 3 hours prior to RNA extraction), heavy metal stress (7mM of $CuCl_2$) and biotic stress (*Staphylococcus aureus)*. Under normal conditions, the expression of isoform R13H8.1b is already higher than the other isoforms, but the level of this isoform increases dramatically during heat shock (Figure 46). In contrast, the other isoforms (with the exception of R13H8.1d that slightly increases is expression) showed similar levels of expression under control and heat-shock conditions. Isoform R13H8.1b also came up as the only variable in our other studies when worms were exposed to heavy metal stress (7mM $CuCl_2$) (Figure 47) and biotic stress (kept on a diet of *Staphylococcus aureus)*.

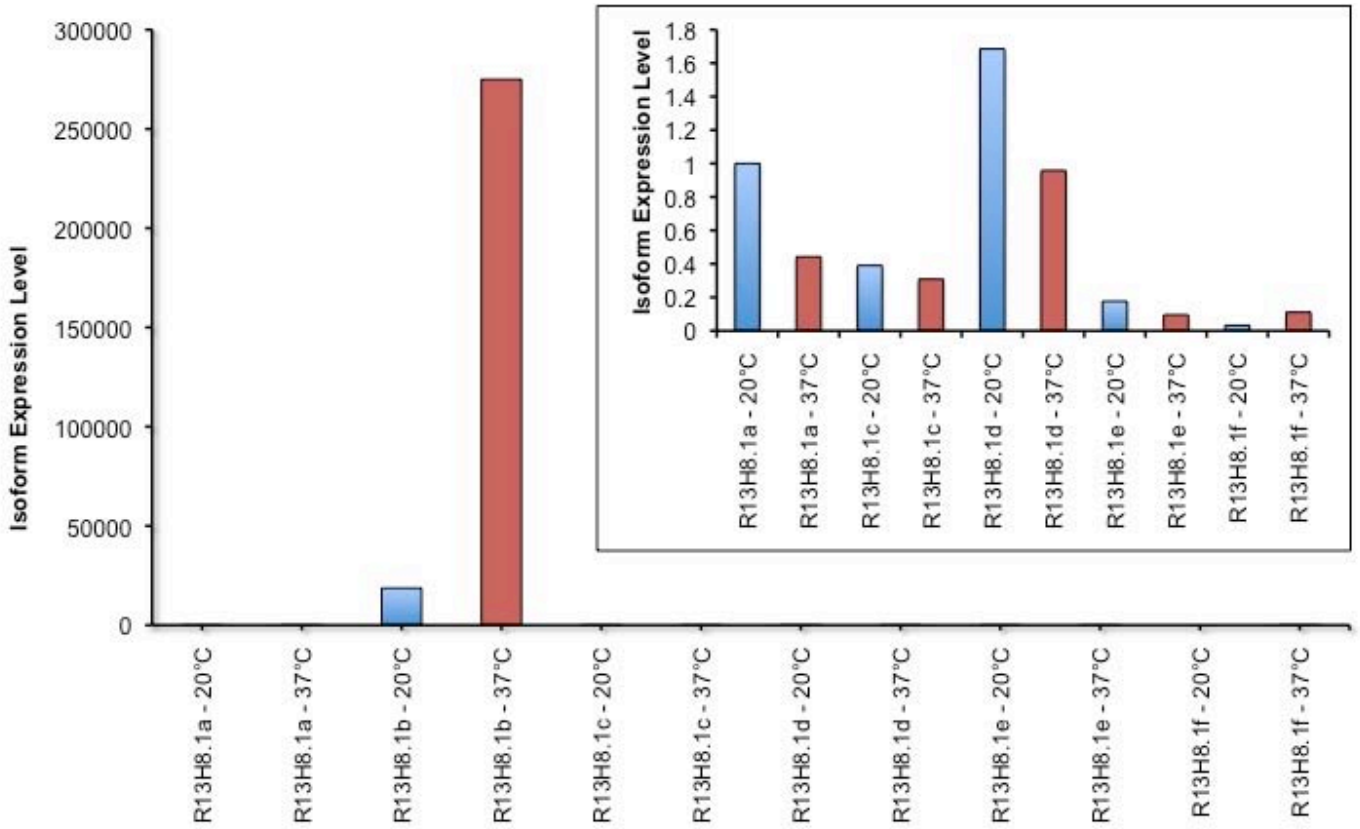

Figure 46 - Expression levels of *daf-16* isoforms (normalized to the reference gene *gpd-3*) *among* mixed populations (nematodes at various stages of development) of *C. elegans* subjected to stress heat stress (37°C) in comparison to nematodes grown under normal (blue bars) conditions. Isoform R13H8.1b is drastically over-expressed in conditions of heat stress hence making it impossible to see the expression levels of all the other lower expressing isoforms. Inset - Expression levels of all *daf-16* isoforms (normalized to the reference gene *gpd-3*) other than R13H8.1b, *among* mixed populations (nematodes at various stages of development) of *C. elegans* subjected to heat stress (37°C) in comparison to nematodes grown under normal (blue bars) conditions.

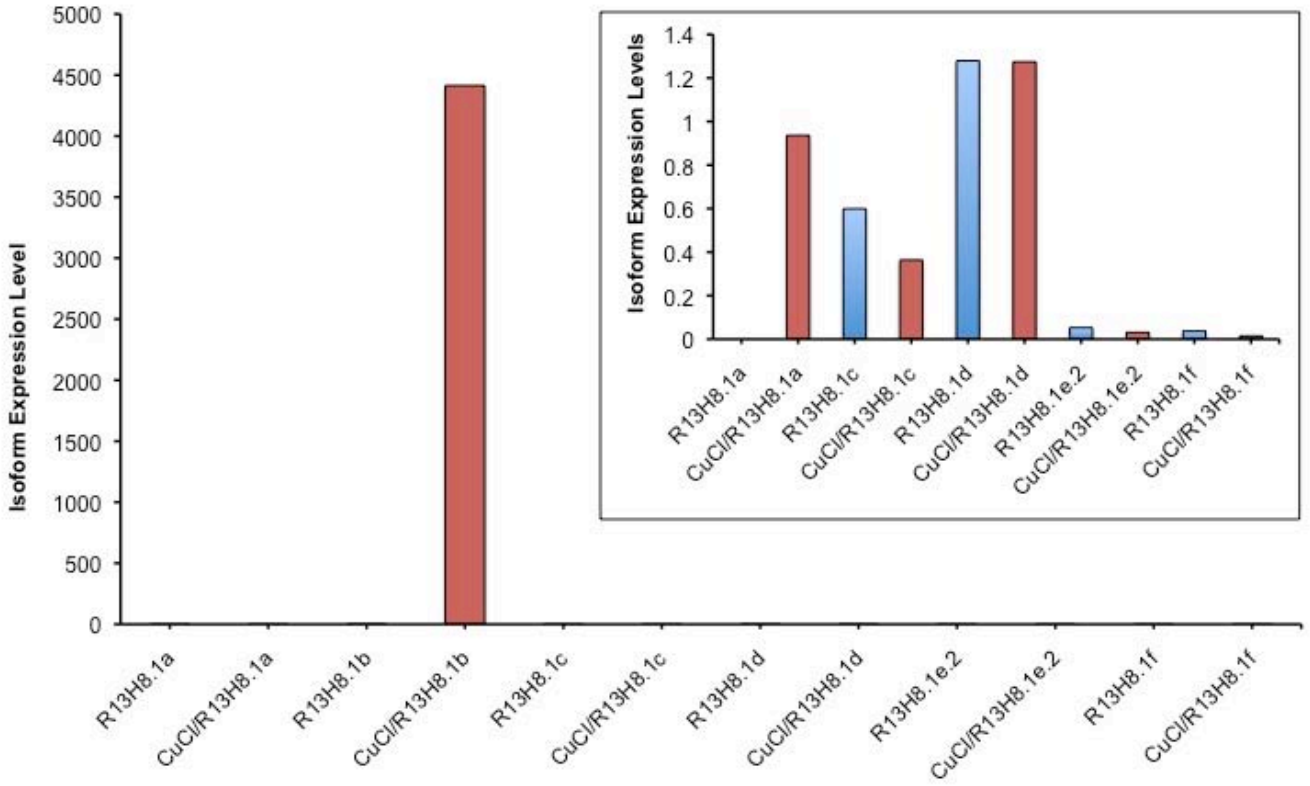

Figure 47 - Expression levels of *daf-16* isoforms (normalized to the reference gene *gpd-3*) *among* mixed populations (nematodes at various stages of development) of *C. elegans* subjected to heavy metal stress (7mM $CuCl_2$) in comparison to nematodes grown under normal (blue bars) conditions. Isoform R13H8.1b is drastically over-expressed in conditions of heavy metal stress hence making it impossible to see the expression levels of all the other lower expressing isoforms. Inset - Expression levels of all *daf-16* isoforms (normalized to the reference gene *gpd-3*) other than R13H8.1b, *among* mixed populations (nematodes at various stages of development) of *C. elegans* subjected to heavy metal stress ($CuCl_2$) in comparison to nematodes grown under normal (blue bars) conditions.

6.5 Discussion

Differential isoform expression is critical for most eukaryotes (van der Vos and Coffer, 2008). From our study we were disappointed not to be have been able to determine the *daf-16* isoforms in the non - *C. elegans* species we studied. Nevertheless, we were able to look at the molecular diversity with regards to expression levels of the various isoforms in *C. elegans*. From our experiments it was evident that isoform R13H8.1b was both the most significantly expressed and most variable isoform, suggesting that this isoform may be the most crucial of all isoforms. The forkhead DNA binding domain of R13H8.1b has been shown to be 50% identical to human FKHR and 46% identical to human AFX with its expression and differential splicing speculated to be functionally important but not implicated in DNA-binding specificity (Ogg et al., 1997). However, we do note that our inference is based on the assumption inspired by previous results that the level of gene expression reflects upon its importance, which in the case of isoforms may not be true.

Whilst R13H8.1b displayed dramatic over expression under conditions of stress, the other Isoforms showed minimal difference in expression. It would have been very interesting to pursue this study, to look at expression in much more detail and perhaps use RNAi to knock down the Isoform R18H8.1b, but time restraints made this an impossibility. Alternative splicing of the *daf-16* homologue, Foxo in mice have indicated that these isoforms are key effectors of Akt-dependent myogenesis (Hribal et al., 2003). Based on such observations, maybe our studies indicate specific isoform driven responses to stress. Perhaps it is safe for us to speculate that differences between the isoforms among species could be the determinant of the varied phenotypes we observed.

Chapter VII

Summary and Future Work

7.1 Fight Harder, Live Longer

The survival of the fittest theory postulated by Charles Darwin has perhaps been the story of life on our planet. The constant pressure on every organism to out-compete its competitors and reproduce has resulted in a diverse array of species and sub species. These organisms occupy complex ecosystems and niches, sometimes having symbiotic relationships with each other, and play a very important part in maintaining the integrity of life as a whole.

For the survival of a species, the ability to evade predators and deal with infections and diseases is vital. Various theories have been postulated to explain survival in terms of lifespan, longevity, immunity and stress tolerance (discussed in the introductory chapter). It is now clear that the lifespan of an organism is determined by a combination of environmental conditions, stochastic factors (such as lifestyle) and genetic background.

Numerous studies have demonstrated that the evolutionarily conserved transcription factor DAF-16 is a critical gene regulator that controls the transcription of hundreds of genes involved in immunity, stress responses and longevity in *C. elegans* (Murphy, 2006). The homologues of *daf-16* in other organisms have been shown to perform similar functions (Holzenberger et al., 2003) and yet species differ significantly in terms of lifespan and immunity, raising the question of how such DAF-16 mediated phenotypes have changed through evolutionary time.

In this thesis I have shown that there exists a covariance of three DAF-16 mediated phenotypes, longevity, immunity and stress response, across the *Caenorhabditis* genus. My studies spanning across the hermaphroditic and gonochoristic *Caenorhabditis* species have also demonstrated that the two gonochoristic species (*C. remanei* and *C. brenneri*) show significantly higher

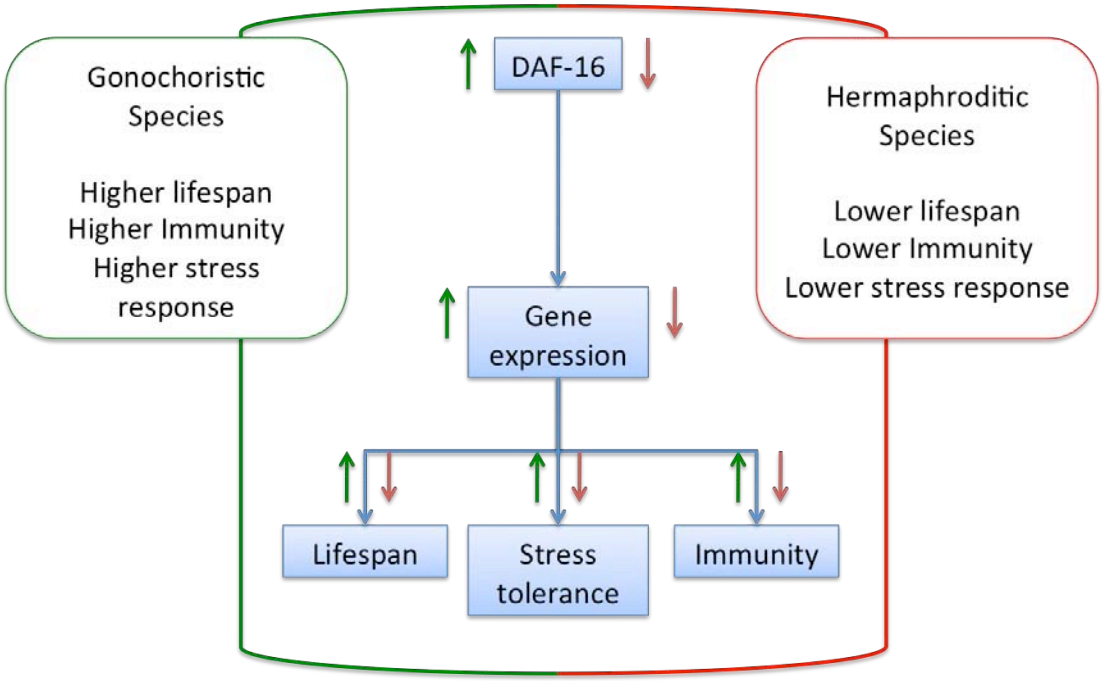

Figure 48 – Figure illustrating the expression of genes leading to increase in phenotype. Arrows beside the boxes indicate high or low activity. Upon high DAF-16 activity as seen among the gonochoristic species, there is an increase in expression of genes that contribute towards an increased lifespan, immunity and stress tolerance with the opposite occurring in the hermaphroditic species.

basal expression of DAF-16 than the shorter-lived hermaphroditic species *C. elegans* and *C. briggsae*. Thus, enhanced expression and/or activation of DAF-16 may be an important mechanism by which species regulate a combination of phenotypes that enhance resistance to abiotic and biotic stresses and hence favour a longer life. The fact that this pattern is seen in multiple isolates of two gonochoristic species may reflect their need to search for a partner to mate, a lifestyle that increases the chance of encountering stressful conditions (eg. pathogens, high temperature) and is likely to favour the evolution of a longer lifespan in order to increase mating opportunities. In addition, since we know very little about the natural ecology of the *Caenorhabditis* nematodes (Kiontke and Sudhaus, 2006), it is possible that differences in the niches inhabited by these species may impose extrinsic stresses that have led to the evolution of improved stress tolerance via the over-expression of DAF-16.

It is interesting to note that susceptibility to several pathogens correlates with other DAF-16 mediated effects, with the exception of the Gram-negative bacterium *S.* typhimurium, which shows similar lethality in all four species and the two *daf*-2 mutants. Since *S.* typhimurium is one of the few human pathogens thus far shown to establish a truly persistent infection in the worm due to its resistance towards antimicrobial peptides (Alegado and Tan, 2008, Aballay et al., 2000), this finding may indicate that DAF-16 plays little or no role in dealing with gut-colonising pathogens.

The insulin-like signalling pathway contributes to both innate immune responses and stress responses in *C. elegans.* Our data suggests that this may also hold true in closely related nematode species. In line with this, we show that the components of this pathway do not show evidence of adaptive sequence evolution during the diversification of these species whereas the complement of putative downstream targets controlled by DAF-16 appear to vary between these species. All three sequenced species share a core DAF-16 regulon comprised of genes functioning in longevity, stress response and other biological processes. However, whilst *C. elegans* and *C. remanei* contain a similar set of target types in their species-specific DAF-16 regulons, the species-specific DAF-16 regulon of *C. briggsae* lacks genes involved in

immunity and stress response. Interestingly, in line with this finding, we observed that a *daf-2* mutant in *C. briggsae* is long-lived and resistant to abiotic stress, but only moderately resistant to killing by a range of pathogens.

Taken together, our data suggest that if you have a immune system / stress response system that fights hard, then you are in turn destined to live longer: Fight Harder, Live Longer.

We also attempted to investigate the *daf-16* gene and its isoforms. There are seven known *daf-16* isoforms in *C. elegans*. Do these isoforms function in response to specific stimuli and are there any key isoforms among these? And does the evolution of *daf-16* at the isoformic level drive variance in phenotypes we observed among the *Caenorhabditis* clade?

Although we were unable to fully investigate this topic given the limited time period, we were able to identify errors in Wormbase with regards to the *C. elegans* isoforms and then identify R12H8.1b to be the most strongly expressed isoform among these under both resting and high-stress (temperature shock) conditions.

In conclusion, we demonstrate covariance of DAF-16 mediated phenotypes in the four most well-characterized species of the *Caenorhabditis* clade. We note that our data are correlative but, as yet, cannot prove a causative influence of *daf-16* expression level on these phenotypes in the gonochoristic species.

Currently, demonstrating a direct role for DAF-16 in phenotypic covariance in the gonochoristic nematodes is not technically feasible. Very few genetic mutants have been made in these species, RNA interference is of low efficiency and no antibodies exist for chromatin immunoprecipitation approaches. However, many groups are currently attempting to develop such tools for these species and, as such, we hope that a full mechanistic

investigation of the IIS pathway in non - *C. elegans* species will be feasible within the next few years.

The future for ageing research is to maximize the experience of life by trying to keep us younger for a longer duration of time, by helping humanity deal with all the age related problems. Lessons learnt from model organisms can be used to reach such a stage: Younger for Longer.

Appendix

List of and legends of supplementary data that have been written onto a CD attached with this thesis.

Appendix 1: Table represents the outcome of the GOTERM BP_2 DAVID analysis performed for the DAF-16 core regulon and the species-specific DAF-16 regulon. The gene count gives the number of genes within the tested gene sets, which are found to be associated with a GO group within GOTERM BP_2. P-value is based on a conservative adjustment of the Fisher's exact probability and was subsequently corrected for multiple testing with Bonferroni correction and the Benjamin-Hochberg method implemented in DAVID. After correction the p-value gets larger. The fold enrichment value measures the magnitude of enrichment.

Appendix 2: Table represents the outcome of the GOTERM BP_ALL DAVID analysis performed for the DAF-16 core regulon and the species-specific DAF-16 regulon. The gene count gives the number of genes within the tested gene sets, which are found to be associated with a GO group within GOTERM BP_ALL. P-value is based on a conservative adjustment of the Fisher's exact probability and was subsequently corrected for multiple testing with Bonferroni correction and the Benjamin-Hochberg method implemented in DAVID. After correction the p-value gets larger. The fold enrichment value measures the magnitude of enrichment.

Appendix 3: Table representing the adjusted DAF-16 core regulon using the GOTERM BP_ALL and GOTERM BP_2 gene categories which revealed significant enrichment for genes involved in the regulation of lifespan, stress response, transport, localization and metabolism.

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