The Ecological Genetics of Senescence and Stress Resistance in *Caenorhabditis elegans*

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Longevity and the rate of senescence are determined by the ecological conditions experienced during a population's recent evolutionary history, and are intrinsically linked to other components of life history and to fitness. These traits should be examined in an ecological context, in which other aspects of the life history are taken into account. However, although many mutations which promote longevity in model organisms disrupt mechanisms that are involved in responding to environmental change, trade-offs associated with increased lifespan have typically been examined in benign laboratory conditions. In the nematode *Caenorhabditis elegans*, long-lived, stress resistant *age-1(hx546)* mutants can compete with wild type worms in favourable growth conditions, but display fitness costs when populations are periodically starved. By monitoring temporal changes in genotype frequencies, I have established that *age-1* mutants can have higher fitness than the wild type strain if mixed genotype populations are exposed to periods of thermal or oxidative stress when food is available. Genotype-by-environment interactions, and spatial and temporal distributions of the FOXO transcription factor DAF-16, suggest that this is because *age-1* mutants are more able to survive, develop and reproduce during and/or after exposure to environmental stress, due to increased expression of genes involved in somatic maintenance and repair. Using population projection matrices, I have demonstrated that the *age-1(hx546)* mutant allele can confer a selective advantage over the wild type genotype when populations experience abiotic stress, even if periods of starvation are frequently endured. This is the first demonstration that a long-lived, laboratory-derived mutant can have higher fitness than a wild type genotype under specific environmental conditions. The results imply that, if genetic variation is present in populations which encounter harsh conditions, increased longevity can evolve as a consequence of selection for greater resistance to stress. I have also established that the effects of mutations which promote longevity on the ability to tolerate environmental stress can be context dependent, and that long-lived *age-1(hx546)* mutants display increased cold tolerance, relative to wild type worms, due to increased expression of Δ^9 desaturase genes and additional transcriptional targets of DAF-16. The results presented in this thesis suggest that genetic and life history responses to environmental stress deserve a more prominent role in evolutionary studies of ageing.

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Chapter 1 - Introduction

1.1 Is death an inevitable consequence of life?

Senescence can be defined as the progressive physiological deterioration and associated declines in fecundity and survival that occur with advancing age (Finch, 1990). Though the term 'ageing' can be applied to any period of life, it is generally used synonymously with senescence and will be used as such throughout this thesis.

1.11 Mortality dynamics and functions of survival

The rate at which mortality increases with age is the principal measure of the rate of senescence. Mortality rates have traditionally been described using the Gompertz-Makeham law (Gompertz, 1825; Makeham, 1860). This incorporates two parameters: the age-independent mortality rate (the Makeham term), and the age-dependent mortality rate (the Gompertz function), which increases exponentially after maturity. Mortality rates are quantified using the equation: $m(t) = Ae^{Gt} + M$, where m(t) is the mortality rate as a function of age (t), A is a constant which describes age-independent mortality, G is the exponential (Gompertz) mortality coefficient, which represents how mortality accelerates with age, and M is the age-independent mortality rate (the Makeham term). In protected environments, the age-independent component is often negligible and mortality rates can be described using the Gompertz function (Ae^{Gt}) alone. An alternative measure is the Weibull function, which can be used to quantify the rate of senescence independently of extrinsic mortality (Weibull, 1951). Whilst the Gompertz function may be appropriate when age-related increases in mortality are caused by increasing vulnerability to extrinsic hazards, the Weibull function may be more suitable when age-related increases in mortality are a result of intrinsic causes (Ricklefs & Scheuerlein, 2002).

1.12 'Somatic slaves' and the immortal germ-line

Most multi-cellular metazoans possess two types of cells: germ (sex) cells, which constitute the germ-line, and somatic (body) cells, which constitute the soma. Whilst the germ cell lineage is passed indefinitely from one generation to the next, somatic cells are required only for the maintenance and propagation of the germ-line during a single generation. The distinction between the germ-line and the soma, which was first recognised by August Weismann (1882), has had a profound impact upon current understanding of the biology of ageing, and is fundamental to the disposable soma theory (Kirkwood, 1977) which is described in 1.22.

1.13 Diversity in longevity and the rate of senescence

By considering the diversity in patterns of age-dependent mortality among different species, it becomes clear that senescence is not necessarily inevitable. Some groups of simple organisms, including certain Cnidarians, such as *Hydra*, do not appear to age at all and this can typically be attributed to a lack of distinction between the germ-line and the soma (Finch, 1990; Martinez, 1998; Kirkwood & Austad, 2000). Variation in longevity is extensive among complex multicellular eukaryotes and there are numerous examples of organisms which survive for longer or shorter periods than one might predict. For instance, certain species of rockfish, such as *Sebastes aleutianus,* can survive for over 200 years (Cailliet *et al*., 2001), and some vascular plants, such as the Great Basin bristlecone pine, *Pinus longaeva*, can survive for millennia (Lanner & Connor, 2001). In contrast, some semelparous organisms die almost immediately after reproduction. Pacific salmon (genus *Oncorhynchus*), for example, stop feeding during the reproductive period and die very soon after spawning has ceased (Finch, 1990). Such variation is intriguing to evolutionary biologists and the extent to which different ecological and evolutionary processes influence longevity, and the relative contributions that genes and the environment make in shaping senescence, have been subjects of intense debate.

1.14 A demographic time bomb: longevity in the 21st century

During the past 200 years, human life expectancy has steadily increased in the developed world. This was initially attributed to a decline in age-independent mortality (the Makeham component), due to factors such as improved sanitation, the availability of vaccinations and the widespread use of antibiotics (Olshansky *et al*., 2001). However, in the past few decades the decline in mortality rate has been age-dependent (the Gompertz component), characterised by an increased probability of survival at late ages (Olshansky *et al*., 2001). This may be due, at least in part, to increased medical care for the elderly. Concurrent with the increase in life expectancy, birth rates have declined in many countries (Christensen *et al*., 2009). These changes have led to considerable modifications in the age structure of populations in the developed world. With an increase in the number of elderly people requiring treatment for age-related diseases, and a decline in the proportion of the population being of working age, these demographic transitions may have major socio-economic consequences in the coming decades. Biogerontology, the study of biological processes which contribute to senescence, is thus of great contemporary importance. Though current medical intervention focuses on the treatment of age-related diseases, insights into the

mechanisms which underly ageing suggest that it may become possible to target the process of senescence itself.

1.2 Ageing – an evolutionary enigma

The concept of senescence presents a paradox to evolutionary biologists. This is because traits which have been moulded by natural selection should theoretically maximise individual fitness, yet age-related reductions in fertility and survival cause individual fitness to decline. Although some researchers argue that individuals may senesce and die to provide space and resources for subsequent generations (Longo *et al*., 2005; Dytham & Travis, 2006), the vast majority of researchers maintain that ageing has not arisen as a product of selection. Opinion on this matter is so strong that 52 biologists signed a statement insisting that 'ageing is a product of evolutionary neglect, not evolutionary intent' (Olshansky *et al*., 2002).

1.21 Ageing as an adaptive trait

Senescence was first considered from an evolutionary perspective by Alfred Russel Wallace (quoted in Weismann, 1889). He reasoned that senescence may have evolved as an adaptive trait which enhances the fitness of subsequent generations by reducing competition for limited resources.

"…. when one or more individuals have provided a sufficient number of successors they themselves, as consumers of nourishment in a constantly increasing degree, are an injury to their successors. Natural selection therefore weeds them out, and in many cases favours such races as die almost immediately after they have left their successors."

Wallace's views were later reiterated and formalised by August Weismann (1882). However, Weismann reversed his initial claims following his realisation that traits may degenerate if they are no longer beneficial (Kirkwood & Cremer, 1982). Several problems have been identified with adaptive explanations of senescence. Perhaps the most prominent is that, due to extrinsic hazards such as predation or disease, senescence is rarely regarded as a significant source of mortality in nature (Kirkwood & Austad, 2000). There is, therefore, little need for selection to 'weed out' old individuals which would otherwise compete with younger kin, and little opportunity for genes which cause senescence to evolve (Kirkwood, 2005). Although recent studies have reported that senescence may constitute a source of mortality in some wild

populations (reviewed in Nussey *et al*., 2008), consequences for population level fitness may be negligible and too indirect to have considerable impacts upon evolutionary dynamics. Another flaw is that adaptive theories of ageing require selection to work on a kin or group basis. To exemplify why this is an issue, Kirkwood (2005) described a scenario in which a non-ageing mutant produces more offspring than other individuals; eventually its descendants would succeed the ageing members of the species. Finally, adaptive theories assume that old individuals are already in some way less fit than their younger successors without explaining how or why this has become the case (Kirkwood, 2005).

Despite these issues, several theoretical biologists have recently attempted to reinstate adaptive theories of ageing (Goldsmith, 2004; Travis, 2004; Mitteldorf, 2004, 2006; Longo *et al*., 2005; Dytham & Travis, 2006). This has been motivated, at least in part, by the realisation that numerous conserved genes modulate longevity in a variety of model organisms (reviewed in Kenyon, 2005). Some theoreticians have interpreted the existence of so called 'gerontogenes' as evidence that a conserved program has evolved specifically to cause senescence (Travis, 2004; Longo *et al*., 2005). Theoretical studies have been used to examine associations between longevity and dispersal. For instance, using spatially explicit patch occupancy models, Travis (2004) and Dytham & Travis (2006) demonstrated that, when fecundity declines with increasing age, longevity can be correlated with dispersal distance. When dispersal events occur at a local scale, longevity may be constrained because individuals who benefit from senescent deaths are likely to be closely related kin. In contrast, greater dispersal distances may lead to the evolution of increased longevity as individuals who benefit from increased space and resources are unlikely to be related. Interestingly, Dytham and Travis (2006) made an attempt to integrate controversial adaptive views of ageing with more accepted non-adaptive theories, suggesting that, as these are non-mutually exclusive, both may play a role in the evolution of senescence in natural populations.

1.22 Ageing as a non-adaptive by-product of evolution

"Senescence has no function; it is the subversion of function."
Alex Comfort (1956)

In age structured populations, an individual's expected contribution to lifetime fitness at a given age changes throughout life and declines beyond maturity (Fisher, 1930; Hamilton, 1966). This is because, in wild populations, individuals succumb to extrinsic mortality sources, such as predation, starvation and disease, and become

progressively less likely to survive and reproduce with increasing age. To maximise lifetime fitness, traits which influence age-specific survival and fecundity should thus be under stronger selection early in life, when future fitness contributions are likely to be high, than late in life, when future fitness contributions are likely to be low. This constitutes the basis of the non-adaptive evolutionary theories of ageing (Medawar, 1952; Williams, 1957; Kirkwood, 1977), and is discussed in more detail in 1.23.

The concept of ageing as a non-adaptive trait was first discussed by Medawar (1952). He suggested that senescence may be caused by late-acting, deleterious mutations which have not been removed by natural selection and, subject to genetic drift, have accumulated within the germ-line throughout time. The development of Medawar's mutation accumulation theory was instigated by Haldane's (1941) notes regarding Huntington's disease, a fatal neurodegenerative disorder which is caused by a dominant mutation and does not affect carriers until they reach 30-35 years of age. Haldane (1941) suggested that the disease has not been eliminated from the human population because carriers are likely to pass the mutant allele to subsequent generations prior to the onset of the disease. Support for the mutation accumulation theory is mixed and controversial. If ageing results from an accumulation of random deleterious mutations then additive genetic variation in fertility and survival should increase with age. However, whilst some studies have provided evidence for mutation accumulation in *Drosophila melanogaster* (Hughes & Charlesworth, 1994; Tatar *et al*., 1996; Hughes *et al*. 2002) and in wild populations of red deer, *Cervus elaphus*, and soay sheep, *Ovis aries* (Wilson *et al*., 2007), another study using *D. melanogaster* found no association between genetic variation and age (Rose & Charlesworth, 1980). Though Medawar's theory has lost favour in recent years due to discoveries of single genes and conserved pathways which modulate longevity, it remains plausible that mutation accumulation may represent a 'private' mechanism of senescence which contributes to ageing in specific evolutionary lineages (Partridge & Gems, 2002).

Later non-adaptive theories of ageing take into account the fundamental concept of life history theory, which stipulates that life-history strategies, i.e. age-specific schedules of survival and fecundity, are characterised by co-variances among traits which must be optimised according to the conditions of a particular ecological niche (Stearns, 1992; Roff, 2002). These theories predict that trade-offs should arise which favour early life fitness components over longevity. The antagonistic pleiotropy theory (Williams, 1957) predicts that senescence occurs because mutations with late-acting, detrimental effects may be selected and maintained in populations if they occur in genes with pleiotropic effects and confer benefits to fitness earlier in life. The related disposable soma theory (Kirkwood, 1977), which is based upon the well supported idea

that during an individual lifetime senescence occurs due to the accumulation of un-repaired damage to somatic molecules and cells, considers that trade-offs arise because metabolic resources must be optimally allocated among growth, reproduction and somatic maintenance and repair mechanisms. Because the soma is required purely for the protection and propagation of the germ-line, the disposable soma theory predicts that resources should only be invested in somatic maintenance and repair functions whilst individuals are likely to survive and reproduce in wild populations. Senescence thus occurs when individuals survive beyond such an age. Support for the antagonistic pleiotropy and disposable soma theories of senescence is discussed throughout the remainder of this chapter.

An important prediction of the evolutionary theories of ageing is that rates of intrinsic mortality (senescence) should be modified according to the rates of extrinsic mortality experienced during a population's recent evolutionary history (Williams 1957). This provides a convenient testable hypothesis for assessing the validity of the non-adaptive theories of ageing and is supported by numerous comparative studies. For instance, bats and birds, which through flight can avoid hazards such as predation and starvation, tend to live longer than non-volant mammals of a similar size (Holmes & Austad, 1994). Additionally, Virginia opossums, *Didelphis virginiana*, from a predator-free island age more slowly than conspecifics inhabiting the more hazardous mainland (Austad, 1993). The hypothesis is also supported by empirical evidence as imposing high levels of extrinsic mortality over multiple generations led to more rapid rates of intrinsic mortality and reduced longevity in populations of *D. melanogaster* relative to controls (Stearns *et al*., 2000). However, the relationship between extrinsic mortality and the rate of senescence may not be quite so straight forward. Individuals are not all equally susceptible to mortality sources regardless of age and condition, and the effects of extrinsic mortality may be altered when density dependence is taken into account (Abrams, 1993; Graves & Mueller, 1993). Consistent with this, Reznick *et al*. (2004) demonstrated that the rate of ageing in guppies, *Poecilia reticulata*, derived from rivers with high levels of predation was lower than that of guppies from rivers with low levels of predation. This may be because high levels of predation reduce population density and thereby decrease competition for resources among surviving individuals. Furthermore, age-specific fecundity and mortality rates are likely to vary according to population density (Williams *et al*., 2006), and, as described in 1.23, changes in these traits modify fitness and the rate at which the efficiency of selection declines with increasing age.

1.23 The dynamics of selection across the lifespan

The intrinsic growth rate (r) of an age (or stage) structured population at a stable distribution is typically used as a measure of that population's fitness (Fisher, 1930). Fisher demonstrated that r, which he referred to as the Malthusian parameter, is a function of age-specific schedules of survival and reproduction and can be quantified using the following equation, where l(x) is the probability of survival to age x, and m(x) is the expected number of daughters produced by a female at age x:

$$\int_0^\infty e^{-rx} l(x)m(x)dx = 1$$

This has since become known as the Euler-Lotka equation, based upon the earlier work of Euler (1760) and Sharpe and Lotka (1911). By modifying this equation, Fisher (1930) developed the concept of the reproductive value, a measure of an individual's expected contribution to future fitness at a given age, which he considered to be directly proportional to the intensity of selection at that age. As individuals become progressively less likely to survive and reproduce with increasing age, Fisher argued that reproductive values, and thus the intensity of selection, should progressively decline beyond maturity.

Using Fisher's Malthusian parameter as a measure of fitness, Hamilton (1966) formulated scaling functions to represent the intensity of selection acting upon age-specific fecundity and survival by quantifying the sensitivity of fitness to perturbations in these traits. He demonstrated that reproductive values do not always provide an appropriate measure of the intensity of selection at a particular age, but provided rigorous quantitative support for the decline in the efficiency of selection after the onset of reproduction. Hamilton (1966) conveyed that selection acting on survival is maximal during development and progressively declines throughout the reproductive period, reaching zero at the last age of reproduction. Consequently, with the possible exception of species which exhibit grand maternal care (Lahdenperä *et al*., 2004), selection does not act directly upon post reproductive lifespan and longevity is expected to evolve in response to selection upon associated traits (Parsons, 1993; Parsons, 2002; Kenyon, 2010). This principle is explored in chapters 4, 5 and 7 of this thesis. Empirical support for Hamilton's scaling functions and the influence of selection dynamics on senescence has been derived from artificial selection experiments using *D. melanogaster* (Rose & Charlesworth, 1980; Rose, 1984). By discarding eggs from young mothers for many generations to obtain flies with a late age at maturity, these experiments demonstrated that longevity can be substantially increased if the onset of the decline in selection is delayed.

1.24 Ageing and life history theory

The evolutionary theories of senescence form a subset of a set of concepts, collectively termed life history theory, which address age-specific variation in and interactions among the demographic (life-history) traits which determine fitness. Life history theory forms a fundamental component of evolutionary biology because the traits which it concerns, including the age at maturity and age-specific schedules of reproduction and survival, constitute the basis upon which selection acts. Roff (2002) outlined three major assumptions underlying life history theory: i) that there is some measure of fitness which is maximised by selection, ii) that the variety of possible life-history strategies is limited by constraints and trade-offs, and iii) that populations harbour sufficient genetic variation for the optimal life-history strategy to be attained.

As growth, reproduction and somatic maintenance and repair mechanisms require metabolic resources, physiological constraints and trade-offs are typically considered to arise because resources must be partitioned among different life-history traits (Stearns, 1992; Roff, 2002). Several studies have investigated resource allocation trade-offs (negative co-variances) by manipulating one trait and looking for a response in another. For instance, by modifying the number of eggs in each clutch, Lack (1954) exemplified that infant mortality increases as brood size rises above an assumed optimum in the common starling, *Sternus vulgaris*. A well established resource allocation trade-off arises from the cost of reproduction. Consistent with the disposable soma theory of ageing, increased reproductive output is associated with low subsequent fecundity and/or accelerated senescence and reduced longevity in a range of organisms (Williams, 1966; Reznick, 1985; Sgro & Partridge, 1999; Barnes & Partridge, 2003). Furthermore, the act of mating itself appears to reduce lifespan in a variety of organisms (Chapman *et al*., 1995; Gems & Riddle, 1996).

Phenotypic correlations do not necessarily imply causation, particularly when observed in quantitative traits that are influenced by a large number of genes (Reznick, 1985; Roff, 2002). Methods which investigate trade-offs by examining both phenotypic and genetic correlations between traits are considered to be more robust. Genetic correlations are thought to occur as a result of pleiotropic interactions or linkage disequilibrium, and can be examined via pedigree analyses or artificial selection (Roff, 2002). Artificial selection experiments have been used to investigate the antagonistic pleiotropy theory of ageing. For instance, in a long term experiment selecting *D. melanogaster* for late-life fecundity and increased longevity, early fecundity initially declined in long lived lines relative to the controls (Rose, 1984). Remarkably, after several years of continued selection, the decline in early fecundity was obscured when populations were maintained in favourable growth conditions, suggesting that the

manifestation of trade-offs between longevity and early components of fitness can be context dependent (Leroi *et al*., 1994). Negative genetic correlations can also be assessed by examining the consequences of single gene mutations which modify life-history traits in model organisms. As described in 1.32, trade-offs consistent with the antagonistic pleiotropy and disposable soma theories of ageing have been observed between longevity and early fitness components in numerous long-lived mutants (reviewed in Van Voorhies *et al*., 2006). Life history consequences of various mutations which promote longevity in the nematode *Caenorhabditis elegans* are investigated in chapter 3 of this thesis, and context dependent trade-offs are examined in chapters 4, 5 and 7.

To maximise fitness, resource allocation strategies must be optimised according to current ecological conditions. Consequently, life-history traits differ independently of genotype across a variety of environmental gradients. For instance, longevity can be modified in many organisms in response to environmental factors, such as food availability (reviewed in Guarente *et al*., 2008) and temperature (reviewed in Munch & Salinas, 2009). There are also extreme examples in which longevity differs dramatically among individuals regardless of genotype. For instance, in social insect colonies, queens can survive for extremely long periods relative to other castes (Keller & Genoud, 1997), and in the nematode *Strongyloides ratti*, free-living females live for only a few days whilst parasitic morphs can survive for over a year (Gardner *et al*., 2004). When traits exhibit discrete or continuous variation independently of genotype in response to different environmental cues, this is referred to as phenotypic plasticity. The set of phenotypes expressed by a single genotype across an environmental gradient is known as the reaction norm (Schmalhausen, 1949), and when reaction norms differ among genotypes a genotype-by-environment interaction (GEI) becomes manifest (Falconer, 1952). Understanding the genetic basis of phenotypic plasticity and GEI can provide important insights into the mechanisms underlying the evolution of longevity and other life-history traits in heterogeneous environments.

1.3 Phenotypic plasticity and the genetics of ageing

“Developmental biologists are prying open the black-box of life history; evolutionary biologists should rejoice”. Armand Leroi, 2001

1.31 From proximate mechanisms to ultimate causation

Biogerontology has traditionally been divided into two interrelated categories which focus on the causes of ageing from the molecular to the population level.

Mechanistic approaches focus on proximate causes during an individual lifetime, i.e. *how* ageing occurs, whereas evolutionary approaches attempt to explain ultimate causes, i.e. *why* ageing occurs. However, it is clear that in order to gain a comprehensive understanding of senescence both approaches must be considered simultaneously. The disposable soma theory provides a platform for integrating an evolutionary explanation for ageing with a more mechanistic understanding of the processes involved in senescence during an individual's lifetime.

There is considerable evidence to suggest that proximal causes of ageing during an individual lifetime involve the progressive accumulation of stochastic damage to cellular components, including nucleic acids, proteins and lipids (reviewed in Nemoto & Finkel, 2004; Kirkwood, 2005; Partridge & Gems, 2006). Though exposure to different endogenous and exogenous damaging agents can be species specific and/or context dependent, several basic principles are relevant to most eukaryotes. For instance, all aerobic organisms are exposed to reactive oxygen species (ROS) as a consequence of mitochondrial respiration. These constitute a major source of cellular and molecular damage (Finkel & Holbrook, 2000), and may play an important role in senescence (Harman, 1956). Furthermore, all organisms have the ability to produce a variety of highly conserved protective proteins, such as anti-oxidant enzymes, DNA repair enzymes and heat-shock proteins/molecular chaperones (Kültz, 2005). Longevity and the rate of senescence may thus be largely determined by the balance between the occurrence of damage and the ability to protect and repair somatic molecules and cells.

During the past two decades it has become increasingly apparent that a variety of interventions increase lifespan in model organisms ranging from yeast to mice, and that certain 'public' mechanisms which modulate the rate of senescence have been conserved among distinct eukaryotic lineages (reviewed in Partridge & Gems, 2002; Kenyon, 2005). These interventions are diverse and modify factors such as stress resistance, caloric intake, sensory perception, protein synthesis, mitochondrial function and telomere attrition (Guarente *et al*., 2008). Consistent with the disposable soma theory of ageing, several methods which can be applied to extend lifespan in model organisms appear to increase the allocation of metabolic resources to somatic maintenance and repair mechanisms.

1.32 Stress and the molecular determinants of longevity

Hutchinson (1957) made the distinction between the realized niche, the range of biotic and abiotic conditions to which an organism is most well adapted, and the fundamental niche, the range of conditions under which an organism can potentially

exist. Ecological stress can be regarded as a condition in which an organism is brought near to or over the edge of its fundamental niche (Van Straalen, 2003). Though the term 'stress' can refer to either an external factor or to an organism's internal state, from an ecological and evolutionary perspective these definitions are often viewed as integrative (Bijlsma & Loeschcke, 1997). In this thesis, 'environmental stress' is used synonymously with 'ecological stress' to refer to abiotic factors and/or an organism's response to adverse conditions. To distinguish between abiotic stress and nutritional stress, for the purposes of this thesis the latter is typically referred to as resource limitation, caloric restriction or starvation.

To maintain cellular homeostasis and the integrity of essential physiological functions, organisms possess a variety of cellular defence systems which, within limits, promote survival during periods of stress (Kültz, 2005). Whilst some of these are specific to particular forms of stress, others are more general and different systems may cooperate as a 'single, integrated, cellular stress-defence system' (Korsloot *et al*., 2004). Genetic manipulations which inappropriately activate stress response mechanisms in favourable growth conditions can extend longevity in a variety of model organisms (reviewed in Kenyon, 2005, 2010). These include mutations which disrupt sensory perception (Apfeld & Kenyon, 1999), or signal transduction and transcriptional regulation (reviewed in Kenyon, 2005; Baumeister *et al*., 2006), and modifications which cause genes encoding stress response proteins to be over-expressed (Orr & Sohal, 1994; Tissenbaum & Guarente, 2001; Walker & Lithgow, 2003). By inducing physiological shifts which enhance the protection and repair of somatic cells at the expense of growth and reproduction, such modifications typically incur trade-offs, consistent with the antagonistic pleiotropy and disposable soma theories of ageing, which reduce lifetime fitness (reviewed in Van Voorhies *et al*., 2006; Kenyon, 2010). However, although genetic manipulations which promote longevity by activating cellular defence mechanisms disrupt wild type responses to environmental change, trade-offs associated with increased lifespan have rarely been examined in heterogeneous environments which are likely to be experienced in nature. For a comprehensive understanding of the consequences of increasing longevity by manipulating such mechanisms, long-lived mutants should ideally be examined in an ecological context. This principle forms the basis of the studies described in this thesis.

Several genes which modulate longevity and the rate of senescence in model organisms encode components of signal transduction pathways which respond to environmental change. The most extensively characterised of these is the highly conserved insulin/insulin growth factor-1 (IGF-1) signalling (IIS) pathway. The role of this endocrine signalling pathway in the determination of lifespan first became apparent

in a study using *C. elegans* (Kenyon, 1993). It later became clear that the IIS pathway influences lifespan in the budding yeast, *Saccaromyces cerevisiae* (Fabrizio *et al*., 2001), *D. melanogaster* (Tatar *et al*., 2001), and the mouse, *Mus musculus* (Holzenberger *et al*., 2003), and there is evidence to suggest that it may also play an important role in human longevity (Bonafe *et al*., 2003; van Heemst *et al*., 2005). In distinct eukaryotic lineages, the IIS pathway regulates the activity of FOXO family transcription factors and the expression of genes involved in metabolism and cellular defence mechanisms (reviewed in Kenyon, 2005, 2010). Mutants which are defective in components of the IIS pathway are long-lived and stress resistant (Johnson *et al*., 2001), and typically display trade-offs consistent with the antagonistic pleiotropy and disposable soma theories of senescence (reviewed in Van Voorhies *et al*., 2006). Though it could be argued that the pathway evolved because its ability to limit lifespan has some adaptive benefit, it is more likely that it arose to promote survival during periods of environmental stress (Kenyon, 2010).

In *C. elegans*, the IIS pathway modulates development, metabolism, stress resistance and longevity. In conditions which are favourable for growth and reproduction, insulin-like ligands bind to the DAF-2 insulin/IGF-1 receptor on the cell membrane and initiate a kinase signalling cascade which negatively regulates the FOXO transcription factor DAF-16 (Lin *et al*., 1997; Ogg *et al.*, 1997). When the pathway is disrupted by low food availability, exposure to environmental stress or a mutation, DAF-16 translocates to the nucleus and activates the transcription of genes involved in cellular defence, metabolism and detoxification (Henderson & Johnson, 2001; Lin *et al*., 2001; Lee *et al*., 2003; Murphy *et al*., 2003; McElwee *et al*., 2004, 2007). By modifying DAF-16 activity, transitions in insulin/IGF-1 signalling thus appear to generate physiological shifts which either promote growth, reproduction and ageing or increase lifespan and stress resistance by enhancing the protection and repair of somatic molecules and cells (Henderson & Johnson, 2001) (Figure 1.1). Life history consequences of mutations which disrupt insulin/IGF-1 signalling in *C. elegans* are examined in favourable growth conditions in chapter 3, and in a variety of heterogeneous environments in chapters 4-7.

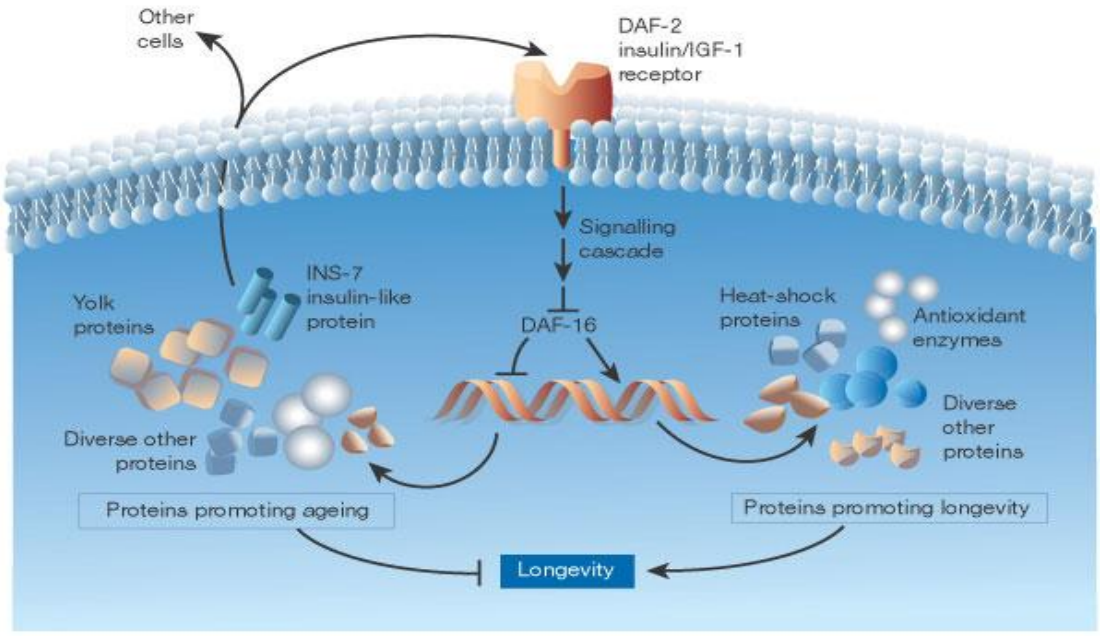


Figure 1.1: The insulin/IGF-1 signalling (IIS) pathway and DAF-16. In *Caenorhabditis elegans*, the IIS pathway regulates the activity of the transcription factor DAF-16. In favourable growth conditions, DAF-16 is inactive and genes encoding proteins which promote ageing are expressed. In harsh conditions, or when the IIS pathway is disrupted by a mutation, DAF-16 translocates into the nucleus and activates the expression of genes encoding proteins which promote longevity. From Gems and McElwee (2003).

1.33 Stress response hormesis

"That which does not kill us makes us stronger". Friedrich Nietzsche.

The term 'hormesis' is derived from toxicology and refers to the beneficial effects of low doses of toxins which are harmful at higher levels (Calabrese & Baldwin, 2003). In ecology, hormesis describes the phenomenon in which exposure to a mild stress during early life can enhance a trait such as fecundity, stress resistance or longevity at a later age (Forbes, 2000; Mangel, 2008). Hormetic effects upon longevity can be induced in a variety of organisms in response to environmental factors such as high temperatures or oxidative stress (reviewed in Gems & Partridge, 2008; Le Bourg, 2009). This may be because exposure to non-lethal stressors stimulates the expression of stress response proteins which enhance the protection of somatic molecules and cells (Gems & Partridge, 2008). It is important to make a distinction between the effects of mild stress and chronic stress. Whilst brief exposure to mild

forms of stress can promote longevity, prolonged exposure to stress is likely to have the opposite effect (Gems & Partridge, 2008; Monaghan *et al*., 2008). Few studies which have examined the effects of mild stress on lifespan have assessed how additional traits are modified (Forbes, 2000). However, it seems likely that treatments which temporarily increase the allocation of resources to somatic maintenance and repair functions should be associated with reductions in growth rate and/or reproduction. Consistent with this, relative to un-stressed controls, fecundity declined in *D. melanogaster* females following exposure to brief periods of heat-shock (Krebs & Loeschcke, 1994; Hercus *et al*., 2003).

1.34 Caloric restriction

Moderate reductions in caloric intake extend lifespan in a wide range of eukaryotes, including *S. cerevisiae* (Lin *et al*., 2000), *C. elegans* (Klass, 1977), *D. melanogaster* (Chippindale *et al*., 1993), rodents (Weindruch & Walford, 1988) and primates (Mattison *et al*., 2003). It has been proposed that this highly conserved form of hormesis may have evolved to promote survival and future reproductive capacity during periodic fluctuations in resource availability (Harrison & Archer, 1988; Holliday, 1989). Like other forms of mild stress, caloric restriction appears to induce the expression of genes involved in somatic maintenance and repair (reviewed in Kenyon, 2010). Consistent with the disposable soma theory, increased lifespan mediated by caloric restriction is often associated with trade-offs such as delayed maturation and reduced fecundity (Shanley & Kirkwood, 2000; Partridge *et al*., 2005). The life history consequences of a mutation which induces caloric restriction in *C. elegans* by reducing the rate of feeding are examined in chapter 3.

Research in model organisms has identified several genes which are required for life extension under calorie restricted feeding regimes. For instance, in *S. cerevisiae* this appears to be regulated, at least in part, through the action of *Sir2* (Lin *et al*., 2000). This gene encodes a histone deacetylase enzyme which physically modifies DNA by removing specific acetyl groups from histones surrounding the DNA molecules, and is thought to mediate transcriptional silencing (Hekimi & Guarente, 2003). Homologues of the *Sir2* gene also influence lifespan in *C. elegans* (Tissenbaum & Guarente, 2001), *D. melanogaster* (Rogina & Helfand, 2004), and possibly mice (Bordone *et al*., 2007). Interestingly, in *C. elegans*, lifespan extension caused by over-expression of *sir-2.1* is dependant upon *daf-16* (Tissenbaum & Guarente, 2001), and SIR-2.1 physically interacts with DAF-16 to modulate responses to certain forms of stress (Berdichevsky *et al*., 2006).

1.4 The role of ecology in ageing research

Contemporary biogerontology is largely biased towards research focusing on a small number of model organisms which are observed in laboratory conditions. Though remarkable advances have been made regarding our understanding of the molecular determinants of longevity, there are of course limitations associated with this approach. For instance, we cannot extrapolate much from model organisms about the extensive variation in longevity that exists within and among populations and species in nature (Partridge & Gems, 2007; Monaghan *et al*., 2008; Nussey *et al*., 2008). Furthermore, longevity and the rate of senescence are products of an organisms' ecological niche and are intrinsically linked to other components of life history and to fitness. These traits should therefore be examined in an ecological context, ideally in natural environments or in conditions which are representative of nature, in which other aspects of the life history are taken into account.

1.41 Senescence and selection in wild populations

Organisms live in heterogeneous environments which are likely to vary over time and space. Such variation can have considerable repercussions for population dynamics and impose different selection pressures on different components of life history. During an individual's lifetime, longevity can be affected by a variety of environmental factors, whether experienced directly at an early age or throughout life, or indirectly through the maternal environment (Metcalf & Monaghan, 2001). For instance, in a wild population of red deer, *Cervus elaphus*, females which were born when population density was high displayed more rapid rates of senescence than females which were born when population density was low (Nussey *et al*., 2007). Similarly, rates of reproductive senescence in the common guillemot, *Uria aalge*, were increased when harsh conditions were encountered early in life (Reed *et al*., 2007).

Environmental heterogeneity is also likely to have an impact upon longevity over multiple generations. As previously described, non-adaptive evolutionary theories of ageing predict that longevity can evolve in response to the level of extrinsic mortality experienced during recent evolutionary history due to changes in the dynamics of selection across the lifespan. Another theory, referred to as the ecological stress theory of ageing (Parsons, 1995, 2002), predicts that increased longevity may evolve in wild populations which experience harsh environmental conditions as a consequence of selection for increased resistance to stress. Consistent with this, variation in the ability to tolerate environmental stress appears to be correlated with differences in longevity among wild populations of *D. melanogaster* (Nevo *et al*., 1998), the nematode

Heterorhabditis bacteriophora (Grewal *et al*., 2002), and the gypsy moth *Lymantria dispar* (Lazarevic *et al*., 2007), and among closely related *Caenorhabditis* species (Amrit *et al*., 2010). Furthermore, artificial selection for resistance to stress has been associated with increased lifespan in the parasitic wasp *Aphytis lingnanensis* (White *et al*., 1970), *D. melanogaster* (Rose, 1992; Hoffman & Parsons, 1993) and the butterfly *Bicyclus anynana* (Pijpe *et al*., 2008). The ecological stress theory of ageing emphasises the importance of the energetic costs of living in heterogeneous environments, and considers that genotypes which utilise metabolic resources most efficiently over a range of environmental conditions should be long-lived and display greater survival than other genotypes during periods of stress (Parsons, 1995). The potential for the evolution of increased longevity in heterogeneous environments as a consequence of selection acting upon genetic variation in the ability to tolerate stress is examined in chapters 4, 5 and 7 of this thesis.

1.42 Ecological genetics: from molecules to populations

Recent advances in genetics and genomics have led to the emergence of a new integrative discipline, referred to as evolutionary and ecological functional genomics (EEFG), which 'focuses on the genes that affect evolutionary fitness in natural environments and populations' (Feder & Mitchell-Olds, 2003). This field aims to investigate the functional significance of genetic and genomic variation among individuals and populations in their natural environments and to elucidate the evolutionary processes which create and maintain this variation. As some important genetic and genomic technologies are currently limited to a few model organisms which are not well characterised in an ecological setting, considerable effort is required to establish EEFG research in non-model organisms (Van Straalen & Roelofs, 2006). However, there is also potential for using laboratory model systems to examine the functional relevance and fitness consequences of genetic variation in environments which may be representative of natural conditions. Although many genes which modulate longevity in model organisms modify responses to environmental change, fitness costs in long-lived mutants have typically been examined in benign laboratory conditions. In this thesis, an ecological genetics approach is used to examine how a mutation which increases lifespan in *C. elegans* alters molecular, individual and population level responses to environmental conditions which may be encountered by wild populations.

1.5 Aims and thesis structure

The studies which are described in this thesis were performed with the overall objective of investigating the life history consequences of mutations which extend longevity in an ecologically relevant context. These studies encompass a variety of methods, from genetic approaches which were used to examine the underlying molecular basis of life history variation in different environments, to demographic approaches which were used to make inferences regarding the conditions under which increased longevity can evolve.

Chapter 2 introduces the study system, *C. elegans*, and describes some of the attributes which make this organism of great value in contemporary biology and in ageing research. It provides an overview of the life history and discusses what is known regarding the natural habitat and diversity of *C. elegans* outside of the laboratory. It finishes with a description of standard methods which are applicable to chapters 3-6.

Chapter 3 describes a comprehensive comparison of age-specific changes in fecundity and survival, in favourable growth conditions, among a variety of long-lived mutants and the wild type strain. Differences between the genotypes are discussed in the context of selection and the evolutionary theories of senescence.

Using a multidisciplinary approach, chapters 4 and 5 examine the manifestation of trade-offs in long-lived *age-1(hx546)* mutants in ecologically relevant conditions, and consider the potential for the evolution of increased longevity in environments in which resource availability and abiotic factors vary over time. To do this, molecular to population level responses to environmental and nutritional stress were compared between *age-1(hx546)* mutants and wild type worms. Chapter 4 considers responses to thermal stress and chapter 5 deals with responses to oxidative stress.

Chapter 6 describes a previously unreported phenomenon, that long-lived *age-1(hx546)* mutants display increased resistance to low temperatures relative to wild type worms. A combination of loss-of-function mutations and RNA interference was used to examine the contribution of desaturase enzymes to the cold tolerant phenotype observed in these mutants.

Chapter 7 uses a demographic framework to assess how exposure to stochastic environments can influence the evolution of longevity in *C. elegans*. Using data which were reported in chapters 4 and 5 to construct population projection matrices, the invasion potential of the *age-1(hx546)* mutant allele was examined under a variety of conditions in which periods of resource limitation and environmental stress were encountered at random intervals.

Chapter 8 summarises the results presented in the five data chapters and discusses the implications of these with respect to the evolution of senescence and stress resistance in natural populations. This chapter also reiterates the importance of evolutionary ecology and ecological genetics in the future of ageing research.

Chapter 2 – The study system

2.1 *C. elegans* as a model organism

"You have made your way from worm to man, and much within you is still worm."
Friedrich Nietzsche

Caenorhabditis elegans is a simple, free living nematode worm which feeds on micro-organisms and is found in temperate regions and some tropical regions throughout the world. Populations are androdioecious, i.e. consist of both males and hermaphrodites; however, males are rarely encountered and hermaphrodites reproduce mainly through self fertilisation. *C. elegans* was first established as a model organism in the late 1960s, by the acclaimed geneticist Sydney Brenner, primarily due to its simplicity and potential for addressing questions regarding development, neurobiology and genetics (Brenner, 1974). It has since become perhaps the most well characterised multi-cellular eukaryote and, during the past two decades, has acquired a fundamental role in ageing research. Numerous attributes contribute to its suitability as a model organism. It is small in size (mature adults are ~1mm in length), has a rapid life-cycle (~3 days at 20°C) and short lifespan (~3 weeks at 20°C), and requires only a humid environment, a bacterial food source and atmospheric oxygen for growth (Hope, 1999). Furthermore, it is possible to maintain stocks indefinitely by freezing in liquid nitrogen (Brenner 1974).

C. elegans is the only metazoan in which the entire cell lineage (959 cells in hermaphrodites and 1031 cells in males) has been traced from egg to adult (Sulston & Horvitz, 1977), a feat made possible by complete transparency at all stages of development. This is relevant to the study of vertebrate development as *C. elegans* contains highly differentiated and specialised cell types, such as neurons and muscle cells, of a similar form to those found in higher eukaryotes (Hope, 1999). *C. elegans* was the first multi-cellular eukaryote for which the entire genome was sequenced (The *C. elegans* genome consortium, 1998), and 60-80% of human genes have *C. elegans* orthologues (Kaletta & Hengartner, 2006). Forward and reverse genetics approaches are well established in *C. elegans* research and provide the means to functionally annotate many of the approximately 22,000 protein-coding genes, in the 100 megabase (Mb) genome, through phenotypic observations. Knockout mutations can be generated using methods such as chemical mutagenesis, ionising radiation or transposon hopping, and the attainment of homozygous mutants is facilitated by the hermaphrodite mode of reproduction following mendelian segregation of the diploid

genome. RNA interference (RNAi), a method of post-transcriptional gene silencing which was initially discovered in the worm (Fire & Mello, 1998), can be administered by feeding, injection or immersion to suppress the expression of specific genes, and the relative ease of germ-line transformations can be exploited to create transgenic lines for a variety of purposes. The standard wild type genotype, N2, was isolated from mushroom compost in Bristol in 1956. Except when stated otherwise, information presented throughout this thesis refers to observations made using N2 and mutant derivatives of this strain.

2.2 Life history and phenotypic plasticity

2.21 Growth in favourable environments

In favourable conditions the *C. elegans* lifecycle is rapid, taking approximately three days from egg to reproductive adult at 20°C (Figure 2.1). Embryogenesis begins within the hermaphrodite uterus and is completed externally. After hatching, larvae progress through four stages (L1-L4) which are separated by successive moults then mature as fertile adults (Figure 2.1). In hermaphrodites, a limited number of sperm are produced during L4 then, from the same germ-line tissue, a larger number of oocytes. Approximately 200-300 offspring are produced over 3-4 days. After sperm has been depleted, post-reproductive adults continue to lay unfertilised oocytes for several days (Kadandale & Singson, 2004), then can survive for an additional 1-2 weeks before dying of old age.

Males arise at a very low frequency (~ 0.2%), following non-disjunction of the X chromosomes during meiosis (Hodgkin, 1983), but can substantially increase hermaphrodite fecundity. Following a successful mating, the larger male sperm out-competes the hermaphrodite self-sperm (LaMunyon & Ward, 1997), and 50% of the resulting offspring are male. However, the increased proportion of males rapidly declines over a few generations (Stewart & Phillips, 2002). This may be because hermaphrodites that are mated make use of their own sperm, in addition to the more competitive male sperm, and thus a greater number of hermaphrodite than male offspring are produced (Wegewitz *et al*., 2008).

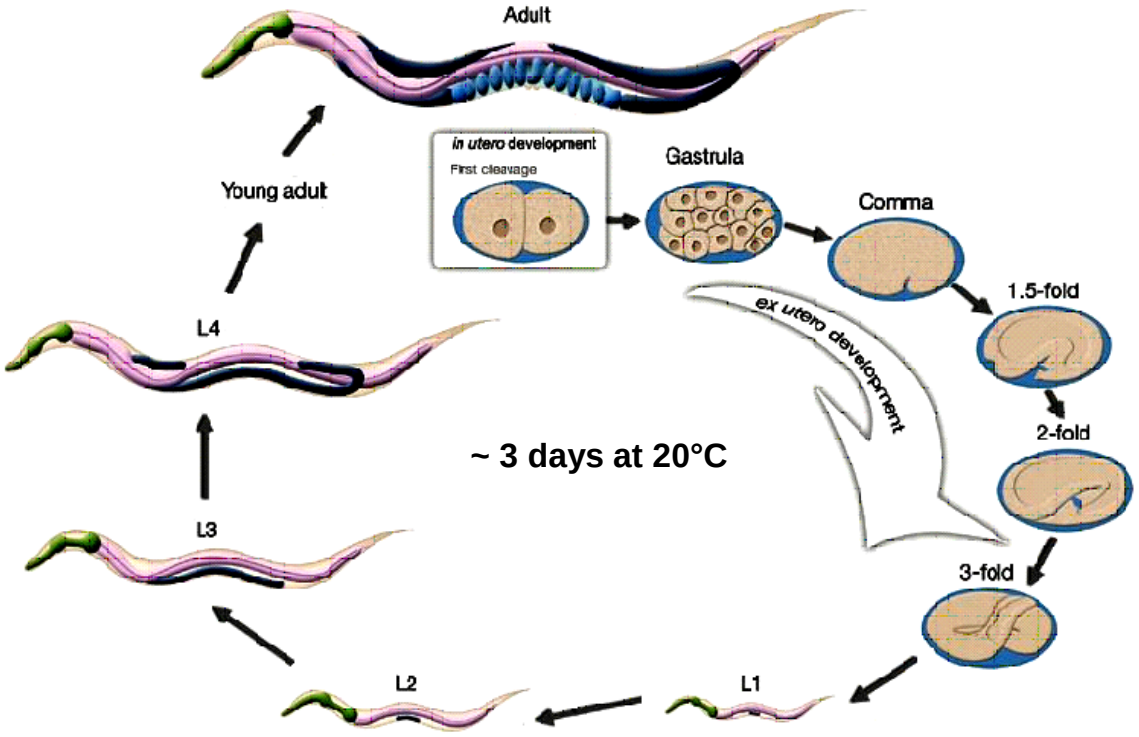


Figure 2.1: The *C. elegans* life-cycle in favourable growth conditions

(adapted from www.wormatlas.org)

As it is likely that the majority of hermaphrodites progress throughout life without encountering a single male, it could be suggested that producing a larger number of self-sperm may confer a selective advantage by increasing fecundity. However, additional spermatogenesis delays the onset of oogenesis and the number of self-sperm has likely been optimised for early maturity (Hodgkin and Barnes, 1991). Though the continued production of oocytes after the depletion of self-sperm may reflect a 'bet hedging' strategy, whereby if a male is encountered hermaphrodites can recommence reproduction at a later age, it is perhaps more likely that the excess number of oocytes produced in laboratory conditions reflects upon the environment. Indeed, there is evidence to suggest that reproduction is only sperm limited when excess food is available (Goranson *et al*., 2005).

2.22 Dealing with stress

In unfavourable growth conditions, *C. elegans* display several discrete forms of phenotypic plasticity (Figure 2.2). Some of these involve entrance into states of diapause during which survival is dramatically prolonged beyond the 'normal' lifespan observed in favourable conditions. Studies of the molecular basis of phenotypic

plasticity in *C. elegans* have become prominent in biogerontology, and have provided many important insights regarding the determinants of longevity.

i) L1 arrest

If eggs hatch in the absence of food, development arrests and starved L1s can survive in a state of diapause for several weeks until conditions improve (Johnson *et al*., 1984). Starved L1s respond rapidly when food becomes available (Baugh *et al*., 2009), which likely reflects strong selection for rapid growth when conditions are permitting. L1 arrest is, at least in part, regulated by insulin/IGF-1 signalling and the FOXO transcription factor DAF-16 (Baugh & Sternberg, 2006). Consistent with the role of insulin/IGF-1 signalling and DAF-16 in stress resistance as described in chapter 1, starved L1s display increased tolerance to various environmental challenges relative to fed L1s (Derry *et al*., 2001; Baugh & Sternberg, 2006; Weinkove *et al*., 2006). Baugh and colleagues (2009) identified additional signalling pathways and transcription factors which are activated during L1 arrest. These include the target-of-rapamycin (TOR) pathway and SIR-2.1, both of which are known to interact with insulin signalling and DAF-16 (Jia *et al*., 2004; Berdichevsky *et al*., 2006), and NHR-49, a key regulator of fatty acid metabolism. Importantly, all of these have been implicated in stress responses and/or the maintenance of energy homeostasis, and are known to play a role in the determination of longevity (Tissenbaum & Guarente, 2001; Vellai *et al*., 2003; Van Gilst *et al*., 2005a).

ii) Dauer formation

In response to low food availability, high population density and high temperature, larvae can arrest in a morphologically distinct state of diapause known as the dauer stage (Cassada & Russell, 1975; Golden & Riddle, 1982; Golden & Riddle, 1984). Population density is perceived via concentrations of a pheromone which is constitutively produced by all worms, and entry into the dauer stage is largely determined by the ratio of this pheromone to food availability (Golden & Riddle, 1982). The dauer state is a non-feeding alternative to the third larval stage and is specialised for endurance in harsh environments. Dauers are highly resistant to various forms of stress and can survive for several months in anticipation of more favourable conditions (Golden & Riddle, 1984). Fat reserves are stored during entry into the dauer stage and metabolism is suppressed to promote long-term survival (Holt & Riddle, 2003). Dauers are thought to be analogous to the infective larvae of many parasitic nematodes (Burglin *et al*., 1998; Ogawa *et al*., 2009), and, though they often appear to be

motionless, are likely to be important for dispersal to new food patches (Cassada & Russell, 1975).

The decision to enter the dauer stage is made early in development and is under neuroendocrine control. Mutant analyses have identified 36 'daf' genes which are involved in dauer formation, including *daf-23* which is more often referred to as *age-1* and is discussed throughout this thesis. Epistatic analyses of these genes have revealed that dauer formation is regulated by conserved signal transduction cascades, including the insulin / IGF-1 signalling (IIS) pathway (Kimura *et al*., 1997), a transforming growth factor (TGF-β) pathway (Ren *et al*., 1996), a guanylyl cyclase pathway (Birnby *et al*., 2000) and a steroid hormone pathway (Jia *et al*., 2002), which control the activation of transcription factors, including the aforementioned DAF-16 (Kimura *et al*., 1997), and the nuclear hormone receptor DAF-12 (Antebi *et al*., 2000). Prolonged survival is dependent on an additional signalling cascade, known as the adenosine monophosphate-activated protein kinase (AMPK) pathway, which down-regulates lipid hydrolysis in adipose-like tissues and prevents the rapid depletion of energy reserves (Narbonne & Roy, 2009). Investigating the molecular basis of dauer formation and survival has been a major focus of ageing research and several of the genes involved have been implicated in the determination of longevity (reviewed in Fielenbach & Antebi, 2008).

iii) Reproductive diapause

The adult reproductive diapause state is a form of arrest observed in young adults which have been starved during L4 (Angelo & Van Gilst, 2009). Development of the first few fertilised embryos arrests within the hermaphrodite uterus and the onset of reproduction is delayed until conditions improve. A striking feature of reproductive diapause is the apoptotic loss of the germ-line (Angelo & Van Gilst, 2009). A small population of germ-line stem cells is maintained from which the germ-line can be regenerated when food becomes available. Angelo and Van Gilst (2009) proposed a 'disposable germ-line' hypothesis, whereby autophagy and/or phagocytosis of oocytes and meiotic germ cells provide the nutrients necessary for survival and viability during diapause. As somatic cells are post-mitotic in adult worms, germ cells are the only cells which can provide a source of nutrition whilst retaining the ability to regenerate. Angelo and Van Gilst (2009) demonstrated that the nuclear hormone receptor gene *nhr-49* is required for entry into reproductive diapause. This transcription factor is known to be a key regulator of glucose and fat metabolism during starvation and, as previously mentioned, has a role in L1 arrest and longevity (Van Gilst *et al*., 2005a).

iv) Facultative vivipary

When gravid hermaphrodites are deprived of food, eggs are retained until they hatch internally. Larvae consume the parents' body contents and, depending on the number developing within a single adult, can use the resources to reach the dauer stage (Chen & Caswell-Chen, 2003). This switch from ovipary (egg laying) to facultative vivipary (internal hatching) is commonly referred to as 'bagging' because the adult body resembles a bag of worms. Internal hatching has been implicated in the evolution of lifespan in *C. elegans* hermaphrodites (McCulloch & Gems, 2003a). This is because internal hatching represents an important sex-specific source of mortality which may increase the age-specific rate of decline in the efficiency of selection in hermaphrodites relative to males. Consistent with this, males from a variety of wild populations are longer lived than isogenic hermaphrodites (McCulloch & Gems, 2003a).

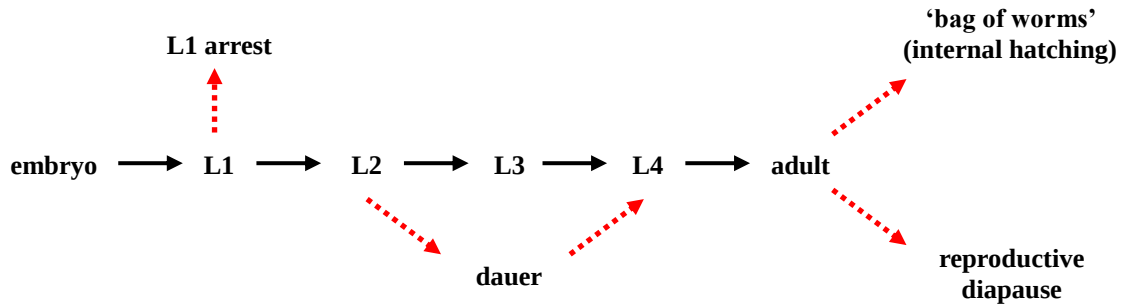


Figure 2.2: Plasticity in response to nutritional and environmental stress. Solid black arrows represent lifecycle stages in favourable conditions whilst broken red arrows represent forms of plasticity observed in harsh environments (adapted from Angelo & Gilst, 2009).

2.23 Plasticity in quantitative traits

C. elegans demonstrate continuous plasticity in quantitative traits such as the age at maturity, body size at maturity, fecundity and lifespan, in response to nutrient availability, temperature and other environmental variables (Goranson *et al*., 2005; Gutteling *et al.*, 2007; Harvey *et al*., 2007, 2008). If genetic variation in the phenotypes expressed in different conditions exists in nature, plasticity in quantitative traits can have an important role in the evolution of life-history strategies in heterogeneous environments. This is discussed further in section 2.32.

2.3 Beyond the laboratory

Very little is known about *C. elegans* natural ecology. Though a few attempts have been made to simulate natural conditions (Goranson *et al*., 2005; Van Voorhies *et al*., 2005) and to observe populations in nature (Barrière & Félix, 2007), *C. elegans* is typically observed in benign laboratory conditions. Whilst this does not necessarily present a problem for disciplines concerned with highly conserved developmental processes, it can become an issue for studies of a more ecological nature. Furthermore, as the expression and/or function of many genes may be context dependent, this lack of knowledge presents a hindrance to ongoing attempts to annotate the genome. As most of the major laboratory model organisms are not well characterised in their natural environments (Peňa-Castillo & Hughes, 2007), this issue is not specific to *C. elegans*.

2.31 Natural habitat and diet

Wild strains have been isolated from anthropogenic habitats such as garden soil, compost heaps and mushroom beds, in geographic locations as diverse as Hawaii, the UK, continental Europe, North America, South Africa, Japan and Australia (Barrière & Félix, 2005a). They have also been collected from terrestrial arthropods and gastropods, suggesting either a phoretic (aiding dispersal) or necromenic (providing a food source following death of the host) association or both (Baird, 1999). Worms have generally been isolated from nature in the dauer stage (Barrière & Félix, 2005b), consistent with the role of dauers in dispersal and indicative of 'boom and bust' population dynamics. Free-living nematodes such as *C. elegans* are likely to be susceptible to a variety of extrinsic hazards which may have an impact upon the dynamics of selection across the lifespan. These include predation by micro-invertebrate predators and nematode trapping fungi, pathogen infections, intra and interspecific competition for limited resources, and frequent fluctuations in abiotic conditions, such as temperature, oxygen concentrations and humidity (Neher, 2010).

In the laboratory *C. elegans* is typically provided with *Escherichia coli* as a food source. However, this bacterium does not constitute a natural food source and many different species of bacteria are likely to be encountered by *C. elegans* in nature. Whilst some of these will be nutritious and readily digested, others may not support rapid growth and many could be pathogenic. The nutritional content and/or pathogenicity of different species of bacteria are likely to have an impact upon *C. elegans* life history. Consistent with this, Coolon *et al*. (2009) recently demonstated that several genes involved in metabolism and immunity, which are differentially regulated in response to

feeding upon different soil bacteria, have functional significance in the determination of life-history traits in specific bacterial environments.

2.32 Intra-specific variation

It has been suggested that the standard wild type stain, N2, may inadequately represent a natural population because it passed through a large number of generations in laboratory conditions before a freezing protocol was developed (Reznick & Gershon, 1999; Gershon & Gershon, 2002; Chen *et al*., 2005). Being subject to inadvertent selection and genetic drift, and passing through numerous population bottlenecks, it is plausible that some genetic and phenotypic characteristics may have diverged from their original states. This may be particularly true of life-history traits as these are influenced by many genes and, therefore, constitute a large mutational target (Delattre and Felix, 2001). Indeed, there is evidence to suggest that the N2 lifespan, though variable among different laboratories, is short relative to other wild isolates (Gems & Riddle, 2000). Making use of the available wild isolates provides the means to extrapolate information regarding intra-specific variation in genetic and phenotypic characteristics.

Numerous phenotypic characteristics vary among wild isolate genotypes. For instance, wild populations exhibit either ‘solitary’ or ‘social’ foraging behaviours, in which individuals either forage in isolation or cluster together in groups around the edges of the bacterial food source (De Bono & Bargmann, 1998). Several aspects related to mating also differ among wild populations. The proportion of males varies (Teotónio *et al*., 2006), as does male mating efficiency (Wegewitz *et al*., 2008), and males of some wild strains deposit a copulatory plug onto the hermaphrodite vulva after mating (Hodgkin & Doniach, 1997). Age at maturity, lifetime fecundity and adult body size differ among wild isolates in constant laboratory conditions (Hodgkin & Doniach, 1997). There is also considerable variation in lifespan among different wild genotypes (McCulloch & Gems, 2003b). These disparities may reflect upon different selection pressures in the ecological conditions to which the wild populations were adapted in nature. Genotype-by-environment interactions have also been observed in several ecologically relevant traits. For example, different genotypes display variation in pathogen resistance (Schulenberg & Ewbank, 2004; Schulenberg & Muller, 2004), in the propensity to form dauers when exposed to different concentrations of dauer pheromone (Viney *et al*., 2003), and in life history responses to different temperatures (Harvey *et al*., 2007). Using recombinant inbred lines (RILs) derived from crosses between different wild isolate genotypes, the molecular basis of such plasticity has

been examined through the identification of quantitative trait loci (QTL) (Shook *et al*., 1996; Kammenga et al., 2007; Gutteling *et al*., 2007; Harvey *et al*., 2008).

Considerable genetic and genomic variation exists among wild isolate genotypes. For instance, some wild genotypes have a high copy number of active Tc1 transposons present in the genome relative to others (Hodgkin & Doniach, 1997). Wild isolates also vary in susceptibility to RNAi inactivation of genes expressed in the germ-line (Tijsterman *et al*., 2002), genome size (Maydan *et al*., 2007), and copy number of over 5% of genes in the genome (Maydan *et al*., 2010). Furthermore, some strains display evidence of genetic incompatibility (Dolgin *et al*., 2007; Seidel *et al*., 2008).

2.33 Population structure

The same mitochondrial (Denver *et al*., 2003), microsatellite (Sivarsundar & Hey, 2003) and AFLP (Barrière & Felix, 2005b) variants have been found in North America and in Europe. Such patterns of variation are indicative of metapopulation dynamics, characterised by low global diversity, long distance migrations and frequent local extinctions (Sivasundar & Hey, 2003; Cutter, 2005). Given the anthropogenic habitat of *C. elegans* wild isolates, it is concievable that dispersal between continents may be facilitated by human activity. Outbreeding frequencies are determined by the rate of spontaneous production of males and male mating efficiency, and can be investigated by examining levels of heterozygosity or linkage disequilibrium. Though males occur at a very low frequency in laboratory conditions, the rate at which outbreeding occurs in nature remains unclear. Depending upon the methods and populations used for analyses, estimates have ranged from 0.1% (Barrière & Félix, 2005b) to 20% (Sivasundar & Hey, 2005). Morran *et al*. (2009) recently suggested that outcrossing may be facultative in some environmental conditions because, in some wild isolates, the proportion of males can be increased substantially following periods of starvation. Furthermore, *C. elegans* appear to exhibit plasticity in sex determination as, in specific bacterial environments, hermaphrodite offspring derived from a mating event can lose an X chromosome during the L1 stage and subsequently develop into males (Prahlad *et al*., 2003).

2.4 *C. elegans* in ageing research

C. elegans is often considered to be the premier model system used in ageing research (Johnson, 2008). In addition to the rapid generation time and short lifespan, this is, at least in part, due to the many single gene mutations which increase lifespan that have been identified and characterized in this organism. *C. elegans* exhibit several

phenotypes associated with senescence which are observed in other organisms, including humans. For example, feeding and mobility decline (Klass, 1977), muscle mass degenerates (Herndon *et al*., 2002), an ageing pigment known as lipofuscin accumulates (Klass, 1977) and susceptibility to infection increases (Herndon *et al*., 2002). *C. elegans* also display symptoms of reproductive senescence which occur even when sperm is not limited (Hughes *et al*., 2006).

Although *C. elegans* has proved to be an excellent system for examining the molecular determinants of longevity, there are disadvantages associated with using this model in ageing research. The most apparent of these are the poor understanding of *C. elegans* ecology and concurrent lack of knowledge regarding the selective pressures which are relevant in natural populations. There is also a distinct lack of information regarding how genes which modulate longevity influence fitness in ecologically relevant conditions. Due to the major emphasis on a single wild type genotype, there is currently no information available concerning natural variation in candidate genes involved in the determination of longevity. It is thus unclear how epistatic interactions between these genes and other components of the genome may influence lifespan in different wild isolate strains. This may be important as studies in *Drosophila* have indicated that the effects of single gene mutations upon longevity may be genotype and/or sex dependent (Spencer *et al*., 2003; Burger & Promislow, 2004). Despite these issues, the value in obtaining data from different wild populations is increasingly being acknowledged. For instance, there has recently been a study investigating the life extending effects of caloric restriction in wild isolate genotypes (Sutphin & Kaeberlein, 2008), and efforts are currently underway to obtain complete genome sequences for a variety of wild strains.

2.5 Standard methods in *C. elegans* research

This section describes some basic procedures which are relevant for chapters 3-6.

2.51 Obtaining and storing strains

The Caenorhabditis Genetics Centre (CGC) at the University of Minnesota stores an abundance of different genotypes, including the standard *C. elegans* wild type (N2) and mutant derivatives of this strain, transformed lines expressing reporters fused to genes of interest, wild isolates from various geographic origins, and a variety of closely related *Caenorhabditis* species. Any of these can be requested by email and received by post within 2-3 weeks.

Strains were stored at -80°C and in liquid nitrogen. To prepare strains for freezing, populations had to be recently starved. Populations were washed off agar plates in 1 ml of M9 buffer solution (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1 ml $MgSO_4$, diluted to 1 litre with H_2O and sterilised by autoclaving) and centrifuged briefly at 2000rpm. After removing 0.5 ml of the supernatant, 0.5 ml of Liquid Freezing Solution (129 ml 0.05 M K_2HPO_4, 871 ml 0.05 M KH_2PO_4, 5.85g NaCl, pH 6 + 30% glycerol (v/v) and sterilised by autoclaving) was added and the mixture was vortexed (Stiernagle, 1999). Aliquots of 0.2 ml were then placed into two cryovial tubes and three standard Eppendorf tubes. These were placed in styrofoam boxes to prevent rapid freezing and were maintained at -80°C overnight. The following day, one Eppendorf tube was removed and the worms were defrosted to ensure that some had survived. Remaining eppendorf tubes were then transferred to permanent locations at -80°C and cryovial tubes were placed in liquid nitrogen.

2.52 Preparation of NGM agar plates and the food source

Worms were maintained on Nematode Growth Medium (NGM) agar in petri dishes which were 6cm in diameter. To prepare the NGM, 3g NaCl, 17g agar and 2.5g peptone were dissolved in H_2O and diluted to 1 litre then autoclaved. After cooling to approximately 55°C, 1 ml 1 M MgSO4, 1 ml 1M CaCl, 1 ml 5 mg/ml cholesterol in ethanol, and 25 ml 1 M KPO4 buffer were added and the solution was mixed (Stiernagle, 1999). For the experiments described in this thesis, 50 mg/l streptomycin and 10 mg/l nystatin were also added to the NGM before plates were poured.

Worms were provided with *E. coli* as a food source. Though the OP50 strain is most frequently used, the experiments described in this thesis were performed using a streptomycin and nystatin resistant strain known as HB101, which was kindly provided by Dr Jolanta Polanowska. It was chosen over OP50 due to problems with contamination during multi-generation experiments involving large populations. Brooks *et al*. (2009) recently demonstrated that HB101 has higher levels of monounsaturated fatty acids and higher carbohydrate content than OP50, but that the different diets have no significant effects upon lifespan. However, wild type worms grow more rapidly on HB101 than OP50 (Soukas *et al*., 2009). Stocks of HB101 were maintained on LB agar plates (10g Bacto-tryptone, 5g Bacto-yeast, 5g NaCL and 15g agar, diluted to 1 litre with H_2O then autoclaved) at 4°C (Stiernagle, 1999). Liquid media stocks were obtained by inoculating L Broth (10g Bacto-tryptone, 5g Bacto-yeast and 5g NaCl, diluted to 1 litre with H_2O and brought to pH 7.0 using 1 M NaOH then autoclaved) with a few colonies of HB101, and incubating at 37°C overnight (Stiernagle, 1999). These solutions were used to seed NGM agar plates, with added streptomycin and nystatin,

by pipetting approximately 200 µl onto the centre of the agar and allowing a bacterial lawn to grow overnight.

2.53 Observing, culturing and transferring *C. elegans*

For the experiments described in this thesis, worms were typically observed using a stereomicroscope with a zoom magnification system. Strains were maintained at 20°C except when stated otherwise. A platinum 'worm pick' which was shaped into a microspatula and was regularly sterilised in a Bunsen burner flame was used to transfer *C. elegans* from one agar plate to another.

2.54 Age synchronisation

Age-synchronised larvae were typically obtained by transferring large numbers of eggs, from well fed young adults, onto new seeded plates and returning after 1-2 hours to collect the hatched L1s. Whilst transferring the eggs, care was taken to avoid picking up young larvae which had already hatched. Larvae can also be synchronised using a bleaching protocol to isolate eggs then allowing L1s to hatch in the absence of food (Sulston & Hodgkin 1988).

2.55 Fluorescence microscopy

Populations were washed off NGM plates in M9 buffer solution and transferred to sterile eppendorf tubes. After allowing 1-2 minutes for worms to settle at the bottom of the tubes, worms were spotted onto 8-well slides and were paralysed to facilitate observations by adding 5mM levamisole. Glass coverslips were placed over each slide. Epifluorescence imaging was performed using a Leica DMR HC confocal microscope and photographs were taken using a Photometrics CoolSNAP camera and Improvision Openlab software.

2.56 Obtaining males and crossing genotypes

Males can be easily identified by tail shape due to the presence of posterior rays. Males were obtained by exposing young adult hermaphrodites to heat-shock at 30°C for approximately 6 hours. This treatment increases the likelihood of non-disjunction of the sex chromosomes and typically 2-5% of the offspring are male (Hodgkin, 1999). When large numbers of males were required, several males were transferred onto an agar plate containing a small lawn of bacteria, 1-2 cm in diameter, and two to three hermaphrodites at the L4 stage. After successful matings, typically 50% of the offspring in the next generation were male.

Crosses between hermaphrodites and males of different genotypes were performed by setting up mating plates with a small lawn of bacteria as described above. F1 generations were monitored for the presence of males to determine if mating had occurred, then hermaphrodites were transferred onto seperate plates to self-fertilise. F2 generations contained a mixture of homozygote and heterozygote individuals for the locus of interest. When possible, homozygote mutants were identified by screening individuals for a particular phenotype (many of the crosses performed for experiments in this thesis could be examined by monitoring dauer formation at 27ºC) and successful crosses were later confirmed using PCR.

2.57 Single worm PCRs

Immediately prior to use, 1 µl of 50 µg/µl proteinase K was added to 100 µl Lysis buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol). For each PCR reaction, 2 µl of this solution was then pipetted into a 200 µl PCR tube and a single worm was picked into the solution. Worms were tapped to the bottom of the PCR tubes then were placed in a freezer at -80°C for approximately 30 minutes. Tubes were transferred to a PCR machine and were heated to 60°C for 1 hr, then to 95°C for 15 mins to lyse the worms and release the genomic DNA. 28 µl of PCR mix (18.95µl distilled H_2O, 3µl PCR buffer, 1.2µl $MgCl_2$, 0.6µl of each 10 µM dNTP, 1.5µl of each 10pM primer (Integrated DNA Technologies) and 1.25µl red *Taq* DNA polymerase) was then added to each tube, giving a total volume of 30 µl per PCR reaction. PCR reactions were run for 35 cycles with an extension time of 1 min at 50°C (times and temperatures varied for some reactions). 6µl of each PCR product and 6µl of a 1 kb DNA ladder were run on a 1% (w/v) agarose gel (0.55g agarose, 55 ml TAE, 3µl 10 mg/ml ethidium bromide) using electrophoresis (~100 volts) for 20-30 mins. DNA bands were then viewed under UV light. For deletions it was possible to detect the presence of a mutation by comparing band sizes with wild type controls. With single base mutations it was necessary to prepare the DNA for sequencing. DNA bands were cut out under UV light using a sterile scalpel. PCR products were then purified from agarose gels using a PCR Purification Kit (QIAGEN Ltd) and the DNA was diluted to appropriate concentrations with distilled H_2O and sent to the University of Leeds sequencing centre.

2.58 RNAi by feeding

RNAi was induced by feeding using clones obtained from the Ahringer library (Kamath *et al*., 2003). Worms were provided with HT115, an IPTG inducible and RNase III deficient *E. coli* strain which has been transformed with the vector L4440 to contain

specific *C. elegans* genome fragments. The bacteria express double-stranded (ds) RNA corresponding to the transcript sequence of the gene of interest. Worms provided with HT115 containing empty vectors (L4440) were used as negative controls in each RNAi experiment.

a) Restriction digests

To ensure that the RNAi clones were correct, the presence of specific genomic fragments was assessed using restriction digest. After removal from -80°C, clones were streaked onto LB agar plates (see 2.52) containing 50 µg/ml ampicillin and 12.5 µg/ml tetracycline and were left to grow overnight at 37°C. A single colony was then transferred into 3 mls LB solution (see 2.52) containing 50 µg/ml ampicillin and was left to grow overnight in an incubator shaker at 37°C. Plasmid DNA was extracted using a QIAprep *Spin Miniprep* Kit (QIAGEN Ltd). For each digest, 3 µl of DNA was mixed with 1.5 µl Bovine Serum Albumin (BSA), 0.5 µl restriction enzyme, 1.5 µl buffer and 8.5 µl distilled H_2O. Restriction enzymes and corresponding buffers were selected using NEB cutter. After incubating for 2-3 hours at 37°C, solutions containing the digested plasmids were run on a 1% (w/v) agarose gel (see 2.57) with a 1kb DNA ladder using electrophoresis (~100 volts) for 20-30 mins. DNA bands were viewed under UV light and sizes were assessed to confirm the presence of the appropriate genomic fragment.

b) Peparation of RNAi agar plates

RNAi agar was prepared by adding 50 µg/ml ampicillin, 12.5 µg/ml tetracycline and 1 mM IPTG to standard NGM (see 2.52). Plates were always poured at least 4 days before seeding.

c) Feeding protocol

Liquid solutions were obtained for each gene by transferring colonies from LB agar plates to L Broth (see 2.52) containing 50 µg/ml ampicillin and allowing the colonies to grow overnight (fresh solutions were prepared for each batch of plates). Plates were then seeded with 400-500 µl of the relevant bacterial solution and left to dry overnight. The following day a few young adults were transferred to each plate. To minimize the amount of HB101 that was transferred from the original plates, only worms which had moved off the bacterial lawn were selected. After several hours these worms were removed and their progeny were left to develop for several days (times differed according to genotype). When these worms had attained maturity, progeny from the next generation were transferred to new RNAi plates seeded with the same bacteria and were left to develop before being screened for the relevant phenotype.

Chapter 3 – Demographic consequences of increased longevity in *Caenorhabditis elegans*

3.1 Abstract

Mutations which activate stress responses, reduce caloric intake or impair mitochondrial function promote longevity in model organisms ranging from yeast to mice. To assess how these processes alter additional components of life history, and how variation in age-specific fecundity and survival modify fitness and the dynamics of selection, in this study a range of long-lived *C. elegans* mutants were compared with the wild type genotype in favourable growth conditions. Consistent with the antagonistic pleiotropy and disposable soma theories of ageing, trade-offs including delayed maturity and reduced early fecundity were observed in stress resistant *daf-2* mutants with reduced insulin/IGF-1 signalling, calorie restricted *eat-2* mutants and *clk-1* mutants with impaired mitochondrial function. Relative to the wild type genotype, these trade-offs reduced lifetime fitness by 18% in *daf-2* mutants, by 30% in *eat -2* mutants and by 37% in *clk-1* mutants. In contrast, long-lived, stress resistant *age-1* mutants with reduced insulin/IGF-1 signalling exhibited similar patterns of growth and reproduction to wild type worms and displayed no fitness cost in favourable growth conditions. Variation in age-specific survival and fecundity among the different genotypes altered additional demographic parameters and the manner in which the intensity of selection changes across the lifespan. Whilst selection dynamics in *age-1* mutants were similar to those observed in wild type worms, the onset of the age-specific decline in selection acting upon survival was delayed in the other long lived mutants due to delayed maturity. Additionally, selection continued to act until a later age in these genotypes due to delayed reproductive senescence. Mutations which promote longevity thus modify life-history strategies to different extents depending upon the mechanism involved and mutations which increase lifespan via the same mechanism can have dramatically different consequences for fitness and the dynamics of selection.

3.2 Introduction

Life-history strategies are subject to physiological constraints and are characterised by co-variances among traits which have been optimised to maximise fitness in a particular ecological niche (Stearns, 1992; Roff, 2002). By disrupting optimal patterns of growth and/or reproduction, interventions which promote longevity in model organisms typically incur trade-offs, consistent with the antagonistic pleiotropy (Williams, 1957) and disposable soma (Kirkwood, 1977) theories of senescence (reviewed in Van Voorhies *et al*., 2006). Furthermore, life history perturbations, induced directly by extending longevity or indirectly via associated trade-offs, are likely to have considerable repercussions for the dynamics of selection across the lifespan (Lande, 1982; Benton & Grant, 1999; Caswell, 2001). There has been a lack of comparative studies exploring the life history consequences of disrupting different mechanisms which modulate lifespan in model organisms. Consequently, although understanding the impacts of life history variation upon fitness and the intensity of selection at a given age is fundamental to the evolutionary theories of senescence, it is unclear how different processes which are used to increase lifespan modify these parameters.

Several genetic, hormetic and pharmacological modifications which promote longevity act through mechanisms that appear to have been conserved among distinct eukaryotic lineages (Guarente *et al*., 2008). For instance, numerous single gene mutations increase lifespan in a range of model organisms by inappropriately activating conserved stress response mechanisms, reducing caloric intake or disrupting mitochondrial function (reviewed in Wolff & Dillin, 2006; Kenyon, 2010). Responses to environmental stress and/or low food availability are thought to enhance survival during harsh conditions by inducing physiological shifts from growth and reproduction to somatic maintenance and repair (Sinclair, 2005; Kenyon, 2010). Mutations which activate such responses in favourable conditions thus appear to increase lifespan by modifying the expression of genes involved in cellular defence and metabolism (reviewed in Kenyon, 2010). In accordance with the free radical theory of ageing (Harman, 1956), it is conceivable that mutations which disrupt mitochondrial function may promote longevity by reducing the production of reactive oxygen species (ROS). However, several studies have suggested that this may not be the case (Braeckman *et al*., 1999; Yang *et al* 2007; Christina *et al*., 2009). Christina *et al*. (2009) recently demonstrated that several long-lived *C. elegans* mitochondrial mutants display a transcriptional profile analogous to that of the yeast retrograde response (Kirchman *et al*., 1999), which promotes the expression of nuclear genes involved in metabolism and protection of somatic molecules and cells (Butow & Avadhani, 2004). Consequently,

interventions which activate stress responses, reduce caloric intake or disrupt mitochondrial function may all promote longevity by up-regulating the expression of genes which reduce the rate at which somatic damage accumulates with age, albeit via different mechanisms.

In this study, a variety of long-lived *C. elegans* mutants were selected to examine the demographic consequences of increasing longevity via different mechanisms. Mutations in *daf-2*, which encodes the insulin / IGF-1 receptor homologue, and *age-1*, which encodes a phosphatidylinositol 3-kinase catalytic subunit, extend lifespan by disrupting insulin / IGF-1 signalling and activating the expression of stress response proteins (reviewed in Kenyon, 2010). Mutations in *eat-2*, which encodes a subunit of a ligand-gated ion channel required for stimulation of the pharyngeal muscle (McKay *et al*., 2004), promote longevity by reducing the rate of pharyngeal pumping and restricting caloric consumption (Lakowski & Hekimi, 1998). Mutations in *clk-1*, which encodes an enzyme required for the synthesis of ubiquinone, an important component of the electron transport chain, increase lifespan by reducing mitochondrial function (Wong *et al*., 1995). Although trade-offs associated with increased longevity have been identified in a range of long-lived mutants (reviewed in Van Voorhies *et al*., 2006), the majority of studies have focussed upon single components of life history rather than the set of traits which determine fitness (though see Walker *et al*., 2000; Jenkins *et al*., 2004; Chen *et al*., 2007). In contrast, this study compares a range of traits, including age-specific fecundity, lifetime fecundity, and egg size and viability, and uses population projection matrices, or Leslie matrices (Leslie, 1945), to examine how variation in age-specific schedules of survival and reproduction modify fitness and additional demographic properties of populations. Population projection matrices are constructed using values obtained from a life-cycle graph, a schematic representation of the life-cycle which defines the projection interval (the time necessary for a transition from one age or stage to the next), probabilities of survival from one age or stage to the next (P_x), and the expected number of offspring produced at each reproductive age (F_x). In *C. elegans*, the wild type life-cycle in favourable growth conditions is characterised by rapid maturity, high fecundity during a relatively short reproductive period and a short lifespan (Figure 3.1a). In contrast, a typical long-lived mutant life-cycle is characterised by delayed maturity, lower fecundity during a prolonged reproductive period and a longer lifespan (Figure 3.1b).

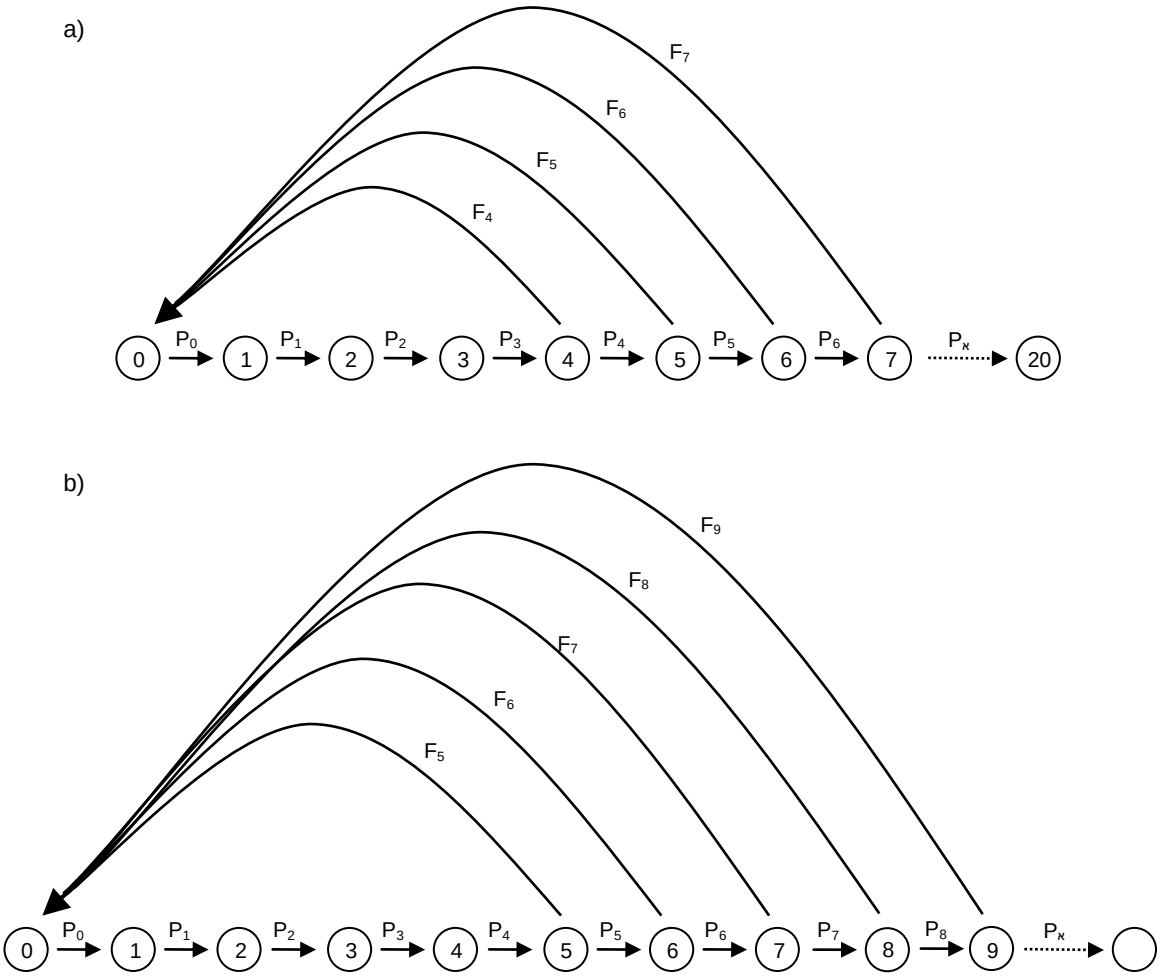


Figure 3.1: Life-cycle graphs for wild type worms and long-lived mutants. Life-cycle graphs for a) the *C. elegans* wild type genotype, and b) a typical long-lived mutant maintained in favourable conditions at 15ºC. Circles represent age classes in 1 day increments and arrows connecting them represent probabilities of survival (P_i) from one age class to the next. Loops to the first age class (eggs) represent age-specific fecundities (F_i). These parameters are used to construct population projection matrices to examine the demographic consequences of life history variation.

3.3 Methods and Materials

3.31 Strains and Culture Conditions

The following genotypes were obtained from the Caenorhabditis Genetics Centre: N2 Bristol (wild type), TJ1052: *age-1(hx546)*, CB1370: *daf-2(e1370)*, DA465: *eat-2(ad465)*, and MQ130: *clk-1(qm30)*. Strains were cultured on nematode growth media (NGM) plates, containing 10 µg/ml nystatin and 50 µg/ml streptomycin, and seeded with *E. coli* (HB101). Strains were cultured and monitored at 15°C because *daf-2(e1370)* mutants arrest development at higher temperatures. During life history assays, individuals were maintained on separate plates with *ad lib* food.

3.32 Age specific fecundity and survival

Generation times were estimated for each genotype at 15°C by collecting small samples of newly hatched larvae then monitoring the time taken until larvae in the next generation began to hatch. Young adult hermaphrodites were then obtained for each genotype by collecting eggs several days in advance depending on developmental rate. Age-synchronised cohorts were initiated for each genotype by transferring as many eggs as possible from these young adults onto empty seeded plates and then returning after 1-2 hours to collect the newly hatched larvae. Although this method does not account for differences among the genotypes in the rate of embryogenesis, it provides a convenient approach to age-synchronise cohorts for demographic analyses (Chen *et al*., 2006, 2007; Muschiol *et al*., 2009).

Before reaching maturity, L4 larvae of each genotype were separated onto individual NGM plates with *ad lib* food. To monitor age-specific fecundity, worms were then transferred to new seeded plates daily throughout the reproductive period. Transferrals began approximately 24 hours after reproduction had commenced in the majority of wild type worms. After allowing time for development (times varied according to genotype), offspring were placed in a cold room at 4°C ± 0.5°C and were stored until time was available for counting. When the number of offspring on a plate was relatively low, counting was performed simply by eye. When many offspring were present on a plate, worms were removed individually using a platinum pick. Age-specific adult survival was monitored daily during and after the reproductive period. If survival status was not obviously apparent, worms were gently touched with a platinum wire in order to stimulate a response. Age-specific fecundity and survival was monitored in approximately 50-60 worms per genotype over 9 separate blocks.

3.33 Egg size and viability

Egg lengths were measured to determine if genotype had an effect upon per offspring investment. Young adults were obtained for each genotype as described in 3.32. Samples of eggs were then collected from these adults and separated onto plates containing 8-10 eggs each. Egg lengths were measured from tip to tip, at a magnification of x 40, using a Nikon SMZ1500 stereomicroscope and LENet imaging software. After allowing sufficient time for embryogenesis, the proportion of these eggs which were viable was determined by counting the number of hatched larvae and the number of un-hatched eggs on each plate. The timing of embryogenesis is prolonged and highly variable in *clk-1(qm30)* mutants (Wong *et al*., 1995). Consequently, *clk-1* mutants were monitored for up to 3 days after eggs had been laid. Egg lengths were measured and viability was monitored in approximately 150 embryos per genotype over 5 separate blocks.

3.34 Construction of population projection matrices

Projection matrices with 1 day increments were constructed for each genotype in R version 2.10.1 (R Development Core Team, 2010). Matrices contained mean daily fecundity values (F_i) on the first row and daily probabilities of transition (survival) from one age class to the next (P_i) on the sub-diagonal. Wong *et al*. (1995) demonstrated that completion of embryogenesis from the 2-cell stage at 15ºC takes 23.6 ± 1.8 hours (mean ± standard deviation) in N2 and 46.1 ± 12.1 hours (mean ± standard deviation) in *clk-1(qm30)* mutants. There is no evidence to suggest that the timing of embryonic development in *age-1*, *daf-2* and *eat-2* mutants differs from wild type worms. Embryogenesis was thus assumed to take 1 day in N2, *age-1*, *daf-2* and *eat-2* mutants and 2 days in *clk-1* mutants. As described in 3.32, age-specific fecundities had been obtained by counting viable offspring only. The mean number of eggs laid per day was thus extrapolated for each genotype by dividing the mean number of viable offspring by the mean proportion of viable eggs. The proportion of viable eggs was then placed in the P_0 element of each projection matrix.

3.35 Eigen analyses

The algebraic properties of a projection matrix describe demographic parameters of a population or genotype (Caswell, 2001). When a population is growing at a stable age distribution, the dominant eigenvalue (λ) provides an estimate of the population growth rate in each time step. This is the natural logarithm of the intrinsic rate of increase ($r = \log(\lambda)$), and is typically used as a measure of fitness (Lande, 1982; Benton & Grant, 1999). The right eigenvector (w) describes the stable age

distribution and the left eigenvector (v) corresponds to age-specific reproductive values, the expected contributions to future fitness for an individual at a given age (Caswell, 2001). These parameters can be used to infer how perturbations in age-specific survival or fecundity modify fitness. Whilst sensitivities represent how absolute changes in age-specific survival or fecundity alter λ (Caswell, 1978), elasticities represent how proportional changes in age-specific survival or fecundity alter λ (De Kroon *et al*., 1986). As selection acts upon changes which modify fitness, sensitivity and elasticity values are used to quantify the intensity of selection acting upon genes which influence survival or fecundity at a specific age (Lande, 1982; Benton & Grant, 1999). Population growth rates (λ), reproductive values (v) and elasticities of λ to changes in age-specific survival and fecundity were calculated for each genotype using the eigen.analysis function in the DemogR package (Jones, 2007), and stable age distributions (w) were determined using the popbio package (Stubben & Milligan, 2007) in R version 2.10.1. To examine variation in population growth rate, populations of each genotype were projected for 500 iterations (days). Populations were initially projected from a predefined starting population vector (N_0), then from a new population vector which was derived at each subsequent time step using the equation $N_{t+1} = MN_t$, where M is the projection matrix and N_t is the population vector at time t (Benton & Grant, 1996). Each starting population vector contained 100 individuals which were spread according to the stable age distribution (SAD) for the relevant genotype. Relative proportions in each age-class were obtained from the right eigenvector (w) of an eigenanalysis of the projection matrix for each genotype.

3.36 Analysis

All analyses were performed in R version 2.10.1.

a) Survival analysis

Individuals which had crawled off the agar or died from mortality sources other than old age (e.g. internal hatching or rupturing of the vulva) were removed from the analysis. Parametric survival models were compared using all possible error distributions and a Weibull error structure was chosen following examination of the plotted residuals. A parametric survival model with a Weibull error structure was then fitted to the data using the psm function in the Hmisc and Design packages.

b) Age-specific fecundity and lifetime fecundity

Data for age-specific changes in fecundity and lifetime fecundity of individuals which had died due to internal hatching were only included in the analysis if death

occurred during the last 1-2 days of reproduction. Changes in age-specific fecundity were compared among the different genotypes using a mixed-effects model. To account for repeated measures on the same individuals over time, the data were first defined as a groupedData object. A linear mixed-effect model with random effects terms for individual and block was then fitted to the data using the nlme package. A linear mixed-effect model was also used to determine how genotype influenced lifetime fecundity. To allow for non-independence of individuals observed within the same block, replicates were nested within blocks and these were fitted as random effects.

c) Associations between lifespan and lifetime fecundity

To examine if an association exists between lifespan and lifetime fecundity within and among the different genotypes, linear mixed effects models were fitted to the data using the nlme package. Block was fitted as a random effect and only data from individuals which had not died due to internal hatching were included in the analysis.

d) Egg size and viability

To determine how genotype affected egg size, a linear mixed-effect model was fitted to the data using the nlme package. Block was fitted as a random factor to correct for non-independence of eggs obtained from hermaphrodites within the same population. To assess how genotype influenced the proportion of eggs which were viable, a generalized linear mixed effect model was fitted to the data using the lmer function in the lme4 package. This model was fitted using the laplace approximation method (Pinheiro & Bates, 1995) and had a binomial error structure and a logit link function. Again, block was fitted as a random factor to account for non-independence of eggs obtained from hermaphrodites within the same population.

e) Stage structure

Differences among genotypes in the proportions of a population at a stable (st)age distribution which are made up by different stages (eggs, larvae, reproductive adults and post-reproductive adults) were examined using Chi-squared goodness of fit tests. For each long-lived mutant genotype, the observed counts in each stage were compared with the wild type (expected) proportions.

3.4 Results

3.41 Age-specific survival

As expected, survival was prolonged in all of the long-lived mutant genotypes compared to the wild type genotype N2 (Figure 3.2). Relative to N2, mean survival times were increased by 71 % in *age-1* mutants ($z = 17.5$, $p < 0.001$), by 69 % in *daf-2* mutants ($z = 16.0$, $p < 0.001$), by 45 % in *eat-2* mutants ($z = 11.4$, $p < 0.001$), and by 50 % in *clk-1* mutants ($z = 13.4$, $p < 0.001$). Whilst *age-1* and *daf-2* mutants were considerably longer-lived than *eat-2* and *clk-1* mutants, survival times of *daf-2* mutants were not significantly different from *age-1* mutants ($z = -1.79$, $p = 0.073$), and survival times of *clk-1* mutants were not significantly different from *eat-2* mutants ($z = 1.39$, $p = 0.165$).

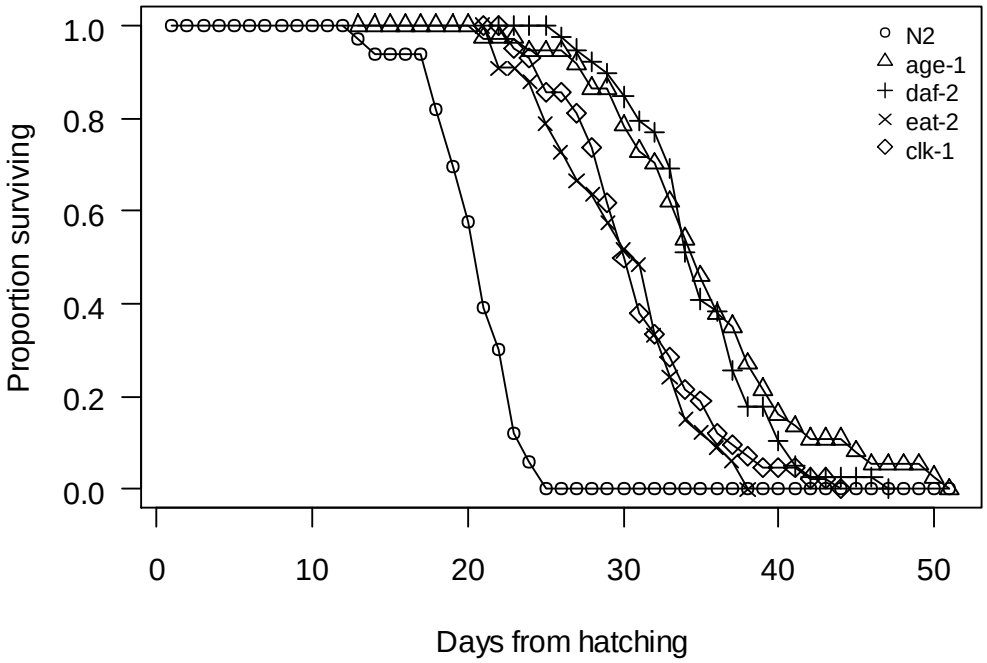


Figure 3.2: Variation in survival among long-lived mutants and wild type worms. Survival curves for wild type worms (N2), and for long-lived *age-1* mutants, *daf-2* mutants, *eat-2* mutants and *clk-1* mutants. Survival was monitored in approximately 40 replicates per genotype, over 9 separate blocks, after removal of individuals which had died from causes other than old age.

3.42 Age-specific fecundity schedules

Remarkably, age-specific schedules of fecundity did not differ between wild type worms (N2) and *age-1* mutants (Figures 3.3a and 3.3b). Both genotypes began to produce offspring 4 days after hatching and both displayed high fecundity during the first 3 days of the reproductive period which peaked 5 days after hatching. However,

although the subsequent decline in fecundity occurred at a similar rate in N2 and *age-1* mutants, mean fecundity was slightly higher in *age-1* mutants 7 and 8 days after hatching (Figure 3.3f). Consequently, whilst schedules of fecundity did not differ between the two genotypes, the mean number of offspring produced during the entire reproductive period was slightly higher in *age-1* mutants ($t = 2.27$, $p = 0.024$) (Figure 3.3f and Table 3.1). Reproduction commenced approximately 5 days after hatching in *daf-2* mutants and, after rapidly reaching a peak, the daily production of offspring declined more gradually than in N2 ($t = 9.45$, $p < 0.001$) (Figure 3.3c). However, the mean number of offspring produced by *daf-2* mutants throughout the reproductive period was relatively low compared to N2 ($t = -10.88$, $p < 0.001$) (Figure 3.3f and Table 3.1). Reproduction began 5-6 days after hatching in *eat-2* mutants and also peaked rapidly then declined more gradually than in N2 ($t = 14.61$, $p < 0.001$) (Figure 3.3d). Mean lifetime fecundity was extremely low in *eat-2* mutants relative to wild type worms ($t = -25.57$, $p < 0.001$) (Figure 3.3f and Table 3.1). In *clk-1* mutants, reproduction commenced 6-7 days after hatching and patterns of fecundity throughout the reproductive period were considerably different from N2 ($t = 16.57$, $p < 0.001$) and the other long-lived genotypes (Figure 3.3e). The rate of reproduction increased more slowly in *clk-1* mutants, and reached a relatively flat peak 8-9 days after hatching before declining gradually. This pattern of reproduction can explain the more sigmoidal curve in cumulative fecundity observed in *clk-1* mutants than in the other genotypes (Figure 3.3f). Mean lifetime fecundity was considerably lower in *clk-1* mutants than in wild type worms ($t = -20.34$, $p < 0.001$) (Figure 3.3f and Table 3.1). Despite similarities between wild type worms and *age-1* mutants, these results suggest that mutations which promote longevity in *C. elegans* are often associated with delayed maturity, reduced fecundity across the lifespan and delayed reproductive senescence. However, the life history consequences of mutations which increase lifespan clearly differ according to the mechanism involved.

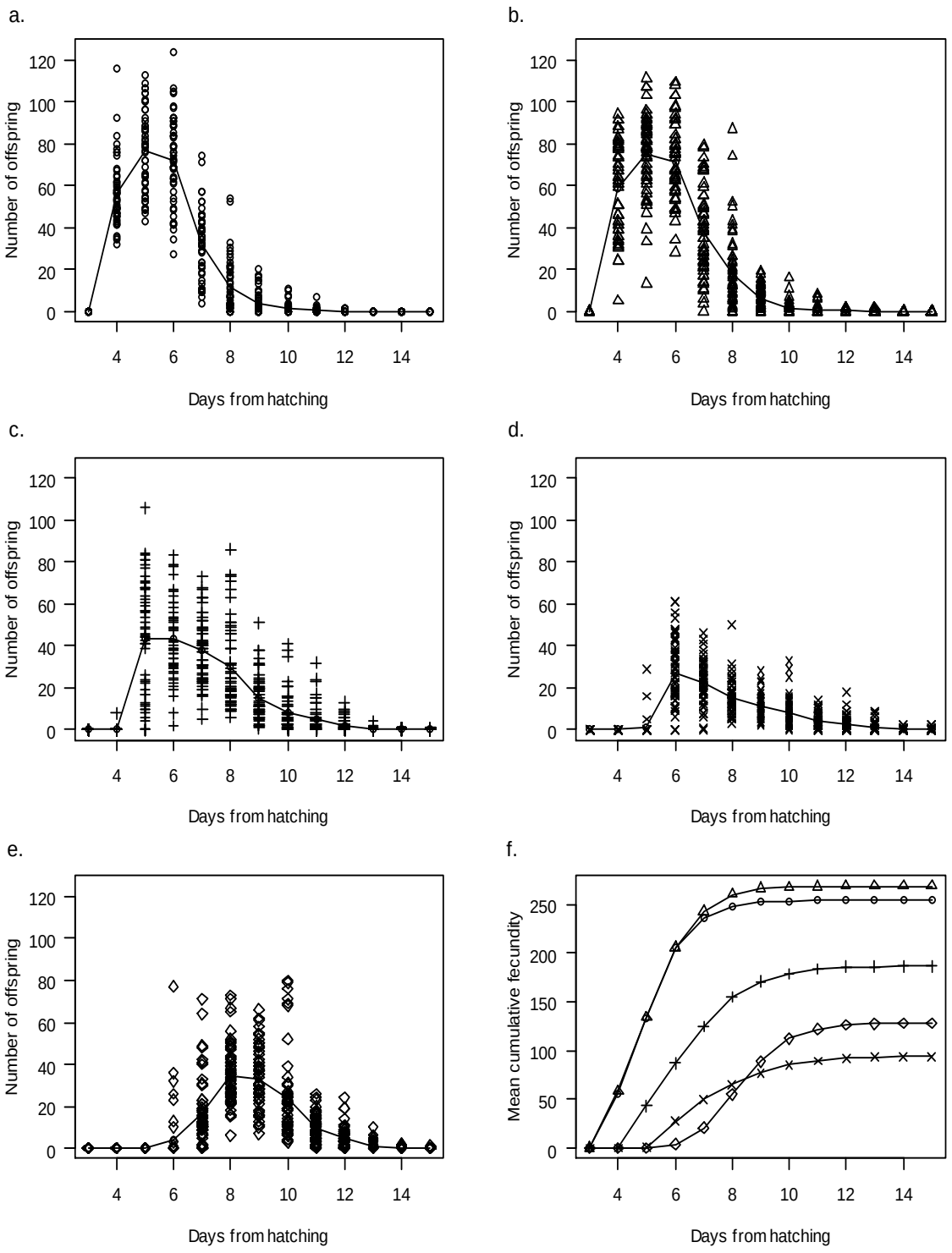


Figure 3.3: Fecundity schedules and mean cumulative fecundity. Age-specific fecundity schedules for a) N2, b) *age-1* mutants, c) *daf-2* mutants, d) *eat-2* mutants and e) *clk-1* mutants, and f) mean cumulative fecundity for N2 (○), and *age-1* mutants (Δ), *daf-2* mutants (+), *eat-2* mutants (x) and *clk-1* mutants (◊). Fecundity was monitored in approximately 50 replicates per genotype over 9 separate blocks.

3.43 Associations between lifespan and lifetime fecundity

After removing individuals which had died due to internal hatching or additional sources of mortality other than old age (10-15 worms per genotype), no significant association was observed between lifespan and lifetime fecundity when data for all of the genotypes were considered (Figure 3.4). However, this was primarily due to the high fecundity and long-lifespan of *age-1* mutants because a highly significant negative association was observed between lifespan and lifetime fecundity when data from *age-1* mutants had been removed, ($t = -5.79$, $p < 0.001$). Within genotypes the association between lifespan and fecundity was consistently negative but was not significantly different from zero in any strain.

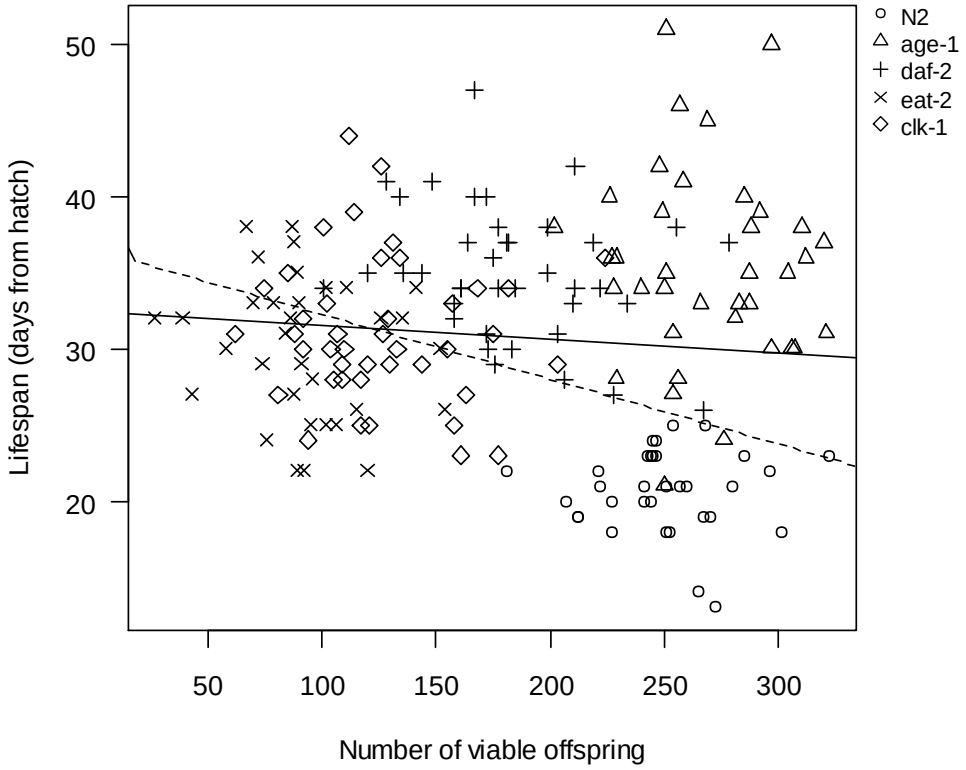


Figure 3.4: Associations between lifespan and lifetime fecundity. Lifespan and lifetime fecundity were monitored in 30-40 replicates per genotype after removal of individuals which had died due to internal hatching. The solid line represents the correlation between lifespan and fecundity when all genotypes were considered. As a trade-off between lifespan and fecundity became apparent when data for *age-1* mutants had been removed, the dashed line represents the correlation between lifespan and fecundity when only N2, *daf-2* mutants, *eat-2* mutants and *clk-1* mutants were considered.

3.44 Egg size and viability

Although wild type worms produce a higher number of offspring during the reproductive period than *daf-2* mutants and *clk-1* mutants, investment in terms of size per egg is comparatively low. Relative to N2, larger eggs were produced by *age-1* mutants ($t = 6.69$, $p < 0.001$), *daf-2* mutants ($t = 24.80$, $p < 0.001$), and *clk-1* mutants ($t = 17.84$, $p < 0.001$) (Table 3.1). This may suggest that per egg investment is higher in these genotypes than in wild type worms. In contrast, egg size did not differ between N2 and *eat-2* mutants. No differences were observed in the proportion of viable eggs produced by wild type, *age-1* mutants, *daf-2* mutants and *eat-2* mutants. However, egg viability was considerably lower in *clk-1* mutants than in the wild type strain ($z = -4.27$, $p < 0.001$) (Table 3.1).

Genotype	Egg length (µm)		Proportion egg viability		Lifetime fecundity		Fitness (λ)	
	mean ± S.D	p*	mean ± S.D	p*	mean ± S.D	p*	absolute	relative
N2	49.99 ± 3.05	n/a	0.985 ± 0.02	n/a	254.2 ± 29.0	n/a	2.51	1
age-1	52.27 ± 2.73	< 0.001	0.976 ± 0.02	0.58	268.5 ± 31.0	0.024	2.52	1.00
daf-2	58.53 ± 3.04	< 0.001	1.000 ± 0.00	0.99	186.4 ± 38.9	< 0.001	2.07	0.82
eat-2	50.33 ± 2.49	0.817	0.996 ± 0.01	0.97	93.9 ± 26.7	< 0.001	1.75	0.70
clk-1	56.11 ± 3.73	< 0.001	0.866 ± 0.05	< 0.001	128.0 ± 38.4	< 0.001	1.59	0.63

p* = p –value relative to the wild type genotype (N2).

Table 3.1: Variation among genotypes in egg size, egg viability, fecundity and fitness. Mean egg lengths (µm) ± standard deviations (S.D), mean proportions of viable eggs ± standard deviations (S.D), mean lifetime fecundity ± standard deviations (S.D), and absolute and relative fitness values for each genotype. Egg lengths and viabilities were monitored in approximately 150 eggs per genotype over 5 separate blocks and, after removal of individuals which died early during the reproductive period due to internal hatching, lifetime fecundity was monitored in approximately 50 individuals over 9 separate blocks. Absolute fitness was calculated as λ, the dominant eigenvalue of the projection matrix for each genotype, and relative fitness was obtained by dividing absolute values for each genotype by the wild type value.

3.45 Fitness and demographic properties of populations

Absolute fitness estimates were highest for wild type worms (N2) and *age-1* mutants (λ = 2.51 and 2.52 respectively) and relative fitness did not differ between these genotypes (Table 3.1). Consequently, population growth rate was equal for wild type and *age-1* mutants in favourable conditions (Figure 3.5). Due to prolonged development and reduced early fecundity, absolute fitness estimates were considerably lower for *daf-2* mutants (λ = 2.07), *eat-2* mutants (λ = 1.75) and *clk-1* mutants (λ = 1.59), and relative fitness/population growth rate was reduced in each of these genotypes compared to the wild type strain (Table 3.1 and Figure 3.5). These results imply that fitness costs typically arise in long-lived *C. elegans* mutants, but that *age-1* mutants display no fitness deficit relative to wild type worms in favourable growth conditions.

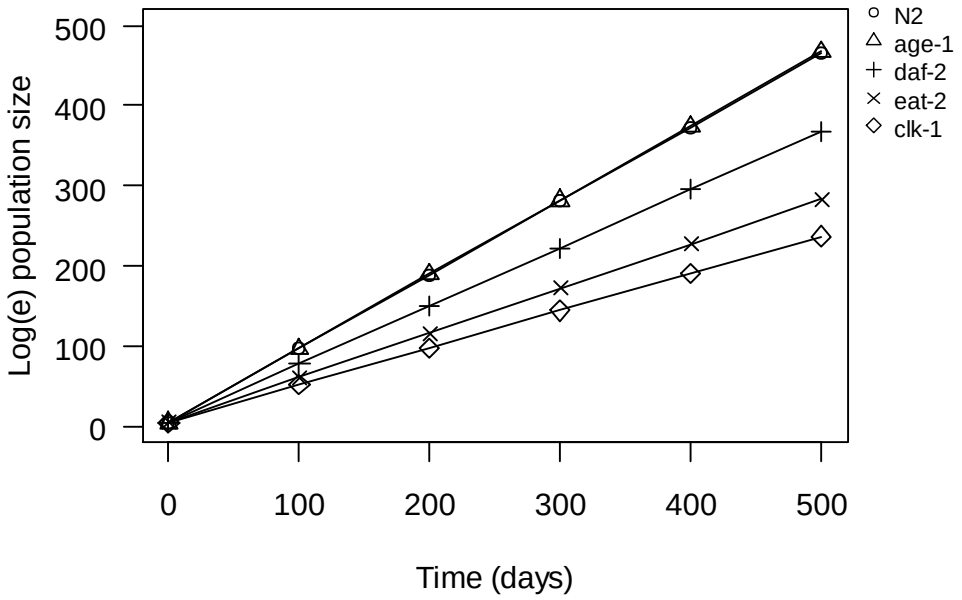


Figure 3.5: Variation among genotypes in population growth rate. Population growth (fitness), over 500 days in favourable conditions, for wild type worms (N2), *age-1* mutants, *daf-2* mutants, *eat-2* mutants and *clk-1* mutants. N2 and *age-1* mutant populations grow at equal rates (overlapping lines). However, growth rates of *daf-2* mutant, *eat-2* mutant and *clk-1* mutant populations are considerably reduced relative to these genotypes.

The proportions of different stages which make up a population growing at a stable (st)age distribution were significantly different from the wild type in the *eat-2* (X^2=19.26, $p < 0.001$) and *clk-1* mutant (X^2= 45.4, $p < 0.001$) genotypes (Figure 3.6a). In *eat-2* mutants this was primarily due to differences in the proportions of eggs and larvae, with eggs constituting approximately 40% of a stable population and larvae

constituting approximaly 57%, compared to 60% and 35% in the wild type respectively. Similar differences were observed in *daf-2* mutants, but these were not statistically significant. Although the proportion of post-reproductive adults which made up a stable (st)age population was extremely low in all of the genotypes, a higher proportion was observed in *clk-1* mutants, with post-reproductive adults constituting 8.1 x 10^{-4}% compared to approximately 1.5x10^{-6}% in the wild type and the other strains. These results suggest that trade-offs associated with increased lifespan can modify the proportions of each stage in populations growing at a stable distribution, but that variation in longevity does not substantially alter the proportions of reproductive and post-reproductive adults in the majority of strains.

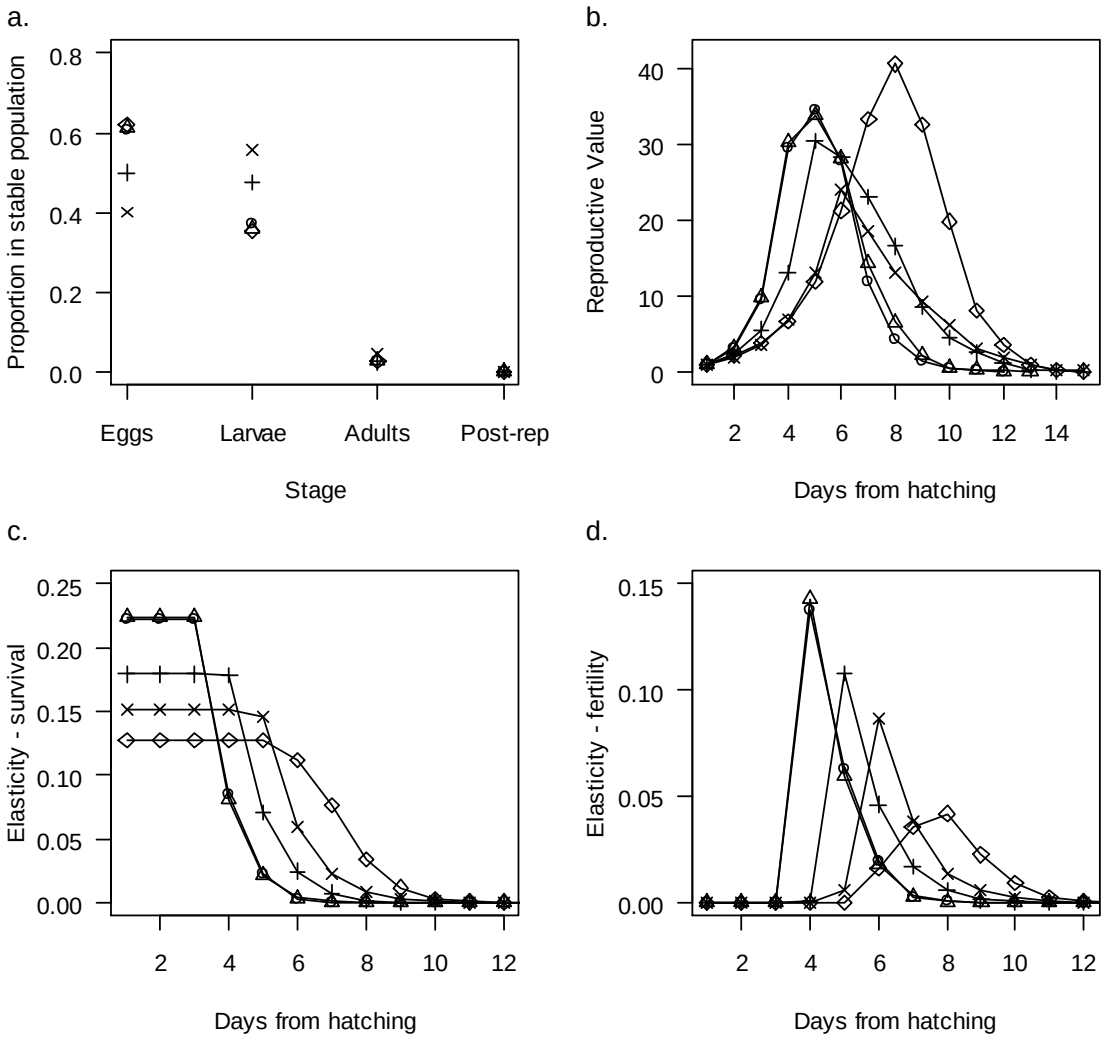


Figure 3.6: Variation among genotypes in the demographic properties of populations. a) Stable stage distributions, b) age-specific reproductive values (v), c) elasticities of λ to changes in age-specific survival, and d) elasticities of λ to changes in age-specific fecundity in N2 (○), *age-1* mutants (∆), *daf-2* mutants (+), *eat-2* mutants (x) and *clk-1* mutants (◊).

Whilst reproductive values (v) varied during development and reproduction in a similar manner in all of the genotypes, the age at which reproductive values peak and the magnitude of the peaks differed among strains (Figure 3.6b). In wild type, *age-1* mutants and *daf-2* mutants, the highest values were observed 5 days after hatching and correspond to the peak in reproductive output displayed in figure 3.3. In *eat-2* mutant and *clk-2* mutants, the peak in reproductive value was delayed until 6 and 9 days after hatching respectively. Elasticities of λ to changes in age-specific survival were highest in all genotypes before reproduction begins; however, elasticity values were higher in N2 and *age-1* mutants than in the other genotypes during the first 3 days after hatching (Figure 3.6c). An increase in survival during this time would thus have a larger impact upon the fitness of wild type and *age-1* mutants than on the fitness of any other genotype. Conversely, an increase in survival 5 or 6 days after hatching would increase fitness in the other genotypes, particularly in *eat-2* and *clk-1* mutants, considerably more than in wild type worms or *age-1* mutants. The elasticities of λ to changes in age-specific fertility were highest during the beginning of the reproductive period for each genotype (Figure 3.6d). Consequently, whilst an increase in fertility 5 days after hatching would have the highest impact on the fitness of *daf-2* mutants, an increase on day 8 would have the highest impact on the fitness of *clk-1* mutants.

3.5 Discussion

Mutations which activate stress responses, reduce caloric intake or impair mitochondrial function promote longevity in model organisms ranging from yeast to mice (Barbieri *et al*., 2003; Bishop & Guarente, 2007; Christina *et al*., 2009). To assess how mutations which increase lifespan via these different mechanisms alter additional components of life history, and how resulting variation in age-specific fecundity and survival modify fitness and the dynamics of selection, in this study a variety of long-lived *C. elegans* mutants were compared with the wild type genotype using a demographic approach. The majority of the long-lived mutants displayed trade-offs which reduced fitness to different extents relative to the wild type strain. Furthermore, variation in age-specific survival and fecundity among these genotypes altered additional demographic parameters and the manner in which the intensity of selection changes across the lifespan. However, long-lived *age-1* mutants displayed remarkably similar patterns of growth and reproduction to wild type worms and had equal fitness in favourable growth conditions.

Long-lived *age-1* mutants and *daf-2* mutants with reduced insulin / IGF-1 signalling displayed similar patterns of age-specific survival. Despite considerable variation in schedules of fecundity between these genotypes, this similarity may reflect the common mechanism of life extension which is activated in these worms. Long-lived *eat-2* mutants and *clk-1* mutants also displayed similar patterns of age-specific survival. Whilst the mechanisms by which *eat-2* and *clk-1* modulate longevity are distinct from the insulin / IGF-1 signalling pathway (Lakowski & Hekimi, 1996, 1998; Houthoofd *et al*., 2003), simultaneous mutations in *eat-2* and *clk-1* do not have additive effects upon lifespan (Lakowski & Hekimi, 1998). It is, therefore, unclear if caloric restriction and *clk-1* mediated mitochondrial dysfunction modify longevity by completely independent processes. The lack of distinction in longevity between *eat-2* mutants and *clk-1* mutants may thus reflect similarities in the mechanisms by which lifespan is increased in these genotypes. Though a clear negative correlation between lifespan and lifetime fecundity was observed among the different genotypes when *age-1* mutants were not considered, the lack of an association between these traits within individual genotypes is consistent with a previous study (Chen *et al*., 2007).

Although age-specific fecundity was similar between wild type worms and *age-1* mutants, mean lifetime fecundity was slightly higher in *age-1* mutants. This difference appeared to arise because a small number of *age-1* mutants displayed comparatively high fecundity 7 and 8 days after hatching, suggesting that *age-1* mutants exhibit a moderate reduction in the rate of reproductive senescence relative to wild type worms.

Consistent with this, the insulin/IGF-1 signalling pathway has previously been acknowledged as a determinant of reproductive senescence (Hughes *et al*., 2007). Despite this difference, *age-1* mutants displayed equal fitness to the wild type genotype in favourable growth conditions. Though this anomaly may initially appear to contradict evolutionary theory, it has previously been reported that *age-1* mutants exhibit fitness costs when exposed to nutritional stress (Walker *et al*., 2000). The relative fitness of *age-1* mutants in additional environmental conditions which are likely to be ecologically relevant is examined in chapters 4, 5 and 7 of this thesis.

Relative to wild type worms, maturity was slightly delayed in *daf-2* mutants and *eat-2* mutants and was considerably delayed in *clk-1* mutants. Furthermore, early fecundity was reduced in all of these genotypes compared to wild type worms. Consistent with the antagonistic pleiotropy and disposable soma theories of senescence, *daf-2*, *eat-2* and *clk-1* mutants, therefore, had considerably lower fitness than the wild type genotype in favourable growth conditions. The most severe fitness deficit was observed in *clk-1* mutants. Although the number of offspring produced by these worms throughout the entire reproductive period was higher than in *eat-2* mutants, *clk-1* mutants attain maturity at a later age and have lower early fecundity. As these traits have a major impact upon the rate of increase of a population (Birch, 1948; Roff, 2002), these results exemplify the importance of considering the entire set of traits which determine fitness when investigating trade-offs in long-lived mutants. Chen *et al*. (2007) compared the fitness of *daf-2 (e1368)* mutants and *clk-1 (qm30)* mutants with wild type worms and obtained higher absolute λ values for all three genotypes than in this study. However, the parameters that Chen *et al*. (2007) used to construct projection matrices were obtained from variation in age-specific survival and fecundity observed at 20ºC. Furthermore, Chen *et al*. (2007) did not take account of the variation in the timing of embryogenesis among the different genotypes and investigated consequences of a *daf-2* mutant allele which has less severe reproductive defects than the one examined in this study (Gems *et al*., 1998).

In a common environment, larger eggs are expected to give rise to higher quality offspring (Roff, 2002). Although relationships between the ‘quality and quantity’ of eggs were not examined within genotypes in this study, there does not appear to be a consistent association between egg size and lifetime fecundity or fitness among the different genotypes. The increased egg length observed in *daf-2* mutants and *clk-1* mutants relative to the wild type is consistent with the larger body sizes observed in adults of these genotypes (McCulloch & Gems, 2003b; personal observations). Conversely, though lengths of wild type and *eat-2* mutant eggs were similar in this study and in a previous study (Mörck & Pilon, 2006), adult body lengths in *eat-2*

mutants are reduced by approximately 34% relative to wild type worms (Mörck & Pilon, 2006).

Despite considerable variation in life-history strategies among wild type, *age-1* mutants and *clk-1* mutants, populations at a stable age distribution contain similar proportions of eggs, larvae and reproductive adults in these genotypes. This may be because the rapid growth and high fecundity of wild type worms and *age-1* mutants, and the prolonged embryogenesis of *clk-1* mutants, ensure that a high proportion of eggs is present in populations at a stable distribution at any point in time. The lower proportion of eggs and the higher proportion of larvae in *daf-2* mutant and *eat-2* mutant populations may reflect the slower rate of post-embryonic development and reduced fecundity in these genotypes relative to wild type worms. Though the genotypes display considerable variation in post-reproductive longevity, little difference in the proportion of post-reproductive adults in stable populations was observed among long-lived *age-1*, *daf-2* and *eat-2* mutants and the wild type strain.

Fisher (1930) proposed that the reproductive value at age x is directly proportional to the intensity of selection at age x and argued that both decline beyond maturity because individuals become progressively less likely to survive and reproduce with increasing age. As described in chapter 1, current understanding of the evolution of senescence has been derived from these concepts. However, though Hamilton (1966) provided rigorous quantitative support for the decline in selection throughout adult life, he demonstrated that reproductive values do not provide an appropriate measure of the intensity of selection acting on survival at a particular age. Consistent with Hamilton's (1966) findings, whilst reproductive values increased during development and peaked at the beginning of the reproductive period in each genotype, elasticity values indicated that the intensity of selection acting on survival is highest before the onset of reproduction then progressively declines until the last age of reproduction. These results are intuitive because, in order for an individual to mature and contribute offspring to future generations, there must be strong selection acting on genes which influence survival during development. Reproductive values followed a similar pattern to the elasticities of λ to changes in fertility and thus more accurately represent the intensity of selection acting on age-specific fecundity.

Though selection acting on survival was most intense during development in all genotypes, elasticities of λ to changes in age-specific survival were highest in wild type worms and *age-1* mutants. As the values represent proportional contributions of changes in age-specific survival to fitness, this may be because the reproductive period is shorter in these worms than in the other genotypes. Although *clk-1* mutants are not the longest lived of the genotypes examined in this study, elasticities of λ to changes in

age-specific survival indicate that, after the onset of reproduction, the rate of decline in selection acting upon survival is slower in these worms than in the other long-lived mutants. This is because *clk-1* mutants display higher fecundity than any of the other genotypes late in life and thus the rate of decline in selection acting on survival is reduced because surviving individuals retain the capacity to contribute to fitness at a late age. Whilst reproductive values represent age-specific contributions to future fitness, elasticities of λ to perturbations in fertility represent proportional contributions of fecundity to fitness at each reproductive age. Though reproductive values begin to rise during development and elasticities of λ to changes in fertility begin to rise at the onset of reproduction, the values of these two parameters are therefore similar in each genotype. Elasticities of λ to changes in age-specific fertility indicate that the intensity of selection acting on fecundity is highest at the beginning of the reproductive period in wild type worms and in *age-1*, *daf-2* and *eat-2* mutants. Conversely, in *clk-1* mutants the intensity of selection reaches a maximum two days after reproduction has commenced. This is likely to reflect the slow rate of increase in fecundity at the beginning of the reproductive period in these worms. The elasticity values which were obtained in this study do not take account of co-variances between different components of life history. In reality an increase in one matrix element may have a negative impact on another. However, although methods have been developed to account for associations between traits (Benton *et al*., 1995; Van Tienderen, 1995), the approach used in this study is useful for making basic inferences regarding selection dynamics in the different genotypes.

The methods which were used in this study to quantify fitness and additional demographic parameters assume constant, unlimited conditions which may not be relevant in nature. However, the approach provides a convenient way to make simple comparisons among different genotypes in a common environment (Caswell, 2001), and has previously been used to examine demographic variation between wild populations of *C. elegans* (Chen *et al*., 2006) and *C. remanei* (Diaz *et al*., 2008), and among long-lived *C. elegans* mutants (Chen *et al*., 2007). The results in this study suggest that in favourable conditions, in which population growth is not constrained and age-specific changes in survival and reproduction do not change over time, mutations which activate stress responses, reduce caloric consumption and impair mitochondrial function reduce fitness to different extents relative to wild type worms. Furthermore, disrupting the different mechanisms produces considerable variation in life-history strategies which alter additional demographic properties of populations.

Chapter 4 – Fitness costs are context dependent in long-lived *age-1* mutants: Part 1 - Thermal stress

4.1 Abstract

Long-lived mutants typically display trade-offs, consistent with the antagonistic pleiotropy and disposable soma theories of ageing, which ultimately reduce lifetime fitness. However, though many mutations promote longevity by disrupting mechanisms which regulate responses to environmental change, fitness costs have rarely been examined in an ecological context. In *C. elegans*, *age-1(hx546)* mutants are long-lived and stress resistant yet have equal fitness to the wild type genotype when maintained in favourable growth conditions. Fitness costs arise when these mutants are exposed to repeated cycles of starvation. In this study, fitness was compared between *age-1* mutants and wild type worms by monitoring temporal changes in genotype frequencies when mixed genotype populations, maintained at low densities with excess food or at high densities with limited food, were periodically exposed to mild (27°C) or intense (30°C) thermal stress. Although fitness costs were observed in *age-1* mutants when populations were maintained at high densities regardless of temperature, remarkably frequencies of *age-1* mutants rapidly increased in low density populations which were periodically exposed to intense thermal stress. This clearly demonstrates that *age-1* mutants can have a selective advantage over the wild type genotype in harsh environments if excess food is available. Consistent with this, spatial and temporal distributions of the FOXO transcription factor DAF-16 indicate that protection of somatic cells is enhanced in *age-1* mutants before, during and after exposure to thermal stress, and *age-1* mutants displayed higher survival and fecundity and matured more rapidly than wild type worms after exposure to high temperatures when food was not limited. This is the first study to demonstrate that a long-lived, laboratory-derived mutant can have higher fitness than a wild type genotype under specific environmental conditions. The results exemplify the importance of investigating trade-offs in an ecological context, and imply that harsh environments may lead to the fixation of stress resistant alleles and indirectly promote the evolution of increased longevity.

4.2 Introduction

Numerous genes have been identified which modulate longevity in model organisms ranging from yeast to mice (reviewed in Kenyon, 2005; Partridge, 2010). Loss-of-function mutations in many of these genes appear to promote longevity by activating highly conserved stress response mechanisms which enhance the protection of somatic molecules and cells (reviewed in Kenyon, 2005). Consequently, genetic manipulations which increase lifespan are often associated with a concurrent increase in resistance to environmental stress. Long-lived mutants typically display trade-offs consistent with the antagonistic pleiotropy (Williams, 1957) and disposable soma theories of ageing (Kirkwood, 1977), but these have rarely been examined in an ecologically relevant context. It thus remains unclear how mutations which promote longevity and increase stress resistance modify fitness in heterogeneous environments which are representative of natural conditions. This is remarkable given that many mutations which extend lifespan disrupt mechanisms which are involved in responding to environmental change (reviewed in Kenyon, 2005).

The antagonistic pleiotropy and disposable soma theories of senescence are based upon the principle that traits are under stronger selection early in life than late in life (Medawar, 1952; Williams, 1957; Hamilton, 1966), and predict that trade-offs should arise which favour early fitness components over longevity. Both entail the fundamental concept of life history theory that life-history strategies are subject to physiological constraints and are characterised by co-variances among traits which have been optimised to maximise fitness in a particular ecological niche (Stearns, 1992; Roff, 2002). However, the nature and magnitude of co-variances among life-history traits can differ depending upon the context in which they are observed (Sgro & Hoffman, 2004). In some cases, trade-offs which are apparent in harsh environments may be obscured in more favourable conditions (Reznick *et al*., 2000; Tessier *et al*., 2000). For instance, Messina and Fry (2003) demonstrated that a trade-off between fecundity and longevity arises in the seed beetle, *Callosobruchus maculatus*, only when individuals are nutritionally stressed. Though the majority of long-lived mutants display distinct trade-offs in benign laboratory environments, some appear to have equal fitness to wild type genotypes when maintained in such conditions (Rogina *et al*., 2000; Walker *et al*., 2000; Marden, *et al*., 2003; Scheckhuber *et al*., 2007). These mutants typically display trade-offs when resources are limited (Walker *et al*., 2000; Marden *et al*., 2003), but it is unclear how fitness is modified by additional ecological factors.

Throughout evolutionary history, mutations which promote longevity in model organisms may have arisen in natural populations. As these are expected to disrupt

optimal life-history strategies, such mutations would likely confer a disadvantage under at least some environmental conditions and eventually be purged by selection (Van Voorhies *et al*., 2006). With the possible exception of species which exhibit grand maternal care, selection does not act upon post reproductive lifespan (Williams, 1957; Hamilton, 1966; Lahdenperä *et al*., 2004). However, given the association between longevity and stress resistance, it has been suggested that selection may indirectly influence post reproductive lifespan by acting upon genetic variation in the ability to tolerate harsh conditions (Parsons, 1995, 2002; Kenyon, 2010). Consistent with this, artificial selection for resistance to environmental stress has been used successfully to increase lifespan in *Drosophila melanogaster* (Rose, 1992) and in the butterfly *Bicyclus anynana* (Pijpe *et al*., 2008). This implies that mutations which promote stress resistance and longevity may not necessarily incur fitness costs under all environmental conditions.

In *C. elegans*, the conserved insulin / IGF-1 signalling (IIS) pathway modulates development, metabolism, stress resistance and longevity by regulating the cellular localisation of the FOXO transcription factor DAF-16. In conditions which are favourable for development and reproduction, the IIS pathway negatively regulates DAF-16 (Lin *et al*., 1997; Ogg *et al.*, 1997), and the transcription factor is distributed evenly throughout somatic cells (Henderson & Johnson, 2001). In harsh environments, insulin signalling is disrupted and DAF-16 molecules accumulate within nuclei and promote the expression of genes involved in cellular defence and metabolism (Henderson & Johnson, 2001; Lin *et al*., 2001; Lee *et al*., 2003; Murphy *et al*., 2003; McElwee *et al*., 2003). The IIS pathway is involved in regulating entry into the long-lived, stress resistant dauer stage, a facultative state of diapause formed in response to low food availability, high population density and/or high temperatures (Golden & Riddle, 1984), which is dependent upon *daf-16* (Kenyon *et al*., 1993). Transitions in insulin signalling thus appear to generate physiological shifts which either promote growth and reproduction or enhance protection of somatic cells (Henderson & Johnson, 2001). Mutants of the IIS pathway are long lived and display increased resistance to various forms of environmental stress (reviewed in Kenyon, 2005), perhaps due to partial activation of the dauer program throughout life (Kenyon *et al*., 1993; McElwee *et al*., 2004, 2006). Consistent with this, whilst severe loss-of-function mutations induce constitutive dauer arrest, many long-lived IIS mutants form dauers at temperatures which are permissive for wild type development and reproduction (Malone *et al*., 1996; Gems *et al*., 1998). At temperatures which do not induce dauer arrest, IIS mutants typically display other trade-offs, such as delayed maturity and/or low fecundity which

reduce fitness relative to wild type worms (Tissenbaum & Ruvkun, 1998; Gems *et al*., 1998; Jenkins *et al.,* 2004).

Mutations in the *age-1* gene, which encodes a phosphatidylinositol 3-kinase (PI3K) that constitutes a central component of the IIS pathway, increase lifespan by up to 80% (Friedman & Johnson, 1988). Remarkably, *age-1*(*hx546*) mutants have equal fitness to wild type worms when excess food is available (Walker *et al*., 2000). However, fitness costs arise when these mutants are exposed to repeated cycles of starvation (Walker *et al*., 2000). As *age-1* mutants exhibit increased resistance to challenges such as high temperatures (Lithgow *et al*., 1994, 1995) and oxidative stress (Larsen, 1993; Vanfleteren 1993), it is conceivable that exposure to additional forms of stress may also alter fitness relative to wild type worms. However, the manner in which fitness could be modified is unclear. Whilst Gershon & Gershon (2002) predicted that exposure to additional stresses may reduce fitness in *age-1* mutants further than starvation conditions alone, Kenyon (2005) noted that wild type worms may not always have a selective advantage as *age-1* mutants are more likely to survive during periods of stress.

In this study, fitness was compared between *age-1* mutants and wild type worms by monitoring temporal changes in genotype frequencies when mixed genotype populations of different density were exposed to either mild or intense thermal stress. As *C. elegans* have been isolated from temperate and tropical regions throughout the world (Barrière & Félix, 2005a), thermal stress is likely to present an important selection pressure in wild populations. Whilst low density populations were always maintained with excess food, high density populations were maintained in more ecologically relevant conditions in which worms had to compete for limited resources. To determine why observed differences in fitness arose between the two genotypes in each condition, post stress survival, times to maturity and fecundity were monitored in each genotype, and spatial and temporal distributions of a DAF-16::GFP fusion protein were compared during and after exposure to thermal stress. This study therefore uses an integrative approach to assess how a laboratory generated mutation which extends lifespan and confers increased resistance to environmental stress modifies fitness in different ecological conditions.

4.3 Methods

4.31 Strains and Culture Conditions

The following genotypes were obtained from the Caenorhabditis Genetics Centre: N2 Bristol (wild type), TJ1052: *age-1(hx546)* and TJ356: N2; zIs356 [*daf-16p::daf-16::gfp*; *rol-6*(*su1006*)]. The latter strain carries an integrated *daf-16::gfp* translational fusion construct, in which GFP is fused to the last predicted amino acid of the DAF-16a2 isoform (Henderson & Jonson, 2001). Expression of the DAF-16::GFP fusion protein is driven by 6 kb upstream of the *daf-16* gene and is sufficient to restore a wild type phenotype in *daf-16(mu84)* null mutants. To obtain the *age-1(hx546)*; zIs356 [*daf-16p::daf-16::gfp*; *rol-6*(*su1006*)] genotype, *age-1(hx546)* mutant males were crossed with young adult hermaphrodites of the TJ356 strain as described in chapter 2. After allowing F1 hermaphrodites to self-fertilise, successful crosses were initially identified by scoring F2 and F3 progeny for dauer formation at 27°C, and for a roller locomotion phenotype resulting from the *rol-6*(*su1006*) mutation. The presence of the *age-1(hx546)* non-synonymous substitution (c→t, causing amino acid change P→S) was later confirmed by PCR and automated DNA sequencing, using the primer sequences 5' CCAGTATTATGCCTGCTTCA and 3' TGCGTACGGGTTCAAACAGC, as described in chapter 2. Strains were cultured on nematode growth media (NGM) plates, containing 10 µg/ml nystatin and 50 µg/ml streptomycin, seeded with *E. coli* (HB101). Strains were maintained at 20°C except when stated otherwise.

4.32 Laboratory selection experiments.

To establish if changes in fitness induced by exposure to thermal stress differ between wild type worms and *age-1* mutants, mixed genotype populations were either maintained at the control temperature (20°C) or periodically exposed to mild (27°C) or intense (30°C) heat shock treatments, and genotype frequencies were monitored over time. To determine if observed differences were dependent upon nutritional status and/or an interaction between temperature and nutritional status, populations were kept either at low densities, with excess food, or at high densities, representing more ecologically realistic conditions where food was limited.

a) Preparation and maintenance of mixed genotype populations

Age-synchronised worms were obtained by transferring eggs from well fed young adults onto empty seeded plates and then returning after 1-2 hours to collect the newly hatched larvae. When these had reached the fourth larval stage (L4), mixed genotype populations were established by transferring 2 individuals of each genotype on to each

NGM plate (24 populations were prepared on each occasion in two separate blocks). Half of the replicate populations were selected for low density treatments and half for high density treatments. To maintain the required densities, populations were washed off the NGM plates, in 500µl M9 buffer solution, and transferred to new seeded plates every second day. Low densities were maintained by transferring approximately one fifth of each original population in 100µl M9 buffer solution, and high densities were maintained by transferring the entire populations. For low density populations this procedure began on day 3, before food became limited, and for high density populations it began on day 5, when food had become limited or had been depleted. After transferrals populations were briefly left to dry in an extractor cabinet.

To monitor the occurrence of mating between the two genotypes, populations were intermittently examined for the presence of males. These arose at a very low frequency, even in populations which had been periodically exposed to thermal stress, and mating was not considered to influence the results.

.

b) Stress treatments

In each block, 4 low density and 4 high density replicate populations were allocated to each temperature treatment. Whilst control populations were constantly maintained at 20ºC, stressed populations were placed in Sanyo incubators at either 27ºC or 30ºC for 24 hours on days 6, 12 and 18.

c) Genotype frequencies

To monitor changes in genotype frequencies, approximately 50 eggs were collected from each population on days 6, 12 and 18, before stress treatments commenced, and on day 24. To ensure that sufficient numbers of eggs were available in the high density populations, eggs were always collected less than 24 hours after populations had been transferred to a new food source. Eggs were placed onto seeded NGM plates then were transferred to 27°C to develop for 3 days. Whilst *age-1* mutants arrest as dauers at 27°C, wild type develop into adults at this temperature (Malone *et al*., 1996). The frequency of each genotype in the populations could thus be determined by counting the number of dauers and adults on each plate. Eggs of known genotype were also placed at 27°C as positive controls. On a few occasions early in the project a small proportion of these controls did not arrest or mature as expected and the experiments were terminated. This problem was not encountered after a different, and likely more reliable, incubator became available for use.

4.33 Post-stress survival, times to maturity and fecundity

To determine why potential differences in fitness arose between the genotypes in each condition, post-stress survival, post-stress times to maturity and post-stress fecundity were compared after well fed and starved larvae had been maintained at 20°C or stressed at 27°C or 30°C for 24 hours. Whilst fed larvae were stressed during either the first or third larval stage (L1s or L3s), starved larvae were stressed as either arrested L1s or dauers. To obtain fed L1s, larvae were age-synchronised, as described in 4.32a, then separated onto seeded NGM plates, containing approximately 20 individuals each, immediately before stress treatments commenced. To obtain fed L3s, this procedure was performed 24 hours in advance. Arrested L1s and dauers were obtained from high density populations which had been starved for approximately 24 hours before stress treatments were implemented. As worms are awkward to transfer in the absence of bacteria, entire populations were stressed or maintained at 20°C. Immediately after the 24 hour stress period, arrested L1s and dauers were selected at random and separated, according to temperature treatment and stage, onto seeded NGM plates containing approximately 20 individuals each. Whilst post-stress survival and times to maturity were monitored in 5 separate blocks, post-stress fecundity was examined in 3 separate blocks.

a) Post-stress survival

Survival status was examined after the stress period then intermittently until maturity (worms which matured were considered to have survived even if they later died as young adults). If survival status was unclear, worms were gently touched with a platinum wire to stimulate a response.

b) Post-stress times to maturity

Before reaching maturity, surviving larvae were separated onto individual plates and developmental stage was monitored regularly until late L4. To compare maturity times, individuals were subsequently examined every 1-2 hours until reproduction commenced (worms were considered to have matured when the first egg had been laid). Worms which had matured during periods when they could not be monitored were removed from the analysis. These occurrences were minimised by performing late evening and early morning checks when necessary and, to ensure that sufficient data were obtained for each genotype, treatment and stage, different blocks commenced at different times of day.

c) Post-stress fecundity

To monitor post-stress fecundity, adults were transferred regularly to new seeded plates until reproduction had ceased (to facilitate counting, transferral frequency varied depending upon the rate at which eggs were being laid). After allowing 1-2 days for development at 20°C, plates containing offspring were placed in a cold room at 4°C ± 0.5°C and were stored at this temperature until time was available for counting. Offspring were counted soon after removal from the cold room (prolonged periods at 4°C ± 0.5°C reduce the rate of movement). When the number of offspring on a plate was relatively low, counting was performed simply by eye. When many offspring were present on a plate, worms were removed individually using a platinum pick.

4.34 Spatial and temporal expression of DAF-16::GFP

To examine how changes in the activity of DAF-16 contribute to the observed phenotypes in each condition, spatial and temporal distributions of a DAF-16::GFP fusion protein were compared between fed and starved TJ356 and *age-1(hx546)*; zIs356 [*daf-16p::daf-16::gfp*; *rol-6*(*su1006*)] L1s, during and after exposure to thermal stress, using methods described in chapter 2. DAF-16::GFP localisation was categorised from 1-4, where 1 represents a uniform distribution throughout cells and 2, 3 and 4 represent increasingly nuclear distributions (see 4.43 for examples of each category).

Several low density populations, which had been maintained with excess food, and several high density populations, which had been starved for approximately 24 hours, were obtained for each genotype. As multiple populations were required at each temperature, these were divided onto additional plates as necessary (low density populations were divided onto seeded plates whilst high density populations were divided onto un-seeded plates). Immediately before temperature treatments were imposed, DAF-16::GFP localisation was recorded in approximately 40 L1s from low density populations and 40 arrested L1s from high density populations for each genotype. For each genotype and density, 4-5 populations were then either maintained at 20°C or placed at 27°C or 30°C for 24 hours. DAF-16::GFP localisation was recorded as above for each genotype, density and treatment after 6 hours and 24 hours. After the 24 hour stress period, populations were washed off their original plates, in 500µl M9 buffer solution, and were transferred to new seeded plates. To prevent high density populations from becoming re-starved only a proportion of each of these populations was transferred, in 100ml M9 buffer solution. After plates had dried in an extractor cabinet they were placed at 20°C. DAF-16::GFP localisation was then scored

as above after 6 hours and 24 hours recovery. Observations were recorded under these conditions in three separate blocks.

4.35 Analysis

All analyses were performed in R version 2.10.1.

a) Laboratory selection experiments

To account for variation in sample size among populations, the analysis was performed on the binomial count data (numbers of each genotype in each population) rather than genotype frequencies. A generalised linear mixed effects model (GLMM) with a binomial error distribution and a logit link function was fitted to the data using the penalised quasi-likelihood (PQL) method (Breslow & Clayton, 1993) in the nlme library. The model was fitted using PQL to allow an auto-correlation function to be included to account for repeated measures on the same populations over time. The model contained a random effects term for block and all explanatory variables (day, temperature and density) and their interactions.

b) Post-stress survival, times to maturity and fecundity

Differences in survival at 30°C were compared between genotypes and between fed L1s and L3s using a generalized linear model (GLM) with a quasi-binomial error distribution and a logit link function. The model was fitted using a quasi-binomial error distribution because the data were under-dispersed. After removal of non-significant terms (stage and an interaction between genotype and stage), the minimum adequate model contained only genotype as an explanatory variable.

As post-stress times to maturity were dependent upon the stage which had been attained before stress treatments commenced, each stage was analysed separately. Times were compared between genotypes and among temperature treatments with generalised linear mixed effects models. These models, which were fitted using the penalised quasi-likelihood (PQL) method, had gamma error distributions and random effects terms to account for variation among blocks.

Post-stress fecundity was compared between genotypes and among stages and treatments using a linear mixed effects model. This model was used to account for variation in sample sizes and random effects of block and contained all explanatory variables (genotype, stage and temperature) and their interactions.

c) DAF-16 localisation

Cellular distributions of DAF-16::GFP were compared between genotypes and among treatments before and during exposure to stress (0 hours, 6 hours stress, 24 hours stress), and during recovery from stress (24 hours stress, 6 hours recovery, 24 hours recovery), using ordinal multinomial continuation-ratio logit models (Agresti, 2002; Thompson, 2009). These models were fitted using the VGAM library (Yee & Wild, 1996) and took account of the ordering in the categorical response (i.e. 1 < 2 < 3 < 4). Continuation-ratio logit models were used to determine the likelihood of continuing past a certain category level given that that category has been reached in the first place. Increased nuclear localisation of DAF-16::GFP was modelled before and during stress periods using the forward argument and translocation of DAF-16::GFP out of the nucleus during recovery from stress was modelled using the reverse argument, which fits the corresponding logits in reverse order (i.e. 4 < 3 < 2 < 1). Minimal adequate models were obtained based upon AIC values. The minimal model used to compare DAF-16::GFP distributions before and during exposure to stress contained all explanatory variables and interactions except for a four way interaction between time, genotype, treatment and density, and a three way interaction between genotype, treatment and density. The minimal model for recovery from stress contained all explanatory variables and interactions except for a four way interaction between time, genotype, treatment and density, and three way interactions between genotype, treatment and density, time, genotype and density, and time, genotype and treatment. When certain categories were not displayed by one or both genotypes in a particular density, or following a particular thermal treatment, it was not possible to obtain parameters to describe all of the observed category transitions. These situations are described within parenthesis in 4.43. P-values were obtained using likelihood ratio tests to compare between models following sequential removal of explanatory variables and interactions.

4.4 Results

4.41 Fitness costs are context dependent in *age-1* mutants.

In low density populations which had been maintained at 20°C, genotype frequencies remained relatively constant over time (Figure 4.1a). However, in high density populations which had been maintained at 20°C, the frequency of *age-1(hx546)* mutants declined over time relative to in low density populations ($t = -17.25$, $p < 0.001$, based upon binomial count data rather than frequencies), from 0.5 to 0.17 ± 0.04 (mean ± standard deviation) by day 24 (Figure 4.1b). These results clearly indicate that there is a fitness cost associated with the *age-1(hx546)* mutant allele when populations are maintained in putatively resource limited conditions at 20°C.

Although *age-1* mutant larvae arrest as dauers at 27°C when food is available whilst wild type larvae do not, frequencies of *age-1* mutants in low density populations which had been periodically exposed to this temperature did not differ over time relative to controls maintained at 20°C (Figure 4.1c). This suggests that life-history strategies may also be modified in wild type worms under these conditions. In high density populations which had been stressed at 27°C, frequencies of *age-1* mutants did not differ from those observed in high density control populations (Figure 4.1d). This is perhaps because larvae arrest development in both genotypes when resources are limited regardless of temperature.

Remarkably, in low density populations which had been periodically stressed at 30°C, frequencies of *age-1* mutants increased considerably over time relative to in low density populations which had not been stressed ($t = 12.69$, $p < 0.001$), from 0.5 to 0.90 ± 0.06 (mean ± standard deviation) by day 24 (Figure 4.1e). However, in high density populations which had been periodically stressed at 30°C the frequencies of *age-1* mutants did not differ from high density controls (Figure 4.1f). These results suggest that *age-1* mutants can have a selective advantage over wild type worms when populations are periodically exposed to intense thermal stress if resources are abundant. However, exposure to thermal stress does not modify the fitness cost observed in *age-1* mutants when populations are maintained in resource limited conditions.

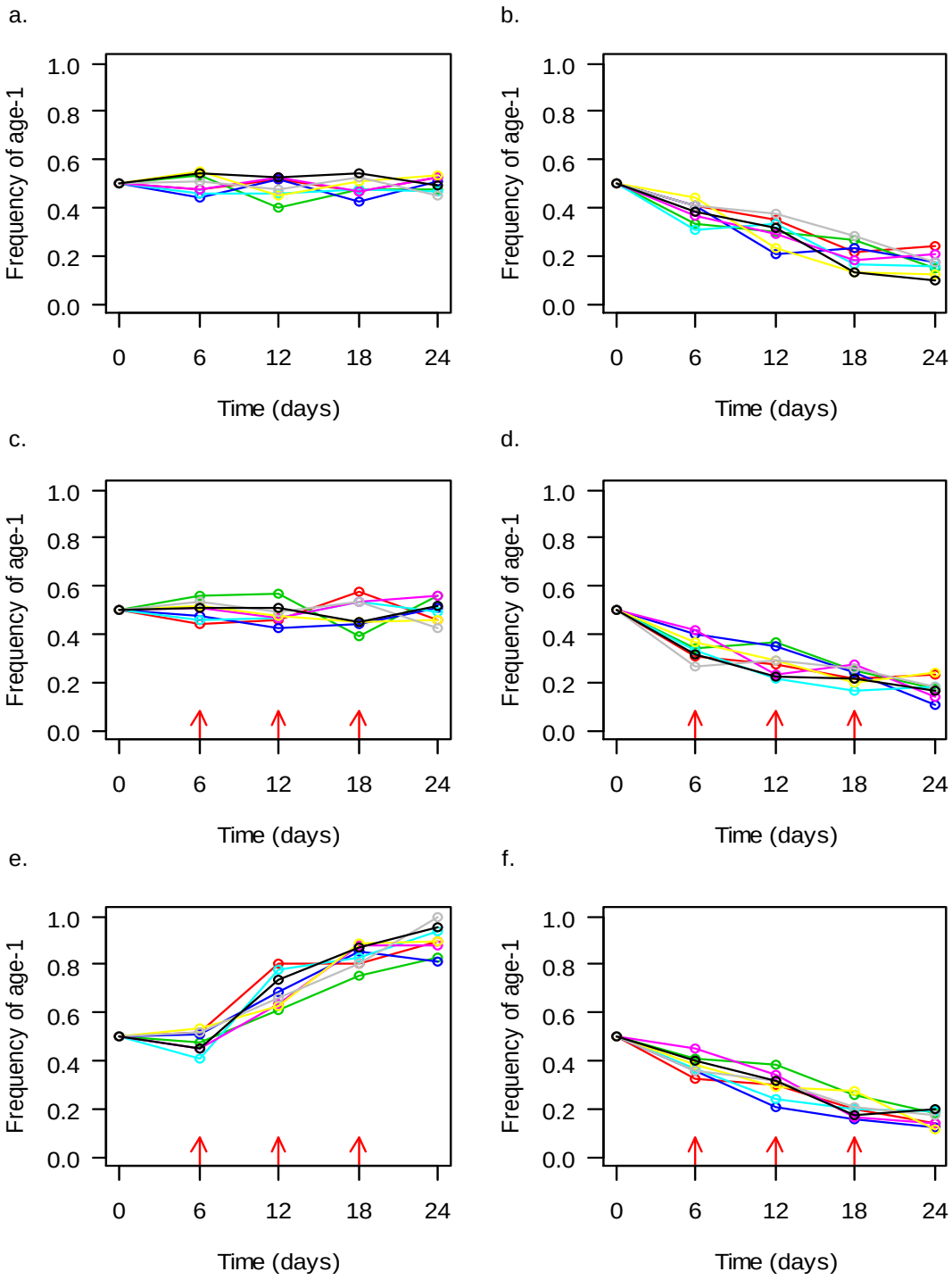


Figure 4.1: Temporal changes in *age-1(hx546)* frequencies. Plots represent *age-1(hx546)* frequencies relative to wild type in low density (a, c and e) and high density (b, d and f) populations maintained at 20°C (a and b) or periodically stressed at 27°C (c and d) or 30°C (e and f). Red arrows indicate days on which stress treatments were implemented and coloured lines represent replicate populations (4 populations were exposed to each treatment in 2 separate blocks).

4.42 Thermotolerance in *age-1* mutants is dependent upon nutritional status

To determine why fitness differences arise between the two genotypes in each condition, post stress survival, times to maturity and fecundity were examined in each genotype. No mortality was observed in either genotype when fed larvae, arrested L1s or dauers had been maintained at 20°C or stressed at 27°C. Survival was reduced in both genotypes when fed larvae had been stressed at 30°C. However, the proportion of individuals which survived was considerably higher in *age-1* mutants than in wild type worms ($F = 197.68$, $p < 0.001$) under these conditions (Figure 4.2). Remarkably, no differences in survival were observed between the two genotypes when starved larvae had been stressed at 30°C (Figure 4.2). These results indicate that the *age-1(hx546)* mutant allele may only enhance resistance to thermal stress when food is available.

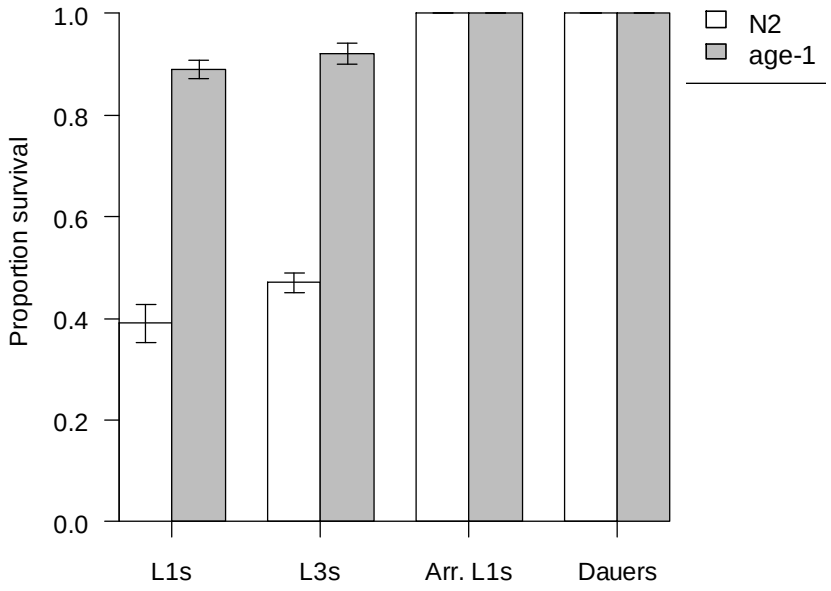


Figure 4.2: Survival following exposure to thermal stress. Bar plots represent the mean proportion of individuals, from 5 separate blocks, which survived after being stressed as L1s, L3s, arrested L1s (Arr. L1s) or dauers at 30°C. Error bars represent standard errors of the means. Survival was monitored in approximately 100 individuals per genotype, treatment and stage.

When fed larvae or arrested L1s had been maintained at 20°C, no differences were observed between genotypes in times to maturity (Figures 4.3a and 4.3b). However, times to maturity were considerably prolonged in *age-1* mutants relative to wild type worms ($t = -26.79$, $p < 0.001$) after larvae had been maintained in the dauer stage at 20°C (Figure 4.3b). This difference may explain why fitness costs arise in *age-*

1 mutants in resource limited conditions. Intermittent observations after food had been provided indicated that this was clearly due to a delay in exit from the dauer stage in *age-1* mutants. Times to maturity were also delayed in *age-1* mutants relative to wild type worms ($t = -20.6$, $p < 0.001$) after fed L1s had been stressed at 27°C for 24 hours (Figure 4.3a). This discrepancy is likely to have arisen because *age-1* larvae arrest as dauers at 27°C. Though wild type worms develop into adults at 27°C, after exposure to this temperature maturity was slightly delayed compared to wild type controls which had been maintained at 20°C ($t = -5.34$, $p < 0.001$). No differences in times to maturity were observed between the two genotypes when fed L3s or arrested L1s had been stressed at 27°C (Figure 4.3a). Although maturity was slightly delayed in both genotypes under these conditions relative to controls maintained at 20°C, these differences were not significant. When dauers had been stressed at 27°C, *age-1* mutants again matured considerably later than wild type worms after food became available.

Relative to controls which had been maintained at 20°C, times to maturity were substantially delayed in both genotypes after fed L1s and L3s had been stressed at 30°C (Figure 4.3a). However, though times were highly variable in both genotypes, *age-1* mutants consistently attained maturity more rapidly than wild type worms after exposure to this temperature (L1s: $t = 8.04$, $p < 0.001$; L3s: $t = 8.88$, $p < 0.001$). Remarkably, no differences in times to maturity were observed between wild type and *age-1* mutants that had been stressed at 30°C as arrested L1s (Figure 4.3b). Whilst wild type worms which had been stressed at 30°C as dauers again matured more rapidly than *age-1* mutants, maturity times were not significantly different from those observed at 20°C (Figure 4.3b). Consistent with the survival data described above, these results suggest that *age-1* mutants may display increased resistance to high temperatures only when food is not limited.

Although individuals which had arrested as L1s or dauers had been starved for approximately 48 hours, remarkably no differences in fecundity were observed between genotypes or among stages after worms had been maintained at 20°C (Figures 4.3c and 4.3d). However, post-stress fecundity was considerably higher in *age-1* mutants than in wild type worms ($t = 17.07$, $p < 0.001$) when larvae had been stressed at 27°C as fed L1s (Figure 4.3c). This is perhaps because *age-1* mutants had arrested as dauers under these conditions and were thus more protected against the damaging effects of high temperatures. Post-stress fecundity was extremely low in both genotypes after worms had been stressed at 27°C as fed L3s. However, no significant difference between *age-1* mutants and wild type worms was observed (Figure 4.3c).

Similarly, no differences in fecundity were observed between the two genotypes after worms had been stressed at 27°C as either arrested L1s or dauers.

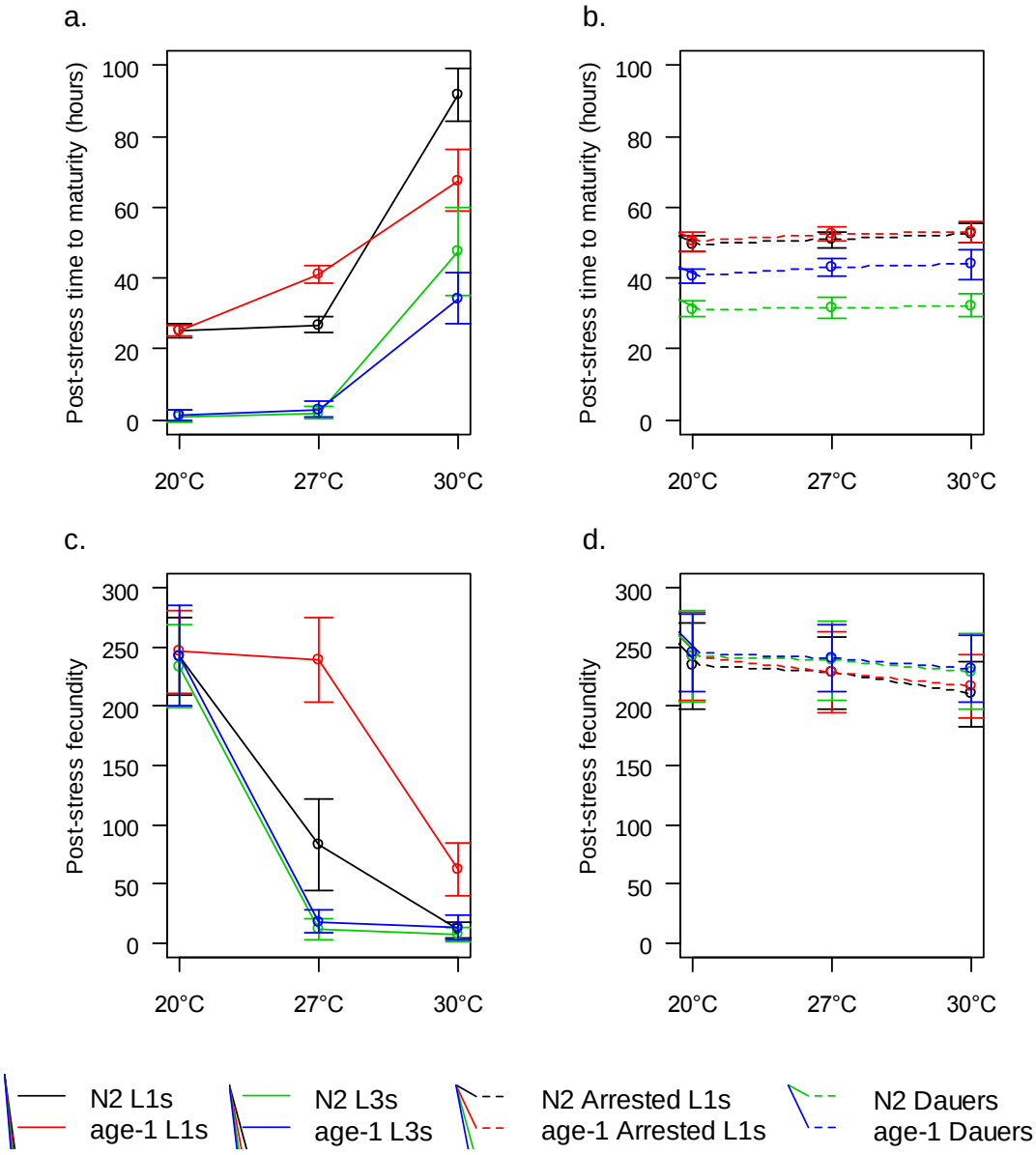


Figure 4.3: Post-stress times to maturity and fecundity. Plots represent mean times to maturity, from 5 separate blocks, in fed (a) and starved (b) larvae, and mean fecundity, from 3 separate blocks, in fed (c) and starved (d) larvae which had been maintained at 20°C or stressed at 27°C or 30°C for 24 hours. Error bars represent standard deviations of the means. Times to maturity were monitored in 80-100 individuals per genotype, stage and treatment, and lifetime fecundity was monitored in approximately 50 individuals per genotype, stage and treatment. In both cases the number of replicates was lower for fed wild type worms which had been stressed at 30°C during L1 or L3 due to mortality.

Though fecundity was dramatically reduced in both genotypes after fed L1s had been exposed to 30°C (Figure 4.3c), *age-1* mutants produced a larger number of viable offspring than wild type worms ($t = 4.80$, $p < 0.001$). However, no differences were observed between the two genotypes when worms were exposed to 30°C as fed L3s (Figure 4.3c). Relative to controls which had been maintained at 20°C, fecundity was slightly reduced in both genotypes after worms had been stressed at 30°C as either arrested L1s or dauers; however, no significant differences were observed between the two genotypes in these conditions (Figure 4.3d). Although *age-1* mutants displayed similar reductions in fecundity to wild type worms after being exposed to either mild or intense thermal stress as fed L3s, the differences observed between the two genotypes are broadly congruent with the suggestion that *age-1* mutants may only display increased resistance to thermal stress when food is available.

4.43 DAF-16 activity protects *age-1* mutants during and after thermal stress.

Spatial distributions of a DAF-16::GFP fusion protein were categorised on a continuum from 1 to 4, where 1 represents a uniform distribution throughout cells and 2, 3 and 4 represent increasingly nuclear distributions (Figure 4.4). In L1s that had been maintained at 20°C with excess food, DAF-16::GFP was mainly distributed throughout somatic cells in wild type, but was more nuclear localised in *age-1* mutants (no fed wild type displayed categories 3 or 4; categories 1-2: $t = 13.62$, $p < 0.001$) (Figures 4.5a and 4.5d). Relative to fed L1s, an increase in nuclear localisation was observed after 24 hours in starvation conditions in wild type L1s (no fed wild type displayed categories 3 or 4; categories 1-2: $t = 13.66$, $p < 0.001$) and in *age-1* mutant L1s (no starved *age-1* mutants displayed category 1; categories 2-3: $t = 14.85$, categories 3-4: $t = 9.02$, $p < 0.001$) (Figures 4.5g and 4.5j, time 0). However, nuclear localisation remained more intense in *age-1* mutants under these conditions (no starved *age-1* mutants displayed category 1; categories 2-3: $t = 9.65$, categories 3-4: $t = 4.71$, $p < 0.001$). Whilst transitions in cellular distributions of DAF-16::GFP were similar in the two genotypes during prolonged starvation (Figures 4.5g and 4.5j, times 6 and 24), DAF-16::GFP distributions appeared to reverse more rapidly in wild type worms than in *age-1* mutants after food had been provided (Figures 4.5g and 4.5j, times 6R and 24R). However, the recovery model described in 4.35c was not sensitive enough to demonstrate this response.

Although the proportion of fed L1s which displayed slight nuclear localisation of DAF-16::GFP increased when wild type were stressed at 27°C (no fed wild type displayed categories 3 or 4 at either 20°C or 27°C; categories 1-2: $t = 2.74$, $p < 0.001$), a more dramatic transition towards intense nuclear localisation was observed in *age-1*

mutants under these conditions (no *age-1* mutants displayed category 1 at 27°C; categories 2-3: t = 8.00, categories 3-4: t = 3.31, $p < 0.001$) (Figures 4.5b and 4.5e, times 6 and 24). This difference may underlie the constitutive dauer arrest observed in *age-1* mutants at 27°C. Furthermore, whilst wild type DAF-16::GFP distributions had returned to those observed in control conditions after 6 hours recovery at 20°C, in *age-1* mutants this response was delayed until after 24 hours recovery (Figures 4.5b and 4.5e, times 6R and 24R). However, the recovery model described in 4.35c was not sensitive enough to reveal this response. Remarkably, in starved L1s which had been stressed at 27°C, no significant differences in cellular distributions of DAF-16::GFP were observed in either genotype relative to starved L1s which had been maintained at 20°C.

When fed L1s were stressed at 30°C, the proportion of individuals which displayed nuclear localisation of DAF-16::GFP increased dramatically in both wild type worms (no wild type displayed categories 3 or 4 at 20°C; categories 1-2: t = 4.17, categories 2-3: t = 1.43, $p < 0.001$) and *age-1* mutants (no *age-1* mutants displayed category 1 or 2 at 30°C; categories 2-3: t = 5.42, categories 3-4: t = 4.56, $p < 0.001$), and nuclear localisation remained intense in both genotypes during the stress period (Figures 4.5c and 4.5f, times 6 and 24). However, DAF-16::GFP distributions appeared to reverse more rapidly in wild type than in *age-1* mutants after removal from the stress. However, the recovery model described in 4.35c was not sensitive enough to demonstrate this response. In starved L1s which had been stressed at 30°C no differences were observed in either genotype relative to starved L1s maintained at 20°C. Though spatial and temporal distributions of DAF-16::GFP differ between the two genotypes in starvation conditions, these results suggest that neither genotype responds to high temperatures via DAF-16 when worms are nutritionally stressed.

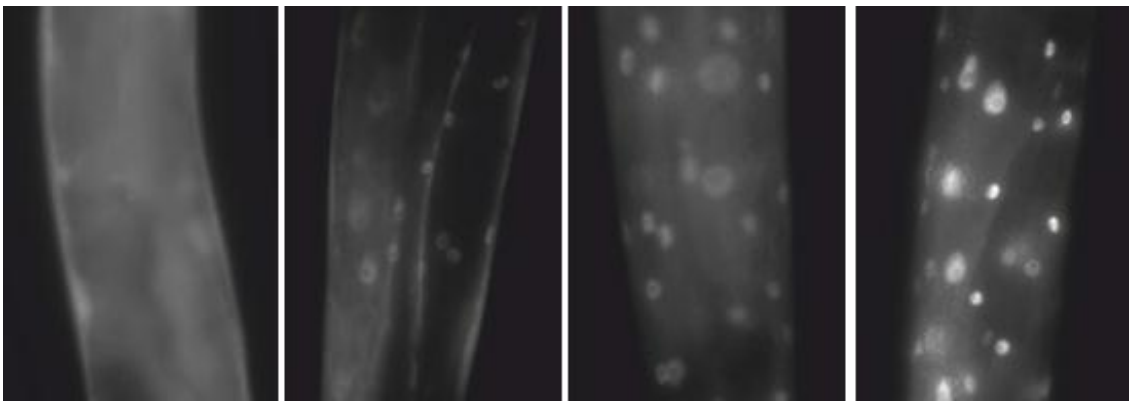
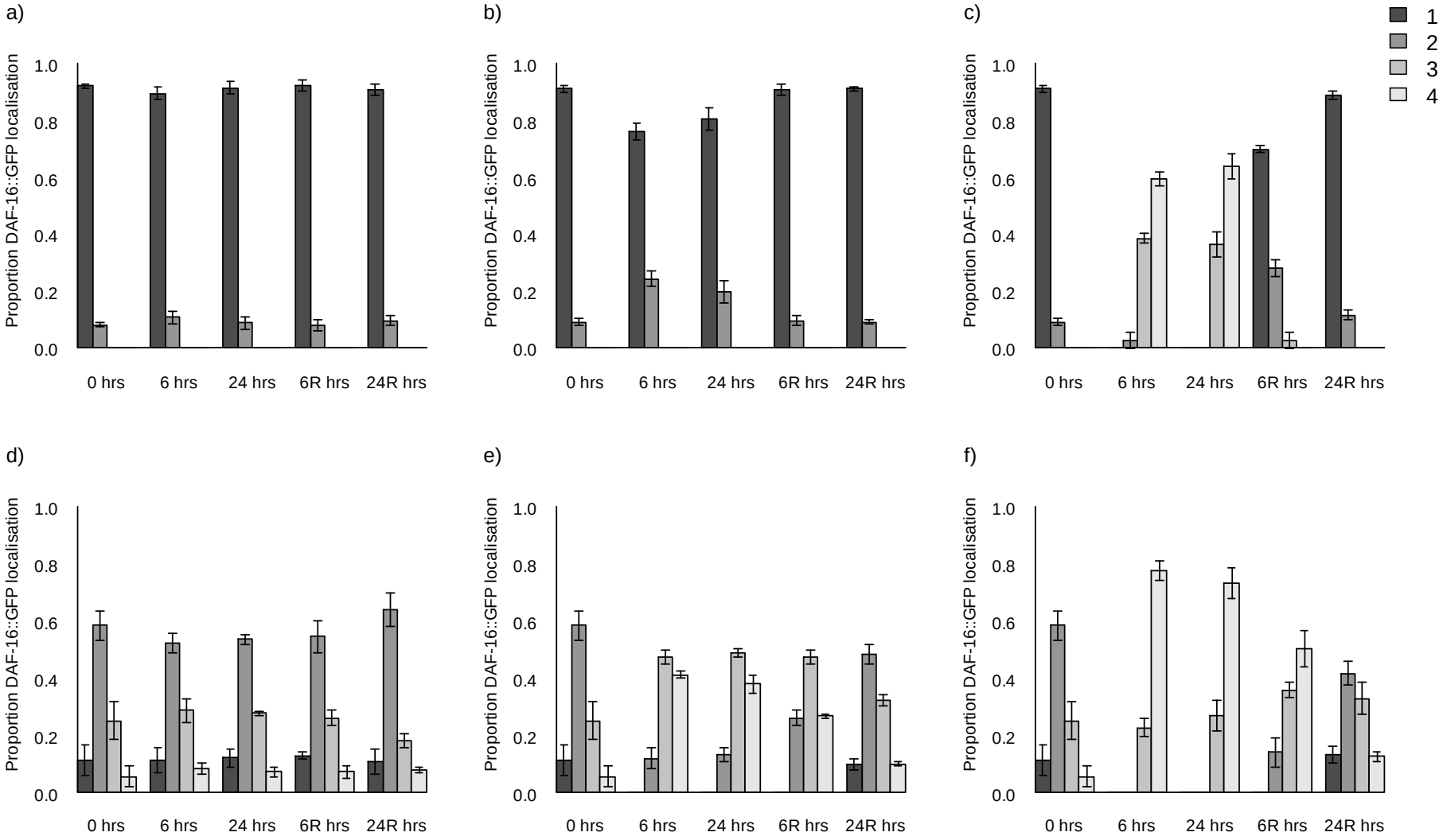


Figure 4.4: Cellular distributions of a DAF-16::GFP fusion protein. From left to right photos represent categories 1 (uniform distribution), 2, 3 and 4 (nuclear localisation).


a)
b)
c)
d)
e)
f)
Proportion DAF-16::GFP localisation
1.0
0.8
0.6
0.4
0.2
0.0
0 hrs
6 hrs
24 hrs
6R hrs
24R hrs
1
2
3
4

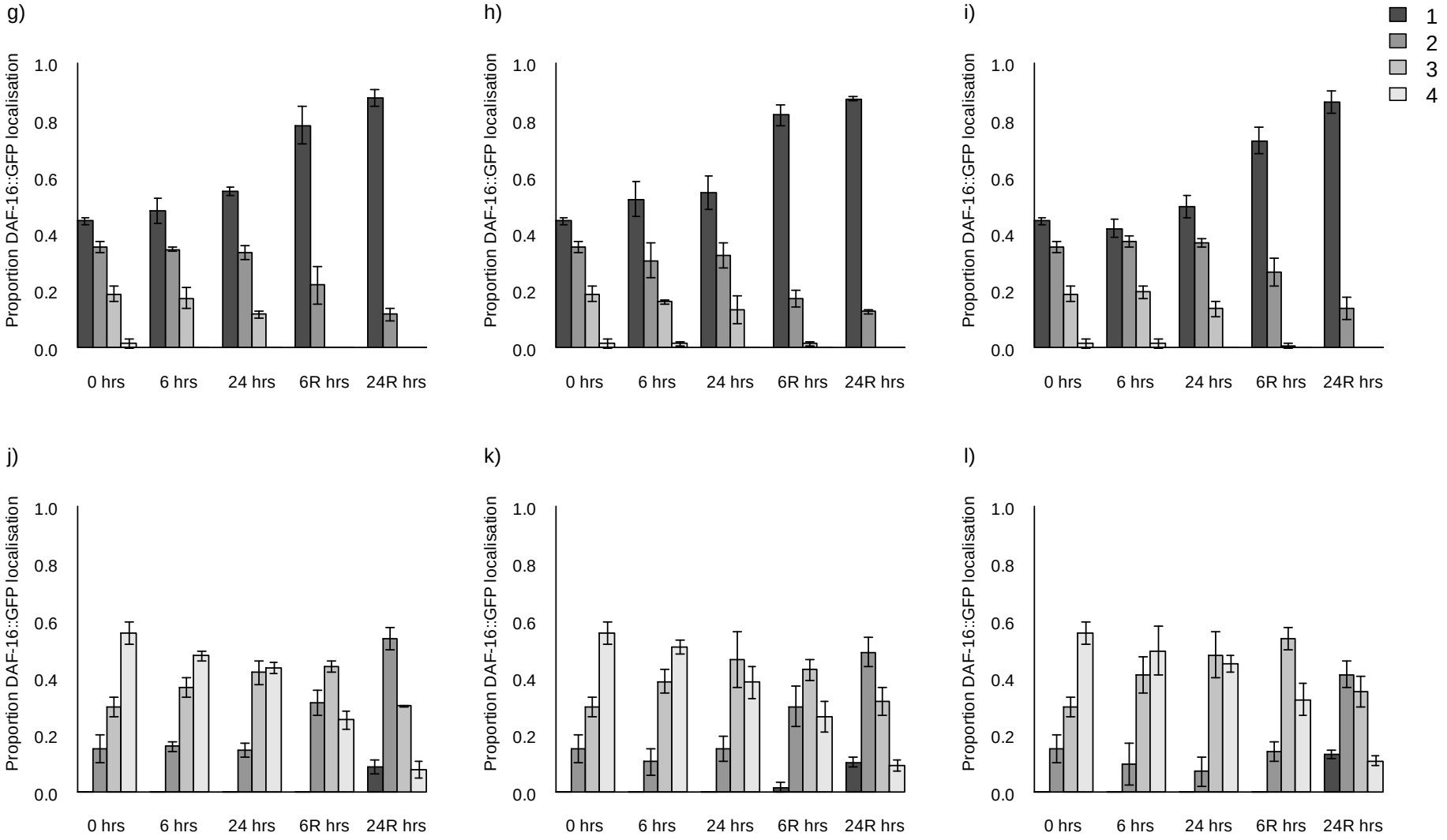

g)
h)
i)
j)
k)
l)
Proportion DAF-16::GFP localisation
1.0
0.8
0.6
0.4
0.2
0.0
0 hrs
6 hrs
24 hrs
6R hrs
24R hrs
1
2
3
4

Figure 4.5: Effects of genotype and treatment on DAF-16::GFP localisation. DAF-16::GFP is more nuclear localised in *age-1* mutants than in wild type worms when food is available (a and d) and in starvation conditions (g and j) at 20°C. When food is available, exposure to 27°C induces intense nuclear localisation of DAF-16::GFP in *age-1* mutants (e) and exposure to 30°C induces intense nuclear localisation of DAF-16::GFP in wild type worms (c) and in *age-1* mutants (f). However, exposure to high temperatures during starvation does not considerably alter DAF-16::GFP distributions in either genotype relative to starved worms maintained at 20°C (g-l). During recovery from starvation and/or exposure to stress, DAF-16::GFP distributions appeared to reverse more rapidly in wild type worms than in *age-1* mutants. Bar plots represent the mean proportion of individuals, from 3 separate blocks, which displayed categories 1-4 of DAF-16::GFP localisation before stress treatments were implemented (0 hrs), during stress treatments (6 and 24 hrs) and after removal from stress and/or after food had been provided (6R and 24R hrs). A-c represent fed wild type L1s, d-f represent fed *age-1* mutant L1s, g-i represent starved wild type L1s and j-l represent starved *age-1* mutant L1s which had been maintained at 20°C (a, d, g and j), stressed at 27°C (b, e, h and k) or stressed at 30°C (c, f, i and l). Error bars represent standard errors of the means. DAF-16::GFP localisation patterns were scored in approximately 120 fed and starved L1s per genotype, treatment and time. The number of replicates was lower for fed wild type L1s which had been stressed at 30°C due to mortality.

4.5 Discussion

In this study an integrative approach was used to examine how increased longevity and resistance to environmental stress can modify fitness in ecologically relevant conditions. Whilst fitness costs consistently arise when *age-1* mutants are exposed to nutritional stress, I have demonstrated that the *age-1(hx546)* mutant allele can confer a selective advantage if populations encounter periods of intense thermal stress when excess food is available. This result, which has not previously been demonstrated for any long-lived mutant, does not refute the antagonistic pleiotropy and disposable soma theories of senescence. Instead it implies that, given a specific ecological niche, mutations which promote longevity could be selected and maintained in wild populations and an optimal life history strategy which is characterised by long life and an ability to tolerate harsh environments may evolve.

As previously demonstrated by Walker *et al*. (2000), *age-1* mutants had equal fitness to wild type worms when populations were maintained in favourable growth conditions. Consistent with this, no differences were observed between the two genotypes in survival to adulthood, age at maturity or lifetime fecundity in these conditions. However, cellular distributions of DAF-16::GFP clearly indicate that the *age-1(hx546)* allele induces constitutive expression of genes involved in somatic maintenance and repair. As the two genotypes appear to invest equally in growth and reproduction, this raises the possibility that wild type worms may store metabolic resources that are unused in favourable growth conditions, or that *age-1* mutants are more efficient at converting food into energy. When populations were maintained at high densities with limited access to food, *age-1* mutants displayed reduced fitness relative to wild type worms. This has also been previously reported (Walker *et al*., 2000), and indicates that the *age-1(hx546)* mutant allele disrupts the optimal response to nutritional stress. This is perhaps because times to maturity were considerably prolonged in *age-1* mutants after worms had been maintained as dauers. *C. elegans* has typically been isolated from nature in the dauer stage (Barrière & Felix, 2005b), suggesting that resource limited conditions and/or high population densities are frequently encountered in nature. If the fitness costs observed in *age-1* mutants primarily arise due to the delay in exit from the dauer stage, this may explain why the *hx546* mutant allele has not been found in wild populations.

Nuclear localisation of DAF-16::GFP is induced in wild type worms when starvation conditions are imposed (Henderson & Johnson, 2001). However, Weinkove *et al*. (2006) demonstrated that DAF-16::GFP translocates to the cytoplasm after prolonged starvation in wild type worms but remains nuclear localised in *age-1*

mutants. Consistent with this, a considerable proportion of starved wild type L1s did not display nuclear localisation of DAF-16::GFP when observed after 24 hours in starvation conditions, and nuclear localisation of DAF-16::GFP was considerably more intense in *age-1* mutants throughout the starvation period. Weinkove *et al*., (2006) proposed that the wild type response may prevent excessive expenditure of resources on somatic maintenance and repair during prolonged periods of starvation, and permit the retention of energy reserves for development and/or reproduction when conditions improve. They also suggested that the disruption of this response may explain why *age-1* mutants display reduced fitness when exposed to starvation conditions. It is possible that prolonged nuclear distribution of DAF-16::GFP both during starvation and after a food source becomes available may explain why exit from the dauer stage is delayed in *age-1* mutants.

Remarkably, no differences in fecundity were observed in either genotype after starved worms had arrested in the L1 diapause stage or as dauers relative to worms which had unlimited access to food throughout development. This is consistent with previous studies (Kim & Paik, 2008; Morran *et al*., 2009), and could imply that no trade-off arises between fecundity and increased investment in somatic maintenance and repair during these states of diapause. However, this may reflect upon the relatively brief duration of the starvation period. Indeed, Kim and Paik (2008) demonstrated that fecundity declines with increased time spent in the dauer stage, but is not altered when dauer arrest has been prolonged only for a few days. It remains possible that a trade-off may become apparent in worms which arrested as L1s or dauers if less favourable conditions are encountered during adulthood.

Although *age-1* mutants arrest as dauers at 27°C, no differences in fitness were observed between the two genotypes when low density populations were periodically exposed to this temperature. This may be because population growth rate is reduced to a similar extent in each genotype, due to low fecundity in wild type worms and to arrested development in *age-1* mutants, when larvae were exposed to 27°C early in development. It is conceivable that a difference in fitness may have arisen between the two genotypes if populations had been stressed at 27°C for prolonged periods of time. Whilst all *age-1* mutants would eventually arrest as dauers or become post-reproductive, the low fecundity of wild type worms would ensure slow but positive population growth. It has previously been demonstrated that hermaphrodite fecundity declines in wild type worms when growth temperatures exceed 24°C (Hirsh *et al*., 1976). Harvey and Viney (2007) demonstrated that, at 25°C, this may be primarily due to a reduction in the number of viable self-sperm. As hermaphrodites produce sperm during the fourth larval stage and larvae which had been placed at 27°C as L3s would

have reached this stage during the 24 hour stress period, the lack of distinction in post-stress fecundity between wild type worms and *age-1* mutants in these conditions may be because high temperatures damage sperm in both genotypes. A high proportion of fed *age-1* mutant L1s displayed intense nuclear localisation of DAF-16::GFP at 27°C, but an increase in nuclear localisation was observed in only a small proportion of wild type worms under these conditions. This is consistent with a previously study (Henderson & Johnson, 2001), and may explain why *age-1* mutant larvae arrest as dauers at 27°C. Several studies have reported that wild type larvae occasionally arrest as dauers at 27°C (Ailion & Thomas, 2000; Morley & Morimoto, 2004; Lee *et al*., 2009). However, Ailion and Thomas (2000) demonstrated that wild type worms rapidly exit the dauer stage at this temperature and continue developing into mature adults.

In low density populations which had been periodically stressed at 30°C, *age-1* mutants clearly displayed higher fitness than wild type worms. Consistent with the ecological stress theory of ageing (Parsons, 1995, 2002), this implies that if sufficient genetic variation exists in populations which frequently encounter harsh conditions, an optimal life history strategy may evolve which is characterised by long life and an ability to tolerate environmental stress. Thermal stress and other environmental challenges present important selection pressures acting upon life-history strategies in nature (Hoffman & Hercus, 2000), and variation in stress resistance has been associated with differences in longevity among wild populations of *D. melanogaster* (Nevo *et al*., 1998), the nematode *Heterorhabditis bacteriophora* (Grewal *et al*., 2002) and the gypsy moth *Lymantria dispar* (Lazarevic *et al*., 2007), and among closely related *Caenorhabditis* species (Amrit *et al*., 2010). This is the first study to demonstrate that a mutation which promotes longevity can confer a selective advantage over a wild type genotype under certain environmental conditions. Scheckhuber *et al*. (2007) claimed that a mutation which extends lifespan in *Podospora anserine* and *Saccaryomyces cerevisiae* increases fitness in both of these organisms. However, their conclusion was based upon differences in the replicative capacities of old cells observed in benign laboratory conditions. Given that early life-history traits have a greater impact upon population growth rates than those expressed late in life, it is not clear if the differences observed by Scheckhuber *et al*. (2007) would in fact increase the relative fitness of these long-lived mutants in any environmental condition.

The higher fitness of *age-1* mutants may be principally due to the previously reported increase in survival during exposure to intense thermal stress (Lithgow *et al*., 1994, 1995). However, *age-1* mutants also attained maturity more rapidly than wild type worms, and post-stress fecundity was moderately higher in *age-1* mutants than in wild type when worms had been stressed as L1s. Intense nuclear localisation of DAF-

16::GFP was induced in *age-1* mutants and in wild type worms at 30°C, suggesting that stress response proteins are up-regulated in both genotypes under these conditions. However, Walker *et al*. (2001) demonstrated that higher levels of the heat-shock protein HSP-16 accumulate in *age-1* mutants than in wild type worms during exposure to high temperatures. Furthermore, the *age-1(hx546)* mutant allele clearly promotes the expression of genes involved in somatic maintenance and repair in control conditions, and nuclear localisation of DAF-16::GFP appeared to be prolonged relative to wild type worms after removal from the stress. As DAF-16 target genes are thus likely to be up-regulated before, during and after exposure to high temperatures, fitness differences may arise between the genotypes because the increased activity of heat-shock proteins/molecular chaperones and other stress response proteins alleviate symptoms associated with thermal stress in *age-1* mutants. Consequently, these worms are more able to survive and to resume development and/or reproduction when conditions improve.

The relative fitness of *age-1* mutants in high density populations which were periodically exposed to 27°C or 30°C did not differ from controls which had been maintained at 20°C. Whilst considerable differences in post-stress survival, developmental rate and fecundity were observed between *age-1* mutants and wild type worms which had been stressed when food was available, differences observed in these traits when larvae had been starved prior to and during the stress period were similar to those observed between starved controls maintained at 20°C. Furthermore, cellular distributions of DAF-16::GFP in starved L1s during and after exposure to thermal stress did not differ considerably in either genotype relative to controls which had been maintained at 20°C. These results suggest that the discrepancy between the two genotypes in resistance to high temperatures does not exist under starvation conditions. The long-lived, stress-resistant phenotype of IIS mutants is thought to arise due to partial activation of the dauer expression profile throughout life (Kenyon *et al*., 1993; McElwee *et al*., 2004). Consequently, the lack of distinction between the two genotypes when larvae were stressed as dauers may be because the up-regulation of DAF-16 target genes is equivalent during this stage. As dauers are highly resistant to several forms of stress, including high temperatures (Anderson, 1978), and it has been reported that arrested L1s are resistant to at least some forms of stress (Baugh & Sternberg, 2006; Weinkove *et al*., 2006), it could be argued that starvation-induced thermotolerance may be specific to these forms of developmental arrest. However, it has also been reported that survival at high temperatures is enhanced in wild type adults under starvation conditions, and that this is at least partially independent of *daf-16* (Henderson *et al*., 2006; Steinkraus *et al*., 2008).

It has been suggested that DAF-16 acts as a nutrient sensor which is primarily activated during starvation conditions, and that additional transcription factors may be more important for responses to other forms of stress (Henderson *et al*., 2006). Though DAF-16 is clearly activated in response to high temperatures when food is available, as the cellular distributions of DAF-16::GFP observed in starved larvae did not vary substantially in either genotype among the different thermal treatments, the results of this study are broadly consistent with this hypothesis. To maintain cellular homeostasis following a rapid increase in temperature, cells elicit a highly conserved response which is regulated by heat-shock factor-1 (HSF-1) and involves the rapid transcription of genes encoding a variety of heat-shock proteins/molecular chaperones (Feder & Hoffman, 1999). Remarkably, the *C. elegans hsf-1* gene is required for the increased lifespan of IIS mutants and for temperature-induced dauer formation in *age-1* mutants (Hsu *et al*., 2003; Morley & Morimoto, 2004). This suggests that DAF-16 and HSF-1 work together to activate the expression of specific genes and indicates that an interesting association exists between the heat-shock response and longevity. Furthermore, whilst starvation induced thermotolerance is at least partially independent of *daf-16* (Henderson *et al*., 2006), the response is dependent upon *hsf-1* (Steinkraus *et al*., 2008). This may explain why no differences in resistance to thermal stress were observed between *age-1* mutants and wild type worms in starvation conditions, and why the relative fitness of *age-1* mutants in high density populations did not vary among thermal treatments.

Whilst daily and seasonal fluctuations in ambient temperature require short term ecological responses, global climate change may necessitate long term evolutionary responses. It is possible that adaptive selection in genes involved in signal transduction pathways which respond to changes in ambient temperature by regulating the expression of molecular chaperones and other stress response proteins may facilitate the ability of species to adapt to new climatic conditions. Consistent with this, studies in *D. melanogaster* have identified natural variation in genes encoding components of the IIS pathway (Williams *et al*., 2006; Paaby *et al*., 2010), and other genes which modulate longevity and/or resistance to high temperatures (Krebs & Feder, 1997; Schmidt *et al*., 2000; Bettencourt *et al*., 2002), which appear to be associated with different latitudinal clines. Adaptive selection in such genes may promote transitions in optimal life-history strategies that modify longevity in natural populations which experience different thermal conditions. Consistent with this, whilst flies from temperate regions have rapid development, high fecundity and short life spans, flies from equatorial regions display slower development, lower fecundity and increased longevity (Edgar, 2006).

Natural populations of *C. elegans* are likely to experience fluctuations in resource availability which either provide opportunities for rapid population growth or promote developmental arrest and dispersal. Furthermore, periods of thermal stress of equal intensity are unlikely to recur on a predictable temporal scale. The likelihood that *age-1* mutants would prevail or decline in more stochastic environments is explored using population projection matrices in chapter 7. The results of this study provide the first demonstration that a long-lived, laboratory-derived mutant can potentially have higher fitness than a wild type genotype, and imply that selection acting upon the ability to tolerate harsh conditions may indirectly promote the evolution of increased longevity in populations which experience periods of intense environmental stress.

Chapter 5 – Fitness costs are context dependent in long-lived *age-1* mutants: Part 2 - oxidative stress

5.1 Abstract

In *C. elegans*, long-lived *age-1(hx546)* mutants display increased resistance to a variety of environmental challenges, including oxidative stress. These mutants display trade-offs in resource limited conditions, but have equal fitness to the wild type genotype when food is available, and can have higher fitness when populations are exposed to intense thermal stress. In this study, fitness was compared between *age-1* mutants and wild type worms by monitoring temporal changes in genotype frequencies when mixed genotype populations of different density were periodically exposed to juglone, a superoxide generator which induces oxidative stress. Remarkably, frequencies of *age-1* mutants increased over time when low density populations which had constant access to food were periodically exposed to either 50μM or 100μM juglone. Although frequencies of *age-1* mutants consistently declined when populations were maintained at high densities with limited resources, exposure to 100μM juglone reduced the rate at which this occurred. These results demonstrate that *age-1* mutants can have a selective advantage over the wild type genotype if exposed to oxidative stress when resources are abundant, and that exposure to intense oxidative stress can reduce the fitness deficit observed in *age-1* mutants in resource limited conditions. Consistent with this, *age-1* mutants displayed higher survival and fecundity and matured more rapidly than wild type worms after exposure to oxidative stress except when stressed during the dauer stage. Spatial and temporal distributions of the FOXO transcription factor DAF-16 suggest that these differences arise because stress response proteins, including antioxidant enzymes which degrade reactive oxidants into non-reactive compounds, are expressed at higher levels in *age-1* mutants than in wild type worms before, during and after exposure to oxidative stress. The results in this study indicate that *age-1* mutants can exhibit higher fitness than wild type worms in a range of harsh conditions, and that genetic variation in the ability to tolerate environmental challenges may promote the evolution of increased longevity in populations which frequently experience periods of oxidative stress.

5.2 Introduction

Free radicals, such as the superoxide ($O^{\bullet}_{2}^{-}$), hydroxyl ($OH^{\bullet}$) and nitric oxide ($NO^{\bullet}$) radicals, and non-radical oxidants, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), are collectively referred to as reactive oxygen species (ROS). These can generate damage in a variety of cellular components, including nucleic acids, proteins and lipids. Endogenous ROS are predominantly produced as a by-product of mitochondrial metabolism and, to a lesser extent, as a consequence of peroxisome metabolism and of certain cytosolic enzyme reactions (Finkel & Holbrook, 2000; Thannickal, 2009). ROS can also be generated in response to exogenous factors such as ultraviolet (UV) radiation, hyperoxia, industrial pollution, traffic exhaust fumes and certain heavy metals, xenobiotics and pesticides (Finkel & Holbrook, 2000; Schröder & Krutman, 2005; Van Straalen & Roelofs, 2006). Although certain ROS play an important role in cellular processes, such as host defence and signal transduction (Finkel & Holbrook, 2000; Thannickal, 2009), the ubiquitous presence of these damaging oxidants in aerobic organisms has ensured that cells can generate a range of anti-oxidant enzymes, including superoxide dismutases (SOD), catalases and glutathione peroxidases, which degrade ROS into non-reactive compounds (Finkel & Holbrook, 2000; Thannickal, 2009).

According to the free radical theory of ageing (Harman, 1956), the accumulation of oxidative damage to macromolecules constitutes a major mechanism contributing to senescence. Consistent with this, certain loss-of-function mutations which promote longevity also confer resistance to oxidative stress in *C. elegans* (Larsen, 1993; Vanfleteren, 1993), *Drosophila melanogaster* (Lin *et al*., 1998; Clancy *et al*., 2001) and mice (Migliaccio *et al*., 1999). In *C. elegans*, the FOXO transcription factor DAF-16 regulates the expression of a variety of genes encoding antioxidant enzymes and other stress response proteins (Lee *et al*., 2003; Murphy *et al*., 2003; McElwee *et al*., 2003, 2004). DAF-16 also regulates the activities of SKN-1, a transcription factor known to play an important role in responding to ROS (Tullet *et al*., 2008), and SMK-1, a transcriptional co-activator required for the expression of DAF-16 target genes involved in resistance to oxidative stress (Wolff *et al*., 2006). As described in chapter 4, DAF-16 target genes are up-regulated in mutants of the insulin / IGF-1 signalling (IIS) pathway (Murphy *et al*., 2003; McElwee *et al*., 2004). Consequently, some of these mutants, including long-lived *age-1(hx546)* mutants, display increased resistance to a variety of forms of oxidative stress (Larsen, 1993; Vanfleteren, 1993; Duhon *et al*., 1996; Honda & Honda, 1999; Yanase *et al*., 2002; de Castro *et al*., 2004; Przybysz *et al*., 2009). As I have previously demonstrated that *age-1(hx546)* mutants can exhibit higher fitness

than the wild type genotype when populations are exposed to high temperatures, it is conceivable that the *age-1(hx546)* mutant allele may also confer a selective advantage when populations experience periods of oxidative stress.

There are distinctions between responses to thermal stress and ROS which make it difficult to predict how relative fitness may be modified in *age-1* mutants under oxidative stress conditions. Firstly, whilst *age-1* mutants are more thermotolerant than wild type worms throughout life when food is available (Lithgow *et al*., 1994), several studies have implied that increased resistance to ROS may only occur with advancing age (Larsen, 1993; Vanfleteren, 1993; Przybysz *et al*., 2009). As selection is ineffectual late in life (Williams, 1957; Hamilton, 1966), a relative increase in survival or fecundity at late ages is not likely to have a considerable impact upon fitness. Secondly, the association between nutritional status and stress resistance may differ depending upon the nature of the stress. Young adults with a wild type background are more resistant to thermal stress when starved than when fed, but are more sensitive to oxidative stress under these conditions (Henderson *et al*., 2006). Consequently, if environmental sources of ROS are encountered by populations maintained in resource limited conditions, a mutation which enhances resistance to oxidative stress when wild type worms are highly susceptible may be favoured by selection.

In this study, fitness was compared between *age-1(hx546)* mutants and wild type worms by monitoring temporal changes in genotype frequencies when mixed genotype populations of different density were exposed to oxidative stress. Whilst low density populations had constant access to food, high density populations were constrained by resource limitation. As populations are likely to encounter periodic fluctuations in resource availability in nature, high density treatments may reflect more ecologically relevant conditions than low density treatments. To determine why potential differences in fitness arose between the two genotypes in each condition, post stress survival, post stress times to maturity and post stress fecundity were monitored in each genotype, and spatial and temporal distributions of a DAF-16::GFP fusion protein were compared before, during and after exposure to oxidative stress. As described in chapter 4, when DAF-16 is distributed evenly throughout somatic cells it is considered to be inactive but when nuclear localised it promotes the expression of genes involved in somatic maintenance and repair (Henderson & Johnson, 2001; Lee *et al*., 2003; Murphy *et al*., 2003; McElwee *et al*., 2003). To induce oxidative stress, worms were exposed to juglone (5-hydroxy-1,4-naphthoquinone), a quinone produced by walnut and butternut trees of the *Juglans* genus. Juglone penetrates the cuticle (de Castro *et al*., 2004) and is reduced to the semiquinone radical (·Q-) inside cells (Khare *et al*., 2009). The semiquinone radical is produced as a by-product of the mitochondrial

electron transport chain and reacts with molecular oxygen to generate the superoxide radical (Finkel & Holbrook, 2000). Exposure to juglone thus increases the production of a major endogenous source of ROS.

5.3 Methods

5.31 Strains and Culture Conditions

See chapter 4.

5.32 Laboratory selection experiments.

a) Preparation and maintenance of mixed genotype populations

See chapter 4.

b) Stress treatments

To induce oxidative stress, populations were periodically transferred onto NGM agar plates containing juglone. 10 mM stock solutions were prepared by dissolving juglone powder (Sigma) in 98% ethanol. Final concentrations of 50μM and 100μM were then obtained by diluting the solutions in melted NGM which was prepared as described in chapter 2. NGM containing juglone was poured into petri dishes in a sterile extractor hood and left to set. Juglone loses its toxicity over time in solution and in agar (de Castro *et al*., 2004), so a fresh solution was prepared on each occasion and NGM agar plates were always poured 5-6 hours prior to use.

At the beginning of the stress period, populations were washed off standard NGM plates in 500μl M9 buffer solution and transferred directly to NMG plates containing juglone. To ensure that low density populations did not become starved during the stress period, the bacterial lawn was also washed off and transferred to the new plates. Populations were left to dry in a sterile extractor hood then were placed in an incubator at 20ºC for the remainder of the 24 hour stress period. Although the toxicity of juglone would progressively decline during this time, all replicate populations would experience this at the same rate. After the stress period, populations were washed off NGM plates containing juglone in 500μl M9 buffer solution and transferred to sterile Eppendorf tubes. To remove any traces of juglone, the populations were centrifuged briefly at 2000g then, after removing the supernatant, were washed in 500μl M9 buffer solution and vortexed. Populations were centrifuged as above, then, after removing the supernatant, 500μl M9 buffer solution was added and the populations were vortexed again. Populations were then transferred to seeded NGM plates and were left to dry in a sterile extractor hood before being replaced in an incubator at 20ºC. To account for any effects of the transferral procedures, control populations were treated as above, but were moved to and from standard NGM plates.

c) Genotype frequencies

See chapter 4.

5.33 Post-stress survival, times to maturity and fecundity

Age-synchronised larvae maintained with excess food and high density, starved populations were obtained as described in chapter 4. These were transferred to and from NGM plates containing 50μM or 100μM juglone as described in 5.32b. As larvae could be lost during the transferral procedures, 30-40 fed L1s and fed L3s were stressed in each block (large numbers of arrested L1s and dauers were always available in the high density populations). Approximately 20 worms of each stage were then randomly selected for post-stress observations. Controls were treated as above, but were moved to and from standard NGM plates. Whilst post-stress survival and times to maturity were monitored in 4 separate blocks, post-stress fecundity was monitored in 3 separate blocks.

a) Post-stress survival

See chapter 4.

b) Post-stress times to maturity

See chapter 4.

c) Post-stress fecundity

See chapter 4.

5.34 Spatial and temporal expression of DAF-16::GFP

Low density and high density populations were transferred to and from NGM plates containing 50μM or 100μM juglone as described in 5.32b. Controls were treated in the same manner, but were moved to and from standard NGM plates. Spatial and temporal distributions of a DAF-16::GFP fusion protein were monitored in each genotype as described in chapter 4. However, data were collected in only 2 separate blocks.

5.35 Analysis

All analyses were performed in R version 2.10.1.

a) Laboratory selection experiments

A generalised linear mixed effects model with a binomial error distribution and a logit link function was fitted to the data using the penalised quasi-likelihood (PQL) method as described in chapter 4. The model contained all explanatory variables (day, temperature and density) and their interactions, an auto-correlation function to account for repeated measures on the same populations over time, and a random effects term for block.

b) Post-stress survival and performance

Differences in survival following exposure to 50μM and 150μM juglone were compared between genotypes and among stages using a generalized linear model. The data were under-dispersed so the model was fit with a quasibinomial error distribution and a logit link function. The model contained all explanatory variables (genotype, stage and treatment) and their interactions. Post-stress times to maturity and fecundity were analysed as described in chapter 4.

c) DAF-16 localisation

Cellular distributions of DAF-16::GFP were compared between genotypes and among treatments before and during exposure to stress (0 hours, 6 hours stress, 24 hours stress), and during recovery from stress (24 hours stress, 6 hours recovery, 24 hours recovery), using ordinal multinomial continuation-ratio logit models as described in chapter 4. The minimal model used to compare DAF-16::GFP distributions before and during exposure to stress contained all explanatory variables and interactions, except for a four way interaction between time, genotype, treatment and density, and a three way interaction between genotype, treatment and density. The minimal model for recovery from stress contained all explanatory variables and interactions, except for a four way interaction between time, genotype, treatment and density, and three way interactions between time, genotype and density, and time, genotype and treatment. When certain categories were not displayed by one or both genotypes in a particular density, or following a particular treatment, it was not possible to obtain parameters to describe all of the observed category transitions. These situations are described within parenthesis in 5.43. P-values were obtained using likelihood ratio tests to compare between models following sequential removal of explanatory variables and interactions.

5.4 Results

5.41 Fitness costs are context dependent in *age-1* mutants.

In low density populations which had not been stressed, genotype frequencies remained relatively constant over time (Figure 5.1a). However, in high density populations which had not been stressed, frequencies of *age-1(hx546)* mutants declined over time relative to in low density populations ($t = -9.09$, $p < 0.001$, based upon binomial count data rather than frequencies), from 0.5 to 0.15 ± 0.04 (mean ± standard deviation) by day 24 (Figure 5.1b). In low density populations which had been periodically stressed on NGM plates containing 50μM juglone, frequencies of *age-1* mutants increased slightly over time relative to in control populations which had not been stressed ($t = 3.55$, $p < 0.001$), from 0.5 to 0.57 ± 0.06 (mean ± standard deviation) by day 24 (Figure 5.1c). In contrast, frequencies of *age-1* mutants in high density populations which had been exposed to these conditions declined over time, from 0.5 to 0.18 ± 0.04 (mean ± standard deviation) by day 24 (Figure 5.1d). Though the rate at which this occurred was reduced relative to the decline observed in high density populations maintained in control conditions, this difference was not significant. In low density populations which had been periodically exposed to 100μM juglone, frequencies of *age-1* mutants increased dramatically over time relative to in control populations which had not been stressed ($t = 14.37$, $p < 0.001$), from 0.5 to 0.71 ± 0.04 (mean ± standard deviation) by day 24 (Figure 5.1e). In high density populations which had been exposed to NGM containing 100μM juglone, frequencies of *age-1* mutants again declined over time, from 0.5 to 0.23 ± 0.04 (mean ± standard deviation) by day 24 (Figure 5.1f). However, the rate at which this occurred was reduced relative to in high density populations which had not been stressed ($t = 3.28$, $p = 0.002$). These results indicate that the *age-1(hx546)* mutant allele confers a selective advantage when low density populations are periodically exposed to either 50μM or 100μM juglone, and that the fitness deficit observed in *age-1* mutants maintained in high density populations with limited resources can be reduced if populations are periodically exposed to high levels of oxidative stress.

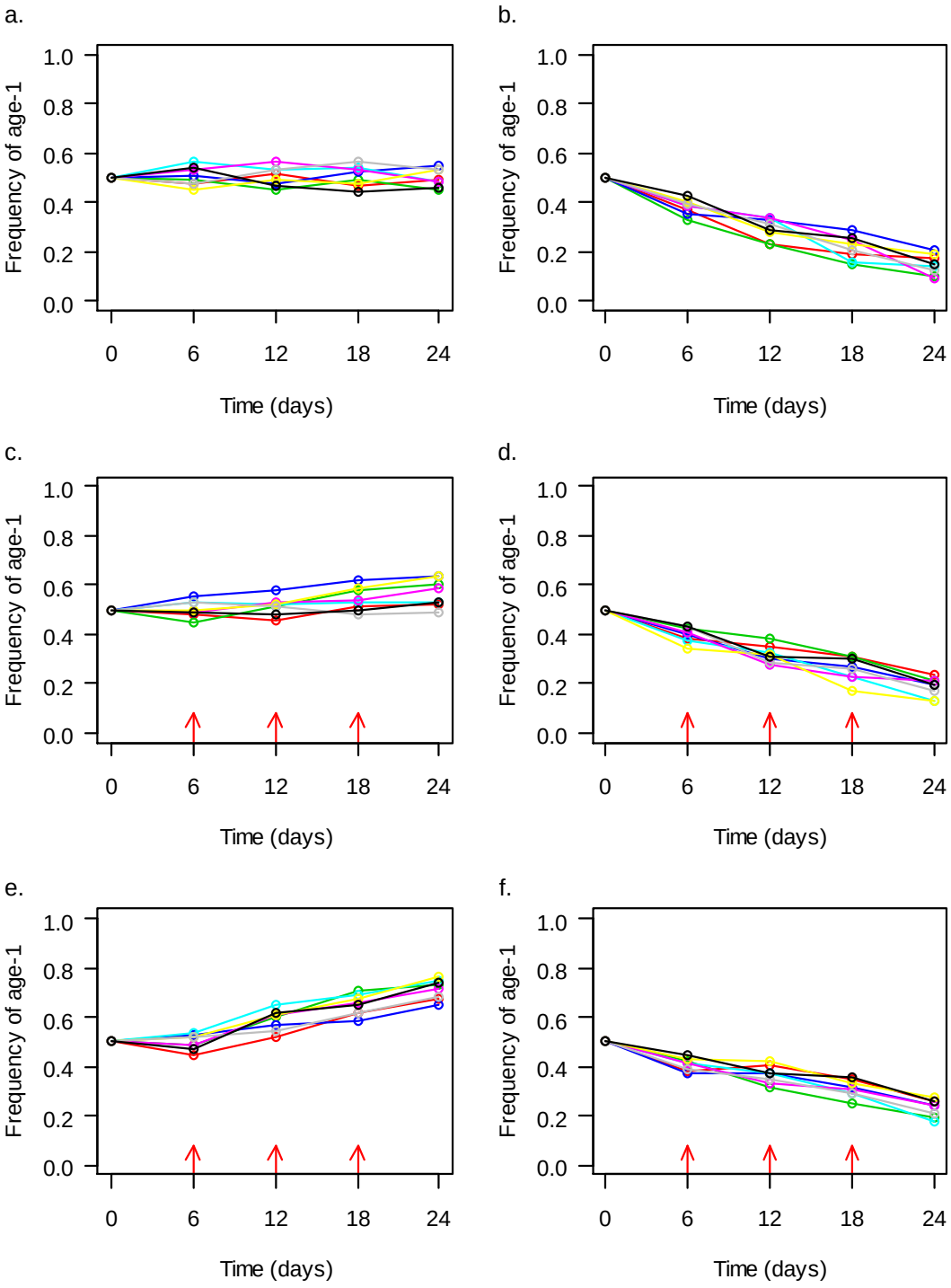


Figure 5.1: Temporal changes in *age-1(hx546)* frequencies. Plots represent *age-1(hx546)* frequencies relative to wild type in low density (a, c and e) and high density (b, d and f) populations maintained in control conditions (a and b) or periodically stressed on NGM agar plates containing 50µM (c and d) or 100µM juglone (e and f). Red arrows indicate days on which stress treatments were implemented and coloured lines represent replicate populations (4 populations were exposed to each treatment in 2 separate blocks).

5.42 Resistance to juglone is higher in *age-1* mutants than in wild type worms except for during the dauer stage

To determine why fitness differences arise between the two genotypes in each condition, post stress survival, times to maturity and fecundity were examined in each genotype. No mortality was observed in either genotype when fed and starved larvae had been maintained in controls conditions. However, survival was reduced in both genotypes relative to unstressed controls when fed L1s, fed L3s or arrested L1s had been exposed to 50μM juglone (Figure 5.2a). Survival was higher in *age-1* mutants under these conditions when larvae had been stressed as fed L1s ($t = 2.46$, $p = 0.018$) or fed L3s ($t = 3.10$, $p = 0.003$). Survival was reduced further in each genotype when fed and starved larvae had been exposed to 100μM juglone (Figure 5.2b). However, whilst survival was considerably higher in *age-1* mutants than in wild type worms when larvae had been stressed as fed L1s ($t = 8.55$, $p < 0.001$), fed L3s ($t = 7.90$, $p < 0.001$) or arrested L1s ($t = 4.35$, $p < 0.001$), no significant difference was observed between the two genotypes when larvae had been stressed during the dauer stage. Interestingly, intermittent observations of mixed genotype populations and populations of transgenic worms expressing DAF-16::GFP during exposure to 100μM juglone indicated that worms which had not arrested as L1s or dauers are highly sensitive to oxidative stress when starved.

When fed larvae or arrested L1s had been maintained in controls conditions, no differences were observed between the two genotypes in times to maturity (Figures 5.3a and 5.3b). However, wild type worms matured considerably earlier than *age-1* mutants after being maintained in the dauer stage ($t = -55.71$, $p < 0.001$) (Figure 5.3b). After exposure to 50μM juglone, maturity was delayed in both genotypes relative to controls except when stressed during the dauer stage (Figures 5.3a and 5.3b). Though *age-1* mutants attained maturity more rapidly than wild type worms when larvae had been stressed as fed L1s ($t = 2.77$, $p = 0.006$) or fed L3s ($t = 4.57$, $p < 0.001$), no significant difference was observed between the two genotypes when larvae had been stressed during L1 arrest. Maturity was further delayed in both genotypes when worms had been stressed on NGM plates containing 100μM juglone (Figures 5.3a and 5.3b). Again *age-1* mutants attained maturity more rapidly than wild type worms when larvae had been stressed as fed L1s ($t = 5.06$, $p < 0.001$) or fed L3s ($t = 9.01$, $p < 0.001$). Though maturity times were slightly delayed in both genotypes when worms had been exposed to 100μM juglone during L1 arrest or the dauer stage, no significant interactions between genotype and treatment were observed.

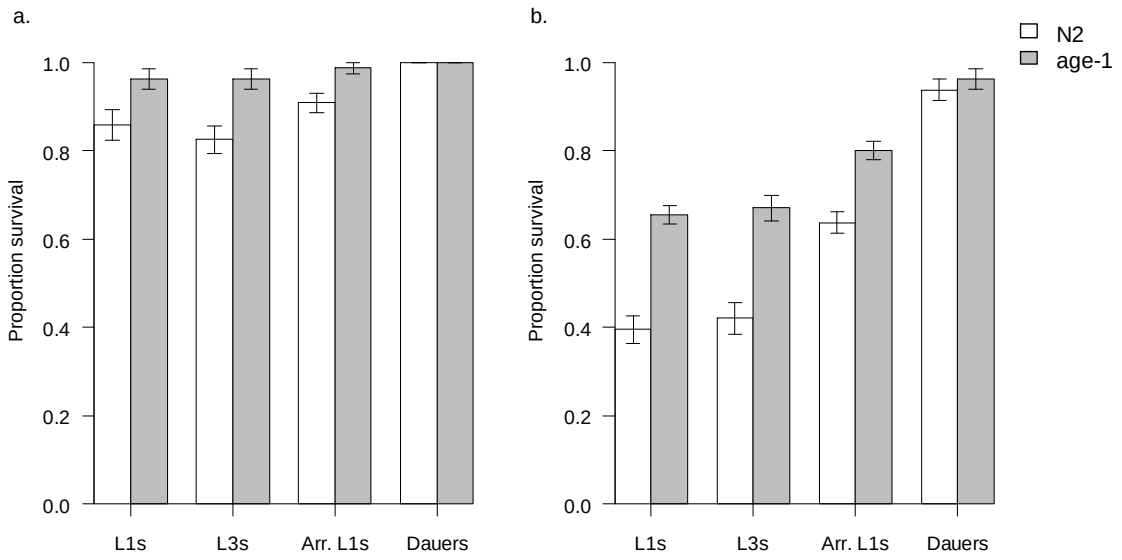


Figure 5.2: Survival following exposure to oxidative stress. Bar plots represent the mean proportion of individuals, from 4 separate blocks, which survived after being stressed as L1s, L3s, arrested L1s (Arr. L1s) or dauers on NGM plates containing a) 50μM or b) 100μM juglone. Error bars represent standard errors of the means and survival was monitored in approximately 75 individuals per genotype, treatment and stage.

In one set of fed controls fecundity was slightly higher in *age-1* mutants than in wild type worms (L1s: $t = 2.19$, $p = 0.028$). However, no other differences in fecundity were observed between the genotypes in control conditions (Figures 5.3c and 5.3d). Post-stress fecundity was reduced in both genotypes relative to controls when worms had been exposed to 50μM juglone as fed L1s, fed L3s or arrested L1s (Figures 5.3c and 5.3d). Though *age-1* mutants produced a larger number of viable offspring than wild type adults when individuals had been exposed to 50μM juglone as fed L1s ($t = 4.18$, $p < 0.001$) or fed L3s ($t = 5.39$, $p < 0.001$), no difference in post-stress fecundity was observed between the genotypes when larvae had been stressed as arrested L1s. More dramatic reductions in fecundity were observed in each genotype when larvae had been exposed to 100μM juglone (Figures 5.3c and 5.3d). However, fecundity remained higher in *age-1* mutants than in wild type worms when larvae had been exposed to these conditions as fed L1s ($t = 7.22$, $p < 0.001$) or fed L3s ($t = 7.80$, $p < 0.001$), and was also higher in *age-1* mutants which had been stressed during L1 arrest ($t = 2.21$, $p = 0.028$). A small decline in fecundity was observed in each genotype when larvae had been exposed to 100μM juglone during the dauer stage, but no significant interaction between genotype and treatment was observed. These results suggest that, relative to wild type worms, post-stress survival, developmental rate and fecundity are substantially higher in *age-1* mutants when larvae are exposed to ROS when food is

available, and that post-stress survival and fecundity are moderately higher in *age-1* mutants when larvae are stressed during L1 arrest.

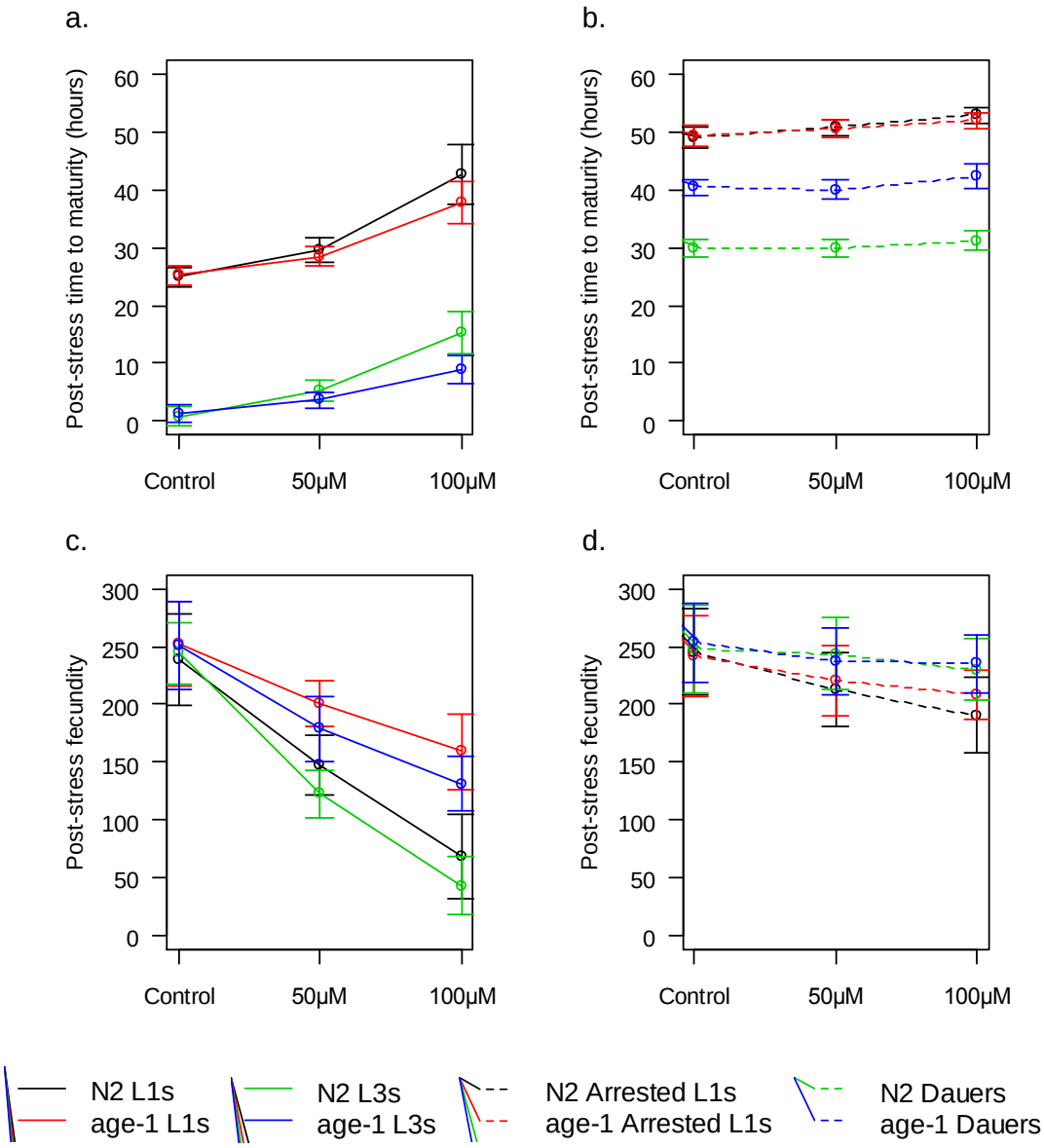


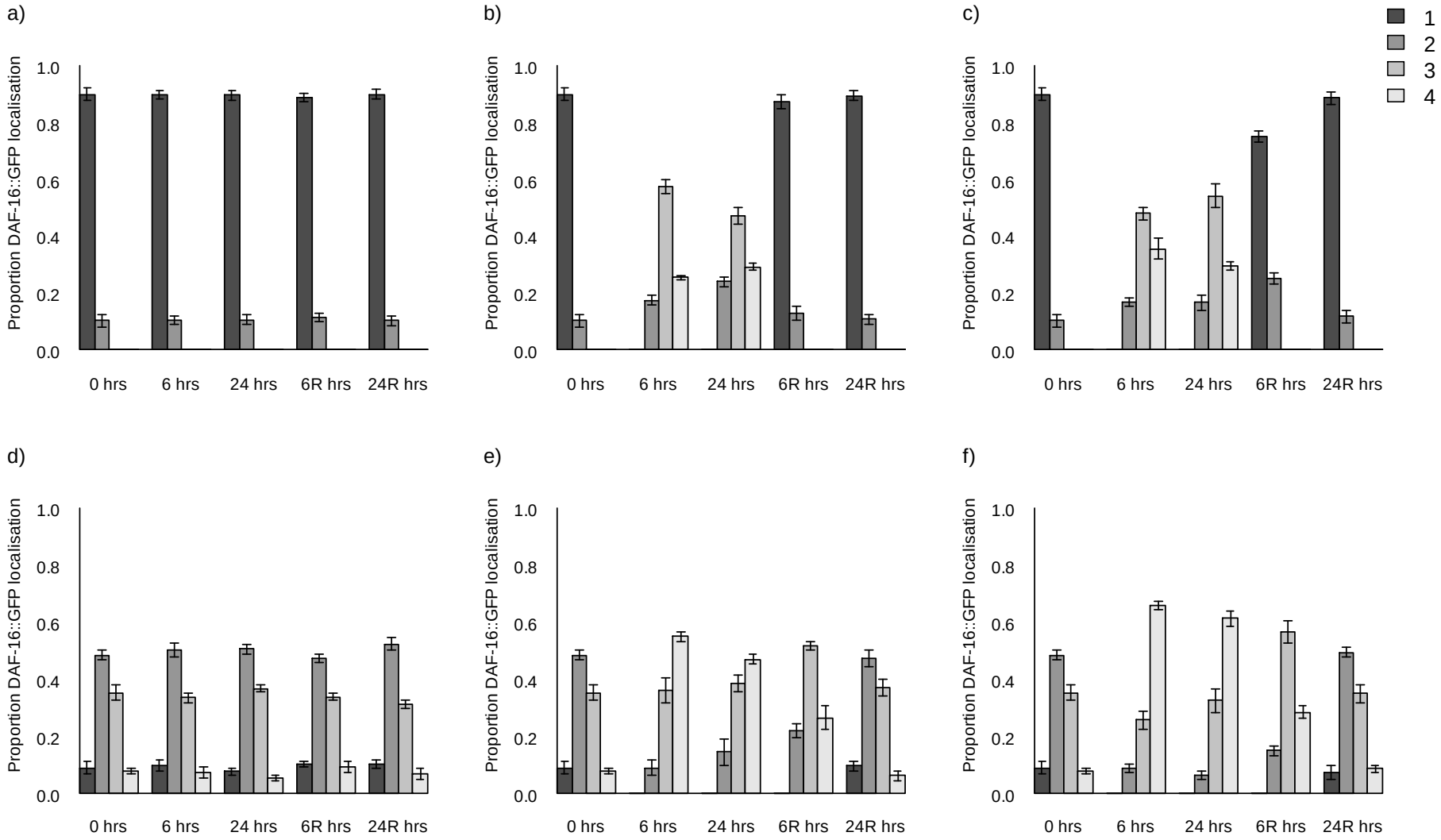
Figure 5.3: Post-stress times to maturity and fecundity. Plots represent mean times to maturity, from 4 separate blocks, in fed (3a) and starved (3b) larvae, and mean fecundity, from 3 separate blocks, in fed (3c) and starved (3d) larvae which had been maintained in control conditions or stressed on NGM plates containing 50μM or 100μM juglone for 24 hours. Error bars represent standard deviations of the means. Times to maturity were monitored in 60-75 individuals per genotype, stage and treatment, and lifetime fecundity was monitored in 40-50 individuals per genotype, stage and treatment. In both cases the number of replicates was lower for 100μM juglone treatments due to mortality.

5.43 DAF-16 activity protects *age-1* mutants during and after oxidative stress.

Spatial distributions of a DAF-16::GFP fusion protein were categorised on a continuum from 1 (uniform distribution) to 4 (nuclear distribution) as described in chapter 4. When L1s were maintained in control conditions with excess food, DAF-16::GFP was mainly distributed throughout somatic cells in wild type, but was more nuclear localised in *age-1* mutants (no fed wild type displayed categories 3 or 4; categories 1-2: $t = 8.58$, $p < 0.001$) (Figures 5.4a and 5.4d). After 24 hours in starvation conditions, an increase in nuclear localisation was observed in wild type L1s (no fed wild type displayed categories 3 or 4; categories 1-2: $t = 6.29$, $p < 0.001$) and in *age-1* mutant L1s (no starved *age-1* mutants displayed category 1; categories 2-3: $t = 5.30$, categories 3-4: $t = 3.68$, $p < 0.001$). However, nuclear localisation remained more intense in *age-1* mutants under these conditions (no starved age-1 mutants displayed category 1 and no wild type displayed category 4; categories 2-3: $t = 9.02$, $p < 0.001$). (Figures 5.4g and 5.4j, time 0). Furthermore, DAF-16::GFP appeared to translocate out of the nucleus more rapidly in wild type worms than in *age-1* mutants after food had been provided (Figures 5.4g and 5.4j, times 6R and 24R). However, this difference was not significant according to the recovery model described in 5.35c, which was not sensitive enough to reveal this response.

Following exposure to 50μM juglone, the proportion of fed L1s displaying nuclear localisation of DAF-16::GFP increased in wild type worms (no fed wild type displayed category 1 when stressed with 50μM juglone or category 4 in control conditions; categories 2-3: $t = 3.42$, $p < 0.001$) and in *age-1* mutants (no *age-1* mutants displayed category 1 when stressed with 50μM juglone; categories 2-3: $t = 5.25$, categories 3-4: $t = 2.50$, $p < 0.001$) (Figures 5.4b and 5.4e, times 6 and 24). However, nuclear localisation remained more intense in *age-1* mutants under these conditions (neither genotype displayed category 1 when stressed with 50μM juglone; categories 2-3: $t = 1.73$, categories 3-4: $t = 3.85$, $p < 0.001$). Furthermore, DAF-16::GFP distributions appeared to reverse more rapidly in wild type worms than in *age-1* mutants after removal from stress. However, the recovery model described in 5.35c was not sensitive enough to demonstrate this response. When starved L1s were exposed to 50μM juglone, spatial and temporal distributions of DAF-16::GFP were similar in both genotypes to those observed in starved L1s maintained in control conditions (Figures 5.4h and 5.4k). Although the intensity of nuclear localisation increased slightly in *age-1* mutants when food was available, DAF-16::GFP responses did not differ substantially in either genotype when fed L1s (Figures 5.4c and 5.4f) or starved L1s (Figures 5.4i and 5.4l) were exposed to 100μM juglone compared to larvae which were exposed to 50μM juglone. These results indicate that nuclear localisation of

DAF-16::GFP can be induced in response to starvation and oxidative stress in both genotypes, but that exposure to juglone has very little effect upon DAF-16::GFP distributions in starvation conditions. Importantly, DAF-16::GFP distributions indicate that DAF-16 target genes may be up-regulated in *age-1* mutants in control conditions, and that nuclear localisation remains more intense than in wild type worms during and after exposure to starvation and/or oxidative stress.


a)
b)
c)
d)
e)
f)
Proportion DAF-16::GFP localisation
1.0
0.8
0.6
0.4
0.2
0.0
0 hrs
6 hrs
24 hrs
6R hrs
24R hrs
1
2
3
4

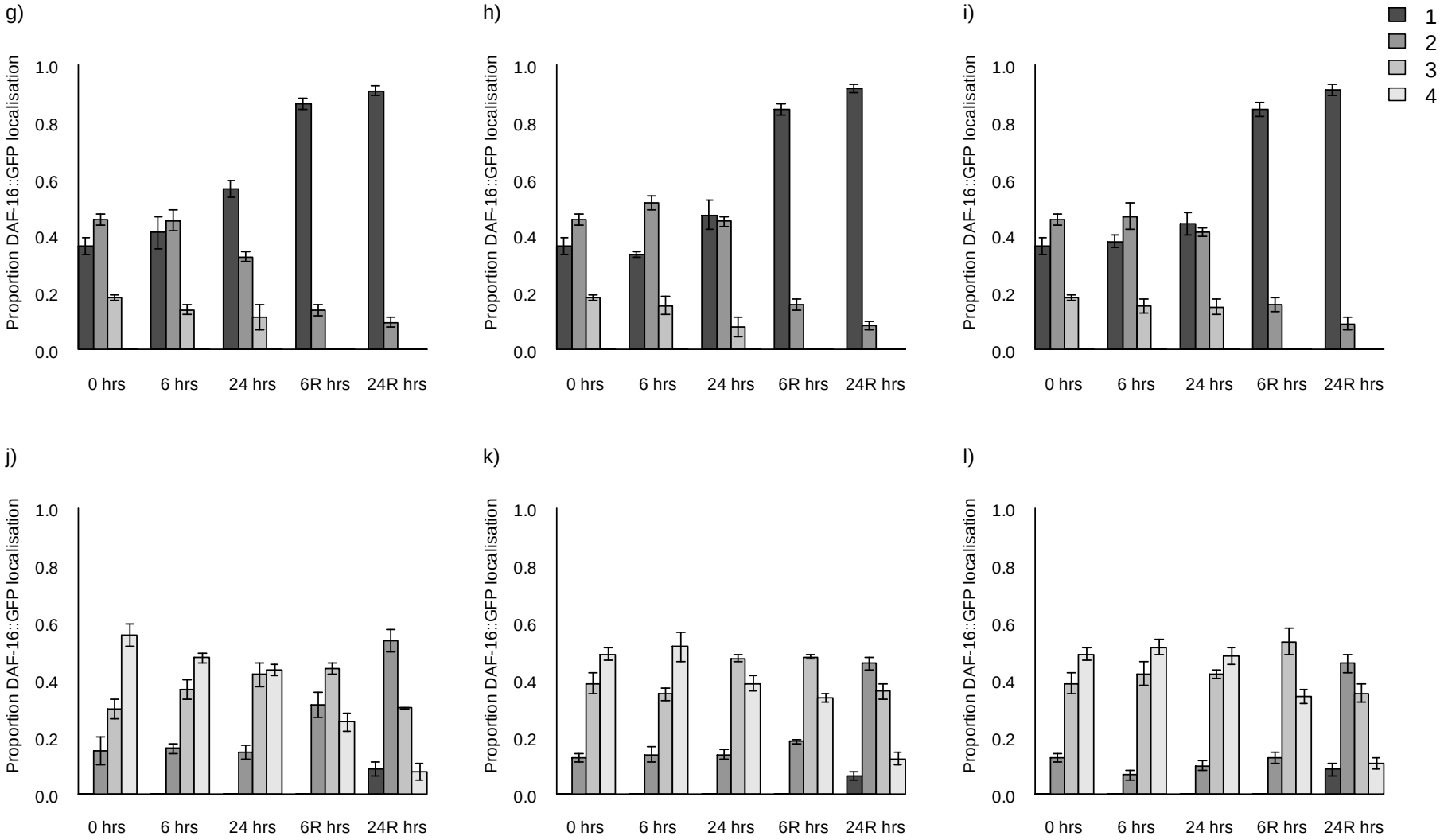

g)
h)
i)
j)
k)
l)
Proportion DAF-16::GFP localisation
1.0
0.8
0.6
0.4
0.2
0.0
0 hrs
6 hrs
24 hrs
6R hrs
24R hrs
1
2
3
4

Figure 5.4: Effects of genotype and treatment on DAF-16::GFP localisation. DAF-16::GFP is more nuclear localised in *age-1* mutants than in wild type worms when food is available (a and d) and in starvation conditions (g and j) when no oxidative stress treatment is applied. When food is available, exposure to 50µM juglone and 100µM juglone induces nuclear localisation of DAF-16::GFP in wild type (b and c) and *age-1* mutants (e and f), but nuclear localisation remains more intense in *age-1* mutants under these conditions. Exposure to juglone during starvation does not substantially alter DAF-16::GFP distributions in either genotype relative to starved controls (g-l). During recovery from starvation and/or exposure to stress, DAF-16::GFP distributions appeared to reverse more rapidly in wild type worms than in *age-1* mutants. Stacked bar plots represent the mean proportion of individuals, from 2 separate blocks, which displayed categories 1-4 of DAF-16::GFP localisation before stress treatments were implemented (0 hours), during stress treatments (6 and 24 hours) and after removal from stress and/or after food had been provided (6R and 24R hours). A-c represent fed wild type L1s, d-f represent fed *age-1* mutant L1s, g-i represent starved wild type L1s and j-l represent starved *age-1* mutant L1s which had been maintained in control conditions (a, d, g and j), exposed to 50µM juglone (b, e, h and k) or exposed to 100µM juglone (c, f, i and l). Error bars represent standard errors of the means. DAF-16::GFP localisation patterns were scored in 60-70 fed and starved L1s per genotype, treatment and time.

5.5 Discussion

In this study an integrative approach was used to examine how molecular to population levels responses to ROS modify the fitness of long-lived *age-1* mutants relative to wild type worms. I have demonstrated that the *age-1(hx546)* mutant allele confers a selective advantage when low density populations which have constant access to food encounter periods of oxidative stress, and that exposure to intense oxidative stress can reduce the fitness deficit observed in *age-1* mutants in resource limited conditions. Given the role of DAF-16 in regulating antioxidant enzymes and other stress response proteins, this may be because *age-1* mutants invest more resources in somatic maintenance and repair than wild type worms before, during and after exposure to oxidative stress and are thus more able to survive, develop and reproduce after the stress has been removed. These results imply that the *age-1(hx546)* mutant allele confers a selective advantage over the wild type genotype under a range of harsh environmental conditions, and that this phenomenon is not specific to thermal stress. Furthermore, if wild populations encounter exogenous sources of ROS when resources are abundant, these results suggest that increased longevity may evolve as a by-product of selection for greater resistance to oxidative stress.

DAF-16::GFP distributions suggest that the *age-1(hx546)* mutant allele promotes the expression of genes involved in somatic maintenance and repair even when no stress is applied. Despite this, no differences in fitness were observed between *age-1* mutants and wild type worms when populations were maintained at low densities with constant access to food. In contrast, frequencies of *age-1* mutants rapidly declined in high densities populations which were maintained with limited food. This is perhaps because nuclear localisation of DAF-16::GFP is more intense in *age-1* mutants than in wild type worms during and after starvation, as these differences may explain why exit from the dauer stage is delayed. As described in chapter 4, these results are consistent with a previous study (Walker *et al*., 2000) and suggest that costs associated with increased lifespan do not necessarily arise in favourable environments, but become apparent in conditions which are likely to be encountered in nature. These results support the antagonistic pleiotropy (Williams, 1957) and disposable soma (Kirkwood, 1977) theories of senescence.

When low density populations were periodically exposed to 50µM juglone, frequencies of the *age-1(hx546)* mutant allele increased slightly over time. As relative fitness is, therefore, moderately higher under these conditions, these results suggest that *age-1* mutants do not only display increased resistance to oxidative stress late in

life. Indeed, post-stress survival and fecundity were higher in *age-1* mutants than in wild type worms when larvae had been exposed to 50µM juglone as fed L1s or fed L3s, and, relative to controls which had not been stressed, post-stress times to maturity were delayed to a lesser extent in *age-1* mutants than in wild type worms. Consistent with this, it has previously been reported that survival is prolonged in *age-1* mutants relative to the wild type when worms are exposed to 240µM juglone on the first day of adulthood (de Castro *et al*., 2004), and *age-1* mutants display higher resistance to the superoxide generator paraquat (Honda & Honda, 1999; Yanase *et al*., 2002) and to hydrogen peroxide (Duhon *et al*., 1996) when stressed during late L4 or as young adults. However, as an association between age and resistance to ROS was not directed examined in this study, it remains possible that the distinction in survival between the two genotypes during and after exposure to oxidative stress may become greater with increasing age.

Spatial distributions of DAF-16::GFP indicate that antioxidant enzymes and other stress response proteins which can be induced in wild type worms in response to stress may be expressed in *age-1* mutants regardless of current environmental conditions. Consistent with this, in the absence of stress, several antioxidant enzymes are expressed at higher levels in *age-1* mutants than in wild type worms during early adulthood (Honda & Honda, 1999; Yanase *et al*., 2002) and late in life (Larsen, 1993; Vanfleteren, 1993). This suggests that *age-1* mutants are prepared in advance to deal with environmental challenges if and when they arise. Exposure to 50µM juglone caused DAF-16::GFP to translocate into the nucleus in wild type worms and intensified the nuclear localisation of DAF-16::GFP observed in *age-1* mutants. These results are consistent with previous studies which have described DAF-16::GFP responses to oxidative stress in wild type worms (Henderson & Johnson, 2001; Kondo *et al*., 2005; Hartwig *et al*., 2009; Przybysz *et al*., 2009; Heidler *et al*., 2010). Although nuclear localisation does not ensure constitutive activation of all DAF-16 target genes (Henderson *et al*., 2006), these results indicate that the expression of proteins which protect cells from damage induced by ROS increases in both genotypes during exposure to oxidative stress. Indeed, Heidler *et al*. (2010) recently demonstrated that exposing wild type worms to 40µM juglone enhances the *daf-16* dependent expression of a superoxide dismutase enzyme, SOD-3, and a heat-shock protein, HSP-16.2, which may promote resistance to oxidative stress (Walker & Lithgow, 2003).

The relative fitness of *age-1(hx546)* mutants increased dramatically when low density populations were periodically exposed to 100µM juglone. Consistent with this, post-stress survival and fecundity were considerably higher and maturity was attained more rapidly in *age-1* mutants than in wild type worms after fed larvae had been

exposed to these conditions. Although survival, developmental rate and fecundity were reduced to a greater extent in both genotypes following exposure to 100µM juglone than to less intense oxidative stress, DAF-16::GFP responses to 100µM juglone were similar to those observed in worms stressed on NGM agar containing 50µM juglone. Despite similarities in cellular distributions of DAF-16::GFP across different concentrations, Heidler *et al*. (2010) recently demonstrated that superoxide dismutase and catalase activities, and the *daf-16* dependent expression of a SOD-3::GFP fusion protein, were substantially higher when wild type worms were exposed to 40µM than to 100µM or 250µM concentrations of juglone. This implies that the integrity of protective mechanisms may be jeopardised when levels of oxidative stress become overly intense, and may suggest that ROS are rarely encountered at such high concentrations in nature. As survival was considerably reduced in *age-1* mutants as well as in wild type worms following exposure to 100µM juglone, it is possible that a reduction in the activity of antioxidant enzymes disrupts the ability of cells to maintain homeostasis in both genotypes under these conditions.

The relative fitness of *age-1* mutants in high density populations which had been periodically exposed to 50µM juglone was similar to the high density populations maintained in control conditions. Consistent with this, differences between the genotypes in molecular and individual level responses to 50µM juglone during starvation were similar to those observed in response to starvation conditions alone. In contrast, periodic exposure of high density populations to more intense oxidative stress reduced the fitness deficit of *age-1* mutants observed in control conditions. Although DAF-16::GFP distributions were similar in starved L1s exposed to 100µM juglone and in starved controls which had not been stressed in both genotypes, nuclear localisation was consistently more intense in *age-1* mutants than in wild type worms in starvation conditions. This distinction may explain why survival, developmental rate and fecundity were slightly higher in *age-1* mutants than in wild type worms when larvae were exposed to 100µM juglone during L1 arrest. Furthermore, this may explain why the fitness deficit of *age-1* mutants in resource limited conditions is reduced when high density populations experience intense oxidative stress. Consistent with these observations, Baugh and Sternberg (2006) demonstrated that starvation does not completely suppress insulin / IGF-1 signalling in wild type worms, and *age-1* mutants also display greater resistance than wild type when starved L1s are exposed to hydrogen peroxide (Weinkove *et al*., 2006). In contrast, no differences in survival or post-stress fecundity were observed between the two genotypes when larvae were stressed during the *daf-16* dependent dauer stage. This may be because the stress resistant phenotype observed in *age-1* mutants is analogous to that observed in wild

type dauers (Kenyon *et al*., 1993; McElwee *et al*., 2004, 2006), and because the up-regulation of DAF-16 target genes is likely to be equivalent in the two genotypes during this stage.

Though it has been reported that starvation reduces the ability to tolerate oxidative stress in wild type worms (Henderson *et al*., 2006), this was not observed in the stages examined in this study. However, it has previously been demonstrated that arrested L1s are more resistant to hydrogen peroxide than fed L1s (Weinkove *et al*., 2006; Kang & Avery, 2009), and dauers are highly resistant to a variety of environmental challenges, including oxidative stress (Anderson, 1982; Larsen, 1993). It is, therefore, possible that resistance to oxidative stress during the L1 diapause and the dauer stage may be specific to these forms of developmental arrest. Indeed, intermittent observations of entire populations during the stress periods suggested that worms of other stages are highly sensitive to oxidative stress when starved.

The results from this study and from chapter 4 indicate that spatial and temporal distributions of DAF-16::GFP are not considerably altered in response to oxidative stress or high temperatures during starvation conditions. Weinkove *et al*. (2006) reported that exposure to hydrogen peroxide causes DAF-16::GFP to translocate out of the nucleus in starved wild type L1s, but does not modify DAF-16::GFP localisation in *age-1* mutants. Consistent with the results in this study, they also reported that neither juglone nor paraquat have any effect upon DAF-16::GFP localisation in wild type worms during starvation conditions. Interestingly, Weinkove *et al*. (2006) demonstrated that starved wild type L1s continue to display increased resistance to hydrogen peroxide relative to fed L1s even after DAF-16::GFP has translocated out of the nucleus following prolonged starvation, and that this is dependent upon *daf-16*. This may suggest that increased transcription of DAF-16 target genes during the first day or two of starvation is sufficient to provide long term stress resistance when starvation is prolonged. If the activity of antioxidant enzymes and other stress response proteins is already enhanced during starvation conditions, this may explain why no additional responses to oxidative or thermal stress were observed. It remains unclear if DAF-16::GFP localisation is modified in response to abiotic stressors in starved stages other than L1 arrest.

In comparison to previous studies which have examined fitness costs in long-lived mutants in benign laboratory conditions, the treatments that were applied in this study may be more representative of natural environments. However, as described in chapter 4, wild populations of *C. elegans* are expected to experience fluctuations in resource availability, and periods of environmental stress are unlikely to recur on a predictable temporal scale. Accordingly, the responses to oxidative stress observed in

this study have been incorporated into population projection models which are used in chapter 7 to examine the invasion success of the *age-1(hx546)* mutant allele in more stochastic environments. This study provides additional evidence to suggest that a long-lived, laboratory derived mutant can have higher fitness than a wild type genotype under certain conditions, and implies that increased longevity may evolve in populations which frequently encounter harsh environments as a consequence of selection for greater resistance to stress.

Chapter 6 - Cold tolerance in long-lived *age-1* mutants is induced by Δ^9 desaturase genes and additional targets of DAF-16

6.1 Abstract

To promote survival at low temperatures, poikilotherms preserve membrane fluidity, and thus the integrity of vital membrane functions, by increasing the proportion of unsaturated fatty acids in membrane phospholipids. This response, homeoviscous adaptation, is primarily mediated by the activity of Δ^9 desaturase enzymes. In *C. elegans*, the expression of the Δ^9 desaturase genes *fat-5*, *fat-6* and *fat-7* is regulated by the insulin / IGF-1 signalling (IIS) pathway and the transcription factor DAF-16. These genes are up-regulated in long-lived IIS mutants and during the facultative, *daf-16* dependent, dauer stage. However, though it is well established that IIS mutants and dauers display enhanced resistance to various forms of stress, no assessment of cold tolerance has previously been reported. In this study I demonstrate that long-lived *age-1(hx546)* mutants are remarkably resilient to low temperature stress relative to wild type worms, following direct transfer from 20°C to 4°C, and that this is dependent upon *daf-16*. I also show that dauers display increased cold tolerance relative to wild type adults under these conditions, suggesting that this state of developmental arrest may be important for survival in natural populations which experience rapid reductions in ambient temperature. Using a combination of loss-of-function mutations and RNA interference, I reveal that the cold tolerant phenotype of *age-1* mutants is predominantly due to the up-regulation of Δ^9 desaturase genes, but that additional transcriptional targets of DAF-16 are also involved. These results indicate that mechanisms other than the preservation of membrane fluidity may play a role in cold tolerance in poikilotherms.

6.2 Introduction

The ability to tolerate and respond to fluctuations in ambient temperature is essential for survival and fitness. This is particularly true for poikilotherms, which cannot regulate their own body temperature yet must be able to function in a range of thermal conditions. A variety of behavioural and physiological strategies have evolved to allow poikilotherms to cope with low temperatures. For instance, Monarch butterflies, *Danaus plexippus*, avoid seasonal temperature declines by migrating thousands of miles south from North America to southern Mexico (Urquhart & Urquhart, 1978), and in response to changes in temperature and/or photoperiod, numerous invertebrates enter states of diapause which facilitate overwinter survival (Denlinger, 2002; Bale & Hayward, 2010). Whilst molecular chaperones are known to play a key role in preventing and repairing damage induced by exposure to high temperatures (Lindquist, 1986), the mechanisms which promote survival at low temperatures are less understood. However, there is substantial evidence to suggest that the preservation of cell membrane fluidity may be an important factor in cold acclimation (the processes involved in physiologically adjusting to cope with conditions following a gradual temperature change) and cold tolerance (the ability to survive at low temperatures) in poikilotherms, and that this can be achieved through alterations in membrane lipid composition (Sinensky, 1974).

At physiological temperatures to which organisms are either adapted or acclimated, membrane lipids are maintained in a fluid or liquid-crystalline phase. However, when temperatures drop below a threshold level, lipid structure changes to a more ordered, rigid gel phase and the integrity of fundamental membrane functions is impaired (Hazel, 1995). To promote survival in cold conditions, poikilotherms can reduce the average temperature at which this transition occurs by increasing the proportion of unsaturated fatty acids in their cell membranes (Cossins, 1994). Numerous studies have revealed that this response, referred to as homeoviscous adaptation (Sinensky, 1974), is at least partially dependent upon the activity of Δ^9 desaturase enzymes (Uemura *et al*., 1995; Tiku *et al*., 1996). Consistent with this, Δ^9 desaturase genes have been implicated in cold tolerance in bacteria (Wada *et al*., 1990), plants (Ishizaki-Nishizawa *et al*., 1996), and poikilothermic animals (Gracey *et al*., 2004; Brock *et al*., 2007; Murray *et al*., 2007). However, a recent study in *C. elegans* demonstrated that the cold tolerant phenotype acquired following a period of acclimation is not exclusively dependent upon the activity of Δ^9 desaturase enzymes (Murray *et al*., 2007). Additional mechanisms may, therefore, be involved in cold adaptation and cold tolerance in poikilothermic animals.

The conserved insulin / IGF-1 signalling (IIS) pathway modulates development, metabolism and longevity in *C. elegans*, in response to environmental heterogeneity, by regulating the activity of the FOXO transcription factor DAF-16. The IIS pathway terminates with the phosphorylation of DAF-16 and, in this state, the transcription factor is uniformly distributed throughout somatic cells (Lin *et al*., 2001). However, when signalling is disrupted, for instance via mutation or exposure to some forms of environmental stress, unphosphorylated DAF-16 accumulates within the nucleus, where it binds to and activates the promoters of genes involved in metabolism and cellular defence (Henderson & Johnson, 2001; Lin *et al*., 2001; Lee *et al*., 2003; Murphy *et al*., 2003). Mutants which are defective in components of the IIS pathway are long lived and display enhanced resistance to a broad spectrum of environmental challenges, including oxidative stress (Larsen, 1993; Vanfleteren, 1993), heat shock (Lithgow *et al*., 1994), ultraviolet light (Murakami & Johnson, 1996), heavy metals (Barsyte *et al*., 2001), hypoxia (Scott *et al*., 2002), microbial infections (Garsin *et al*., 2003), and hypertonic stress (Lamitina & Strange, 2005). The long lived, stress resistant phenotype is also a characteristic of the dauer stage, a facultative state of diapause which is partially regulated by the IIS pathway and is dependent upon *daf-16*. Consistent with this, genome wide microarray analyses have identified numerous genes downstream of DAF-16 which are up-regulated in both IIS mutants and dauers, and among these are the three *C. elegans* Δ^9 desaturase genes: *fat-5*, *fat-6* and *fat-7* (Murphy *et al*., 2003; Wang & Kim, 2003; McElwee *et al*., 2004; McElwee *et al*., 2006). As the proportion of unsaturated fatty acids in cell membranes should, therefore, be higher in these worms than in non-dauer wild type, it is likely that IIS mutants and dauers may also exhibit enhanced resistance to low temperatures, at least in the absence of an acclimation period. Furthermore, given the abundance of genes associated with stress resistance and metabolism which are up-regulated in IIS mutants and dauers, and that cold acclimation is not exclusively dependent upon the activity of Δ^9 desaturase enzymes (Murray *et al*., 2007), it is conceivable that additional transcriptional targets of DAF-16 may contribute to cold tolerance in *C. elegans*.

In this study, survival was compared between wild type and *age-1(hx546)* mutant adults, and between adults and dauers of each genotype, during prolonged exposure to low temperatures. Although the *C. elegans* Δ^9 desaturase enzymes exhibit slight differences in function, with FAT-5 primarily converting palmitic acid to palmitoleic acid whilst FAT-6 and FAT-7 convert stearic acid to oleic acid (Watts & Browse, 2000), there is considerable overlap in biochemical activity and loss-of-function mutations in either *fat-6* or *fat-7* induce compensatory responses in the expression of the remaining Δ^9 desaturase coding genes (Brock *et al*., 2006). Consequently, mutants which are

defective for single Δ^9 desaturase genes display no obvious phenotype (Brock *et al*. 2006). Therefore, to assess the extent to which Δ^9 desaturase genes contribute to survival at low temperatures in wild type and *age-1(hx546)* mutant adults, a combination of loss-of-function mutations and RNAi was used to simultaneously eliminate or presumably reduce their expression. Contributions of Δ^9 desaturase genes to cold tolerance were also assessed in the wild type and *age-1* mutant backgrounds by comparing recovery times among different strains following brief periods at low temperatures. This approach has previously been used to characterise variability in cold tolerance among different populations of *Drosophila melanogaster* (David *et al*., 1998; Hoffman *et al*., 2002; Anderson *et al*., 2005; Burger & Promislow, 2006).

6.3 Methods and Materials

6.31 Strains and culture conditions

The following genotypes were obtained from the Caenorhabditis Genetics Centre: N2 Bristol (wild type), TJ1052 *age-1(hx546)*, CF1038 *daf-16(mu86)*, BX107 *fat-5(tm420)*, BX106 *fat-6(tm331)*, BX153 *fat-7(wa36)*, BX160 *fat-6(tm331)*; *fat-5(tm420)*, BX110 *fat-7(wa36)*; *fat-5(tm420)*, BX156 *fat-6(tm331)*; *fat-7(wa36)* and TJ356 N2; *zIs356* [pGP30 (*daf-16*::GFP)]. Strains with the following genotypes were obtained by crossing *age-1(hx546)* mutant males with young adult hermaphrodites of each relevant strain: *age-1(hx546)*; *daf-16(mu86)*, *age-1(hx546)*; *fat-5(tm420)*, *age-1(hx546)*; *fat-6(tm331)*, *age-1(hx546)*; *fat-7(wa36)*, *age-1(hx546)*; *fat-6(tm331)*; *fat-7(wa36)* and *age-1(hx546)*; *zIs356* [pGP30 (*daf-16*::GFP)]. After allowing F1 hermaphrodites to self-fertilise, homozygous *age-1(hx546)* mutants were identified by scoring F2 and F3 progeny for dauer formation at 27°C as described in chapter 4. To obtain *age-1(hx546)*; *daf-16(mu86)* double mutants, which are dauer defective, 'partial dauers' were selected based on developmental arrest at 27°C, then genotype was confirmed by PCR using the primers listed in appendix 6.1. For the *age-1(hx546)*; *zIs356* [pGP30 (*daf-16*::GFP)] genotype, individuals were also screened for a roller phenotype as described in chapter 4. For the remaining genotypes, hermaphrodites with an *age-1(hx546)* background were separated on to individual NGM plates and allowed to self fertilise. To examine the presence of the *fat-5(tm420)*, *fat-6(tm331)* and *fat-7(wa36)* mutations in these lines, growth rates and survival of progeny were monitored and compared with wild type and *age-1* mutant controls at 10°C. An *age-1(hx546)*; *fat-6(wa36)*; *fat-7(wa36)* triple mutant line was identified based upon reduced growth rates and survival at this temperature, and this was subsequently confirmed by PCR using the primers listed in appendix 6.1. However, the remaining double desaturase mutants were not obtained in the *age-1(hx546)* mutant background. All three single desaturase mutants were obtained in the *age-1(hx546)* mutant background by PCR screening, using the primers listed in appendix 6.1. The presence of the *fat-5(tm420)* or *fat-6(tm331)* deletions were confirmed by comparing band sizes with controls of known genotype following gel electrophoresis. The presence of the *fat-7(wa36)* substitution (c→t, producing a premature stop codon) was confirmed by automated DNA sequencing. Strains were maintained on nematode growth media (NGM) plates, containing 10 µg/ml nystatin and 50 µg/ml streptomycin, and seeded with *E. coli* (HB101) except where stated otherwise. Strains were maintained at 20°C until cold tolerance assays commenced.

6.32 RNAi

As *age-1(hx546)*; *fat-5(tm331)*; *fat-6(wa36)* and *age-1(hx546)*; *fat-5(tm331)*; *fat-7(wa36)* triple mutants were not obtained, these combinations were achieved using RNAi to suppress the expression of *fat-5* in *age-1(hx546)*; *fat-6(tm331)* double mutants and in *age-1(hx546)*; *fat-7(wa36)* double mutants. RNAi was also used to suppress the expression of *fat-5* in the equivalent wild type strains. Simultaneous mutations in all three Δ^9 desaturase genes cause embryonic lethality (Brock *et al*., 2006). Therefore, to obtain individuals in which the expression of all three Δ^9 desaturase genes had been knocked down or knocked out, RNAi was used to: i) reduce *fat-5* expression in *fat-6; fat-7* double mutants and in *age-1; fat-6; fat-7* triple mutants, and ii) reduce *fat-6* and *fat-7* expression in *fat-5* mutants and in *age-1; fat-5* double mutants. As *fat-6* and *fat-7* have ~84% nucleotide homology, expression of both is knocked down when either is targeted by RNAi (Brock *et al*., 2006). Though growth rate was slow, and many eggs were not viable, adults were obtained for each condition using this approach.

RNAi was induced by feeding, using clones from the Ahringer library, as described in chapter 2. N2 and *age-1(hx546)* mutant adults fed on HT115 bacteria containing empty RNAi plasmid vectors were used as negative controls. To ensure that the expression of the relevant genes had been suppressed, strains were maintained on RNAi plates for at least one generation before young adults were collected for cold tolerance assays. Before RNAi agar plates were prepared, the presence of the corresponding genomic fragments in the *fat-5* and *fat-6*/*fat-7* RNAi clones was confirmed using restriction digests as described in chapter 2. Restriction enzymes NdeI and AseI were used for the *fat-5* digests, and AseI and AccI were used for the *fat-6* digests.

6.33 Cold tolerance assays

a) Adults

Cold tolerance assays were performed using well fed young adults which had been synchronised to reach maturity on the day that the experiments began. As the different genotypes grow at different rates (e.g. *fat-6; fat-7* mutants take approximately 2 days longer to mature than wild type), eggs were collected several days in advance depending upon developmental rate. During development the worms were maintained at 20°C. Twenty individuals, on each NGM or RNAi plate, were transferred directly to 4°C ± 0.5°C on the first day of adulthood. This temperature is considerably below the thermal range for wild type growth and activity of 15°C - 25°C (Dusenbery & Barr, 1980). Survival was then monitored regularly (usually daily but in some cases every 2nd day) following approximately 20 minutes recovery at room temperature. If survival

status was not immediately apparent, worms were gently touched with a platinum wire to stimulate a response.

b) Dauers

Populations of N2 and *age-1(hx546)* mutants were initiated with an equal number of age-synchronised adults. These populations, which were maintained at 20°C, grew at similar rates and depleted their food patches at roughly the same time. Dauers were collected approximately 24 hours later and 20 individuals were transferred directly to 4°C ± 0.5°C on each NGM plate. Survival was monitored as above until all of the non-dauer controls (N2 and *age-1(hx546)* mutant adults) had died.

6.34 Cold coma recovery times

Recovery times were monitored in a representative set of genotypes, including N2, *age-1(hx546)* mutants, *fat-6(tm331)*; *fat-7(wa36)* double mutants and *age-1(hx546)*; *fat-6(tm331)*; *fat-7(wa36)* triple mutants, following 6 hours exposure to 4°C ± 0.5°C. Well fed young adults, which had been synchronised to reach maturity on the same day, were separated onto 5 plates containing 10 individuals each. After the stress period, the plates were randomised and divided among four observers who, using stop watches, recorded the time taken for recovery at room temperature (approximately 22°C). Worms were considered to have recovered once they had begun to actively move around the plates. Data were collected in 2 separate blocks.

6.35 DAF-16::GFP localisation

To assess the activity of DAF-16 at low temperatures, cellular distributions of a DAF-16::GFP fusion protein were compared among well fed TJ356 and *age-1(hx546)*; *zIs356* [pGP30 (*daf-16*::GFP)] young adults using methods described in chapter 2. These worms had either been maintained at the control temperature (20°C) or had been exposed to 4°C for 6 hours. DAF-16::GFP localisation was categorised from 1-4, where 1 represents a uniform distribution throughout cells and 2, 3 and 4 represent increasingly nuclear distributions as described in chapter 4. Localisation was scored in approximately 120 individuals per genotype and treatment over three separate blocks.

6.36 Analysis

All analyses were performed in R version 2.10.1.

a) Survival analysis

A small number of individuals which had died from mortality sources other than exposure to low temperatures (e.g. rupturing of the vulva) were removed from the analysis. Due to slight variation in survival times among controls (N2 adults) observed in separate experiments, each experiment was analysed independently. All survival experiments were analysed using parametric regression models with Weibull error distributions (Hmisc and Design libraries).

b) Cold coma recovery times

Cold coma recovery times were compared among genotypes using a generalised linear mixed effects model. The model was fitted using the penalised quasi-likelihood (PQL) method (nlme library) and had gamma error distributions and a random effects term to account for variation among plates and between blocks.

c) DAF-16 localisation

Cellular distributions of DAF-16::GFP were compared between genotypes and among temperatures using an ordinal multinomial continuation-ratio logit model (Agresti, 2002; Thompson, 2009) in the VGAM library (Yee & Wild, 1996). This analysis took account of the ordering in the categorical response (i.e. $1 < 2 < 3 < 4$) and was used to determine the likelihood of continuing past a certain category level given that that category has been reached in the first place. The minimal adequate model was obtained based upon AIC values and, after removal of an interaction between genotype and treatment, contained genotype and treatment as explanatory variables. When certain categories were not displayed by one or both genotypes at a particular temperature, it was not possible to obtain parameters to describe all of the observed category transitions. These situations are described within parenthesis in 6.45. P-values were obtained using likelihood ratio tests to compare between models following sequential removal of explanatory variables.

6.4 Results

6.41 Reduced insulin signalling promotes cold tolerance in a *daf-16* dependent manner

To establish if insulin signalling plays a role in resistance to low temperatures, survival times at 4°C were compared between young wild type (N2) and *age-1(hx546)* mutant adults following direct transfer from 20°C. To assess if differences in cold tolerance were due to differences in the activity of DAF-16, survival times were also monitored in *daf-16(mu86)* null mutants and in *age-1(hx546)*; *daf-16(mu86)* double mutants under these conditions. *age-1* mutants displayed prolonged survival at 4°C relative to the wild type genotype ($z = 12.02$, $p < 0.001$), with mean survival times increased by 85% (Figure 6.1a). However, no significant differences in survival were observed among wild type worms, *daf-16* mutants and *age-1*; *daf-16* double mutants. This suggests that *daf-16*(+) is required for increased cold tolerance in *age-1* mutants, but does not contribute to wild type survival at 4°C. Survival at 4°C was also compared among N2 and *age-1* mutant dauers and fed adults. In both genotypes, more than 90% of dauers survived until after all fed adults had died (Figure 6.1b), and no significant difference between wild type and *age-1* mutants was observed during this stage. This indicates that wild type and *age-1* mutant dauer larvae are remarkably resilient to cold temperatures relative to fed adults (wild type: $z = 13.49$, $p < 0.001$, *age-1* mutants: $z = 8.12$ $p < 0.001$), at least in the absence of an acclimation period. Preliminary observations suggested that, in both genotypes, arrested L1s, adults in reproductive diapause and post-reproductive adults which were maintained without food also survive at low temperatures for longer periods than fed adults (data not shown).

6.42 Cold tolerance in *age-1* mutants is facilitated by Δ^9 desaturases

An *age-1*; *fat-7* double mutant had not yet been obtained when cold tolerance was monitored in single desaturase mutants. However, loss-of-function mutations in *fat-5*, *fat-6* and *fat-7* in the wild type background and in *fat-5* and *fat-6* in the *age-1* mutant background had only marginal effects upon cold tolerance (appendix 6.2). In contrast, simultaneous mutations in *fat-6* and *fat-7* reduced cold tolerance considerably in both the wild type ($z = -7.79$, $p < 0.001$) and *age-1* mutant backgrounds ($z = -14.5$, $p < 0.001$) (Figure 6.2a). Mean survival times at 4°C were reduced by 40% in *fat-6; fat-7* double mutants relative to wild-type and by 57% in *age-1; fat-6; fat-7* triple mutants relative to *age-1* mutants. However, although *age-1; fat-6; fat-7* triple mutants were more sensitive to 4°C than wild type worms ($z = -2.53$, $p = 0.012$), they remained more

cold tolerant than equivalent individuals with a wild type background ($z = 5.27$, $p < 0.001$).

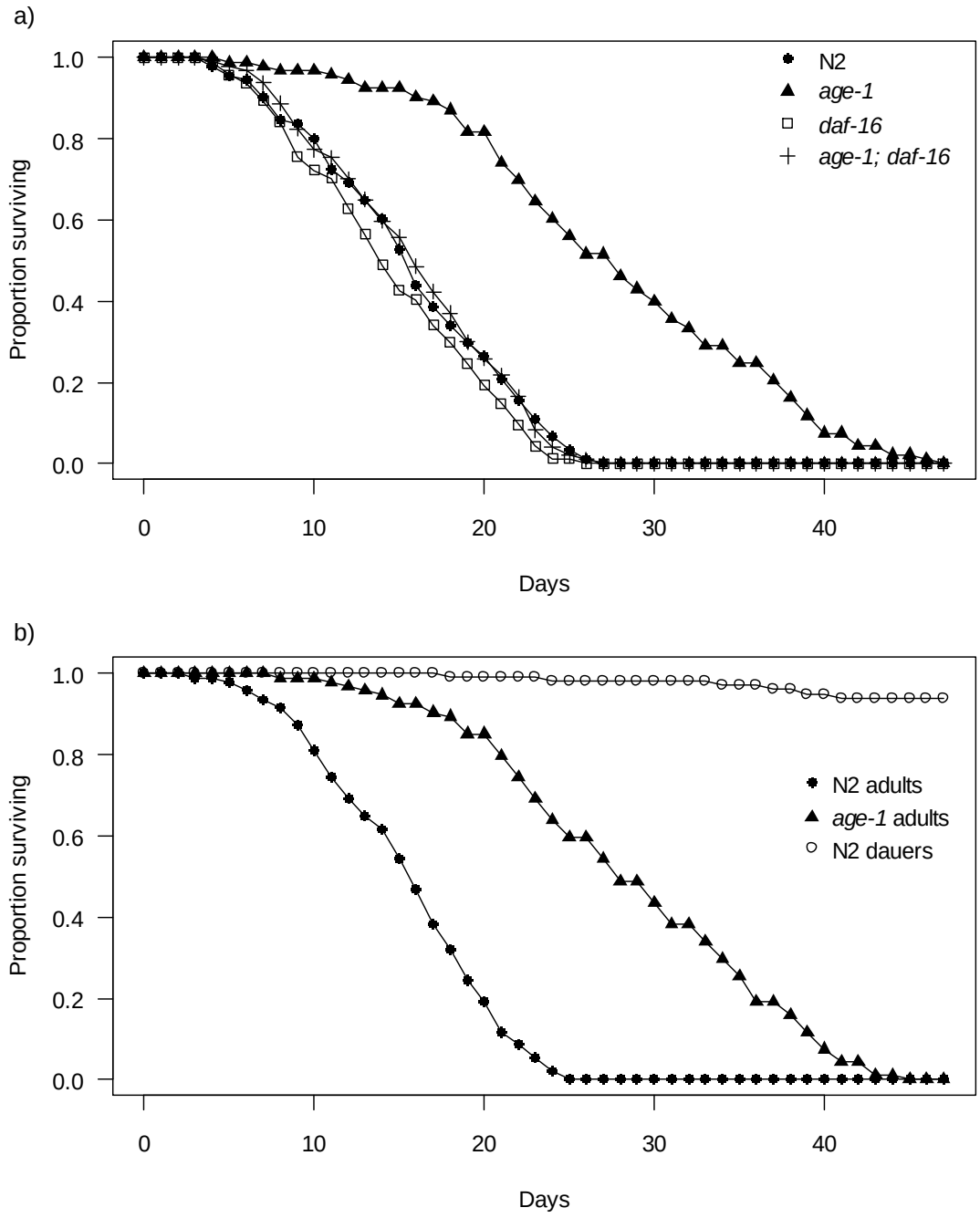


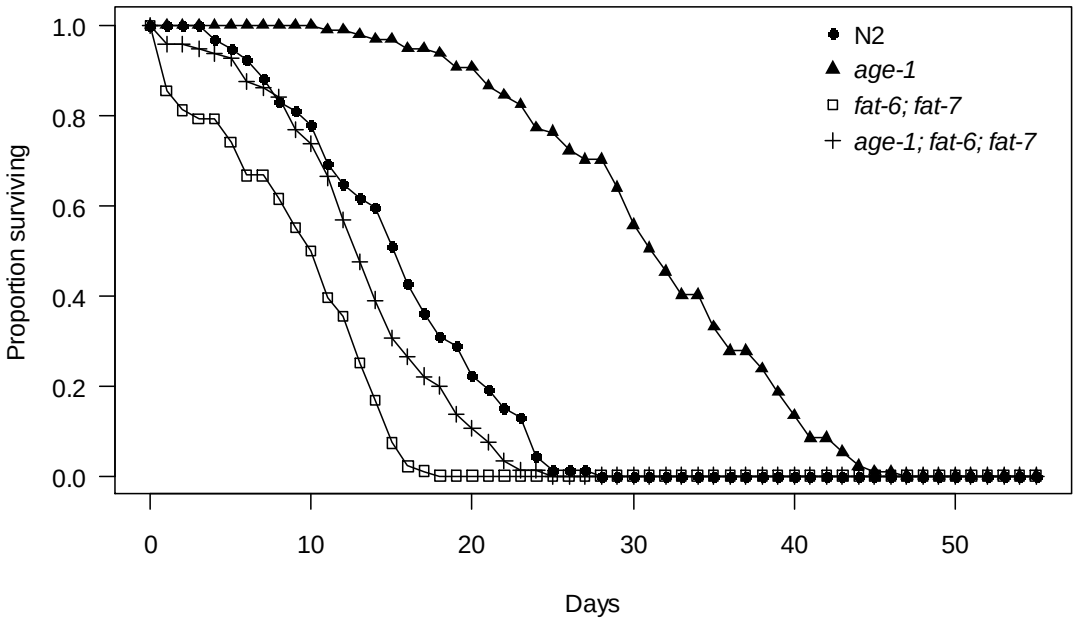
Figure 6.1: Variation in cold tolerance among genotypes and between stages. Survival curves for a) wild type (N2), *age-1(hx546)* mutants, *daf-16(mu86)* mutants and *age-1(hx546)*; *daf-16(mu86)* double mutants at 4°C, and b) wild type (N2) and *age-1(hx546)* mutant adults and dauers at 4°C. Survival was monitored in 90-100 individuals per genotype and stage.

RNAi of *fat-5* in *fat-6* and *fat-7* mutants and in *age-1; fat-6* and *age-1; fat-7* double mutants reduced survival times at 4°C in both the wild type and *age-1* mutant backgrounds (Figure 6.2b). Relative to wild type controls on HT115 bacteria containing empty RNAi plasmid vectors, suppressing the expression of *fat-5* in *fat-6* mutants reduced mean survival times, by 23% ($z = -3.48$, $p < 0.001$), and suppressing the expression of *fat-5* in *fat-7* mutants reduced mean survival times by 30% ($z = -4.84$, $p < 0.001$). Similarly, relative to *age-1* mutant controls on HT115 bacteria containing empty RNAi plasmid vectors, RNAi of *fat-5* in *age-1; fat-6* double mutants reduced mean survival times by 33% ($z = -6.67$, $p < 0.001$), and RNAi of *fat-5* in *age-1; fat-7* double mutants reduced mean survival times by 38% ($z = -7.81$, $p < 0.001$). Although survival times were reduced slightly further in both the wild type and *age-1* mutant backgrounds when *fat-5* was suppressed in *fat-7* mutants than when *fat-5* was suppressed in *fat-6* mutants, these differences were not significant. However, whilst *age-1; fat-6* double mutants on *fat-5* RNAi plates remained more cold tolerant than wild type controls on HT115 bacteria containing empty RNAi plasmid vectors ($z = 2.66$, $p = 0.008$), survival in *age-1; fat-7* double mutants on *fat-5* RNAi plates was not significantly different from wild type controls.

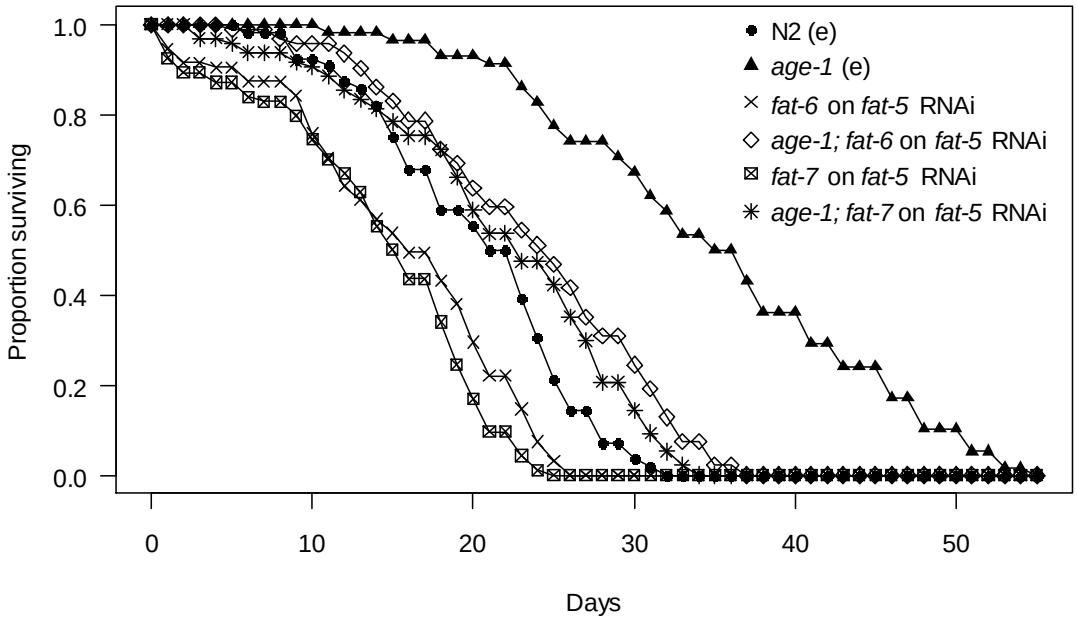
Survival times at 4°C were reduced dramatically in both wild type and *age-1* mutant backgrounds when the function of all three Δ^9 desaturase genes was reduced or eliminated (Figure 6.2c). Relative to wild type controls on HT115 bacteria containing empty RNAi plasmid vectors, RNAi of *fat-5* in *fat-6; fat-7* double mutants reduced mean survival times by 67% ($z = -10.60$, $p < 0.001$), and RNAi of *fat-6* and *fat-7* in *fat-5* mutants reduced mean survival times by 59% ($z = -8.23$, $p < 0.001$). Relative to *age-1* mutant controls on HT115 bacteria containing empty RNAi plasmid vectors, RNAi of *fat-5* in *age-1*; *fat-6; fat-7* triple mutants reduced mean survival times by 69% ($z = -11.40$, $p < 0.001$), and RNAi of *fat-6* and *fat-7* in *age-1; fat-5* double mutants reduced mean survival times by 62% ($z = -9.58$, $p < 0.001$). In both genetic backgrounds, *fat-6; fat-7* mutants on *fat-5* RNAi appeared to be more sensitive than *fat-5* mutants on *fat-6*/*fat-7* RNAi; however, these differences were not statistically significant. Although survival times were reduced dramatically in both genotypes when all three Δ^9 desaturase genes were either knocked out or presumably knocked down by RNAi, individuals with an *age-1* mutant background remained more cold tolerant than equivalent individuals in a wild type background. Mean survival times were 75% higher in *age-1*; *fat-6; fat-7* triple mutants on *fat-5* RNAi than in *fat-6; fat-7* double mutants on *fat-5* RNAi, ($z = 5.35$, $p < 0.001$), and were 66% higher in *age-1*; *fat-5* double mutants on *fat-6*/*fat-7* RNAi than in *fat-5* mutants on *fat-6* / *fat-7* RNAi ($z = 4.92$, $p < 0.001$). These results imply that

additional transcriptional targets of DAF-16 contribute to the cold tolerant phenotype of *age-1* mutants.

a)



b)



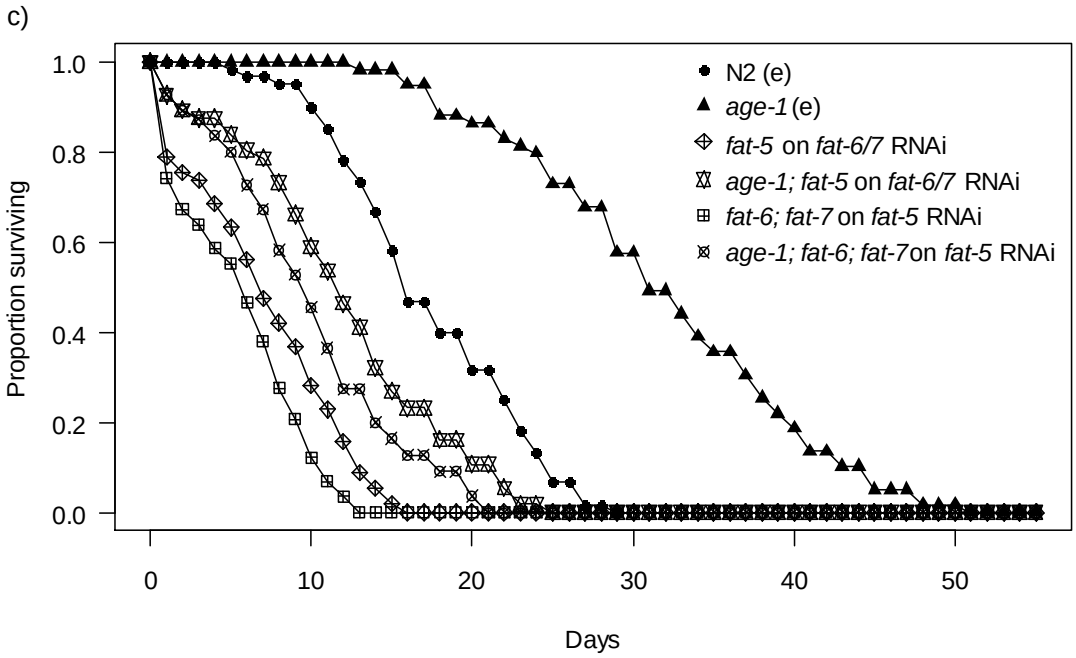


Figure 6.2: Variation in cold tolerance following knockout or knockdown of Δ^9 desaturase genes. Survival curves at 4°C in wild type and *age-1* mutant backgrounds following knockout or knockdown of Δ^9 desaturase genes. a) *fat-6*; *fat-7* double mutants and *age-1*; *fat-6*; *fat-7* triple mutants have reduced survival compared to wild type (N2) and *age-1* mutants on HB101 bacteria. Survival was monitored in 90-100 individuals per genotype. b) *fat-5* RNAi in *fat-6* and *fat-7* mutants and in *age-1*; *fat-6* and *age-1*; *fat-7* double mutants reduced cold tolerance relative to respective controls on HT115 bacteria containing empty RNAi plasmid vectors (N2(e) and *age-1*(e)). Survival was monitored in 50-60 individuals for N2(e) and *age-1*(e) controls and in 90-100 individuals of each mutant genotype on *fat-5* RNAi bacteria. c) Knockdown/knockout of all three Δ^9 desaturase genes reduced survival times at 4°C in the wild type and *age-1* mutant backgrounds. Survival was monitored in 50-60 individuals per genotype/treatment.

6.43 Δ^9 desaturase enzymes promote rapid recovery from cold shock

Considerable variation in cold coma recovery times was observed among the different genotypes following 6 hours exposure to 4°C (Figure 6.3). Although *age-1* mutants recovered more rapidly than wild type worms ($t = 7.18$, $p < 0.001$), differences in recovery between the two genotypes were less apparent than differences in survival following prolonged periods at 4°C. Relative to wild type worms, recovery times were substantially prolonged in *fat-6*; *fat-7* double mutants ($t = -12.37$, $p < 0.001$). Similarly, relative to *age-1* mutants, recovery was delayed in *age-1*; *fat-6*; *fat-7* triple mutants ($t = -12.65$, $p < 0.001$). This indicates that FAT-6 and FAT-7 promote rapid recovery in both genotypes after exposure to low temperatures. Although *age-1*; *fat-6*; *fat-7* triple mutants took longer to recover than wild type worms ($t = -6.07$, $p < 0.001$), they

recovered more rapidly than equivalent individuals with a wild type background ($t = 6.89$, $p < 0.001$).

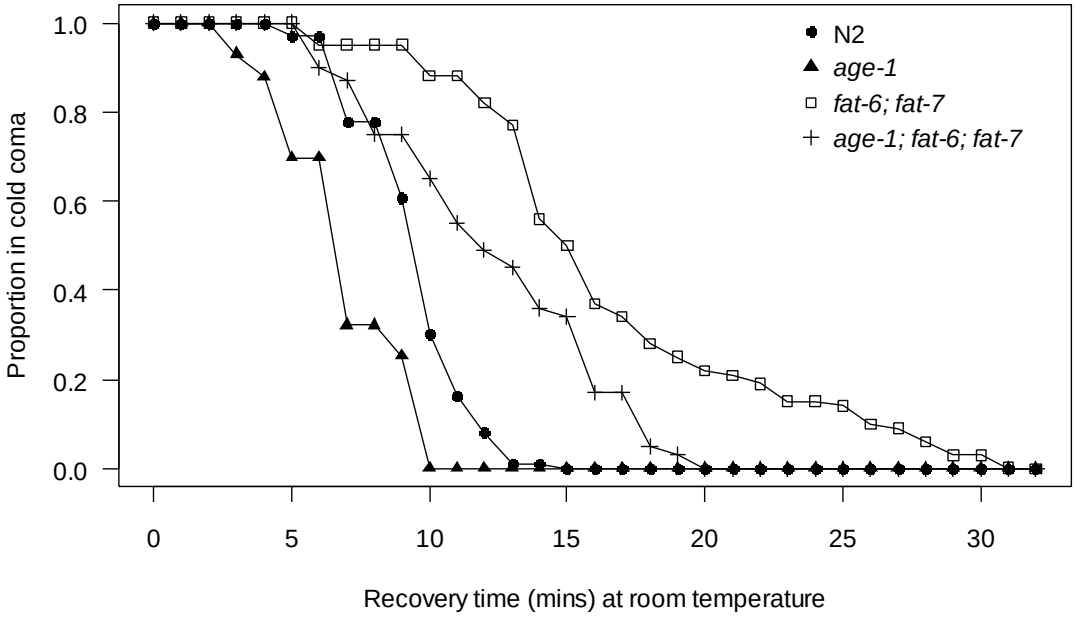


Figure 6.3: Variation in cold coma recovery times. Lines represent variation in recovery times among wild type (N2), *age-1* mutants, *fat-6*; *fat-7* double mutants and *age-1*; *fat-6*; *fat-7* triple mutants following 6 hours cold shock at 4°C. Recovery times were recorded in 95-100 individuals per genotype over 2 separate blocks.

6.45 DAF-16 is not activated during exposure to low temperatures.

Spatial distributions of a DAF-16::GFP fusion protein were categorised on a continuum from 1 (unlocalised) to 4 (nuclear localised) as described in chapter 4. Whilst the cellular distribution of DAF-16::GFP was almost entirely uniform in wild type adults at 20°C, it was more nuclear in *age-1(hx546)* mutant adults (no fed wild type displayed categories 3 or 4; category 1-2: $t = 17.26$, $p < 0.001$) (Figure 6.4). Although there was a slight increase in the intensity of nuclear localisation in *age-1* mutant adults at 4°C (a transition was observed between categories 2 and 3: $t = 2.79$, $p = 0.029$), no significant change in DAF-16::GFP subcellular localisation was observed in wild type adults following exposure to this temperature (Figure 6.4).

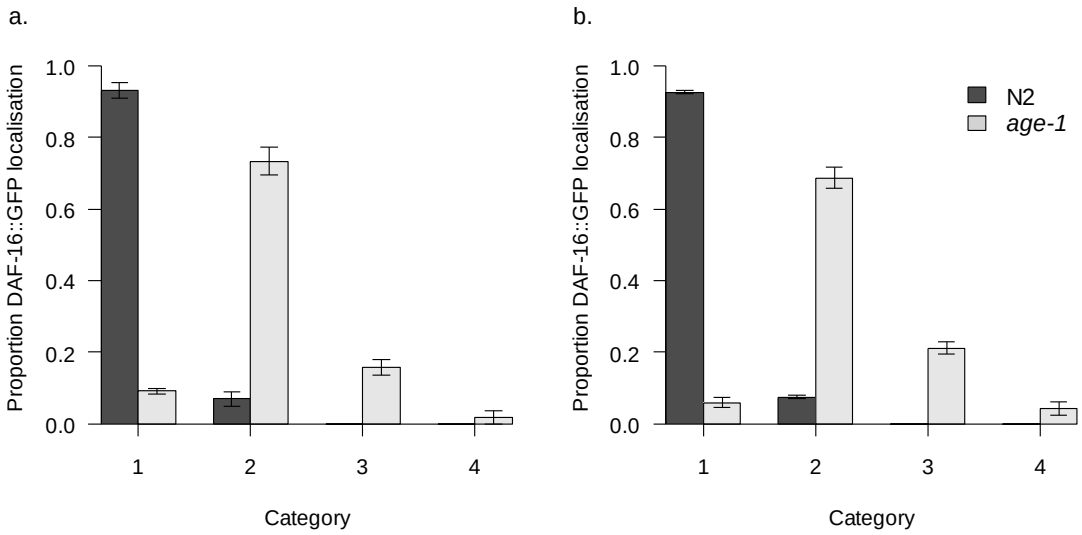


Figure 6.4: Effects of genotype and temperature on DAF-16::GFP localisation. Bar plots represent the mean proportion of wild type adults and *age-1* mutant adults which displayed categories 1-4 of DAF-16::GFP localisation at a) 20°C and b) after exposure to 4°C. Error bars represent standard errors of the means. Subcellular localisation of DAF-16::GFP was scored in approximately 120 individuals per genotype and treatment, over 3 separate blocks.

6.5 Discussion

In *C. elegans*, mutations which disrupt insulin/IGF-1 signalling initiate a conserved stress response due to the inappropriate activation of the transcription factor DAF-16 (reviewed in Kenyon, 2005, 2010). As transcriptional targets of DAF-16 are thus up-regulated in standard growth conditions, *age-1* mutants and other IIS mutants are prepared in advance to deal with various environmental challenges should they arise. Dauers, which in wild type are formed in unfavourable growth conditions, are also protected against various forms of impending stress due, at least in part, to the up-regulation of DAF-16 target genes. However, although it is well established that IIS mutants and dauers display enhanced resistance to several forms of environmental stress relative to non-dauer wild type, no assessment of cold tolerance in these worms has previously been reported. In this study I have demonstrated for the first time that IIS mutants and dauers have increased cold tolerance relative to non-dauer wild type after direct transfer from 20°C to 4°C. Although it remains unclear how mutations which disrupt insulin/IGF-1 signalling modify the ability to tolerate cold temperatures following a more gradual decline in ambient temperature, this implies that long-lived *age-1* mutants display increased resistance to a previously unreported form of environmental stress which may be encountered in nature.

At 20°C, the optimum temperature for population growth, the proportion of unsaturated fatty acids is likely to be higher in IIS mutants or dauers than in wild type adults due to the up-regulation of Δ^9 desaturase genes. Indeed, the proportion of unsaturated fatty-acids in cell membranes is lower in Δ^9 desaturase mutants than in wild type maintained at control temperatures (Brock *et al*., 2007; Murray *et al*., 2007). Furthermore, Hellerer *et al*. (2007) demonstrated that the ordering of stored lipids in the hypodermal cells of *daf-2 (e1370)* mutants, which are defective for the insulin / IGF-1 receptor, is reduced compared to in wild type worms. This reduced order state, which is characteristic of the fluid or liquid-crystalline lipid phase, indicates that a higher proportion of unsaturated fatty acids is present in *daf-2* mutants than in wild type worms. Consequently, the ability to maintain membrane fluidity following a sudden drop in temperature should be greater in IIS mutants and dauers as they are 'prepared' in advance for the impending stress, at least with regard to the threat to membrane function. Though it remains unclear if the observed differences relative to wild type adults would be maintained following a period of acclimation, the results presented in this study support this proposed scenario as the cold tolerant phenotype of *age-1* mutants is substantially reduced, and cold coma recovery times are prolonged, when the expression of Δ^9 desaturase genes is disrupted.

When the expression of two Δ^9 desaturase genes was reduced simultaneously, the largest reduction in survival at 4°C was observed in *age-1; fat-6; fat-7* triple mutants relative to *age-1* mutants. This difference was greater than in *fat-6; fat-7* double mutants relative to the wild type, suggesting that *fat-6* and *fat-7* play a major role in the cold tolerant phenotype of *age-1* mutants. Despite this, *age-1; fat-6; fat-7* triple mutants remained more tolerant than equivalent individuals with a wild type background, suggesting that increased expression of *fat-5* and/or additional targets of DAF-16 contribute to cold tolerance in *age-1* mutants. Consistent with this, *age-1; fat-6*; *fat-7* triple mutants recovered more rapidly than *fat-6*; *fat-7* double mutants after 6 hours cold shock at 4°C. In both genetic backgrounds, the decline in cold tolerance following RNAi of *fat-5* in *fat-6* or *fat-7* mutants was slightly less distinct than that observed in the double desaturase mutants. As these combinations were achieved using both mutation and RNAi, this may simply reflect reduced efficiency of RNAi relative to loss-of-function mutations. However, Brock *et al*., (2007) also demonstrated that, whilst survival to adulthood was reduced in all three double Δ^9 desaturase mutants at 10°C and 15°C relative to the wild type, this was most apparent in *fat-6; fat-7* double mutants. Furthermore, *fat-6; fat-7* double mutants display additional defects, including reduced fat storage, slow growth, reduced fecundity and a high proportion of embryonic lethality, which are not observed in the double mutants involving *fat-5* (Brock *et al*., 2007). Though the expression of *fat-5* is increased > 40-fold in *fat-6; fat-7* double mutants (Brock *et al*., 2007), these observations may reflect the close evolutionary relationship and resulting sequence similarity and substrate specificity of FAT-6 and FAT-7 (Watts & Browse, 2000). As *fat-7* expression is up-regulated in wild type worms during acclimation at 10°C whilst *fat-5* and *fat-6* expression remain unchanged (Murray *et al*., 2007), it is also possible that, of the three Δ^9 desaturase genes, *fat-7* has the most important role in cold tolerance. Consistent with this, although the differences were not statistically significant, survival at 4°C was slightly prolonged in *fat-6* mutants and *age-1*; *fat-6* double mutants on *fat-5* RNAi relative to *fat-7* mutants and *age-1*; *fat-7* double mutants on *fat-5* RNAi respectively.

When the function of all three Δ^9 desaturase genes was targeted, cold tolerance was reduced more dramatically in the *age-1* mutant than in the wild type background. Despite this, however, individuals with an *age-1* mutant background remained more cold tolerant than the equivalent individuals with a wild type background. This suggests that, the cold tolerance in *age-1* mutants is principally due to the up-regulation of Δ^9 desaturase genes, but that additional transcriptional targets of DAF-16 are also involved. This supports the findings of Murray *et al*. (2007), who demonstrated that mechanisms in addition to the preservation of membrane fluidity must be involved in

cold acclimation in *C. elegans*. IIS mutants and dauers do differ from wild type adults in additional aspects of metabolism which may be involved in cold tolerance. For instance, genes involved in the synthesis of trehalose sugars, which have been implicated in cold acclimation and/or survival in *Saccharomyces cerevisiae* (Schade *et al*., 2004), *D. melanogaster* (Overgaard *et al*., 2007), the nematode *Heterorhabditis bacteriophora* (Jagdale *et al*., 2005), and the codling moth, *Cydia pomonella* (Khani *et al*., 2007), are up-regulated in dauers and *daf-2 (e1370)* adults (Wang & Kim, 2003; McElwee *et al*., 2006; Honda *et al*., 2010), and trehalose levels are approximately 2-fold higher in *age-1* mutants than in wild type (Lamitina & Strange, 2005). Glycerol, which has a well established role as a cryoprotectant and has been implicated in rapid cold-hardening in several insect species (Lee *et al*., 1987), is also found at higher levels in *age-1* mutants than in wild type worms (Lamitina & Strange, 2005). In addition, several genes encoding heat-shock proteins, which have a role in cold tolerance in *S. cerevisiae* (Pacheco *et al*., 2009) and in a variety of insect species (Rinehart *et al*., 2007), are expressed at higher levels in IIS mutants and dauers than in wild type adults (Murphy *et al*., 2003; Wang & Kim, 2003; McElwee *et al*., 2004). Furthermore, certain antioxidants are present at higher levels in *age-1* mutants and dauers than in wild type adults (Larsen, 1993), and antioxidant enzymes have been implicated in resistance to low temperatures in insects (Joanisse & Storey, 1996) and plants (Thomashow, 1999; Dai *et al*., 2009). Any of these mechanisms may make a contribution to cold tolerance beyond that provided by the desaturase genes.

Though cold tolerance in *age-1* mutants and dauers is clearly *daf-16* dependent (dauer formation is dependent on functional *daf-16*), *daf-16* mutant adults do not display reduced cold tolerance relative to wild type adults. Furthermore, DAF-16 was not activated at low temperatures in adults with a wild type background in this study. These results suggest that *daf-16* is not required for wild type adult survival at 4°C. Using a transgenic line created with a different DAF-16::GFP fusion construct, Wolf *et al*. (2008) reported activation of DAF-16 in worms with a wild type background following 2 hours exposure to 1°C or 8°C. It is unclear which strain most accurately represents changes in spatial distributions of DAF-16::GPF under different thermal conditions. However, Wolf *et al*. (2008) also reported that DAF-16::GFP was nuclear localised in approximately 40% of adults at 20°C. Given the rapid development and high fecundity of wild type worms at this temperature, it is possible that the construct used in this study provides a more accurate portrayal of DAF-16::GFP distributions. Although DAF-16::GFP becomes nuclear localised during exposure to high temperatures, oxidative stress and starvation (Henderson & Johnson, 2001), DAF-16 is not activated in response to other forms of stress to which IIS mutants display increased resistance,

such as ultraviolet light (Henderson & Johnson, 2001) and hypertonic stress (Lamitina & Strange, 2005). This may be because different forms of stress elicit distinct responses which involve different signalling pathways and transcription factors. Several transcription factors, in addition to DAF-16, are known to be important in the regulation of Δ^9 desaturase genes. These include the nuclear hormone receptors NHR-49 and NHR-80, and SBP-1, the *C. elegans* homologue of sterol-regulatory-element-binding-protein (SREBP) transcription factors which are key regulators of lipid homeostasis in mammals (Ashrafi *et al*., 2003; Van Gilst *et al*., 2005a, 2005b; Brock *et al*., 2006; Yang *et al*., 2006). It is conceivable that one or more of these may play an important role in cold tolerance and/or cold acclimation in *C. elegans*.

C. elegans has been isolated from temperate regions throughout the world (Barrière & Félix, 2005a) and populations of this species are likely to experience daily as well as seasonal fluctuations in temperature. Though cold tolerance in wild type adults can be enhanced following a period of acclimation (Murray *et al*., 2007), these worms also show a degree of resistance following a sudden drop in temperature which is at least partially due to the activity of FAT-5, FAT-6 and FAT-7. However, given that the level of resistance observed in wild type dauers was considerably higher than in wild type adults following direct transfer from 20°C to 4°C, the results presented in this study may elucidate a mechanism that is important for the survival of *C. elegans* populations in natural environments in which rapid changes in temperature occur. Although there is currently no evidence to suggest that dauer formation can be induced by exposure to low temperatures, states of diapause are induced in response to changes in temperature and/or photoperiod in other invertebrates (reviewed in Denlinger, 2002; Bale & Hayward, 2010). For instance, in some insects the adult reproductive diapause state, which is considered to be important for over-winter survival, is formed in response to shortening day length (Kimura, 1984). Furthermore, reproductive diapause appears to be regulated, at least in some species, by the conserved insulin signalling pathway and orthologues of DAF-16 (Tartar *et al*., 2001; Williams *et al*., 2006; Sim & Denlinger, 2008).

In this study I have identified a previously unreported form of stress to which IIS mutants and dauers exhibit increased resistance. I suggest that, relative to wild type adults, these worms display prolonged survival at low temperatures because they are physiologically prepared in advance for the impending stress. In *age-1* mutants this is primarily due to the up-regulation of Δ^9 desaturase genes in standard growth conditions, but additional DAF-16 target genes also appear to be involved. This supports the suggestion that factors other than membrane lipid composition may contribute to cold tolerance in poikilothermic animals.

Chapter 7 – The evolution of longevity in stochastic environments

7.1 Abstract

Environmental stress constitutes a major factor shaping evolutionary trajectories in wild populations and selection acting upon the ability to tolerate harsh conditions may indirectly affect the evolution of associated phenotypes. In this study a demographic framework was used to assess how exposure to stochastic environments can influence the evolution of longevity in *C. elegans*. Using a combination of projection matrices to represent how schedules of fecundity and survival are modified in different conditions, Monte Carlo simulations were used to project populations of wild type worms and long-lived, stress resistant *age-1* mutants in a variety of heterogeneous environments. Stochastic growth rates (λ_s) were used as a measure of fitness and the invasion success of the *age-1(hx546)* mutant allele was determined by comparing these values between the two genotypes in each set of conditions. When populations frequently encountered periods of starvation, relative fitness was reduced in *age-1* mutants. Though *age-1* mutants could coexist with the wild type genotype under these conditions when populations were not subject to density-dependent regulation, frequencies of the *age-1(hx546)* mutant allele, therefore, remained lower than those of the wild type genotype over time. In contrast, the fitness of *age-1* mutants relative to wild type worms was greater when populations experienced periods of environmental stress, even when resource availability varied over time. The magnitude of the discrepancy between the two genotypes was determined by the frequency at which harsh conditions were imposed. These results indicate that the *age-1* mutant life history strategy confers a selective advantage in certain stochastic environments and that the *age-1(hx546)* mutant allele can successfully invade wild type populations over time. Consequently, short term ecological responses to environmental stress can lead to the fixation of stress resistant alleles and indirectly promote the evolution of increased longevity. This may have important implications for the evolution of life-history strategies in an increasingly stressful world.

7.2 Introduction

Evolutionary theories of senescence predict that longevity is determined by the level of extrinsic mortality experienced in a particular ecological niche (Williams, 1957). When mortality is high, longevity is constrained because few individuals survive and reproduce until a late age. Conversely, when mortality is low, longer life-spans may evolve if a considerable proportion of individuals continue to contribute to future generations late in life. It could, therefore, be argued that exposure to stressful environments may inhibit the evolution of increased longevity by imposing a source of mortality which reduces the number of individuals which survive and reproduce until a late age. However, given the association between stress resistance and longevity described throughout this thesis, if sufficient genetic variation exists in populations which experience harsh conditions, it is conceivable that selection may inadvertently promote the evolution of longer life-spans by favouring genotypes with the greatest ability to tolerate stress (Parsons 1995, 2002; Kenyon, 2010).

Exposure to environmental stress is considered to be an important factor shaping evolutionary dynamics in wild populations (Calow, 1989; Hoffman & Parsons, 1991; Bijlsma & Loeschcke, 1997; Hoffman & Hercus, 2000). Stressful conditions may facilitate micro-evolutionary transitions by increasing the rate at which new genotypes arise and by imposing selection pressures which accelerate the rate at which alleles become fixed in a population (Hoffman & Parsons, 1991; Nevo, 2001; Wright, 2004). However, if trade-offs associated with increased stress resistance reduce fitness relative to other genotypes in favourable growth conditions, stress resistant genotypes are only likely to be favoured in certain environments (Hoffman & Parsons, 1991). The ecological stress theory of ageing (Parsons, 1995, 2002) predicts that selection for resistance to environmental stress may underlie evolutionary changes which increase longevity in wild populations. Consistent with this, variation in the ability to tolerate stress has been associated with differences in longevity between populations of *Drosophila melanogaster* from distinct microhabitats (Nevo *et al*., 1998), and with differences in dauer longevity among wild populations of the nematode *Heterorhabditis bacteriophora* (Grewal *et al*., 2002). Furthermore, greater longevity was observed in populations of the gypsy moth *Lymantria dispar* from stressful habitats than from more benign habitats (Lazarevic *et al*., 2007). In laboratory studies, artificial selection for resistance to stress has been associated with increased lifespan in the parasitic wasp *Aphytis lingnanensis* (White *et al*., 1970), *D. melanogaster* (Rose, 1992; Hoffman & Parsons, 1993) and the butterfly *Bicyclus anynana* (Pijpe *et al*., 2008).

This study uses a demographic framework to assess how selection can indirectly influence the evolution of longevity in *C. elegans* by acting upon genetic variation in the ability to tolerate environmental stress. Using a combination of population projection matrices to represent how age-specific fecundity and survival are modified in wild type worms and long-lived, stress resistant *age-1(hx546)* mutants in different environments, the potential for long term evolutionary transitions is examined by investigating the invasion success of the *age-1* mutant life history strategy into a wild type population in a variety of stochastic conditions. As described in chapters 4, 5 and 6, *age-1* mutants display resistance to various environmental challenges. However, it is unclear which of these constitute important selective pressures in nature. In this study I have attempted to simulate natural conditions by projecting populations in stochastic environments which could conceivably be encountered by wild populations. The available data permitted temporal variation in resource availability, temperature and oxidative stress to be considered. Whilst wild populations are likely to experience resource limitation and fluctuations in ambient temperature, the importance of oxidative stress is less clear. However, reactive oxygen species (ROS) can be produced in response to environmental factors such as UV radiation, hyperoxia, industrial pollution and certain heavy metals, xenobiotics and pesticides (Finkel & Holbrook, 2000; Schröder & Krutman, 2005; Van Straalen & Roelofs, 2006). In this study exposure to oxidative stress is thus used as a proxy for the combined effects of these different environmental stressors. Despite temporal variation in resource availability, for simplicity it is assumed that populations occupy a single patch and that population dynamics are not subject to density-dependent regulation.

7.3 Methods

All procedures were performed in R version 2.10.1

7.31 Construction of projection matrices

To simulate responses to different environmental conditions, eighteen irreducible matrices were created for each genotype. Each matrix contained daily fecundity values on the top row and daily probabilities of transition (survival) from one stage/age to the next on the sub-diagonal. The matrices contained 26 different stage/age classes (Figure 7.1) which had been simplified to reflect the life history of each genotype at 20ºC when populations were projected in 1 day increments. When it was necessary to estimate age-specific fecundity or survival values, estimates were identical for the two genotypes unless stated otherwise. Following periods of starvation and/or stress, it was assumed that no effects of the maternal environment arose in subsequent generations.

a) Matrix 1: favourable conditions (deterministic growth)

Data for age-specific fecundity at 20ºC were available for the wild type genotype from experiments which have not been reported in this thesis. These data had been collected as described in chapter 3 except that adults had been transferred every 12 hours during the reproductive period. Reproductive schedules were not available for *age-1(hx546)* mutants at 20ºC. To deal with this issue, mean lifetime fecundity values were determined for each genotype using the data reported for fed controls at 20ºC in chapters 4 and 5. Age-specific fecundities were then extrapolated from these mean values by using the reproductive schedules for the wild type genotype to determine the proportion of total offspring which should be produced each day. With each projection from stage 1 (eggs), worms progress through stages 2 (L1-L2), 6 (L3-L4), 8 (1^{st} day of reproduction), 12 (2^{nd} day of reproduction), 16 (3^{rd} day of reproduction), 20 (4^{th} day of reproduction) then 24 (5^{th} day of reproduction) (Figure 7.1). As very little mortality is observed during development or reproduction in favourable growth conditions (chapter 3), in both genotypes it is assumed that 100% of worms progress to each successive stage. Though the timing required to complete embryogenesis is over estimated for 20ºC, the developmental period of 3 days is approximately correct (times vary according to the food source and when provided with HB101 development is slightly more rapid).

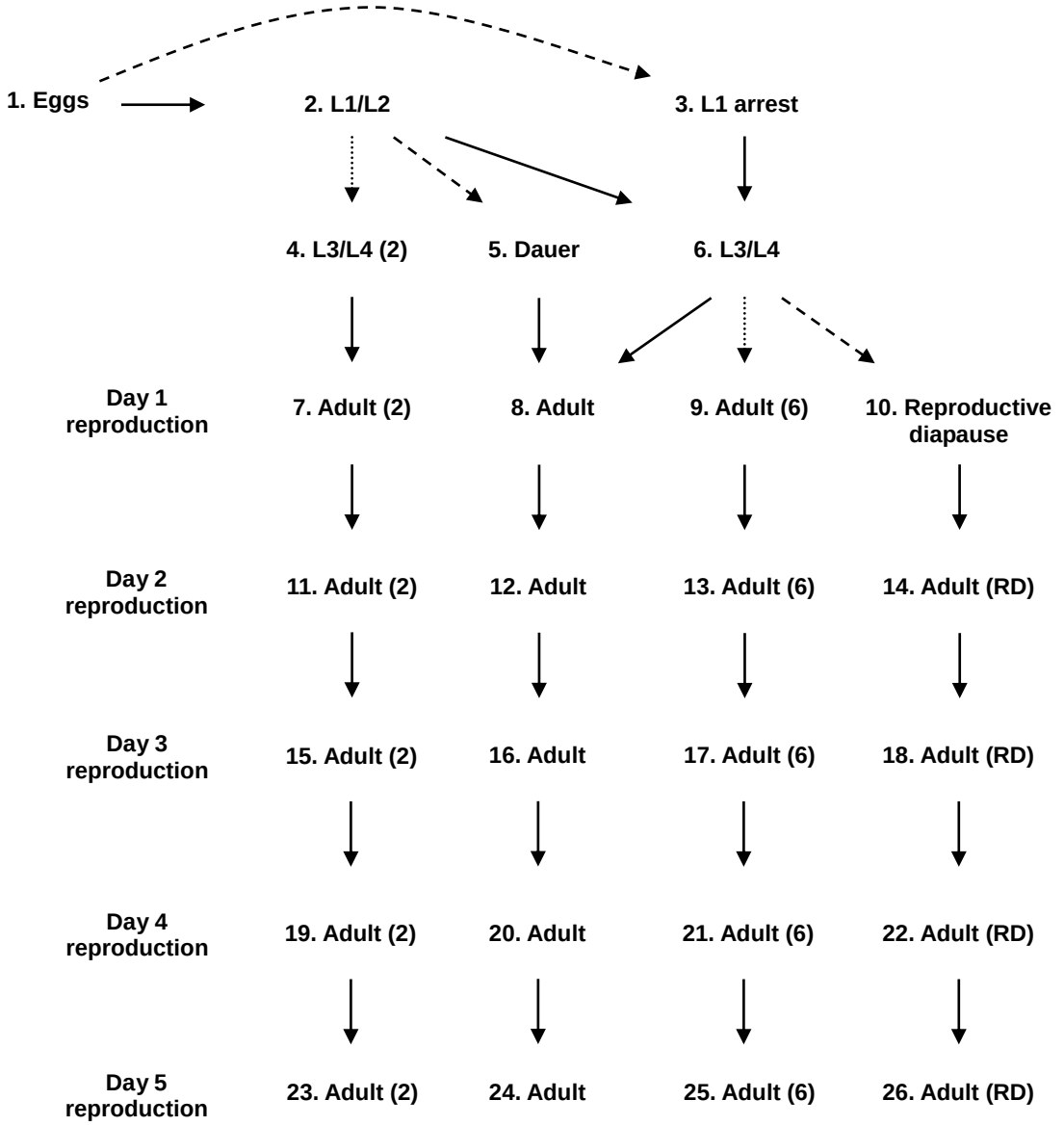


Figure 7.1: Projection matrix classes. Stage/age classes included in each projection matrix to represent responses to different environmental conditions. Dashed arrows represent transitions which occur primarily in starvation conditions. Dotted arrows represent transitions which occur during or after exposure to abiotic stress. In parentheses, '2' refers to worms which were stressed during stage 2 (L1/L2), '6' refers to worms which were stressed during stage 6 (L3/L4), and 'RD' refers to worms which had arrested in the adult reproductive diapause state during starvation.

b) Matrices 2-5: Starvation conditions

Matrix 2: Starvation day 1

Newly hatched larvae arrest development in the L1 diapause state; L1-L2s arrest as dauers; 50% of L3-L4s arrest in reproductive diapause and 50% mature as

reproductive adults (estimated from Angelo & Van Gilst, 2009). At each reproductive age fecundity is reduced to 10% of that observed in favourable conditions (estimated).

Matrix 3: Starvation day 2+

On the first iteration (starvation day 2), 25% of eggs hatch externally and larvae arrest development in the L1 diapause state (stage 3), but 75% of eggs hatch internally and larvae progress to stage 2 (estimated). These larvae have sufficient resources to reach the dauer stage on the second iteration. Arrested L1s, dauers and adults in reproductive diapause remain in these states throughout the starvation period. All reproductive adults die due to internal hatching.

Matrix 4: Recovery day 1

Arrested larvae resume development. To account for the delay in maturity observed in *age-1* mutants relative to wild type worms after larvae had arrested in the dauer stage (wild type mature ~ 30 hours and *age-1* mutants mature ~ 40 hours after food has been provided, chapters 4 and 5), 70% of wild type and 30% of *age-1* mutants attain maturity. Adults in reproductive diapause commence reproduction but fecundity at each reproductive age is reduced to 20% of that observed in favourable conditions regardless of the length of the starvation period (estimated from Angelo & Van Gilst, 2009).

Matrix 5: Recovery days 2-5

All worms which had arrested in the L1 diapause state and all remaining dauers mature. The age specific fecundity of adults which had arrested as L1s or as dauers is equal to that of adults which had developed in control conditions (chapter 4 and 5). After each starvation period populations were projected using this matrix for 4 time steps to ensure that all adults which had arrested in reproductive diapause became post-reproductive.

c) Matrices 6-8: heat shock 27ºC

Matrix 6: heat shock 27ºC (1 day only)

Only 50% of eggs are viable (estimated); wild type L1-L2s progress to stage 4 (L3-L4 when stressed during L1-L2) and *age-1* mutant L1-L2s arrest as dauers; L3-L4s progress to stage 9 (1^{st} day of reproduction when stressed during L3-L4). At each reproductive age fecundity is reduced to 5% of that observed in favourable conditions (estimated). It is assumed that post-stress reproduction is similar in worms that are

stressed during adulthood and in worms that are stressed during L3/L4. Therefore, depending on current age, reproductive adults progress to stages 13, 17, 21 or 25.

Matrix 7: Recovery day 1

All eggs are viable (low fecundity during the stress accounts for reduced reproductive investment and reduced viability) and newly hatched larvae progress to stage 2. Wild type larvae which had been stressed as L1-L2s progress from stage 4 to stage 7. Whilst 70% of *age-1* mutants which had been stressed as L1-L2s remain in the dauer stage, 30% progress to stage 8. When larvae had been stressed as L3-L4s, post-stress fecundity at each reproductive age class was reduced to 4.4% in wild type worms and to 6.9% in *age-1* mutants (these values were determined by dividing the relevant mean post-stress fecundities from chapter 4 by the mean fecundities for each genotype in control conditions). It was assumed that when worms had been stressed during the reproductive period remaining post-stress fecundity was the same as when larvae had been stressed during L3-L4.

Matrix 8: Recovery days 2-6

Remaining *age-1* mutant dauers mature. When wild type larvae had been stressed as L1-L2s, post-stress fecundity at each reproductive age class was reduced to 36% of that observed in control conditions (this was determined by dividing mean post-stress fecundity from chapter 4 by mean fecundity in control conditions). When *age-1* mutants had been stressed as L1-L2s, post-stress fecundity at each reproductive age class was similar to unstressed controls (chapter 4). Populations were projected using this matrix for 5 time steps to ensure that all wild type adults which had been stressed as L1-L2s became post-reproductive.

d) Matrices 9-12: heat shock 30ºC

Matrix 9: heat shock 30ºC (1 day only)

All eggs are non-viable (estimated); 60% of wild type L1-L2s die and 10% of *age-1* mutant L1-L2s die; 50% of wild type L3-L4s die and 10% of *age-1* mutant L3-L4s die (chapter 4). In both genotypes surviving larvae arrest development. Though adult mortality is likely to differ between the two genotypes in a similar manner to larval mortality, reproductive adults are unlikely to contribute further to population growth after the stress period. Accordingly no reproductive adults progress to subsequent ages.

Matrix 10: Recovery day 1

To account for the delay in maturity observed after removal from the stress (chapter 4), all surviving wild type larvae remain arrested. Though surviving *age-1* mutant L1-L2s remain arrested, 20% of *age-1* mutant L3-L4s progress to stage 9.

Matrix 11: Recovery days 2-3 (wild type) or day 2 (*age-1* mutants)

In both genotypes, L1s-L2s progress to stage 4 and remaining L3-L4s progress to stage 9. When larvae had been stressed during L3-L4, post-stress fecundity at each reproductive age class was reduced to 2.5% in wild type worms and to 4.8% in *age-1* mutants (these values were determined by dividing mean post-stress fecundities from chapter 4 by mean fecundities for each genotype in control conditions). To account for the delay in maturity in wild type worms which had been stressed during L1-L2 (chapter 4), wild type populations were always projected for 2 time steps using this matrix.

Matrix 12: Recovery days 4-9 (wild type) or days 3-9 (*age-1* mutants)

In both genotypes worms which had been stressed during L1-L2 progress from stage 4 to stage 7 (this occurs on recovery day 3 for *age-1* mutant populations and on recovery day 4 for wild type populations). When larvae had been stressed during L1-L2, post-stress fecundity at each reproductive age class was reduced to 4.8% in wild type worms and to 26.5% in *age-1* mutants (these values were determined by dividing mean post-stress fecundities from chapter 4 by mean fecundities in control conditions). Populations were always projected using this matrix until all wild type adults which had been stressed during L1-L2 became post-reproductive (6 iterations for wild type populations and 7 iterations for *age-1* mutant populations).

e) Matrices 13-15: Oxidative stress

Matrix 13: Oxidative stress (1 day only)

As it is unclear how likely populations are to encounter oxidative stress in natural conditions, only data for responses to low concentrations of juglone (50μM) are used in this chapter. In both genotypes 50% of eggs are viable (estimated); 14% of wild type L1-L2s die and 4% of *age-1* mutant L1-L2s die; 17% of wild type L3-L4s die and 4% of *age-1* mutant L1-L2s die (chapter 5). Surviving L1-L2s progress to stage 4. To account for the slight delay in maturity observed in both genotypes when larvae are stressed during L3-L4 (chapter 5), 66% of wild type L3-L4s progress to stage 9 whilst 17% remain in stage 6, and 77% of *age-1* mutant L3-L4s progress to stage 9 whilst 19% remain in stage 6. At each reproductive age, 15% of wild type adults die and 4% of *age-1* mutant adults die (estimated). In both genotypes fecundity of surviving adults

is reduced to 10% of that observed in favourable conditions at each reproductive age (estimated).

Matrix 14: Recovery day 1

To account for the delay in maturity after larvae had been stressed during L1-L2 (chapter 5), 75% of surviving wild type larvae progress to stage 7 whilst 25% remain in stage 4, and 80% of surviving *age-1* mutant larvae progress to stage 7 whilst 20% remain in stage 4. In both genotypes, remaining larvae which were stressed during L3-L4 progress to stage 9. When larvae had been stressed during L3-L4, post-stress fecundity at each reproductive age was reduced to 51% in wild type worms and to 71% in *age-1* mutants (these values were determined by dividing mean post-stress fecundities from chapter 5 by mean fecundities in control conditions). It was assumed that when worms had been stressed during the reproductive period post-stress fecundity remained at 10% of that observed in control conditions.

Matrix 15: Recovery days 2-7

In both genotypes, remaining larvae which had been stressed during L1-L2 progress to stage 7. When larvae had been stressed as L1-L2s, post-stress fecundity at each reproductive age class was reduced to 62% in wild type worms and to 80% in *age-1* mutants (these values were determined by dividing mean post-stress fecundities from chapter 5 by mean fecundities in control conditions). Populations were projected using this matrix for 6 time steps to ensure that all adults which had been stressed during L1-L2 became post-reproductive.

f) Matrix 16: 27ºC heat shock - starved (1 day only)

As described in 7.32, matrices 16-18 were only applied after populations had already been projected in starvation conditions using matrices 2 and 3. Consequently, populations were comprised of worms which had arrested in stages 3, 5 and 10. After populations have been projected using one of these matrices, they were always projected using matrix 3 (continued starvation) for at least 1 time step. Recovery matrices were therefore not required. In matrix 16, all arrested L1s and dauers survive and remain in these states (chapter 4). Given that adults have increased resistance to thermal stress when maintained in starvation conditions (Henderson *et al*., 2006), it was assumed that all adults in reproductive diapause should also survive and remain in this state.

g) Matrix 17: 30ºC heat shock - starved (1 day only)

As survival was not reduced in either genotype when arrested L1s or dauers were exposed to 30ºC (chapter 4), this matrix does not differ from matrix 16.

h) Matrix 18: Oxidative stress - starved (1 day only)

10% of wild type arrested L1s die and 1% of *age-1* mutant arrested L1s die (chapter 5). All dauers survive. It was unclear how adults in reproductive diapause respond to oxidative stress so survival was not altered in either genotype.

7.32 Randomisation of matrix sequences

In each set of projections, the *age-1* mutant population was initiated after the wild type population had been growing for 50 days. For simplicity, to ensure that the mutant population always arose in control conditions, the first 50 iterations for the wild type population were defined as follows: 10 days in favourable conditions (matrix 1 x 10), 20 days in starvation conditions (matrix 2 x 1, matrix 3 x 19), then 20 days in favourable conditions (matrix 4 x 1, matrix 5 x 4, matrix 1 x 15). Populations were then projected for 950 time steps using matrix sequences which had been randomised as described below. These were identical for the two genotypes except when stated otherwise.

Step 1

Two vectors were created containing lists of different possible options. The first contained environmental states which could only be encountered when populations had previously been projected in favourable growth conditions or had fully recovered from periods of starvation or stress. These included a) favourable growth conditions (matrix 1), b) starvation conditions (matrix 2), c) 27ºC heat shock (matrix 6), d) 30ºC heat shock (matrix 9) and e) oxidative stress (matrix 13). The second contained states which could only be encountered when populations had previously been projected in starvation conditions. These included a) continued starvation (matrix 3), b) recovery from starvation (matrix 4), c) starvation and 27ºC heat shock (matrix 16), d) starvation and 30ºC heat shock (matrix 17), and e) starvation and oxidative stress (matrix 18).

Step 2

Probability distributions were created for each vector to control the frequency at which different states could arise. These distributions were modified for different sets of projections as described in 7.33.

Step 3

A set of rules was devised to ensure that, after the first 50 iterations, new environmental states were selected at random from the appropriate vector at a frequency determined by the relevant probability distribution. Matrices were placed in a specific order when necessary (Figure 7.2). For instance, when a state of environmental stress was selected after populations had been projected in favourable growth conditions, the stress matrix was used for a single iteration then was followed by a specific sequence of recovery matrices. These sequences were identical for wild type populations and *age-1* mutant populations except after exposure to 30ºC (7.31d). When starvation conditions were selected, populations were first projected using matrix 2 (starvation day 1), then, for subsequent iterations, using matrix 3 (starvation day 2+). After a minimum number of time steps in starvation conditions (7.33), a new environmental state was randomly selected from the starvation specific vector. If starvation and an additional form of stress were selected, populations were projected using the stress matrix for a single iteration then were again projected using matrix 3. If favourable growth conditions were selected, populations were projected using a specific sequence of recovery matrices before a new environmental state could again be selected at random.

7.33 Projection of populations in stochastic environments

Populations do not necessarily converge to a stable age distribution when age-specific fecundities and probabilities of survival vary over time (Benton & Grant, 1996). However, for simplicity, the wild type starting population vector contained 100 individuals which were spread according to the stable age distribution in favourable growth conditions. Relative proportions in each stage/age class were obtained from an eigenanalysis of matrix 1 as described in chapter 3. To simulate a mutant invasion, the *age-1* mutant starting population vector contained a single egg. Though in reality a mutation would arise in only one allele, it was assumed that the mutant was immediately homozygous. After projection from the starting population vector (N_0), a new population vector was derived at each subsequent time step according to the equation $N_{t+1} = MN_t$, where M is the projection matrix at time t and N_t is the population vector at time t (Benton & Grant, 1996).

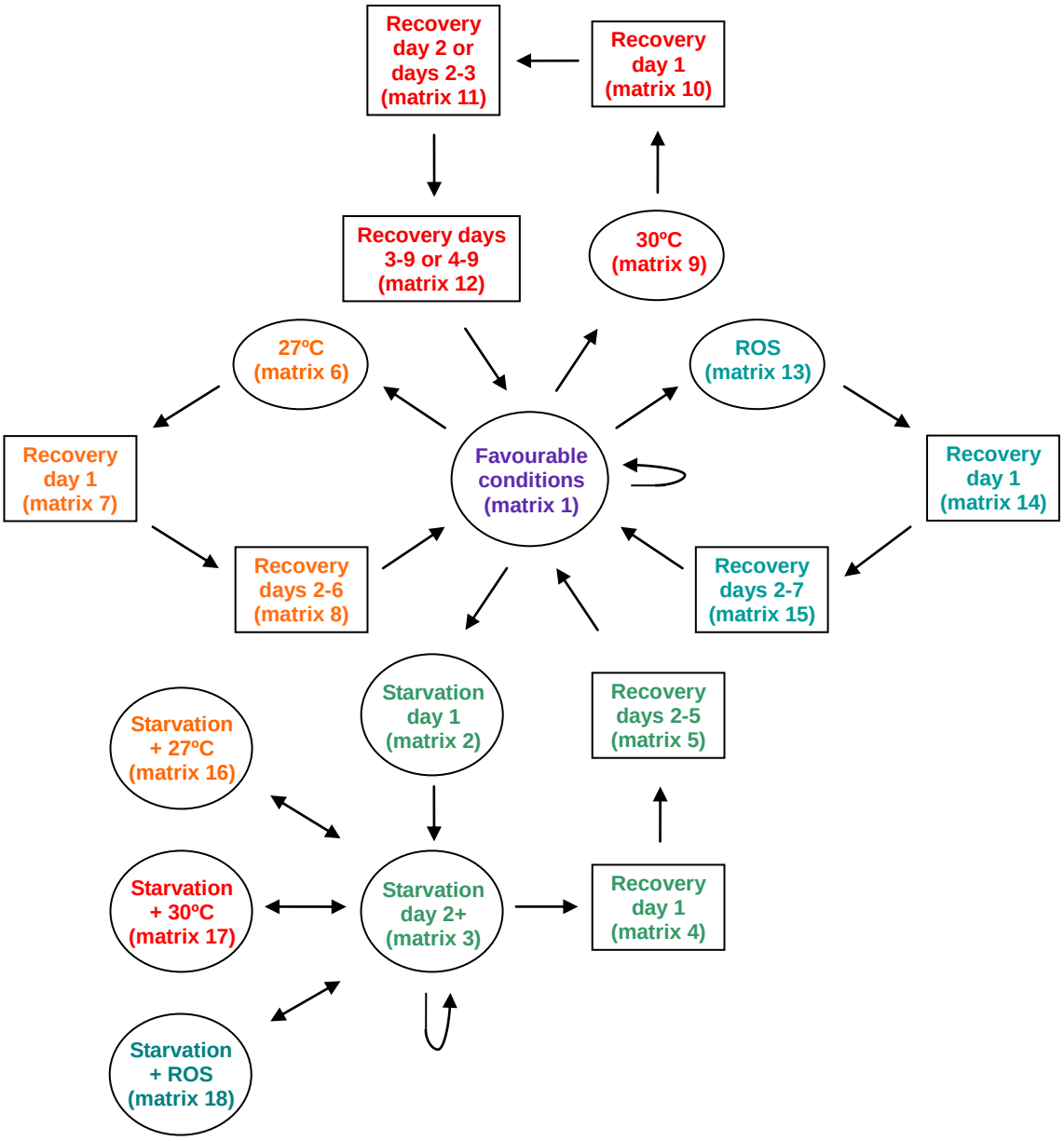


Figure 7.2: Randomisation of matrix sequences. Responses to different environmental conditions were represented by 18 projection matrices per genotype. Purple print corresponds to favourable growth conditions, red corresponds to intense thermal stress (30°C), orange corresponds to intermediate thermal stress (27°C), blue corresponds to oxidative stress (ROS), and green corresponds to starvation conditions. Environmental states were selected at random according to a predefined probability. When necessary, populations were projected using a specific sequence of matrices until favourable conditions were restored.

Populations were projected using the following sets of randomised matrix sequences:

a) Resource fluctuation

Populations were projected in stochastic environments in which only resource availability varied over time. When populations had previously been projected in favourable growth conditions, continued projection in favourable growth conditions (matrix 1) was selected at a frequency of 0.5 and projection in starvation conditions (matrix 2) was selected at a frequency of 0.5. After populations had been projected for a single iteration using matrix 2, matrix 3 (starvation day 2+) was applied for at least 4 time steps before a new matrix could be selected at random. Continued starvation (matrix 3) was then selected at a frequency of 0.8 and recovery (matrix 4) was selected at a frequency of 0.2. As favourable conditions were always restored for at least 5 time steps during recovery from starvation (matrix 4 x 1, matrix 5 x 4), the minimum starvation period of 5 days ensured that populations were not projected in favourable growth conditions more frequently than in starvation conditions.

b) Low frequency stress

Populations were projected in stochastic environments in which resource availability varied over time and periods of environmental stress were encountered at very low frequencies. When populations had previously been projected in favourable growth conditions, continued projection in favourable growth conditions (matrix 1) was selected at a frequency of 0.425, projection in starvation conditions (matrix 2) was selected at a frequency of 0.425, exposure to 27ºC (matrix 6) was selected at a frequency of 0.06, exposure to 30ºC (matrix 9) was selected at a frequency of 0.06, and exposure to oxidative stress (matrix 13) was selected at a frequency of 0.03. After populations had been projected for a single iteration using matrix 2, matrix 3 (starvation day 2+) was applied for at least 9 time steps before a new matrix could be selected at random. Continued starvation (matrix 3) was then selected at a frequency of 0.425, recovery (matrix 4) was selected at a frequency of 0.425, exposure to starvation and 27ºC (matrix 6) was selected at a frequency of 0.06, exposure to starvation and 30ºC (matrix 9) was selected at a frequency of 0.06, and exposure to starvation and oxidative stress (matrix 13) was selected at a frequency of 0.03. As favourable conditions were always restored for at least for 5 time steps during recovery from starvation or periods of environmental stress, the minimum starvation period of 10 days ensured that populations were not projected in favourable growth conditions more frequently than in starvation conditions.

c) High frequency stress

Populations were projected in stochastic environments in which resource availability varied over time and periods of environmental stress were encountered at relatively high frequencies. When populations had previously been projected in favourable growth conditions, continued projection in favourable growth conditions (matrix 1) was selected at a frequency of 0.25, projection in starvation conditions (matrix 2) was selected at a frequency of 0.25, exposure to 27ºC (matrix 6) was selected at a frequency of 0.20, exposure to 30ºC (matrix 9) was selected at a frequency of 0.20, and exposure to oxidative stress (matrix 13) was selected at a frequency of 0.10. After populations had been projected for a single iteration using matrix 2, matrix 3 (starvation day 2+) was applied for at least 24 time steps before a new matrix could be selected at random. Continued starvation (matrix 3) was selected at a frequency of 0.25, recovery (matrix 4) was selected at a frequency of 0.25, exposure to starvation and 27ºC (matrix 6) was selected at a frequency of 0.20, exposure to starvation and 30ºC (matrix 9) was selected at a frequency of 0.20, and exposure to starvation and oxidative stress (matrix 13) was selected at a frequency of 0.10. As favourable conditions were always restored for at least for 5 time steps during recovery from each period of starvation or environmental stress, the minimum starvation period of 25 days ensured that populations were not predominantly projected in favourable growth conditions.

7.34 Estimation of fitness (λ_s) in stochastic environments

To obtain fitness values in favourable conditions, the deterministic growth rate (λ) for each genotype was derived from the dominant eigenvalue of matrix 1 as described in chapter 3. In heterogeneous environments, in which schedules of fecundity and survival vary over time, population growth rates are not predetermined. To obtain fitness values in stochastic environments, Monte Carlo simulations were used to estimate the average per capita growth rate for each genotype. To do this, 1000 randomised matrix sequences for each set of stochastic conditions were used to project populations for 1000 time steps, then the stochastic growth rate (λ_s) was determined for each genotype using the equation $\log \lambda_s \approx 1/950 \log (N_{1000}-N_{50})$, where N_{1000} is the population size after 1000 time steps and N_{50} is the population size after 50 time steps when *age-1* mutants were introduced (Tuljapurkar, 1990; Metz *et al*., 1992; Benton *et al*., 1995; Benton & Grant, 1996). The invasion success of the *age-1(hx546)* mutant allele could then be determined by comparing fitness estimates in each set of stochastic environments and by monitoring the proportion of projections in which the frequency of *age-1* mutants exceeded that of the wild type genotype.

7.4 Results

7.41 Temporal variation in resource availability reduces the relative fitness of *age-1* mutants

In favourable growth conditions, age-specific schedules of fecundity and survival do not vary over time. Consequently, wild type and *age-1* mutant populations increase in size at a steady rate (Figure 7.3a). Under these conditions, fitness (the deterministic growth rate, λ) of the two genotypes is remarkably similar (Table 7.1), and genotype frequencies converge towards a stable equilibrium after the *age-1* mutant allele has been introduced (Figure 7.3b). When resource availability varies over time, age-specific schedules of fecundity and survival are modified accordingly. Fitness (the stochastic growth rate, λ_s) is considerably reduced in both genotypes compared to in favourable growth conditions but is reduced to a greater extent in *age-1* mutants than in wild type worms (Table 7.1). Relative fitness is reduced by approximately 3% in *age-1* mutants; however, $\lambda_s > 1$ in each genotype when periods of starvation occur at the frequency imposed in this study, indicating that both genotypes can persist under these conditions (Figure 7.3c, Table 7.1). Therefore, when resource availability varies periodically, but population dynamics are not subject to density-dependent regulation, *age-1* mutants can coexist with the wild type genotype albeit remaining at lower frequencies throughout time (Figure 7.3d).

7.42 *age-1* mutants can invade in stochastic environments

When resource availability varies over time and periods of environmental stress are encountered at low frequencies, fitness is reduced in both genotypes compared to in favourable growth conditions (Figure 7.4a, Table 7.1). However, though populations frequently encounter periods of starvation and stress is rarely imposed, fitness is reduced to a greater extent in wild type worms than in *age-1* mutants under these conditions (Table 7.1). Relative fitness is increased by approximately 3% in *age-1* mutants, suggesting that the *age-1* mutant life history strategy can successfully invade if populations occasionally encounter periods of environmental stress (Figure 7.4b, Table 7.1). Consistent with this, the frequency of the *age-1* mutant allele exceeded that of the wild type genotype within 950 time steps in approximately 44% of simulations (Table 7.1).

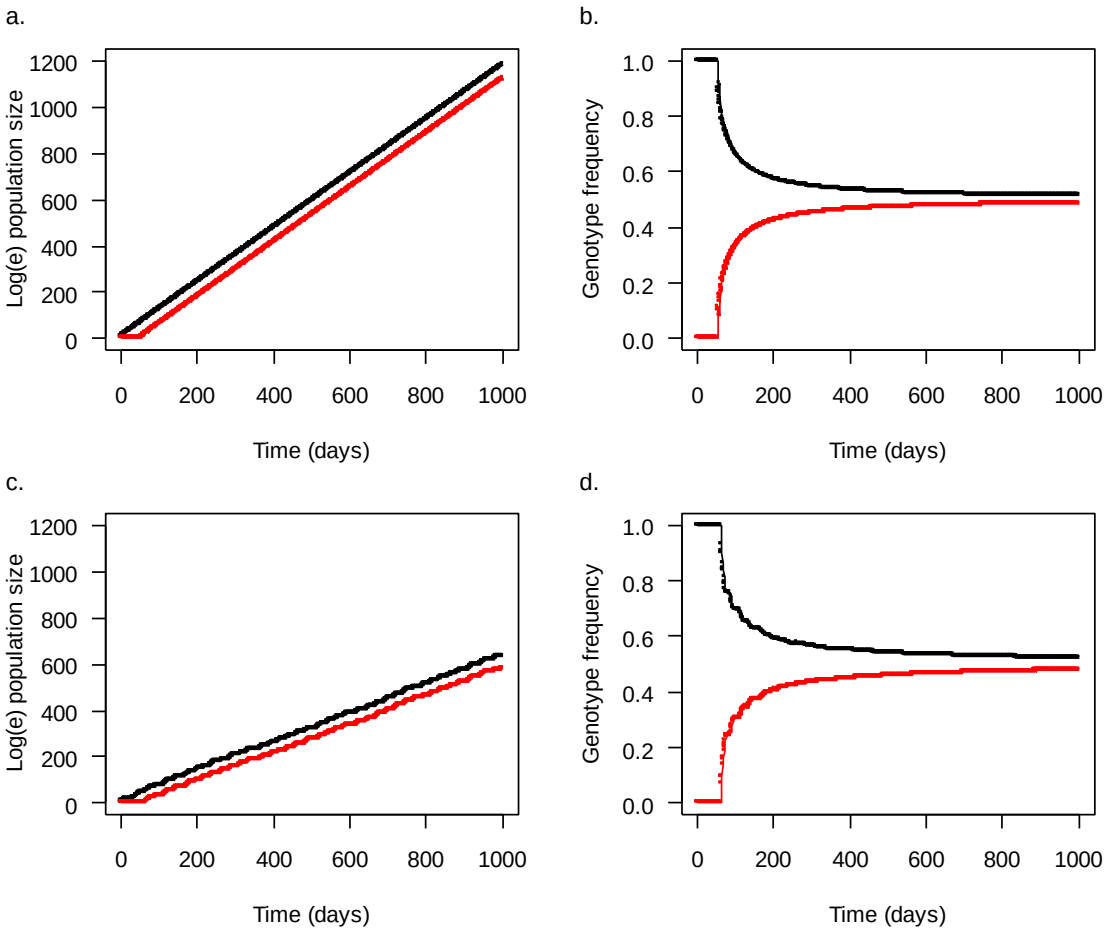


Figure 7.3: Deterministic and stochastic growth rates. Population growth (a and c) and temporal variation in genotype frequencies (b and d) in favourable conditions (a and b) and in a representative stochastic projection in which resource availability varies over time (c and d). Black lines represent the wild type genotype and red lines represent *age-1* mutants. Relative proportions of fed versus starved days in the projection presented in 1c are displayed in appendix 7.1.

When resource availability varies over time and periods of environmental stress are experienced at a relatively high frequency, fitness is considerably reduced in both genotypes compared to in favourable growth conditions (Table 7.1). However, under these conditions, the reduction in fitness is much more apparent in wild type worms than in *age-1* mutants (Figure 7.4c, Table 7.1). Relative fitness was 11% greater in *age-1* mutants, and the frequency of the *age-1* mutant allele exceeded that of the wild type genotype within 950 time steps in approximately 98% of simulations (Figure 7.4d, Table 7.1). The large distinction between the genotypes under these conditions suggests that the mutant allele may replace that of the wild type even when populations are regulated by density-dependent processes. These results indicate that, when responses to stress

vary among different genotypes, increased lifespan can evolve as an indirect consequence of selection for greater ability to tolerate harsh environmental conditions.

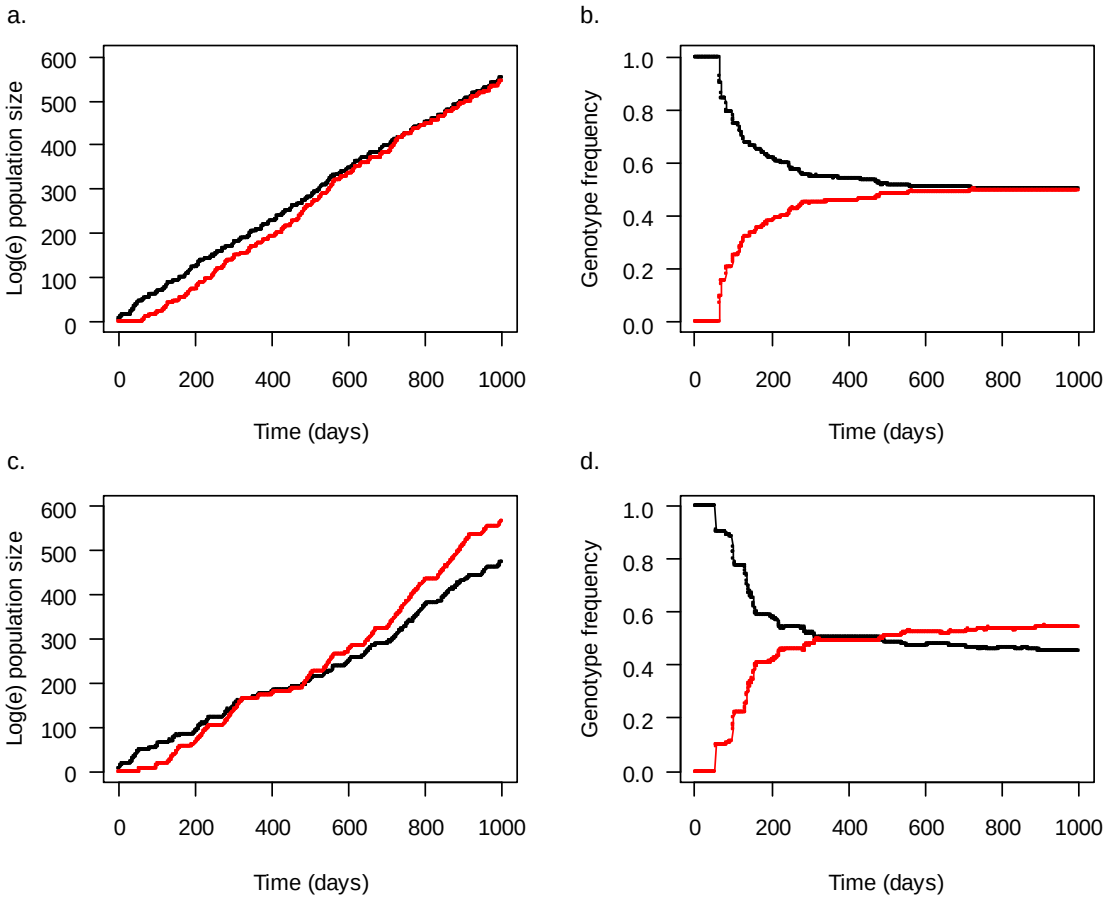


Figure 7.4: Population growth in stochastic environments. Representative projections of population growth (a and c) and temporal variation in genotype frequencies (b and d) in stochastic environments with fluctuating resource availability and low frequency stress (a and b) and in stochastic environments with fluctuating resource availability and high frequency stress (c and d). Black lines represent the wild type genotype and red lines represent *age-1* mutants. The relative proportions of different environmental states applied in the projections presented in a and c are displayed in appendix 7.2 and 7.3 respectively.

Genotype			Wild type (N2)	*age-1*
Favourable environments		**Absolute fitness (λ)**	3.594	3.603
		Relative fitness	1.0	1.002
		I_{50}	-	0
Stochastic environments	**Resource fluctuation only**	**Absolute fitness (λ_s)**	1.850	1.794
		Relative fitness	1.0	0.970
		Environmental impact	0.515	0.498
		I_{50}	-	0
	Resource fluctuation and low frequency stress	**Absolute fitness (λ_s)**	1.754	1.808
		Relative fitness	1.0	1.031
		Environmental impact	0.488	0.502
		I_{50}	-	0.437
	Resource fluctuation and high frequency stress	**Absolute fitness (λ_s)**	1.546	1.703
		Relative fitness	1.0	1.101
		Environmental impact	0.430	0.473
		I_{50}	-	0.985

Table 7.1: Fitness in different environmental conditions. Absolute and relative fitness values in different environmental conditions. Deterministic growth rates (λ) were derived from an eigenanalysis of matrix 1 and stochastic growth rates (λ_s) were estimated from 1000 separate population projections for each set of environmental conditions. Environmental impact values describe the reduction in fitness in each stochastic environment relative to favourable conditions, and I_{50} values represent the proportion of simulations in which the frequency of the *age-1* mutant allele exceeded that of the wild type genotype.

7.5 Discussion

In this study a demographic approach was used to examine how environmental stochasticity can influence the evolution of longevity in *C. elegans*. As selection does not act directly upon longevity, lifespan is expected to evolve in response to selection on associated traits. Consistent with this, I have demonstrated that long-lived *age-1* mutants can invade wild type populations which intermittently experience harsh conditions due to selection for greater resistance to environmental stress. Though *age-1* mutants display higher fitness than the wild type genotype in certain ecological contexts, this does not contest the evolutionary theories of senescence. Instead, it suggests that when sufficient genetic variation is present in populations which encounter periods of environmental stress, an optimal life history strategy may evolve which is characterised by long life and an ability to tolerate harsh conditions.

As previously demonstrated (Walker *et al*., 2000; chapters 4 and 5), no costs were associated with increased longevity in favourable growth conditions, but the relative fitness of *age-1* mutants was reduced when resource availability varied over time. Despite this, *age-1* mutants were able to coexist with wild type worms, albeit at lower frequencies, when populations experienced transient changes in resource availability. However, density dependence was not taken into account in this study and populations were assumed to exist in discrete habitat patches. When resources become limited, populations are unlikely to remain spatially static until conditions improve, and the ability to disperse to and exploit new patches of food is likely to have a considerable influence upon evolutionary dynamics (Hanski & Gilpin, 1997). Given that dauers are highly specialised for dispersal (Cassada & Russell, 1975), and that *age-1* mutants exhibit delayed exit from the dauer stage after food is encountered (chapters 4 and 5), it is conceivable that the *age-1(hx546)* mutant allele may be rapidly purged from natural populations if it arose in environments in which resource availability varies over time but are otherwise relatively benign.

When populations experienced transient changes in resource availability and periods of environmental stress were occasionally encountered, fitness was slightly increased in *age-1* mutants relative to wild type worms. This indicates that *age-1* mutants may be able to invade wild type populations even when starvation conditions arise more frequently than additional forms of stress. However, the relatively small difference in fitness between the two genotypes may suggest that both could be maintained by balancing selection if the *age-1(hx546)* mutant allele arose under such conditions. This could be true at a local scale or on a broader spatial gradient. The reduction in fitness in *age-1* mutants under resource limited conditions has repeatedly

been cited as an important example in which trade-offs consistent with the evolutionary theories of senescence (Williams, 1957; Kirkwood, 1977) arise in a long lived mutant in ecologically relevant conditions. Though the results in this study are of course based upon a simplified model in which density dependent processes are not taken into account, they imply that fitness costs in *age-1* mutants may be insignificant in environments in which additional factors vary over time. This does not contradict the evolutionary theories of senescence. It simply suggests that in certain environmental conditions the *age-1* mutant life history strategy may be favoured over that of the resident wild type. Consistent with this, when periods of environmental stress were encountered at high frequencies and resource availability continued to vary over time, the *age-1(hx546)* mutant allele clearly conferred a selective advantage over the wild type genotype. Under these conditions there is therefore considerable potential for adaptive selection of the *age-1(hx546)* mutant allele, suggesting that increased longevity can evolve when genetic variation in the ability to tolerate harsh conditions is present in populations which frequently experience environmental stress.

Responses to stress have been examined extensively in *C. elegans* and other model organisms. However, some forms of stress may rarely be experienced in nature or may be encountered at lower intensities than imposed in laboratory studies (Van Straalen & Roelofs, 2006). *C. elegans* exhibit phenotypic adaptations which clearly promote survival in resource limited conditions, and worms have been isolated from geographic regions in which high temperatures may be experienced. The stochastic environments imposed in this study are therefore likely to be ecologically relevant. Exposure to low temperatures was not considered because insufficient information was available to create accurate projection matrices. However, as *C. elegans* have been isolated from temperate regions, low temperatures may impose an important selection pressure in wild populations. It is conceivable that wild populations may also experience temporal variation in factors such as pathogen infection, salinity and humidity. Though it has previously demonstrated that *age-1* mutants display increased resistance to pathogen infections (Garsin *et al*., 2003) and hypertonic stress (Lamitina & Strange, 2005), it is unclear if *age-1* mutants have greater resistance than wild type worms to low humidity. However, dauers are resistant to desiccation (Riddle, 1988), suggesting that the ability to tolerate low humidity may be enhanced by the activity of genes which are regulated by DAF-16, and trehalose sugars, which have been implicated in desiccation resistance in a variety of organisms (Crowe *et al*., 1984), are present at higher levels in *age-1* mutants than in wild type worms (Lamitina & Strange, 2005). It is therefore plausible that *age-1* mutants display resistance to several environmental challenges that have not been considered in this study which are likely

to be experienced by wild populations. This implies that selection may act upon genetic variation in *age-1* and other genes associated with stress resistance in nature.

In *C. elegans*, wild isolates display genetic variation for several traits associated with fitness, and the expression of these traits differs across environmental gradients (Shook *et al*., 1996; Gutteling *et al*., 2007; Kammenga *et al*., 2007; Harvey & Viney, 2007; Harvey *et al*., 2008). For instance, the manner in which lifetime fecundity varies across a thermal gradient differs among wild isolate genotypes (Harvey & Viney, 2007). These genotype-by-environment interactions may reflect adaptations to local conditions. It is conceivable that natural variation in *age-1* and/or other genes which modify responses to stress may be observed among wild populations. Whilst certain alleles may be favoured in relatively benign environments, others may be favoured when harsh conditions are frequently encountered. Though longevity differs among wild isolate genotypes in favourable growth conditions (McCulloch & Gems, 2003b), the genetic basis of this variation is currently unclear. Future studies may reveal that variation in *age-1* and/or other genes encoding components of the IIS pathway underlie these differences. Indeed, it has previously been suggested that genetic variation in regulatory pathways which modulate responses to environmental stress may play an important role in the evolution of longevity in nature (Kenyon, 2010). Furthermore, it has recently been reported that differences in lifespan among closely related *Caenorhabditis* species are associated with variation in stress resistance and immunity, and in expression levels of homologues of *daf-16* (Amrit *et al*., 2010).

Stress resistance is clearly associated with increased longevity in many artificially selected genotypes and long-lived mutants (Rose, 1992; Hoffman & Parsons, 1993; Johnson *et al*., 2001). However, it is important to note that these phenotypes are not likely to be expressed simultaneously. It is well established that lifespan can be increased in various organisms following exposure to low intensities of stress (reviewed in Gems & Partridge, 2008), but more extreme forms of stress are likely to inhibit longevity by disrupting mechanisms which maintain cellular homeostasis (Yu, 2004). Consequently, although long-lived genotypes can increase in frequency when populations experience periods of environmental stress, the potential longevity of these genotypes may only become apparent in benevolent conditions. Consistent with this, certain long-lived *C. elegans* and *D. melanogaster* mutants only display increased longevity in benign laboratory conditions (Van Voorhies *et al*., 2005; Baldal *et al*., 2006).

Consistent with the ecological stress theory of ageing (Parsons, 1995, 2002), this study has demonstrated that stochastic exposure to harsh environments can lead to the fixation of novel life-history strategies and promote the evolution of longevity.

Given contemporary transitions in global climate and additional anthropogenic pressures, this may have important implications for the evolution of life-history strategies in an increasingly stressful world.

Chapter 8 – Discussion

Several mutations which promote longevity in model organisms disrupt mechanisms which are involved in responding to environmental change. For a comprehensive understanding of the consequences of manipulating these processes, long-lived mutants should ideally be examined in an ecological context. Using a multidisciplinary approach, I have assessed how a mutation which increases lifespan in *C. elegans* alters molecular to population level responses to different environmental conditions. The results have implications for the evolution of longevity in wild populations, and exemplify the importance of investigating trade-offs associated with increased longevity in ecologically relevant conditions.

8.1 Fitness costs in long-lived mutants

8.11 Fitness costs in favourable growth conditions

The antagonistic pleiotropy (Williams, 1957) and disposable soma (Kirkwood, 1977) theories of ageing predict that senescence occurs as a consequence of reduced selection late in life and that longevity is constrained because life-history strategies are optimised to maximise lifetime fitness in a particular ecological niche. By disrupting optimal life-history strategies, mutations which promote longevity are thus expected to reduce fitness relative to shorter lived controls. Although fitness is a measure of relative population growth rate and is determined by age-specific schedules of survival and reproduction (Fisher, 1930), the majority of studies which have examined fitness costs in long-lived mutants have focussed on single components of life history, such as the age at maturity or lifetime fecundity. In contrast, chapter 3 described a comprehensive comparison of age-specific changes in survival and fecundity among a variety of long-lived mutants and the wild type strain. A demographic approach was used to assess how changes in these traits modify fitness in favourable growth conditions. The mutants were chosen to reflect some of the different mechanisms which modulate longevity and included stress resistant *daf-2* and *age-1* mutants with reduced insulin/IGF-1 signalling, calorie restricted *eat-2* mutants and *clk-1* mutants with impaired mitochondrial function.

In favourable growth conditions, fitness (the deterministic growth rate) was reduced by 18% in *daf-2* mutants, by 30% in *eat -2* mutants and by 37% in *clk-1* mutants relative to the wild type genotype. Although mutations in *age-1* increase lifespan via the same mechanism and to a similar extent as mutations in *daf-2*, no fitness cost was observed in *age-1* mutants when age-specific schedules of survival and fecundity were recorded in benign laboratory conditions. This is consistent with a

previous study (Walker *et al*., 2000), and implies that trade-offs associated with increased longevity do not necessarily arise in constant environments when excess food is available. Mutations which promote longevity, therefore, modify life-history strategies to different extents depending upon the mechanism involved and mutations which disrupt the same pathway can have dramatically different consequences for fitness. Variation in age-specific survival and fecundity among the different genotypes was also used to infer how mutations which extend longevity could in theory modify the intensity of selection at different ages. Whilst selection dynamics in *age-1* mutants were similar to those observed in wild type worms, the onset of the age-specific decline in selection acting upon survival was delayed in the other long lived mutants due to later age at maturity. Furthermore, selection continued to act until a later age in these genotypes due to delayed reproductive senescence. Although the deterministic methods which were used in chapter 3 assume constant, unlimited conditions which are not likely to be relevant in an ecological context, the approach provides a convenient way to make comparisons among different genotypes in a common environment.

8.12 The *age-1* mutant anomaly

As described in chapter 4, co-variances among life-history traits can differ depending upon the context in which they are observed (Sgro & Hoffman, 2004). Trade-offs may not always become apparent in favourable conditions, particularly if excess food is available (Reznick *et al*., 2000; Tessier *et al*., 2000). Consistent with this, a small number of long-lived mutants have been identified which do not display trade-offs in benign laboratory environments (Rogina *et al*., 2000; Walker *et al*., 2000; Marden, *et al*., 2003; Scheckhuber *et al*., 2007). For instance, in *Drosophila melanogaster*, *Indy* mutants live approximately twice as long as wild type flies yet display no reduction in growth rate or fecundity when food is not limited (Rogina *et al*., 2000). To my knowledge, *age-1* mutants are the only long-lived *C. elegans* mutants which have been identified that do not exhibit fitness costs in favourable growth conditions. Despite increased expression of genes involved in somatic maintenance and repair, this suggests that *age-1* mutants invest metabolic resources in processes involved in growth and reproduction equally to wild type worms. This perhaps implies that wild type worms store unused resources in favourable growth conditions and/or when males are not present. As fecundity is sperm-limited, it is conceivable that a fitness cost may become apparent when males are present if reproductive output is increased to a greater extent in wild type worms than in *age-1* mutants. However, it is also possible that *age-1* mutants are more efficient at converting food into energy.

Although studies which have examined metabolic rate in insulin/IGF-1 signalling (IIS) mutants have produced contrasting results (Vanfleteren and De Vreese, 1995; Van Voorhies and Ward, 1999), it is well established that the IIS pathway plays an important role in regulating metabolism. As metabolism is suppressed in dauers to promote long-term survival (Holt & Riddle, 2003), and IIS mutants display similar transcription profiles to wild type dauers (McElwee *et al*., 2004, 2006), the expression of many genes involved in metabolism is modified in these worms (Murphy *et al*., 2003; McElwee *et al*., 2003, 2004, 2006). Using microarray data to examine transcript profiles in *daf-2* mutant adults, McElwee *et al.* (2006) suggested that energy availability is increased in *daf-2* mutants relative to wild type worms due to higher conversion of fats to carbohydrates and higher storage of ATP. As mutations in *daf-2* and *age-1* both disrupt insulin/IGF-1 signalling and activate the transcription factor DAF-16, this may also apply to *age-1* mutants and could potentially explain why fitness costs do not arise in favourable environments despite increased investment of resources in somatic maintenance and repair.

8.13 Fitness costs in resource limited conditions

As fitness is a measure of relative population growth rate, it should ideally be examined in a population context, in which different genotypes compete for resources in conditions which are likely to be experienced in nature. When long-lived *age-1* mutants are maintained in mixed genotype populations with limited resources, fitness is clearly reduced relative to wild type worms (Walker *et al*., 2000; chapters 4 and 5). Context dependent trade-offs have also been observed in long-lived *D. melanogaster Indy* mutants when flies are cultured with low food availability (Marden *et al*., 2003), and are likely to arise because mutations which promote longevity disrupt the optimal partitioning of limited resources among growth, reproduction and somatic maintenance and repair mechanisms.

I have demonstrated that the fitness cost observed in *age-1* mutants in resource limited conditions may be primarily due to a delay in maturity after worms have been maintained in the dauer stage (chapters 4 and 5). This perhaps occurs because nuclear localisation of DAF-16 is more intense and prolonged in *age-1* mutants than in wild type worms during and after starvation (Weinkove *et al*., 2006; chapters 4 and 5). *C. elegans* have mainly been isolated from nature as dauers (Barrière & Felix, 2005b), and this stage is likely to be of major importance for dispersal to new food patches and for the persistence of populations in nature. To maximise fitness and ensure rapid population growth when favourable growth conditions are encountered, exit from the dauer stage is likely to be under strong selection in wild populations (Burnell *et al*.,

2005). This may explain why the *hx546* mutant allele has not (yet) been found in nature.

8.2 Molecular to population level responses to environmental stress

8.21 The insulin/IGF-1 signalling pathway and resistance to stress

Different forms of stress can induce similar forms of damage to essential cellular components including DNA, proteins and membrane lipids (Kültz, 2005). Consequently, although some cellular responses can be specific to particular environmental conditions, highly conserved proteins involved in processes such as DNA and protein repair are often expressed regardless of the source of the stress (Kültz, 2005; Van Straalen & Roelofs, 2006; Roelofs *et al*., 2008). For instance, a variety of different types of stress induce the expression of heat-shock proteins (molecular chaperones) and antioxidant enzymes (Kültz, 2005; Roelofs *et al*., 2008). Nuclear localisation of DAF-16 does not necessarily ensure constitutive expression of all DAF-16 target genes (Henderson *et al*., 2006), and additional transcription factors, transcriptional co-activators and/or other proteins may be required for transcriptional specificity in different environmental conditions (Wolff *et al*., 2006; Berdichevsky *et al*., 2006; Hsu *et al*., 2008; Li *et al*., 2008). However, as described in chapters 4-6, IIS mutants and dauers display increased resistance to several different types of environmental stress, suggesting that reduced insulin/IGF-1 signalling mediates a general rather than a specific cellular stress response. Furthermore, *age-1* mutants display resistance to some forms of stress which do not appear to activate DAF-16 in wild type worms (Henderson & Johnson, 2001; Lamitina & Strange, 2005; chapter 6, though see Wolf *et al*., 2008). This implies that constitutive expression of stress response genes in *age-1* mutants facilitates the prevention and/or repair of somatic damage in these worms regardless of the source of stress.

I have demonstrated for the first time that *age-1* mutants and dauers display increased resistance to cold temperatures relative to wild type adults (chapter 6). Although genes which are known to promote cold tolerance are up-regulated in response to low temperatures in wild type worms (Murray *et al*., 2007), this may not be mediated by DAF-16 activity as exposure to cold conditions did not induce nuclear localisation of a DAF-16::GFP fusion protein in this study (though see Wolf *et al*., 2008). However, the increased cold tolerance observed in *age-1* mutants was clearly *daf-16* dependent, suggesting that constitutive expression of DAF-16 target genes prior to and during exposure to low temperatures promotes resistance to low temperatures in these worms. Using a combination of loss-of-function mutations and RNAi, I have

demonstrated that this is largely due to the up-regulation of Δ^9 desaturase genes, but that additional transcriptional targets of DAF-16 are likely to be involved. Following an assessment of factors known to promote survival at low temperatures in other organisms, it became apparent that numerous genes which are up-regulated in IIS mutants and dauers could potentially contribute to the cold tolerant phenotype. These include genes which are involved in the synthesis of trehalose sugars and glycerol, and genes encoding antioxidant enzymes and heat shock proteins. As DAF-16 activity does not appear to be induced by exposure to low temperatures, this is consistent with the suggestion that reduced insulin signalling mediates a general stress response.

8.22 Stress resistance and nutritional state

Many long-lived mutants display enhanced resistance to various forms of environmental stress (reviewed in Johnson *et al*., 2001; Kenyon, 2010). However, stress resistance in long-lived mutants has rarely been considered in different nutritional conditions. In wild type worms, stress resistance differs according to nutritional status and stage. It is well established that dauers are highly resistant to a variety of environmental challenges (Anderson, 1978, 1982; Larsen, 1993; Lithgow *et al*., 1995), and larvae which have arrested in the L1 diapause stage are more resistant than fed L1s to at least some forms of stress (Baugh & Sternberg, 2006; Weinkove *et al*., 2006, chapters 4 and 5). However, resistance during other stages in starvation conditions appears to be dependent upon the type of stress which is applied. For instance, adults are more resistant to thermal stress when starved but are more resistant to oxidative stress when food is available (Henderson *et al*., 2006).

Given that the long-lived, stress-resistant phenotype of IIS mutants is likely to result from partial activation of the dauer program throughout life (Kenyon *et al*., 1993; McElwee *et al*., 2004, 2006), transcriptional profiles of *age-1* mutants and wild type worms may not differ during the dauer stage. It thus seems intuitive that *age-1* mutant and wild type dauers should display similar abilities to tolerate environmental stress. Consistent with this, no differences in survival were observed between wild type and *age-1* mutants when dauers were exposed to high temperatures (chapter 4), oxidative stress (chapter 5) or low temperatures (chapter 6) for the durations that these stresses were applied. In contrast, although no difference was observed between the two genotypes when arrested L1s were exposed to thermal stress (chapter 4), *age-1* mutants display higher resistance to oxidative stress than wild type worms during this stage (Weinkove *et al*., 2006; chapter 5). It remains unclear how the two genotypes differ in resistance to thermal and oxidative stress during other stages in starvation conditions; however, these results imply that the effects of life-extending mutations on

resistance to environmental stress depend upon the context in which they are observed.

Although exposure to thermal or oxidative stress had little effect upon cellular distributions of DAF-16 in starved worms (chapters 4 and 5), as the transcription factor is activated in starvation conditions it is possible that starvation-induced stress resistance may be partially mediated by DAF-16. Consistent with this, increased resistance to hydrogen peroxide in starved L1s relative to fed L1s is dependent upon *daf-16* (Weinkove *et al*., 2006). This increased tolerance is observed even after prolonged periods of starvation when DAF-16::GFP is no longer nuclear localised (Weinkove *et al*., 2006). Furthermore, Henderson *et al*. (2006) demonstrated that starved *daf-16* mutant adults are considerably more sensitive to oxidative stress than starved wild type adults. This suggests that the expression of DAF-16 target genes is necessary for survival under starvation conditions when oxidative stress is applied. In contrast, starvation-induced resistance to thermal stress is not entirely dependent upon *daf-16* (Henderson *et al*., 2006). As DAF-16 target genes are up-regulated in IIS mutants, this distinction may explain why exposure to oxidative stress reduced the fitness deficit observed in *age-1* mutants maintained in high density populations with limited resources whilst exposure to thermal stress did not.

8.23 Costs and benefits of a long life in heterogeneous conditions

The fitness cost observed in *age-1* mutants under resource limited conditions has repeatedly been cited as an important example in which trade-offs consistent with the evolutionary theories of senescence arise in a long lived mutant in ecologically relevant conditions. However, wild populations are likely to experience spatial and temporal variation in ecological factors in addition to resource abundance, and genetic variation in the ability to survive and reproduce in different environmental conditions is likely to have a major impact upon the evolution of life-history strategies in nature. I have established that *age-1* mutants can have higher fitness than wild type worms if mixed genotype populations are exposed to thermal or oxidative stress when food is available (chapters 4 and 5). This is the first demonstration that a mutation which promotes longevity can confer a selective advantage over a wild type genotype (though see Scheckhuber *et al*., 2007). The results imply that, given a particular ecological niche, selection acting upon genetic variation in the ability to tolerate environmental stress may lead to the fixation of stress resistant alleles and indirectly promote the evolution of increased longevity. This is consistent with the ecological stress theory of ageing (Parsons, 1995, 2002), and with studies which have reported life history

consequences of artificial selection for increased resistance to stress (White *et al*., 1970; Rose, 1992; Hoffman & Parsons, 1993; Pijpe *et al*., 2008).

DAF-16 distributions and genotype-by-environment interactions suggest that *age-1* mutants have higher fitness than wild type worms in low density populations which encounter periods of stress because increased expression of stress response proteins promotes the ability to survive, develop and reproduce during and/or after exposure to harsh conditions. However, in high density populations, frequencies of the *age-1(hx546)* mutant allele consistently declined over time. This occurred despite more intense nuclear localisation of DAF-16::GFP than in wild type worms before, during and after exposure to stress in starvation conditions. This may be because a large proportion of worms of each genotype would have arrested development in the L1 diapause state or as dauers. There appears to be no distinction between the two genotypes in resistance to thermal stress during L1 arrest and in resistance to thermal or oxidative stress during the dauer stage. Furthermore, *age-1* mutants took considerably longer to attain maturity than wild type worms after being maintained as dauers regardless of the environmental conditions which were imposed. This suggests that if the *age-1(hx546)* mutant allele was to arise in a population which experiences periods of environmental stress, its fate may ultimately depend upon the availability of food.

8.4 The evolution of longevity in natural populations

8.41 The evolution of longevity in stochastic environments

The experiments which were reported in chapters 4 and 5 fail to account for environmental stochasticity as populations were maintained either at low densities, with excess food, or at high densities, with limited food, and periods of environmental stress of equal intensity were imposed at regular intervals. In reality, populations are likely to experience fluctuations in resource availability and stochastic variation in factors such as temperature, humidity, oxidative stress and pathogen infection. Using a demographic approach, I examined the conditions under which the *age-1(hx546)* mutant allele can invade a wild type population which has already been established (chapter 7). I have demonstrated that when populations experience stochastic variation in resource availability only, *age-1* mutants are always at a disadvantage and cannot invade a wild type population. In contrast, when resource availability varies over time and populations experience periods of thermal or oxidative stress at random intervals, the *age-1(hx546)* mutant allele confers a selective advantage over the wild type genotype. The relative fitness of *age-1* mutants was slightly higher than wild type

worms when populations experienced periods of environmental stress at very low frequencies but was considerably higher when stress was encountered on a more regular basis. These results are remarkable because they imply that the *age-1(hx546)* mutant allele could invade a wild type population even if periods of starvation are frequently endured. Consequently, although they are based upon a simplified model in which density dependent processes are not taken into account, they suggest that fitness costs in *age-1* mutants may be insignificant in environments in which additional factors vary over time. As the conditions which were considered in this study are likely to be ecologically relevant, the results have implications for the evolution of longevity in nature. This is particularly true given current transitions in global climate and additional anthropogenic pressures.

Non-adaptive evolutionary theories of senescence (Medawar, 1952; Williams, 1957; Kirkwood, 1977) are based upon the principle that the efficiency of selection declines with increasing age (Fisher, 1930; Hamilton, 1966). These theories predict that, in natural populations, longevity and the rate of senescence reflect the level of extrinsic mortality experienced in a particular ecological niche (Williams, 1957). This is because the efficiency of selection at a given age is ultimately determined by the likelihood of survival and reproduction at that age. It would be interesting to ascertain how changes in age-specific schedules of survival and reproduction which are induced by stochastic exposure to harsh conditions modify the dynamics of selection across the lifespan. Although I have demonstrated that a mutation which promotes longevity and confers resistance to environmental stress can increase fitness relative to a wild type genotype in conditions which are likely to be encountered in nature, the results in this study do not contradict the antagonistic pleiotropy and disposable soma theories of ageing. To ensure continued propagation of the germ-line, individuals must invest metabolic resources in somatic maintenance and repair mechanisms to be able to survive in adverse environments, and to retain the ability to develop and reproduce when conditions improve. Genes which influence the ability to tolerate harsh conditions are, therefore, likely to be under strong selection in natural populations. Whilst resource allocation trade-offs associated with increased investment in somatic maintenance and repair may arise in favourable growth conditions and/or in resource limited conditions, fitness benefits over long time scales may outweigh these disadvantages. Consistent with the ecological stress theory of ageing (Parsons, 1995, 2002), and the consequences of artificial selection for increased resistance to stress (White *et al*., 1970; Rose, 1992; Hoffman & Parsons, 1993; Pijpe *et al*., 2008), the results presented in chapters 4, 5 and 7 indicate that, for a comprehensive understanding of the factors which influence the evolution of longevity in natural populations, the effects of

ecological sources of stress which are not necessarily lethal must be considered in addition to levels of extrinsic mortality.

8.42 Ecological genetics in ageing research

The major objective of the majority of studies focussing on ageing in model organisms is to elucidate the processes which underlie senescence in humans (Partridge & Gems, 2007). Given that some of the molecular determinants of longevity appear to have been conserved among distinct eukaryotic lineages (reviewed in Kenyon, 2005, 2010), inferences from research using model organisms such as *C. elegans* may have important medical implications. However, it is less clear how studies using model organisms in benign laboratory conditions can contribute to our understanding of the evolution of senescence and longevity in wild populations. Most of the major model organisms are not well characterised in an ecological setting (Peňa-Castillo & Hughes, 2007), and there is a distinct lack of knowledge regarding the selective pressures which are relevant to these species in nature. Furthermore, the majority of studies in model organisms focus upon single wild type genotypes and mutant derivatives of these strains. Some 'wild type' model organisms may have diverged considerably from their natural states, and it is unclear how epistatic interactions between 'gerontogenes' and other genes which vary among individuals in nature may influence the rate of senescence and longevity in wild populations (Partridge & Gems, 2007). Evolutionary and ecological studies of the factors which influence these traits may benefit from using a variety of wild genotypes and from establishing model systems which are more characterised in an ecological context.

Until relatively recently, ageing was not considered to constitute an important source of mortality in wild populations. However, it is becoming increasingly apparent that senescence occurs in nature more often than previously assumed (reviewed in Nussey *et al*., 2008). Although natural variation in genes which modulate longevity has been associated with entry into diapause, lifespan and adaptation to different climatic regions in *D. melanogaster* (Schmidt *et al*., 2000; Williams *et al*., 2006; Paaby *et al*., 2010), the significance of mutations which increase lifespan in model organisms for senescence in wild populations is unclear (Monaghan *et al*., 2008). Furthermore, longevity is determined by both genetic and environmental factors during an individual lifetime, and certain mutations which increase longevity in model organisms can have opposite effects upon lifespan in different environmental conditions (VanVoorhies *et al*., 2005; Baldal *et al*., 2006). This suggests that the effects of variation in genes which modulate longevity can be context dependent. Consistent with this, I have

demonstrated that the life history consequences of a mutation which increases lifespan differ according to the conditions in which they are observed.

There was previously a distinct lack of information regarding how genes which modulate longevity in model organisms can influence fitness in ecologically relevant conditions. However, I have addressed this issue using empirical and theoretical studies to examine the fitness consequences of the *age-1(hx546)* mutant allele in a variety of conditions which may be experienced by wild populations. Using an ecological genetics approach, I have, therefore, contributed to the understanding of how genetic variation in lifespan and the ability to tolerate environmental stress may influence the evolution of longevity in nature. The studies described in this thesis indicate that important inferences regarding the factors which influence longevity and the rate of senescence in wild populations may be derived from model organisms when these are investigated in an ecologically relevant context.

8.5 Conclusions

Longevity and the rate of senescence are ultimately determined by the ecological conditions experienced during a population's recent evolutionary history, and are intrinsically linked to other components of life history and to fitness. These traits should be examined in an ecological context, ideally in natural environments or in conditions which are representative of nature, in which other aspects of the life history are taken into account. Using a multidisciplinary approach, I have demonstrated that if genetic variation is present in populations which encounter harsh conditions, longevity can evolve as a consequence of selection for greater resistance to environmental stress. I have also established that long-lived *age-1(hx546)* mutants and dauers display increased cold tolerance relative to wild type adults, and that the effects of mutations which promote longevity on resistance to stress can be context dependent. The results presented in this thesis imply that genetic and life history responses to ecological stress deserve a more prominent role in evolutionary studies of ageing.

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Appendices

Appendix 6.1 Primer sequences

age-1(hx546)

Non-synonymous substitution (c→t) causing amino acid change P→S. Primers amplified a 564 bp region containing the substitution.

5’ CCAGTATTATGCCTGCTTCA

3’ TGCGTACGGGTTCAAACAGC

daf-16(mu86)

10980 bp deletion. Primers amplified a 857 bp region in the wild type only.

5’ CATCCATCCATACACCCACA (internal)

3’ CAATTGATCGGTTGGCTTCT (internal)

fat-5(tm420)

779 bp deletion. External primers amplified a 1480 bp region in the wild type and a 701 bp region in the mutant. Internal primer with 5’ external primer amplified an additional 502 bp region in the wild type only.

5’ AGACTCCGCCCCTTCTTTT (external)

3’ AAGTGCTTTAGGCTTGGGCTC (external)

3’ CTGAATTAGGAAACGTAGGC (internal)

fat-6(tm331)

1232 bp deletion. External primers amplified a 1767 bp region in the wild type and a 535 bp region in the mutant. Internal primer with 5’ external primer amplified an additional 905 bp region in the wild type only.

5’ CCAGAGACGCAATATCTCGC (external)

3’ CACATCCATGATTGGATACC (external)

3’ GATGAGCTCCGGCGGTTATT (internal)

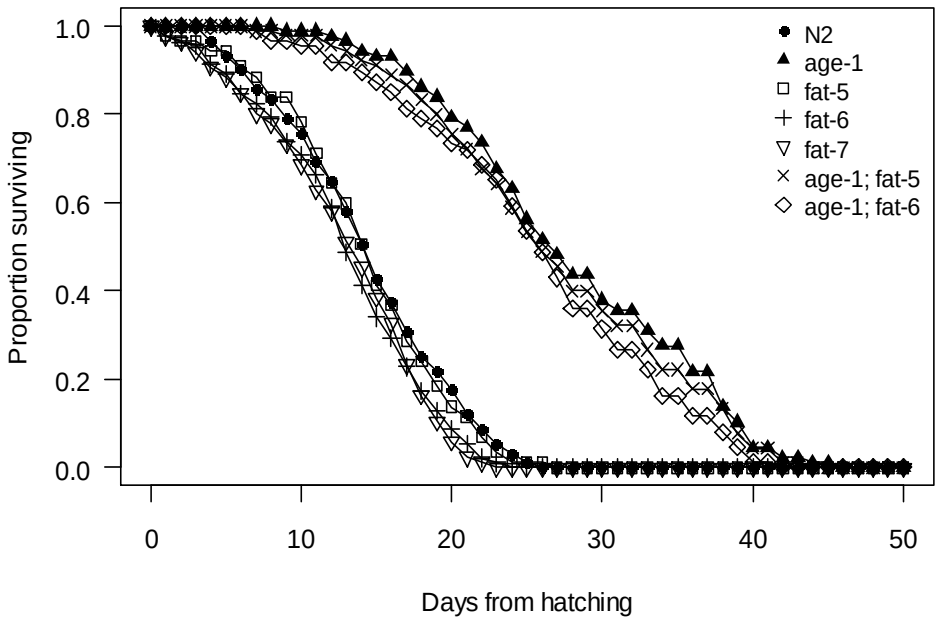
fat-7(wa36)

Substitution (c→t) producing a premature stop codon. Primers amplified a 457 bp region containing the substitution.

5’ ACAAGGCAACCACACCAATG

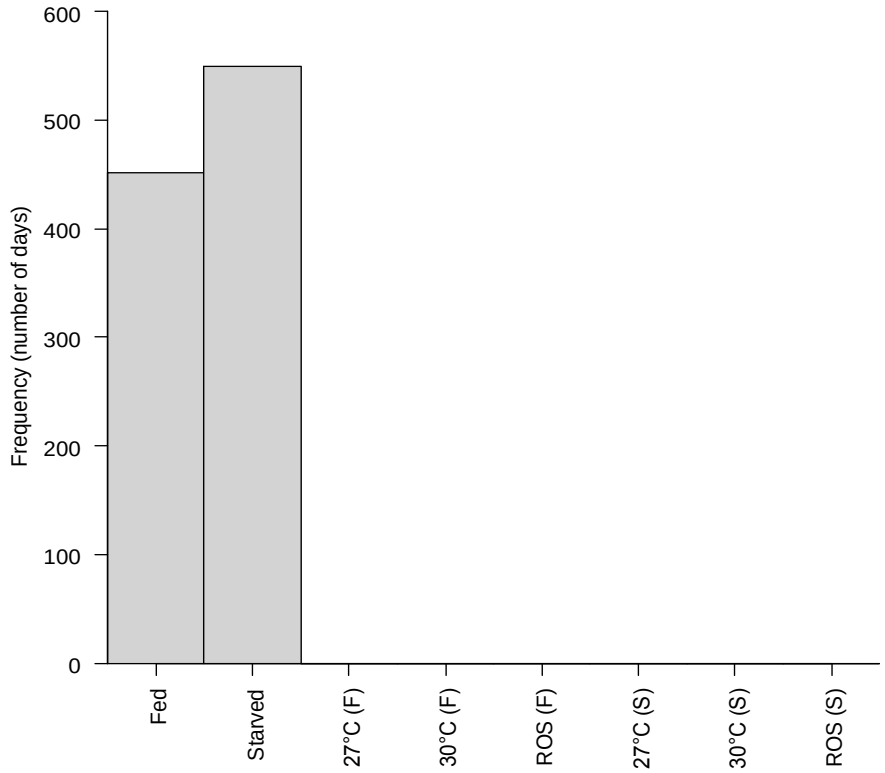
3’ ATGCACCAAGTGGCGTGAAGT

Appendix 6.2 Cold tolerance in single Δ^9 desaturase mutants



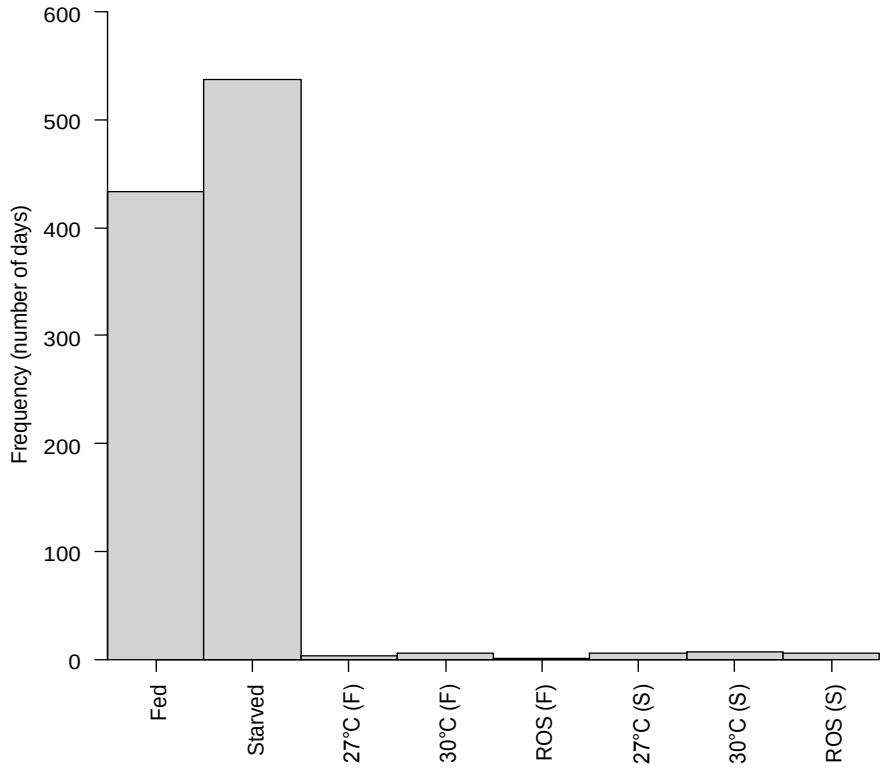
Survival curves at 4°C for wild type (N2), *age-1(hx546)* mutants and single desaturase mutants in each genetic background. Survival was monitored in 90-100 individuals per genotype and stage.

Appendix 7.1 Fluctuating resource availability



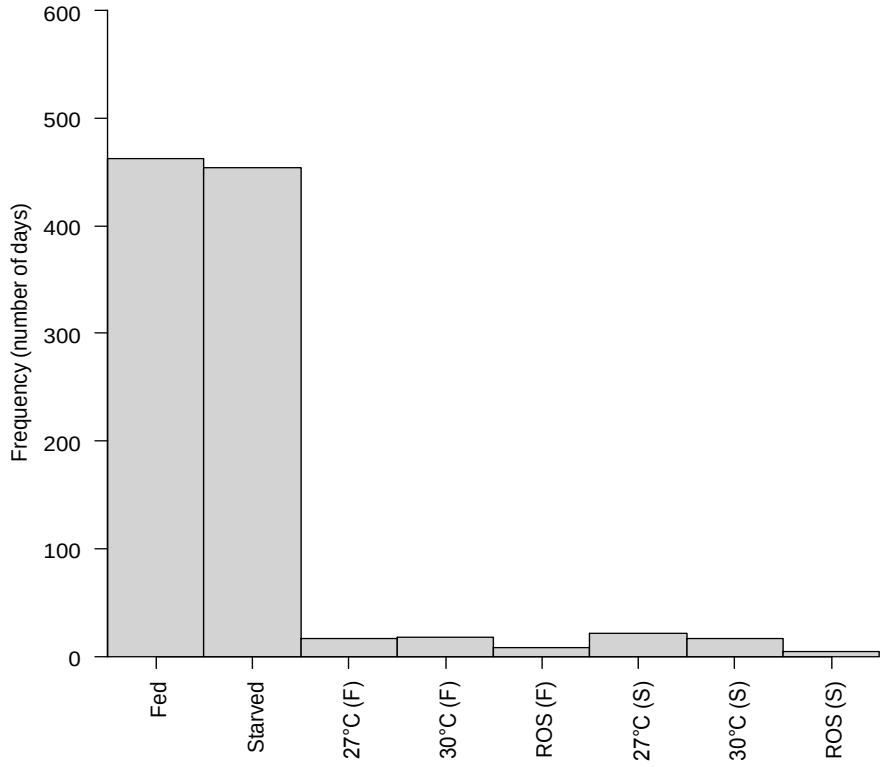
Relative frequencies of fed versus starved days which were applied in the stochastic projection presented in figures 7.3c and 7.3d.

Appendix 7.2 Low frequency stress



Relative frequencies of different environmental states which were applied in the stochastic projection presented in figures 7.4a and 7.4b. F and S refer to fed and starved respectively and ROS refers to oxidative stress.

Appendix 7.3 High frequency stress



Relative frequencies of different environmental states which were applied in the stochastic projection presented in figures 7.4c and 7.4d. F and S refer to fed and starved respectively and ROS refers to oxidative stress.