Sex differences in ageing in the nematode *Caenorhabditis elegans*

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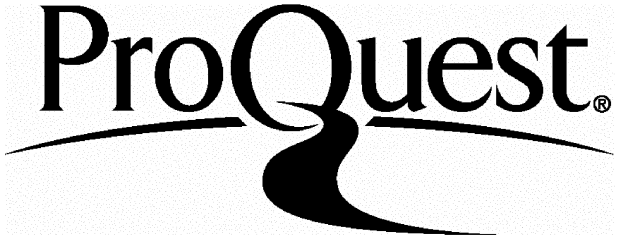
PhD Thesis



Abstract

Males of the nematode *Caenorhabditis elegans* are longer-lived, more stress resistant and more likely to enter an alternative dispersal stage (dauer) than hermaphrodites. Why might this be? Since these traits are suppressed by insulin-/IGF-1-like signalling (IIS), it seemed possible that IIS levels could be lower in males. Increased male lifespan and stress resistance were dependent upon *daf-16*, which encodes the transcription factor negatively regulated by IIS, suggesting that IIS is down-regulated in males. Moreover, the male bias to dauer formation was lost in IIS mutants. However, DAF-16::GFP was more localised to the nucleus (where it is active) in hermaphrodites, hence sex differences in DAF-16 activity may occur downstream of IIS.

Lifespan is extended by certain uncoordinated (*unc*) mutations, which disrupt motility by affecting neurons or musculature. Only neuronal *unc* mutations extended lifespan, suggesting that disruption of neurotransmission and/or neuroendocrine function may be responsible. The *daf-16*-dependence of the lifespan increase due to several *unc* mutations suggests that IIS may be the component affected. Since *unc* mutations increase lifespan more in males than hermaphrodites, sex differences in IIS/ DAF-16 activity may underlie this effect, at least in part.

Hermaphrodite lifespan is regulated by gonadal signalling. Lifespan responses of males to ablation of gonad precursors were reduced relative to hermaphrodites, suggesting reduced lifespan regulation by the male gonad. Moreover, in contrast to hermaphrodites, male lifespan was largely unaffected by mutation of *daf-12*, which encodes a nuclear hormone receptor that interacts with IIS and is involved with transduction of gonadal signals.

Overall, therefore, evidence suggests fundamental sex differences in lifespan regulation. Why might this evolve? Studies of several dioecious and other androdioecious species suggested that increased male lifespan is a general trait among nematodes. Potentially, differential degrees of male rarity and exposure to non-senescent modes of death shape evolution of sex differences in lifespan.

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Abbreviations

ACh	Acetylcholine
Age	Increased adult lifespan phenotype
CI	Confidence intervals around a median
Daf-c	Dauer formation-constitutive phenotype
Daf-d	Dauer formation-defective phenotype
DFR	Dauer formation ratio
DIC	Differential image contrast
DR	Dietary restriction
FuDR	5-fluoro-2'-deoxyuridine
GFP	Green fluorescent protein
Hid	High temperature-induced dauer formation phenotype
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IIS	Insulin-/IGF-1-like signalling
IR	Insulin receptor
ME	Mating efficiency
NGM	Nematode growth medium
NHR	Nuclear hormone receptor
PI3K	Phosphoinositide 3-kinase
rf	Reduction-of-function
Rol	Roller phenotype
s.e.	Standard error of the mean
SOD	Superoxide dismutase
TGF-β	Transforming growth factor-β
ts	Temperature-sensitive
Unc	Uncoordinated phenotype

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Section A: Main Introduction

Contents

A.0 Introduction to ageing

A.0.1 Background

Ageing research is a highly active area of scientific investigation. Its importance to human health and quality of life is clear, with many conditions associated with old age, such as Alzheimer's disease, many cancers, diabetes, heart disease and stroke. The economic cost of supporting a disproportionately large number of elderly individuals in the developed world over the next few decades adds to the need to understand and potentially counter the changes associated with later life. Besides the practical applications of ageing research (the ethics of which will not be considered here), the almost ubiquitous occurrence of ageing among organisms makes it an intriguing subject for evolutionary researchers.

Ageing and the death that follows it are clearly not beneficial to the individual, which is driven by its selfish genes to maximise its own fitness. The phenomenon of ageing therefore requires some explanation. Current theory holds that it is a non-adaptive trait that occurs due to a reduction in selection pressure at older ages (Bidder 1932). Genes that manifest deleterious effects at later ages are less subject to selection than those that harm an individual early in life and reduce its fitness (Haldane 1941; Medawar 1952). A point is reached at which an individual is highly likely to have died through non-senescent events, meaning that there is little selection for alleles that promote survival at such an age. The appearance of deleterious phenotypes has little impact on the fitness of such an old individual, so late-acting harmful alleles can perpetuate free from selection.

There are two main sub-theories concerning the source of late-acting deleterious mutations. The mutation accumulation theory argues that over the generations there is a gradual accumulation of late-acting deleterious mutant alleles. The later the effect of an allele, the less efficiently it will be removed from the population by selection (Medawar 1952). The antagonistic pleiotropy theory suggests the existence of multi-effect alleles which, while being advantageous to an organism early in life when selection pressure is high, are disadvantageous at older ages, resulting in the senescent phenotype (Williams 1957).

Senescence results in an increased chance of death (mortality rate) and reduced reproductive ability with increasing age. Organisms which senesce rapidly often do so after a burst of reproduction (semelparity), and include many spiders, squid, octopus, Pacific salmon, *Antechinus* (marsupial mouse) and many monocarpic angiosperms. Death in such species results mainly from structural damage and physiological shifts, such as wear and tear of body parts and sudden hormonal changes, and is often very uniform in mechanism and time of onset within a species. Gradual senescence is more variable in extent between individuals and tends to be seen in iteroparous species. The causes of senescence in such cases are less clear, but may overlap with those of rapidly senescing organisms. Since gradual senescence is often more variable, it is likely that there are multiple mechanisms underlying the process within each species. Gradually senescing species include parasitic nematodes, many perennial plants and most fish, birds and mammals (see (Finch 1990)). Interestingly, studies in *Hydra* suggest that this organism may not senesce at all (Martínez 1998). In this sense, *Hydra* is similar to the non-senescent germlines of all organisms.

A.0.2 Sex differences in ageing

In addition to the variation in timing and rate of senescence between species, there are often differences between the sexes within a species, as reflected by sex differences in lifespan. Examples of organisms in which females are the longer-lived sex include many fish (Bidder 1932), flour beetles (Park 1945), spiders (Deevey & Deevey 1945) and gerbils (Committee on Animal Models for Research in Aging 1981). This is also the case for humans: females born in 2000 have a life expectancy of 80 years, compared with 75 years for males[1]. Conversely, increased male lifespan is apparent in the housefly (Ragland & Sohal 1973), many birds (see (Smith 1989)), certain mice (Oliff 1953; Holzenberger *et al* 2002), Syrian hamsters and guinea pigs (Committee on Animal Models for Research in Aging 1981). Many such reports, however, are based on anecdotal evidence from wild populations, and may reflect sex differences in exposure to hazards such as predation rather than the rate of ageing. Studies into sex differences in ageing and their underlying genetics are therefore especially relevant in model

[1] National Statistics Online: www.statistics.gov.uk

organisms such as the nematode *Caenorhabditis elegans*, which is the focus of this study. Such organisms can easily be maintained in the laboratory under controlled conditions, thus removing many confounding variables.

What are the mechanisms underlying sex differences in longevity? These are at least partly understood in a few, select organisms, especially in semelparous species. For example, males of the marsupial mouse *Antechinus* die after only one season of intense mating, while females survive to breed another year. The immune system of males is completely depleted due to the high levels of coticosteroids produced during the mating season (Diamond 1982). However, factors underlying sex differences in lifespan are poorly understood in most species, and are likely to involve both senescent and non-senescent components. In humans, for example, the risk of extrinsic death for young men due to accidents and suicide, or as a result of excessive smoking and alcohol consumption, is significantly higher than for women (Holden 1987). However, there is still an element of the sex difference in lifespan that cannot be accounted for by lifestyle, implying that there are intrinsic genetic differences between the sexes that regulate lifespan. For example, a higher risk of cardiovascular disease among men (due to differential modulation of cholesterol levels by the sex hormones) also contributes to the female lifespan advantage.

A mechanism that has been proposed to explain the increased female lifespan of many mammals is the "genotypic sex" model. This postulates that possession of two X chromosomes by the female protects against recessive X-linked disorders, due to compensation for the defective allele by the second X chromosome. There is some indirect evidence that X chromosome heterozygosity may lead to increased female lifespan in mammals, since inbred strains of mice, in which individuals presumably have identical X chromosomes, show no sex differences in lifespan (Committee on Animal Models for Research in Aging 1981). Likewise, as might be predicted (McArthur & Baillie 1932), preliminary evidence in birds (in which males are the homogametic sex) suggests that males are longer-lived than females, although certain reports refute these observations (e.g. (Landauer & Landauer 1931)). The relevance of this theory is unclear, however, since recessive X-linked genetic disorders are rare.

Investigating sex differences in C. elegans *lifespan*

The model organism *C. elegans* provides a useful tool for investigating the genetics underlying sex differences in ageing, since it has been demonstrated that there is a sex difference in lifespan in this well-characterised species under controlled conditions. Maintaining males and hermaphrodites together in mixed-sex populations reduces the lifespans of both sexes due to deleterious effects of mating (Gems & Riddle 1996; 2000b). To avoid these confounding effects, initial studies into sex differences in lifespan used grouped, single-sex cultures, and suggested that hermaphrodites were the longer-lived sex (Gems & Riddle 1996; 2000b). However, subsequent experiments found that if males are maintained in isolation away from other males, they live longer than hermaphrodites, by ~20%. This is because in the absence of hermaphrodites, grouped males attempt to mate with one another (Gems & Riddle 2000b). Thus, both mating with hermaphrodites and attempts at mating with other males have a severe negative impact on male lifespan, reducing it by ~50%. The presence of only one other male results in a significant decrease in lifespan (Gems & Riddle 2000b). Possibly, this is due to mechanical damage to the male tail and perhaps subsequent bacterial infection. In an interesting parallel, males also live longer than females in the housefly *Musca domestica*, but only when maintained away from other males, since male-male interactions also reduce lifespan in this species (Ragland 1973).

In addition to this intrinsic sex difference in wild-type lifespan, *C. elegans* also displays sex differences in response to the uncoordinated (*unc*) class of mutations, which reduce motility. Such mutations extend solitary male lifespan, but have little effect on hermaphrodite lifespan (Gems & Riddle 2000b; see Chapter 3). It was postulated that *unc* mutations could inhibit a deleterious component of male-specific behaviour, and by doing so reveal an underlying male longevity (Gems & Riddle 2000b). However, the fact that certain *unc* mutations are also able to extend hermaphrodite lifespan (Ailion *et al* 1999; Gems & Riddle 2000b; Munoz & Riddle 2002), suggests that the lifespan increase due to *unc* mutations may not be entirely due to behavioural effects, but may involve sex differences in neuroendocrine function.

What factors could underlie the sex differences in wild-type lifespan and the lifespan response to *unc* mutations? There are a number of candidates, since studies on

C. elegans hermaphrodites have shown that lifespan is regulated by several components, including the insulin/ IGF-1-like neurosecretory pathway, gonadal signalling and the nuclear hormone receptor DAF-12. It is therefore possible that sex differences in components such as these may be involved in sex differences in lifespan and response to lifespan-extending mutations. Investigating the role of such factors in sex differences in lifespan was the aim of Chapters 2-5 of this project. Before investigations into sex differences in lifespan could begin, however, it was necessary to develop a suitable culture method for solitary male *C. elegans*. This is because maintaining males in isolation on agar plates results in a high proportion climbing the walls of the Petri dishes and dying from desiccation due to their active mate-searching behaviour. Such animals must therefore be censored from survival analyses, markedly reducing sample size. Development of a suitable culture method for solitary male *C. elegans* is described in Chapter 1.

The existence of sex differences in lifespan raises the interesting question as to what their evolutionary origins might be. Does the reproductive strategy of a species influence which sex is longer-lived, or could sex differences in exposure to non-senescent hazards play a role? The aim of the final chapter of this project was therefore to gain an understanding as to why males might evolve an increased lifespan by comparing *C. elegans* with a range of dioecious and hermaphroditic nematode species.

To aid interpretation of this thesis, the following sections provide some background on what factors may cause ageing and the current status of ageing research in other laboratory model organisms, before introducing *C. elegans* in more detail and discussing its involvement in ageing research to date.

A.1 Mechanistic theories of ageing

Mechanistic theories of ageing aim to describe how the senescent process occurs at the anatomical, cellular or molecular level. It has been estimated that there are over 300 separate mechanistic theories of ageing (Medvedev 1990). However, it is likely that senescence is multi-factorial, involving a combination of different mechanisms. Some of the most popular and best-supported current mechanistic theories of ageing are introduced below.

A.1.1 Free radical theory of ageing (for review see (Beckman & Ames 1998))

Aerobic respiration is never entirely efficient, resulting in the generation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide and hydroxyl radicals. These molecular species are also produced in response to ultraviolet light and free radical generators such as methyl viologen. ROS are highly oxidising, removing electrons from cellular proteins and lipids and resulting in widespread structural and functional damage.

A range of defences has evolved to neutralise ROS, including the antioxidant enzymes catalase, Cu-Zn superoxide dismutase (SOD), MnSOD, glutathione peroxidases and glutathione S transferases. In addition, there are hydrophilic radical scavengers (e.g. glutathione), lipophilic radical scavengers (e.g. flavonoids) and a suite of reducing enzymes responsible for re-reduction of oxidised antioxidants.

The free radical theory of ageing postulates that increased ROS generation and/ or decreased antioxidant capability over time result in accumulated cellular damage and ultimately senescence. Evidence for the free radical theory of ageing is accumulating (selected examples are given below), although it is not always clear whether the effects measured cause senescence or are merely indirect by-products of senescent processes. In addition, negative and conflicting results have also been published which do not support the theory, with a substantial number of inter-species differences. While the role of free radicals in the senescent process remains unclear, their involvement is strongly implicated.

- Oxidative protein modification increases with age by up to 30% in organisms as diverse as humans and houseflies (Sohal *et al* 1993a; Stadtman 1992), while lipid peroxidation increases with age in humans, reducing lipid bilayer fluidity and elasticity (Zarling *et al* 1993).
- Mitochondrial generation of H_2O_2 and O_2•- increases by up to 100% in two-year-old compared with five-month-old gerbils (Sohal *et al* 1995).
- The fluorescent pigment lipofuscin is believed to consist partly of breakdown products from lipid peroxidation. The age-related accumulation of lipofuscin in cell culture depends upon the partial pressure of oxygen (Sohal *et al* 1989). Moreover, species with higher metabolic rates accumulate lipofuscin faster (Nakano & Gotoh 1992).
- Hyperoxia reduces lifespan of *C. elegans* while hypoxia extends lifespan (Honda *et al* 1993). In addition, hyperoxia increases SOD levels in young but not older *C. elegans*, implying that ability to resist oxidative damage decreases over time (Darr & Fridovich 1995).
- Transgenic *Drosophila* over-expressing Cu/Zn SOD are significantly longer-lived than wild type and show reduced protein oxidation (Parkes *et al* 1998; Sun & Tower 1999). In addition, *Drosophila* lines selected for increased longevity have increased SOD and catalase activity (Dudas & Arking 1995; Tyler *et al* 1993).
- *C. elegans* strains with mutations in components of insulin-IGF-1-like signalling show extended lifespan accompanied by increased resistance to oxidative stresses such as H_2O_2, and raised SOD and catalase levels (Larsen 1993; Honda & Honda 1999; Vanfleteren 1993) (see Section A.4.5).

Potentially involved with the free-radical theory of ageing is the fact that dietary restriction, which reduces food intake, increases lifespan by 30-80% in a range of species, including: yeast (Jiang *et al* 2000), waterstriders (Kaitala 1991), *Drosophila* (Chapman & Partridge 1996), spiders (Weindruch 1996), fish, (Weindruch 1996), mice (Weindruch & Walford 1982), rats (McCay, *et al* 1935) and dogs (Kealy *et al* 2002). Animals with reduced dietary intake remain active and healthy for longer and have reduced growth rates and body size compared with animals fed *ad libitum*. In rodents,

DR results in delayed onset of age-related diseases such as certain cancers, and retards development of insulin resistance. Preliminary data indicate that DR has similar effects in primates (Lane *et al* 1996 & 2000; Ramsey *et al* 2000).

Free radical levels may be reduced under conditions of DR, since there is a smaller decline in antioxidant enzyme levels with age in DR animals and less membrane peroxidation (Lee & Yu 1990; Yu 1996). In addition, mice under DR display reduced carbonylation of proteins (Sohal *et al* 1994). Thus, the lifespan increase due to DR may be mediated by effects on oxidative stress resistance (Sohal *et al* 1994).

A.1.2 Rate-of-living theory of ageing

The free radical theory of ageing is often linked to the "rate-of-living theory" (Pearl 1928), which posits that the lifetime energy usage per unit mass is constant across organisms, hence those with a higher metabolic rate senesce and die more quickly. This idea is often invoked to explain the lifespan differences between small and large animal species, since smaller animals must expend more energy in order to maintain body temperature than larger ones. It is true that metabolic rate generally increases as size decreases. However, certain taxa deviate from the lifespan-metabolic rate correlation quite markedly, such as birds, bats and primates. The rate of living theory may therefore ultimately be deemed to be an over-simplification.

However, other research has argued that measurement of oxygen consumption is not necessarily a reliable means of determining metabolic rate, and that if species differ intrinsically in the amount of ROS generated by their mitochondria then this may account for the differences. This was found to be the case in pigeon mitochondria, which consume two to three times as much oxygen as rat mitochondria but generate half the amount of H_2O_2 (Barja *et al* 1994). Moreover, comparisons between two species of mouse have shown that the longer-lived species generates half the amount of $O_2^{-}\bullet$ and three to five times less H_2O_2 (Sohal *et al* 1993b). Thus, the rate of living theory may go some way to explain species lifespans.

A.1.3 Mitochondrial theory of ageing

Also related to the free-radical theory is the mitochondrial theory of ageing. Because mitochondria are major sites of ROS generation (Chance *et al* 1979), and since their DNA is unprotected by histones and shows poor mutation repair, it was proposed that they are especially vulnerable to free radical damage (Wallace, 1992). The rate of oxidative damage of mtDNA is approximately ten times that of nuclear DNA (Ames *et al* 1993). Mitochondria with ROS-induced mutations could lead to disrupted respiratory electron transport and hence increased ROS generation and in turn more mitochondrial damage in a positive feedback loop (Ozawa 1995). The eventual outcome would be cellular senescence through reduced ATP production and through protein and lipid damage by the resulting ROS (Miquel *et al* 1980) or cell death, since free radical generation results in creation of a mt transition pore, releasing cytochrome c and initiating apoptosis (Liu *et al* 1996). Indeed, an accumulation of mutations in mtDNA with age has been found in humans (Cortopassi & Arnheim 1990) and in *C. elegans* (Melov *et al* 1995).

A.1.4 Replicative senescence theory of ageing

Another potential mechanism by which senescence may occur (in organisms that experience somatic cell proliferation during adulthood) is through limitations in cell proliferative capacity. Healthy somatic eukaryotic cells cannot divide indefinitely, and eventually become senescent. Senescent cells arrest in the G1 phase of mitosis and have reduced protein turnover, increased lysosome synthesis and are larger in size (for review see (Stanulis-Praeger 1987)). Once senescent, cells do not die and are in fact more resistant to apoptosis than replicative cells (Wang 1995). Cellular phenotypes also change upon senescence, and protein expression differences have been seen in several studies (e.g. (Maier *et al* 1993)). The proliferative limit may be an anti-cancer mechanism, since the risk of a neoplastic transformation increases with the number of divisions.

The replicative senescence theory of ageing postulates that once cells reach their replicative limit, the capacity for body maintenance and repair would eventually decline, with the result being senescence. For example, accumulation of senescent fibroblasts

may result in loss of dermal collagen due to altered cell differentiation (West *et al* 1989). Accumulation of senescent cells could also lead to late-onset cancer by altering the intercellular environment, allowing previously checked cancer cells to proliferate (Campisi 1997).

A cell's replicative potential is thought to be determined at least in part by the length of its telomeres, which are stretches of highly repetitive DNA at the ends of chromosomes. Telomeres prevent deterioration of coding regions of the chromosome during replication by inhibiting chromosomal non-disjunctions, translocations and fusions (Blackburn 1991). However, telomere length is reduced upon each cell division (Harley *et al* 1990) due to incomplete DNA replication (Levy *et al* 1992; Counter *et al* 1992) until division is no longer possible and the cell becomes senescent (Allsopp *et al* 1992). Telomere length is a predictor of fibroblast replicative capacity *in vitro* (Allsopp *et al* 1992). Proliferative potential in mammals is also regulated genetically, with changes in gene expression occurring in cells approaching senescence. For example, transcription factors such as c-fos, Id and E2F are no longer expressed, probably due to the inhibition of cyclin-dependent kinases (Campisi 1997a).

Certain facts imply the involvement of a proliferative limit in senescence. Firstly, fibroblasts from elderly humans undergo fewer divisions in cell culture than those from young individuals (Hayflick & Moorhead 1961). In addition, the number of population doubling times for fetal fibroblast cells shows a positive correlation with the mean lifespan of the species (Röhme 1981). Finally, premature ageing syndromes are accompanied by premature cell senescence. For example Werner's syndrome, which results in osteoporosis, wrinkled skin, atherosclerosis, diabetes and cancer, is caused by mutation in a DNA helicase gene *WRN* (Yu *et al* 1996). Also, reduced telomere length and proliferative capacity are seen in fibroblasts from patients suffering from Hutchinson-Gilford progeria (Allsopp *et al* 1992). Whether or not replicative senescence is a cause or even an effect of the senescent process remains to be clarified.

A.2 Model organisms used in ageing research

A number of convenient model systems are used to investigate the evolution and mechanisms of ageing. While the major focus of this work is the nematode *Caenorhabditis elegans*, the other laboratory organisms regularly employed in ageing research, and what has been discovered about ageing in each to date, are introduced briefly below.

A.2.1 *Saccharomyces cerevisiae* (budding yeast) (for review see (Sinclair *et al* 1998))

This unicellular eukaryote is inexpensive to maintain and easily manipulated in the laboratory. Its genome has been sequenced and many mutant strains are available. This organism has the added benefit that it can be cultured as either stationary or budding phase cells, allowing chronological and replicative lifespan measurement. Different strains of yeast have different lifespans, implying that there is a strong genetic element regulating longevity.

Cells cultured under glucose starvation cease to ferment and instead begin respiratory metabolism, entering stationary phase. These cells are thermotolerant and appear to maintain themselves actively, since removal of SOD results in cell death, implying that stationary phase cells still generate ROS (Longo *et al* 1996). Eventually, however, these cells lose their ability to resume growth when transferred to nutrient-rich medium.

S. cerevisiae also has a limited budding capacity (Mortimer & Johnston 1959), with the cell cycle declining with age. During replicative ageing, cells increase in size, their surfaces become wrinkled, nucleoli degenerate and there is a redistribution of Sir proteins from telomeres and from mating (*HM)* loci to the nucleolus (Sinclair *et al* 1997). Since Sir proteins are involved with chromatin silencing, the result is age-associated expression of *HM* genes. Loss of silencing of HMRa and HMLα genes results in sterility. Daughter cells from senescing mother cells also have reduced lifespan, implying the existence of a "senescence factor" in the cytoplasm (Kennedy *et al* 1994).

Yeast cells therefore show a limited replicative potential as do mammalian cells. This, along with the fact that there is a high degree of genetic homology between *S.*

cerevisiae and higher organisms, means that this yeast is likely to be of great relevance to ageing research in general. For example, two homologous genes have been found to regulate lifespan, perhaps by linking longevity to nutrient availability in *S. cerevisiae* (*SIR2*) (Kaeberlein *et al* 1999) and *C. elegans* (*sir-2.1*) (Tissenbaum & Guarente 2001). In addition, deletion of *RAS1* or over-expression of *RAS2*, both homologues of mammalian *ras* genes, results in increased yeast lifespan (Sun *et al* 1994).

Lifespan extension of ~25% by dietary restriction has also been shown in *S. cerevisiae*, an effect which is dependent on *SIR2* and NAD (Lin *et al* 2000). This lifespan extension may be the result of increased respiration brought about by DR. Increased respiration appears to result in increased Sir2 activity and hence enhanced gene silencing (Lin *et al* 2002).

A.2.2 *Drosophila melanogaster* (fruit fly)

This is another small, inexpensive organism that is convenient for research into the genetics of ageing. Like *S. cerevisiae* and *C. elegans*, the entire genome has been sequenced and many mutants are available.

Selection experiments using *Drosophila* have produced long-lived lines (Luckinbill *et al* 1984; Partridge & Fowler 1992; Rose *et al* 1984). Interestingly, such lines often show delayed or reduced fertility, implying the potential involvement of a trade-off between reproductive investment and allocation of resources to repair and maintenance (see Chapter 4).

A number of long-lived mutants have also been identified, the first of which was the *methuselah* (*mth*) mutant (Lin *et al* 1998). *mth* encodes a putative GTP-binding protein-coupled seven-transmembrane domain receptor homologue. This class of receptor is involved with a wide range of processes including neurotransmission, light and odour stimulus transduction and hormone functioning. Mutation of *mth* results in an ~35% increase in mean lifespan and increased resistance to starvation, oxidative stress and high temperature. Interestingly, Mth is required for effective neurotransmitter release at the neuromuscular junction, which is an intriguing parallel with the increased lifespan of *C. elegans* neuronal *unc* mutants (see Chapter 3).

Mutation of the gene *Indy* (*I'm not dead yet*) also extends *Drosophila* lifespan. The *Indy* gene product shows homology to a mammalian sodium carboxylate transporter (Rogina *et al* 2000). Such molecules are required to transport intermediates such as citrate and succinate in the Krebs cycle, and the authors speculate that Indy may regulate intermediary metabolism and absorption of metabolites, perhaps in a manner similar to dietary restriction.

There is also some evidence for ROS being involved with senescence in *Drosophila*. As mentioned above, over-expression of Cu/Zn SOD in transgenic *Drosophila* results in increased lifespan and reduced protein oxidation (Sun & Tower 1999). It is also noteworthy that increased SOD and catalase activities are seen in *Drosophila* lines selected for increased longevity (Tyler *et al* 1993; Dudas & Arking 1995).

Much current ageing research in *C. elegans* is focusing on the insulin/ insulin-like growth factor-1 (IGF-1)-like signalling (IIS) pathway, mutations in which have been found to extend lifespan (see Section A.4.5). Like its *C. elegans* equivalent, *Drosophila* IIS shows much homology with the mammalian insulin and IGF-1 signalling pathways. Reduction-of-function mutations in at least two *Drosophila* IIS components- the insulin receptor and insulin receptor substrate homologues- result in increased lifespan (Clancy *et al* 2001; Tatar *et al* 2001; Tu *et al* 2002). *Drosophila* IIS and its role in the regulation of lifespan are described in more detail in Section A.4.5 below and in Chapter 2.

A.2.3 *Mus musculus* (mouse)

Compared with the invertebrate models described above, mice are expensive to maintain in the laboratory, meaning that experimental sample sizes are necessarily smaller, and survival analyses take 2-3 years rather than weeks. The first intervention found to extend mouse lifespan was DR, as described above e.g. (Weindruch & Walford 1982). The genetics of ageing in *Mus* is less well characterised than that of *C. elegans*, *Drosophila* and *S. cerevisiae*, but is a rapidly growing area of research. Significantly, mutation of the IGF-1 receptor extends mouse lifespan as seen in *C. elegans* and *Drosophila* insulin/ IGF-1 receptor homologue mutants (Holzenberger *et al* 2002), as do certain dwarf

mutations affecting pituitary function, and hence secretion of hormones including growth hormone and IGF-1 (see Section A.4.5 below).

A.3 Introduction to *Caenorhabditis elegans*

A.3.1 *Caenorhabditis elegans* as a model genetic system

C. elegans is a particularly convenient model organism for the purposes of ageing research, having a lifespan of approximately two weeks. It measures approximately 1.2mm when adult, hence is easy and cheap to maintain. Large numbers of worms can be grown on the surface of an agar-filled Petri dish with an *Escherichia coli* food source (Brenner 1974). Because hermaphrodites are exclusively self-fertilising, homozygous mutant strains can be maintained with ease, while the existence of males allows back-crossing of strains and multiple mutant strain constructions. Strains and isolates can be frozen at -70°C for many years, allowing convenient storage of stocks.

C. elegans has well-developed genetics: many mutants have been generated and characterised, with strains freely available from the *Caenorhabditis* Genetics Center[2]. The genome has been completely sequenced, identifying approximately 19,000 genes (*C. elegans* Sequencing Consortium 1998), and a public database exists to supply sequence information (www.wormbase.org). Finally, the predictable cell numbers and migrations in this organism (Sulston & Horvitz 1977), along with its transparent body, make it an ideal model for investigating developmental pathways and for visualising gene expression using Green Fluorescent Protein (GFP) and other markers.

A.3.2 Genetics and nomenclature in *C. elegans*

Genetic loci are designated names of three lower-case italicised letters, joined by a hyphen to an Arabic number e.g. *unc-32*. To indicate the linkage group of the locus, an italicised Roman numeral follows e.g. *unc-32 III*. The three-letter abbreviation can refer either to the mutant phenotype of the disrupted gene (in this case uncoordinated) or to the protein product of the wild-type gene e.g. *pdi* (protein disulphide isomerase).

[2] Caenorhabditis Genetics Center, University of Minnesota, 6-6160 Jackson Hall, 321 Church Street S,E, Minneapolis, MN 55455, http://biosci.umn.edu/CGC/CGChomepage.htm

Phenotypes are written as locus names, but are non-italicised and have a capital letter (e.g. Unc), while gene products are written in upper case e.g. UNC-32. Alleles are denoted as specific italicised numbers in brackets following the gene name e.g. *unc-32(e189).*

A.3.3 Biology and anatomy

In nature, *C. elegans* is a free-living soil-dweller, probably feeding on slime moulds and bacteria (Riddle & Albert 1997). Many wild isolates have been collected from around the globe, which vary in a range of behavioural, reproductive and anatomical characteristics (Hodgkin & Doniach 1997).

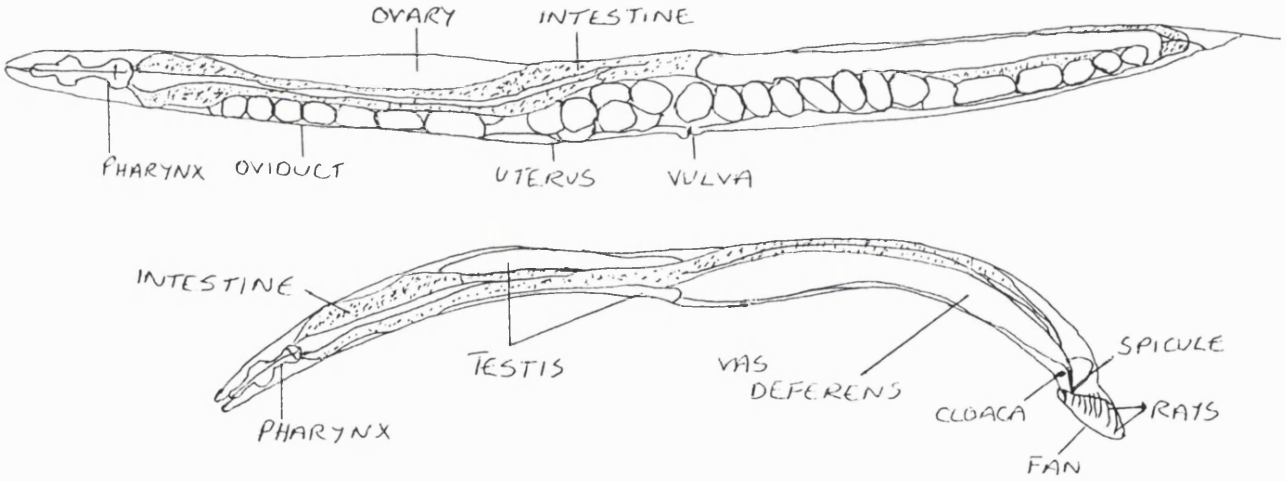
The body of adult *C. elegans* is covered by an acellular outer cuticle composed mainly of cross-linked collagens. Beneath the cuticle lies the hypodermis, which in turn connects to body wall muscles via protruding muscular filaments. The majority of muscles are striated and used for movement, while non-striated muscles are used for pumping of the pharynx, defecation and intestinal contractions. Through the centre of the body runs the intestine, which connects to the pharynx at the anterior and the anus at the posterior. The pharynx pumps food into the buccal cavity of the worm, and bacteria are broken up by contraction of the terminal bulb (Doncaster 1962).

C. elegans is a sexual species composed of protandrous hermaphrodites and males (Figure A.1). Hermaphrodites cannot mate with one another, but can either self-fertilise or out-cross with males (Figure A.2). Where mating of a hermaphrodite with a male does occur, male sperm are used by the hermaphrodite in preference to self-sperm, with physical displacement of hermaphrodite sperm taking place (Ward & Carrel 1979). In a hermaphrodite that has already depleted its store of sperm and hence has stopped producing oocytes, mating with males stimulates the production of more oocytes, and so increases the number of progeny produced above the 300 or so that are obtained from self-fertilisation to over 1000 (Hodgkin & Barnes 1991).

Males occur rarely in laboratory populations, at a frequency of ~0.1% (Hodgkin & Doniach 1997; Ward & Carrel 1979) and are the result of spontaneous non-disjunction of the X chromosome during meiosis (Nigon 1949). Males are shorter and thinner than hermaphrodites, and when adult can be distinguished by their specialised

tail structures designed for copulation and sperm transfer (Figures A.1 & A.3). Males possess certain extra cells that are not present in hermaphrodites, including 79 neurons contributing to male mating behaviours, and 16 extra hypodermal cells, which are required for the male tail structures. Many gene classes involved specifically with male development have been identified.

Figure A.1: Diagrammatic representation of adult hermaphrodite (top) and male *C. elegans*. The hermaphrodite is ~1.2mm long. Reproduced from (Riddle *et al* 1997).



Males also differ from hermaphrodites in that they possess only one gonad arm, compared with two in hermaphrodites (Figure A.1). Hermaphrodites produce a limited number of amoeboid sperm, which are generated during the final larval stage in the proximal gonad, and stored 160 per gonad arm in spermathecae (Hodgkin & Barnes 1991). A switch to oocyte production in the loop region and proximal gonad begins during early adulthood and continues until the store of sperm is depleted. Once the oocytes have matured along the gonad arms they pass through the spermathecae.

Fertilised oocytes then pass into the uterus and leave the body via the vulva on the ventral side of the worm. Embryonic development continues within the egg for approximately 14 hours, after which time a first-stage larva emerges and begins feeding.

The *C. elegans* male lacks the hermaphrodite-specific structures such as spermathecae, uterus, vulva and the associated musculature and nervous tissue. The one-armed somatic gonad comprises a testis, seminal vesicle and valve region. The vas deferens leads away from the gonad to the cloaca, through which the ejaculate leaves the body. Sperm production begins late in the final larval stage and continues throughout life; male worms can produce approximately 3000 sperm.

Figure A.2: DIC image of a male displaying searching behaviour during mating. Scale bar= 100μm

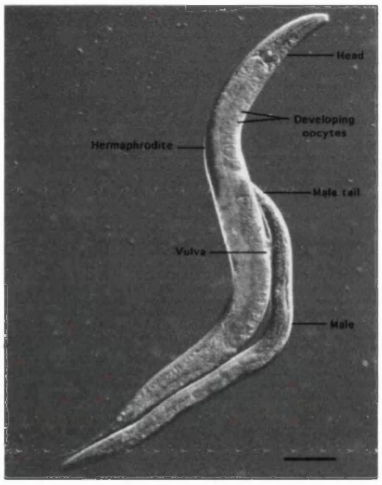
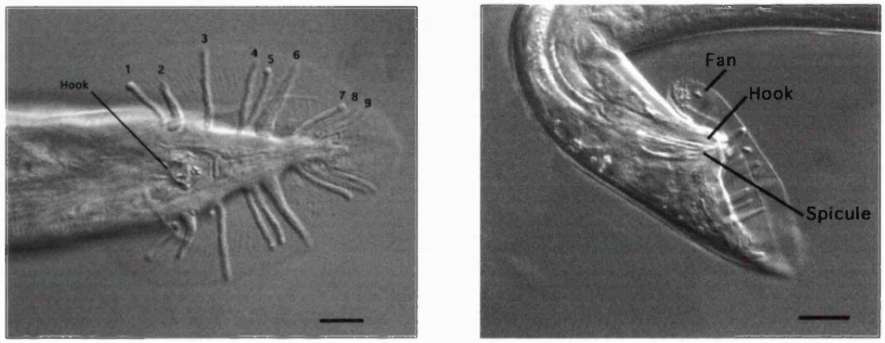


Figure A.3: DIC images of the specialised male tail structures from a young adult (a) Ventral view. 1 -9 label each of the paired rays. Scale bar= 20 μm. (b) Lateral view. Scale bar= 35μm

(a) **(b)**



Gender in *C. elegans* is determined by the ratio of autosomes to sex chromosomes rather than the number of sex chromosomes *per se.* Hermaphrodites possess two X chromosomes and five pairs of autosomes, while males lack a second X chromosome (X0). Detection of the ratio of X:A chromosomes affects a group of regulatory genes which include: *xol-1* (X0 lethal), *sdc-1*, *sdc-2* and *sdc-3* (sex and dosage compensation) (Villeneuve & Meyer 1987; Miller *et al* 1988; Nusbaum & Meyer 1989; Klein & Meyer 1993). These genes act on a suite of downstream targets including *her* (hermaphroditisation) and *tra* (transformer) genes in order to regulate the sexual fate of the somatic cells. They also regulate the activity of a set of dosage compensation genes, which inhibit the expression of X-linked genes.

A.3.4 *C. elegans* life cycle

Post-embryonic development in *C. elegans* begins when a worm hatches from the egg as a stage 1 larva (L1). During times of plentiful food and low population density, development proceeds through three further larval stages (L2-L4) (Figure A.4), with a cuticle moult preceding each new stage. After one final moult, the worm is adult and is soon able to reproduce. The entire life cycle is completed in just 50 hours at 25°C.

C. elegans includes an alternative larval stage in its life cycle, which is selected if conditions are not suitable for reproduction. This dauer larva (*Ger.* "endure") is a developmentally arrested diapausal stage that can survive for several months (Klass & Hirsh 1976) and can endure a wide range of stresses. During development to dauer, animals contract radially due to hypodermal shrinkage (Cassada & Russell 1975) and begin to store lipids, resulting in a dark appearance (Figure A.5). Dauers are non-feeding due to the occlusion of the mouth and suppression of pharyngeal pumping (Cassada & Russell 1975). SOD and catalase levels are increased in dauer larvae (Vanfleteren & De Vreese 1995; Larsen 1993), which could at least partly explain their extreme longevity.

The decision to enter dauer development begins between L1 and L2 (Figure A.4). The main cue from the environment is the detection of a threshold level of constitutively produced dauer pheromone which, along with low food levels, signifies overcrowding and results in development into L2d (Golden & Riddle 1982). Dauer formation also

increases with temperature (Golden 1984b). If conditions do not improve, development continues into dauer.

Figure A.4: *C. elegans* life cycle. Red arrows: low food/ high population density; blue arrows: high food/ low population density

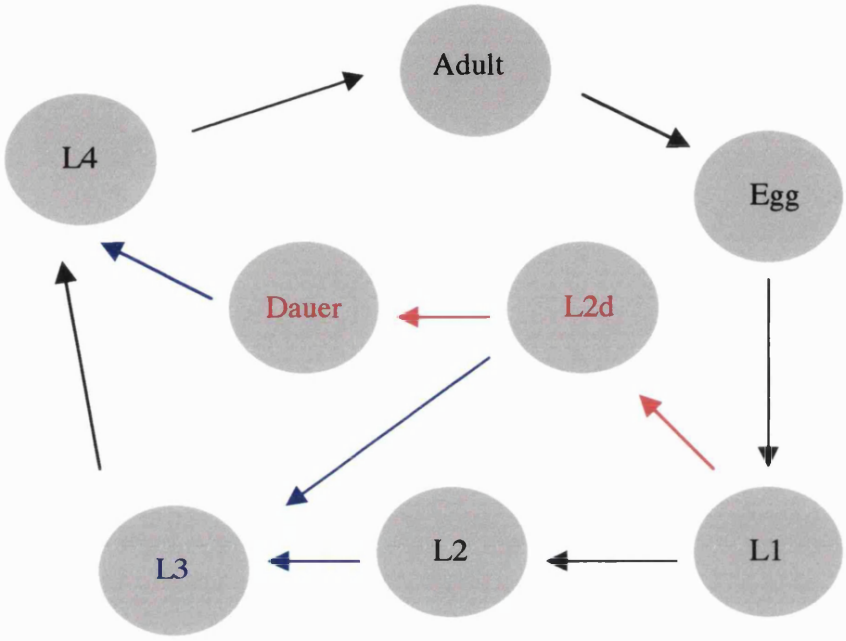
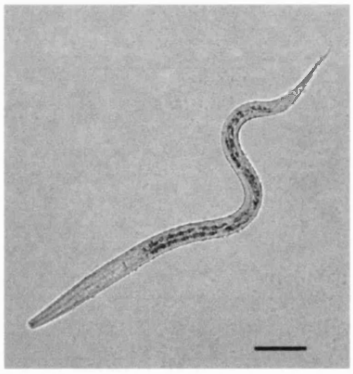


Figure A.5: Bright field image of a dauer larva. Scale bar= 40μm



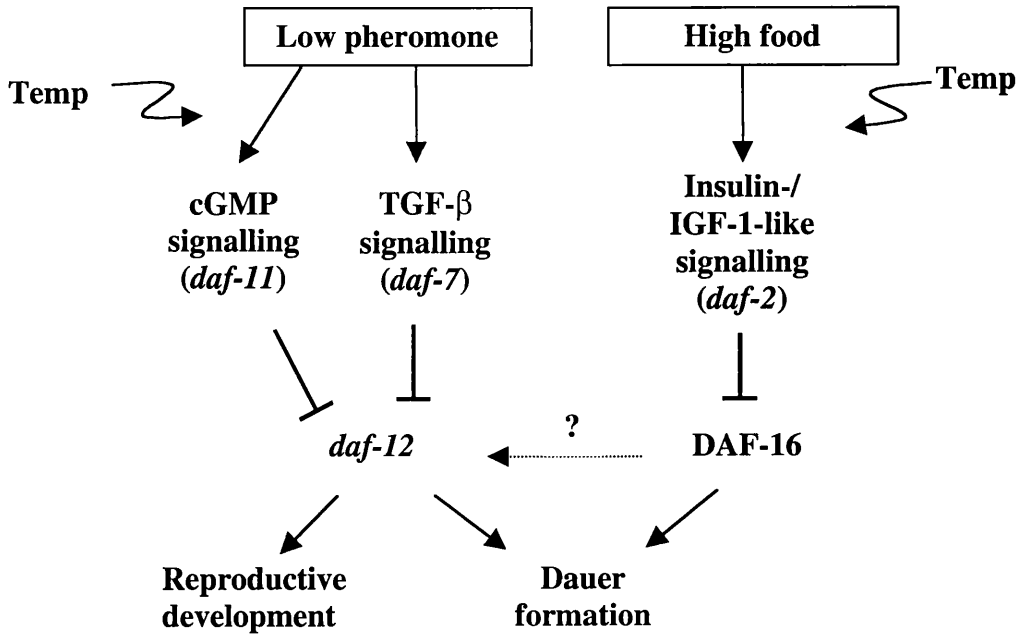
Environmental stimuli are detected via sensory endings of amphidial neurons, which are exposed to the surroundings through paired amphid pores (Ward *et al* 1975). Laser ablation of the amphidial chemosensory neurons ADF, ASG, ASI and ASJ causes

constitutive dauer formation, implying that signalling from these neurons regulates the process (Bargmann & Horvitz 1991). Once dauer or L2d larvae encounter conditions suitable for development to adulthood, they begin to recover and enter L4 (Cassada & Russell 1975).

A.3.5 Genetics of dauer formation

The underlying genetics of dauer formation are well understood due to the identification of many dauer formation (*daf*) mutants and their subsequent classification into three parallel dauer formation pathways (e.g. (Riddle *et al* 1981; Vowels & Thomas 1992)). There are two main classes of *daf* mutant phenotype: dauer defective (Daf-d) and dauer constitutive (Daf-c). Daf-d mutants are unable to form dauers under any conditions, while Daf-c mutants form dauers under inappropriate conditions such as in the presence of ample food and no dauer pheromone. Many Daf-c mutants are temperature-sensitive, being wild type for dauer formation at 15°C, with increasing temperature resulting in increased dauer formation- many Daf-c mutants form 100% dauers at 25°C. The three pathways act to integrate the nutritional, pheromonal and temperature cues from the environment, resulting in an organism-wide commitment either to dauer formation or to adult development (Figure A.6).

Figure A.6: Simplified representation of the three dauer formation pathways



The gene *daf-22* is upstream of at least two of the pathways (cGMP and TGF-β), and is required for production of the dauer pheromone, which shows similarity to hydroxylated fatty acids (Golden & Riddle 1984a). Loss-of-function *daf-22* mutants are Daf-d unless dauer pheromone is added to the medium (Golden & Riddle 1985). *daf-22* is unable to suppress the Daf-c phenotype of mutants with defects in components of the three pathways described below, which implies that Daf-c mutants generate "false" internal signals that lead to dauer formation independent of actual pheromone levels (Golden & Riddle 1985; Riddle *et al* 1981).

cGMP signalling

The cGMP pathway begins with *daf-11*, which encodes a transmembrane guanyl cyclase and hence is involved with cGMP generation. cGMP is required to regulate kinases, cGMP-gated ion channels and other cellular components. *daf-11* reduction-of-function mutants are temperature-sensitive Daf-c, and also form dauers readily when even slightly crowded (Thomas *et al* 1993; Riddle *et al* 1981). *daf-21* has a similar phenotype to that of *daf-11* (Thomas *et al* 1993), and encodes heat-shock protein 90, which is involved with protein stabilisation (Birnby *et al* 2000). *daf-11* and *daf-21* are therefore required for reproductive development. Unlike in all the other Daf-c mutants, it is possible to suppress the *daf-11* and *daf-21* Daf-c phenotypes with dye-filling (*dyf*) mutations affecting sensory cilia (Thomas *et al* 1993). This implies that *daf-11* signalling occurs in the cilia, in response to dauer pheromone levels, and that it mediates an early step in chemosensory regulation of dauer formation (Vowels & Thomas 1992).

TGF-β signalling

The ligand for the TGF-β signalling pathway controlling dauer formation is DAF-7. *daf-7*::GFP expression is seen in the ASI chemosensory neurons of fully fed larvae, but not in starvation dauers (Ren *et al* 1997). This, along with the fact that reduction-of-function mutations in *daf-7* or ablation of ASI and the chemosensory neuron ADF result in a Daf-c phenotype, suggests that *daf-7* function normally acts to promote reproductive development (Swanson & Riddle 1981; Bargmann & Horvitz 1991). Like cGMP signalling, transforming growth factor-β signalling in *C. elegans* mainly responds to

pheromone levels to regulate dauer formation, since L1 larvae raised in M9 buffer and no food still express *daf-7*, while those raised in M9 buffer with pheromone do not (Ren *et al* 1997).

The receptor for DAF-7 is heterodimeric, composed of the transmembrane serine-threonine kinases DAF-1 (type I) (Georgi *et al* 1990) and DAF-4 (type II) (Estevez *et al* 1993). Reduction-of-function mutations in either *daf-1* or *daf-4* result in a Daf-c phenotype. When type II serine-threonine kinases bind ligand they recruit the type I component into the heterodimer. The type I receptor then phosphorylates downstream components of the signalling pathway (Smads), which translocate to the nucleus where they act as transcription factors (see (Inoue & Thomas 2000)). In *C. elegans*, proteins downstream of *daf-1/ daf-4* signalling include DAF-5, which has weak homology with the Sno oncoprotein, and the Smads DAF-3, DAF-8 and DAF-14. Reduction of function mutations in *daf-8* (Riddle *et al* 1981; Estevez *et al* 1996) and *daf-14* (Inoue & Thomas 2000) result in a Daf-c phenotype, while *daf-3* and *daf-5* mutants are Daf-d, and completely suppress the Daf-c phenotypes of *daf-1*, *daf-4*, *daf-8* and *daf-7* (Patterson *et al* 1997; Vowels & Thomas 1992; Riddle *et al* 1981).

It was previously found that cGMP and TGF-β signalling act in parallel to regulate dauer formation (Thomas *et al* 1993). However, it has recently been demonstrated that *daf-11* can also act upstream of TGF-β signalling, since *daf-11* mutants are defective for *daf-7* expression, and *daf-7* cDNA can rescue the *daf-11* Daf-c phenotype (Murakami *et al* 2001).

The downstream target of both TGF-β and cGMP signalling is the nuclear hormone receptor DAF-12, since Daf-d mutations of *daf-12* completely suppress the Daf-c phenotypes of *daf-11*, *daf-7*, *daf-1* and *daf-4* mutants (Antebi *et al* 1998; Antebi *et al* 2000; Riddle *et al* 1981; Vowels & Thomas 1992). Being ubiquitously expressed, *daf-12* is a candidate for receipt and transduction of cell-subset specific dauer formation signals into a whole-organism response (Antebi *et al* 2000). The involvement of *daf-12* in regulation of dauer formation and lifespan is discussed in more detail in Chapter 5.

Insulin-/IGF-1-like signalling

Insulin-/IGF-1-like signalling (IIS) acts in parallel to both cGMP and TGF-β signalling to regulate dauer formation (Riddle & Albert 1997). It is possible that nutritional status is the main cue integrated by IIS, similar to the regulation of glucose homeostasis by insulin in mammals. The *C. elegans* receptor tyrosine kinase DAF-2 is a homologue of both the mammalian insulin receptor and IGF-1 receptor (Kimura *et al* 1997), and regulates a number of branching downstream pathways. The best-characterised of these is the phosphoinositide 3-kinase (AGE-1) branch, which transduces DAF-2 signalling into a phosphorylatory cascade via serine-threonine kinases to a forkhead transcription factor (DAF-16) (Shepherd *et al* 1998). Mutation of *daf-2* or several downstream components of this branch (*age-1*, *pdk-1*, *akt-1*) results in a temperature-sensitive Daf-c phenotype (Riddle & Albert 1997), which is completely suppressed by mutation of *daf-16*. Since IIS plays a major role in *C. elegans* lifespan regulation, it is described in more detail in Section A.4.5 below.

A.4 Understanding ageing in *C. elegans*

Of all the model organisms described above, *C. elegans* is the best characterised with respect to the genetics underlying ageing. A number of classes of mutation and interventions have been identified that extend lifespan, which are introduced below. It should be noted that the previous work described here has been performed exclusively in hermaphrodites unless stated otherwise.

A.4.1 Coenzyme Q

Coenzyme Q (Q) is an electron and proton carrier in the electron transport chain which has antioxidant properties and is a cofactor involved with mitochondrial uncoupling. Although *C. elegans* is able to synthesise the Q_9 isoform of Q, it can also obtain the *E. coli* Q_8 isoform from its food. Both isoforms contribute to electron transport. Growing *C. elegans* on Q-deficient bacteria extends wild-type lifespan by ~60%. It has been

suggested that reducing electron transport chain activity through removal of exogenous Q_8 may reduce damaging ROS generation (Larsen & Clarke 2002).

A.4.2 SOD mimetics

If the free radical theory of ageing is correct, then increased free radical scavenging by antioxidants might be expected to increase lifespan by removing harmful ROS from the cellular environment. In order to test this, *C. elegans* was treated with the salen-manganese SOD mimetics EUK-8 and EUK-134 (Eukaryon Inc, Bedford, MA, USA; Baudry *et al* 1993). After treatment with 0.05-10mM EUK-8 in monoxenic liquid culture, a mean increase in wild-type lifespan of ~40% was seen (Melov *et al* 2000). Moreover, the typically short lifespan of the oxidation-hypersensitive mutant *mev-1(kn1)* was restored to a wild-type level upon treatment with 0.5mM EUK-8. These results provided the first clear evidence of a pharmacological intervention increasing lifespan in *C. elegans*, and moreover supplied evidence to support the free radical theory of ageing. It should be noted, however, that subsequent attempts to repeat lifespan extension in *C. elegans* using EUK-8 have been unsuccessful, in both monoxenic and axenic culture ((Keaney & Gems 2003) and B. Braeckman & J.R. Vanfleteren (pers. comm.)), nor do these compounds increase lifespan in houseflies (Bayne & Sohal 2002) or *Drosophila* (M. West & L. Partridge, pers. comm.).

A.4.3 Dietary restriction

As described above, DR extends lifespan in many species. Although there have been a number of reports of lifespan extension in *C. elegans* upon DR, the magnitude of lifespan increase has been variable, and it is not always clear whether the effects are the result of reduced food intake. The first report of an effect of food concentration on *C. elegans* lifespan came from experiments involving different concentrations of *E. coli* in liquid culture (Klass 1977). Hermaphrodite lifespan at 20°C was increased to 26 days at a bacterial concentration of $1x10^8$ cells ml^{-1}, compared with a normal lifespan on agar plates at that temperature of ~18 days. However, recent attempts to replicate these findings have not been successful: negligible increases in lifespan are seen relative to

agar plates, and the optimal concentrations for brood size and lifespan are the same (M. Keaney & D. Gems, pers. comm).

Later reports documented the finding that a number of *eat* mutants with reduced pharyngeal pumping (Avery 1993) showed lifespan increases of up to 50% relative to wild type (Lakowski & Hekimi 1998). The degree of lifespan extension correlated with the severity of the *eat* mutant allele. Unfortunately, subsequent work by a number of workers has failed to reproduce the increased lifespan of *eat* mutants (M. Keaney & D. Gems, pers. comm.; H. Tissenbaum pers. comm.; P. Larsen pers. comm.; B. Braeckman pers.comm.).

Another technique used to investigate DR in *C. elegans* is reduction of bactopeptone concentration in standard NGM agar, which reduces growth of the bacterial food source. Concentrations of bactopeptone below the standard 2.5g l^{-1} all resulted in extension of lifespan, and lifespan was increased by up to 30% (mean) and 80% (maximum) when bactopeptone was eliminated from the agar altogether (Hosono *et al* 1989).

Another possible means of imposing dietary restriction on *C. elegans* is through the use of axenic medium, which is a solution of soy peptone, yeast extract and precipitated haemoglobin. *C. elegans* lifespan is extended in axenic medium by up to 80%, while brood size is reduced and development time extended (Croll *et al* 1977; De Cuyper & Vanfleteren 1982). However, the mechanism of lifespan extension by axenic medium on lifespan remains unclear. Axenic medium lacks coenzyme Q, an essential requirement of *C. elegans* which, when removed from the food source, extends lifespan (see above). It is therefore unclear how much of the lifespan increase in axenic medium is the result of DR and how much is due to the lack of coenzyme Q.

A.4.4 Clock genes

One class of genes involved with lifespan determination in *C. elegans* is the clock (*clk*) genes. Mutation of *clk-1*, *clk-2*, *clk-3* or another clock gene *gro-1* results in maternally rescued Age and developmental timing phenotypes (Lakowski & Hekimi 1996). Interestingly, *clk* mutants are resistant to U.V. irradiation (Murakami & Johnson 1996), hence increased stress resistance may play a role in the increased lifespan of *clk* mutants

at least to some extent. Since *clk-1* encodes a homologue of a mitochondrial protein in yeast that is involved in coenzyme Q synthesis (Ewbank *et al* 1997), it has been proposed that the increased longevity of *clk-1* may be the result of decreased ROS generation due to decreased activity of the electron transport chain (Larsen & Clarke 2002). Interestingly, *clk-2* encodes a homologue of yeast Tel2p, which is necessary to maintain telomeres (Runge & Zakain 1996). Thus, *clk-2* may be involved with regulation of proliferative senescence (Section A.1.4 above). The *clk* mutants do not feature in this work and therefore will not be considered further.

A.4.5 Insulin/ IGF-1-like signalling

The class of mutations that produce the largest effects on *C. elegans* lifespan are those in *daf-2* and the PI3K branch of insulin/ IGF-1-like signalling (IIS). Mammalian insulin and IGF-1 signalling have been extensively studied due to their roles in many fundamental physiological processes and the associated diseases of signalling dysfunction, such as diabetes and growth retardation. For this reason, before describing the IIS pathways and mutations that affect *C. elegans* and *Drosophila* lifespan, the two homologous mammalian pathways will be introduced. A diagram summarising insulin/ IGF-1 signalling in mammals and IIS in *Drosophila* and *C. elegans* is presented at the end of this section (Figure A.9).

Mammalian insulin and IGF-1 PI3K signalling

Mammalian insulin is an anabolic hormone that promotes synthesis and storage of carbohydrates, lipids and proteins. Production of insulin occurs exclusively in the β-cells of the islets of Langerhans in the pancreas. Insulin signalling via the insulin receptor results in a wide range of cellular effects, including regulation of transcription, protein kinase/ phosphatase activation and promotion of cell growth and differentiation. These diverse effects are the result of a complex branching network of signalling pathways downstream of the insulin receptor (e.g. (Avruch 1998; Hausdorff *et al* 1996)) and lead to uptake of glucose, lipid biosynthesis and metabolism of amino acids in adipocytes, heart, liver and skeletal muscle.

Mammalian IGF-1, produced mainly by the liver but also at various peripheral sites in the body, is required for pre- and post-natal growth and development, with mouse *Igf-1* null mutants being dwarf at birth. Those individuals that survive the neonatal period are infertile as adults, and are 60% of normal size (Baker *et al* 1993; Liu *et al* 1993).

The insulin receptor (IR) is expressed ubiquitously. Mice with homozygous null mutations in the insulin receptor gene develop normally and are carried to term, but shortly after birth develop severe hyperglycaemia and hyperketonaemia, dying within three days (Accili *et al* 1996). Humans lacking insulin receptor function display strong insulin resistance and, in the severest cases, leprechaunism, which results in reduced stature, fasting hypoglycaemia and post-prandial hyperglycaemia (Wertheimer *et al* 1993). The IGF-1 receptor (IGF-1R) is also expressed ubiquitously, although tissue-specific levels of expression relative to the insulin receptor vary. Mouse null mutants for the IGF-1 receptor are small and die shortly after birth (Liu *et al* 1993).

The IR and IGF-1R are transmembrane receptor tyrosine kinases. These are heterodimers composed of two inner α and two outer β chains, with an extracellular ligand-binding domain (LBD) and an intracellular kinase domain linked by a transmembrane region. The LBD contains two homologous domains (L1 and L2) separated by a cysteine-rich region. Ligand binding results in autophosphorylation of the catalytic kinase domain on tyrosine residues.

Many of the effects of mammalian insulin and IGF-1 are the result of signalling to a phosphoinositide 3-kinase (PI3K), which is the best characterised signalling branch to date (for review see (Shepherd *et al* 1998)). PI3K has roles in many cellular functions, including growth and differentiation, carbohydrate, lipid and protein metabolism, platelet activation, cell motility and membrane trafficking. PI3K exists as a heterodimer of a regulatory and a catalytic subunit. The regulatory subunit contains Src homology (SH2) domains specific for YxxM sequences on the tyrosine kinase receptor. Upon receptor activation, SH2 domains on the PI3K dock to the phosphotyrosine residues in the YxxM motifs of the IR or IGF-1R and act to increase the activity of the catalytic subunit of PI3K. The SH2 domains of the regulatory subunit also promote recruitment of the PI3K to complexes in areas where the substrate of the enzyme is found.

Although there is a limited amount of direct recruitment of the PI3K to the IR or IGF-1R in this way (see (Shepherd *et al* 1998)), most recruitment takes place indirectly via insulin receptor substrate (IRS) proteins, which modulate PI3K activity. Four mammalian IRS proteins have been identified, with the most ubiquitously expressed being IRS-1 and IRS-2. IRS proteins are characterised by their C-terminal pleckstrin-homology and phosphotyrosine binding domains, which are required for interaction with phosphoinositides and the tyrosine kinase receptor respectively. They also contain many N-terminal tyrosine phosphorylation sites (Myers & White 1996) which are mainly located in YxxM motifs. YxxM motifs on IRS proteins are auto-phosphorylated by activated receptor kinases, which allows binding to the SH2-containing sub-unit of PI3K and a number of other effector proteins, leading to downstream cascades of intracellular signalling.

Once activated, PI3K catalyses the conversion of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) into phosphatidylinositol 3,4,5-trisphosphate (PIP_3). The production of PIP_3 is countered by the action of the PTEN protein tyrosine phosphatase enzyme, which converts PIP_3 back to $PI(4,5)P_2$ by removing the 3' phosphate. During insulin signalling, production of PIP_3 results in the activation of PI(3,4)P2/ PI(3,4,5)P3-dependent kinase 1 (PDK1), which in turn activates the serine-threonine kinase PKB/Akt.

The effects of PKB/Akt (e.g. up-regulation of glycogen synthesis, uptake of glucose and protein synthesis) are achieved through the regulation of the winged helix/ forkhead transcription factors FOXO1, FOXO3a and FOXO4 (Brunet *et al* 1999; Kops *et al* 1999; Rena *et al* 1999), which regulate transcription of target genes. PKB/Akt moves to the nucleus following insulin or IGF-1 signalling and phosphorylates these transcription factors, resulting in their localisation to the cytoplasm, and thereby their inactivation (e.g. (Biggs *et al* 1999; Nakae *et al* 1999; Tang *et al* 1999)). The different effects of insulin compared with IGF-1 signalling are likely to be at least in part due to differential patterning of forkhead transcription factor phosphorylation (Nakae *et al* 2000).

Insulin/ IGF-1-like PI3K signalling in C. elegans

In *C. elegans*, the transmembrane receptor tyrosine kinase DAF-2 is a homologue of both the mammalian insulin and IGF-1 receptors, showing approximately 30% overall homology to each (Kimura *et al* 1997). As well as conferring a ts Daf-c phenotype (described in Section A.3.5 above), mutation of *daf-2* results in a ts increase in lifespan (Age phenotype) when animals are raised at 15°C and shifted to higher temperatures after the dauer decision point (Kenyon *et al* 1993). The putative null allele, *m65*, is so severe, however, that all animals undergo non-conditional embryonic or L1 arrest or dauer formation (Gems *et al* 1998). It is therefore only possible to study the Age phenotype in reduction-of-function (*rf*) *daf-2* alleles. While *rf* mutations in components of TGF-β and cGMP signalling also result in ts Daf-c phenotypes (Section A.3.5), these mutants are not long-lived, implying that IIS alone is involved with regulation of longevity.

daf-2 mutant alleles can be classified according to the presence or absence of a number of pleiotropic effects seen at non-permissive temperatures (Gems *et al* 1998) (Figure A.7).

Figure A.7: Phenotypes and example alleles of the two classes of *daf-2* mutation (Gems *et al* 1998)

CLASS 1	**CLASS 2**
TEMPERATURE-SENSITIVE	TEMPERATURE-SENSITIVE
Age Daf-c Itt	Age, Daf-c, Itt
	And some or all of:
e.g. *m41*, *m577*, *e1368*	Motility defects, gonadal abnormalities, morphology defects, reduced brood size, embryonic/ L1 arrest, late progeny production, reduced feeding
	e.g. *e1370*, *e979*, *e1391*

The existence of two classes of *daf-2* allele which do not form a single allelic series implies a bi-functionality of the DAF-2 receptor, potentially due to outputs to more than one branch of the IIS pathway. Alternatively, class 2 *daf-2* mutations may simply be more severe than class 1 mutations, resulting in a general down-regulation of cell function and the associated pleiotropic effects. Whichever is the case, it is likely that the different effects of the two classes of *daf-2* allele are the result of differential levels or patterning of forkhead transcription factor phosphorylation.

Mosaic analyses and mutant rescue experiments have shown that *daf-2* does not act cell-autonomously to regulate dauer formation (Apfeld & Kenyon 1998; Wolkow *et al* 2000). Rather, activity of *daf-2* in certain neuronal subsets results in the production of a secondary signal, which is translated into an organism-wide cellular commitment. The ubiquitous expression of *daf-12*, encoding the nuclear hormone receptor downstream of cGMP and TGF-β signalling, makes it a potential candidate for the receipt, integration and transduction of developmental signals from all three dauer formation pathways. *daf-12* appears to act both downstream of and in parallel to *daf-2* with respect to regulation of dauer formation and lifespan, discussed in more detail in Chapter 5.

Mutations in two components of PI3K downstream of *daf-2* also extend lifespan: *age-1* (which encodes the catalytic subunit of the PI3K (Morris *et al* 1996)), and *pdk-1* (which encodes the homologue of mammalian PDK1) (Friedman & Johnson 1988; Paradis *et al* 1999). It would therefore appear that the PI3K branch of IIS in particular is involved with regulation of lifespan. Like dauers, the long-lived IIS mutants show increased resistance to oxidative stress, U.V. irradiation and thermal stress, implying that increased stress resistance may play a role in their increased longevity (Lithgow *et al* 1995; Murakami & Johnson 1996; Honda & Honda 1999; Larsen 1993; Vanfleteren, 1993).

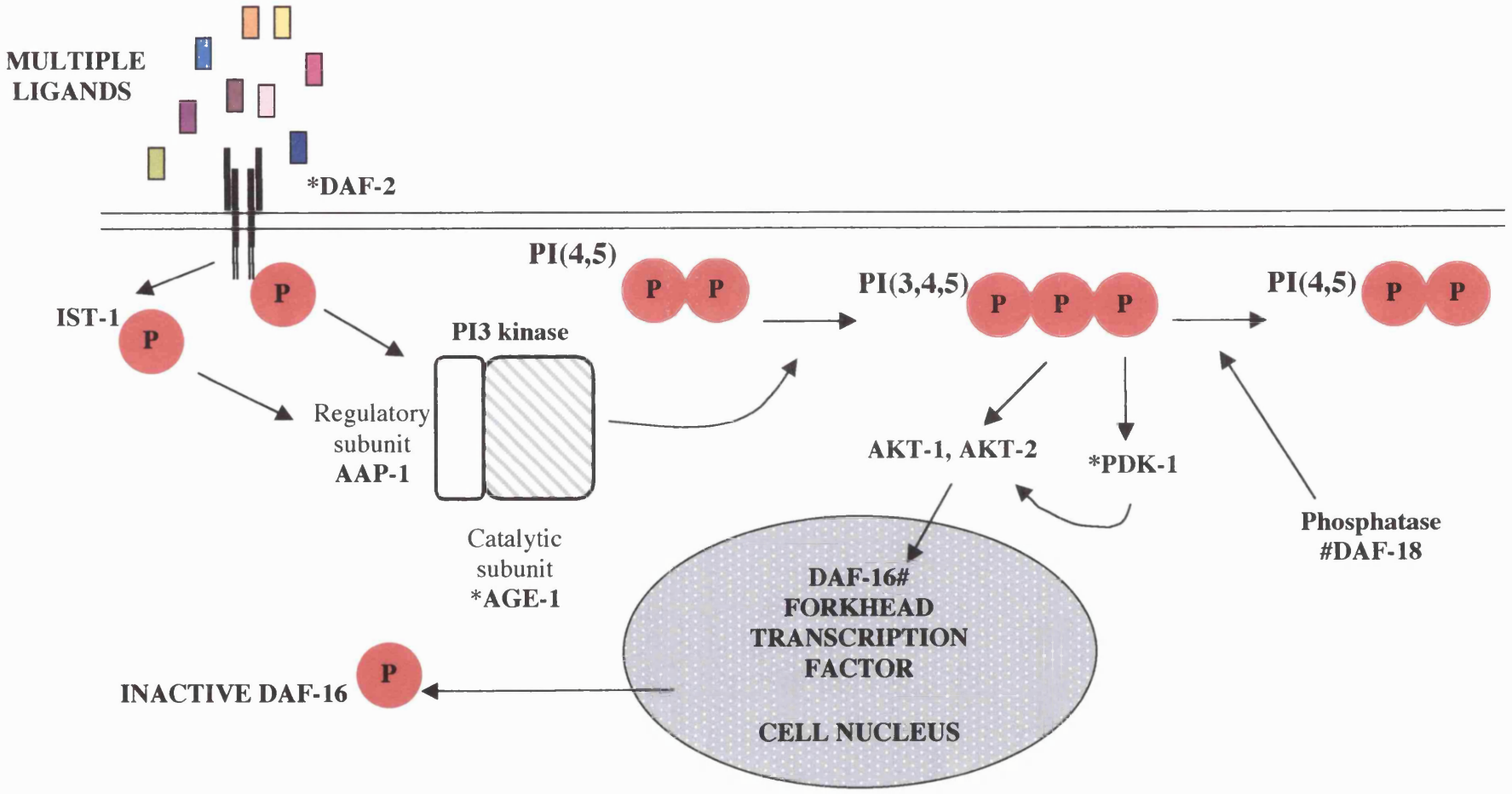
The Daf-c, stress resistance and Age phenotypes of all of the above IIS mutants are completely suppressed by a null mutation in the gene at the base of the pathway, *daf-16* (Gottlieb & Ruvkun 1994; Kenyon *et al* 1993; Larsen *et al* 1995), which encodes a winged helix/ forkhead transcription factor with homology to members of the mammalian FOXO family described above (Ogg *et al* 1997; Lin *et al* 1997). As in the equivalent mammalian system, the DAF-16 protein is active when in the nucleus, and is

inactivated by IIS due to its phosphorylation by AKT proteins (Paradis & Ruvkun 1998), following which it localises to the cytoplasm (Lee *et al* 2001). IIS is likely to respond at least in part to changes in nutrient availability, with decreased food levels resulting in decreased IIS, increased DAF-16 activity and hence transcription of dauer formation genes and/ or repression of transcription of genes for reproductive development. The downstream targets of *daf-16* are as yet unidentified, but have been postulated to include stress resistance genes, due to the heat, oxidative and U.V. stress resistance phenotypes of the Daf-c/ Age IIS mutants. *daf-16* reduction-of-function and null mutants are Daf-d and have slightly reduced lifespans (Kenyon *et al* 1993; Riddle & Albert 1997), suggesting that *daf-16* activity is required for dauer formation and for maintenance of wild-type lifespan (Figure A.8).

Other branches of IIS in *C. elegans* are less well characterised than PI3K signalling but may also play a role in the regulation of dauer formation and/ or lifespan. For example, preliminary evidence suggests a role of RAS signalling from *daf-2* in these processes (M. Nanji, pers.comm.). Like PI3K signalling, RAS signalling phosphorylates DAF-16 (Kops *et al* 1999).

Approximately 40 genes encoding insulin-like ligands have been identified to date in the *C. elegans* genome, but it is not yet clear what, if any, functions the encoded proteins have (Pierce *et al* 2001). Only two (INS-1 and INS-18) are predicted to have a cleaved C peptide as seen in mammalian insulin. Over-expression of both *ins-1* and mammalian insulin in *C. elegans* enhances the Daf-c phenotype of *daf-2* mutants, implying that at least one of these ligands (INS-1) may have an antagonistic effect on IIS (Pierce *et al* 2001), in contrast to the agonistic effects of mammalian insulin and IGF-1 on their respective signalling pathways. However, it is possible that over-expression of these ligands in *C. elegans* may be causing insulin resistance or down-regulation of *daf-2* expression rather than reflecting the true interaction between ligand and receptor. More recently, the *daf-28* gene has been found to encode an insulin-like ligand that, when mutated, results in Daf-c and Age phenotypes similar to those seen in *daf-2(rf)*, *age-1(rf)* and *pdk-1(rf)* mutants, implying that this ligand has a stimulatory effect on IIS as insulin and IGF-1 do in mammals (Li *et al* 2002).

Figure A.8: An outline of the dauer formation (PI3K) branch of IIS in *C. elegans*. *Daf-c, Age; #Daf-d



Insulin/ IGF-1-like PI3K signalling in Drosophila *(see (Garofalo 2002)).*

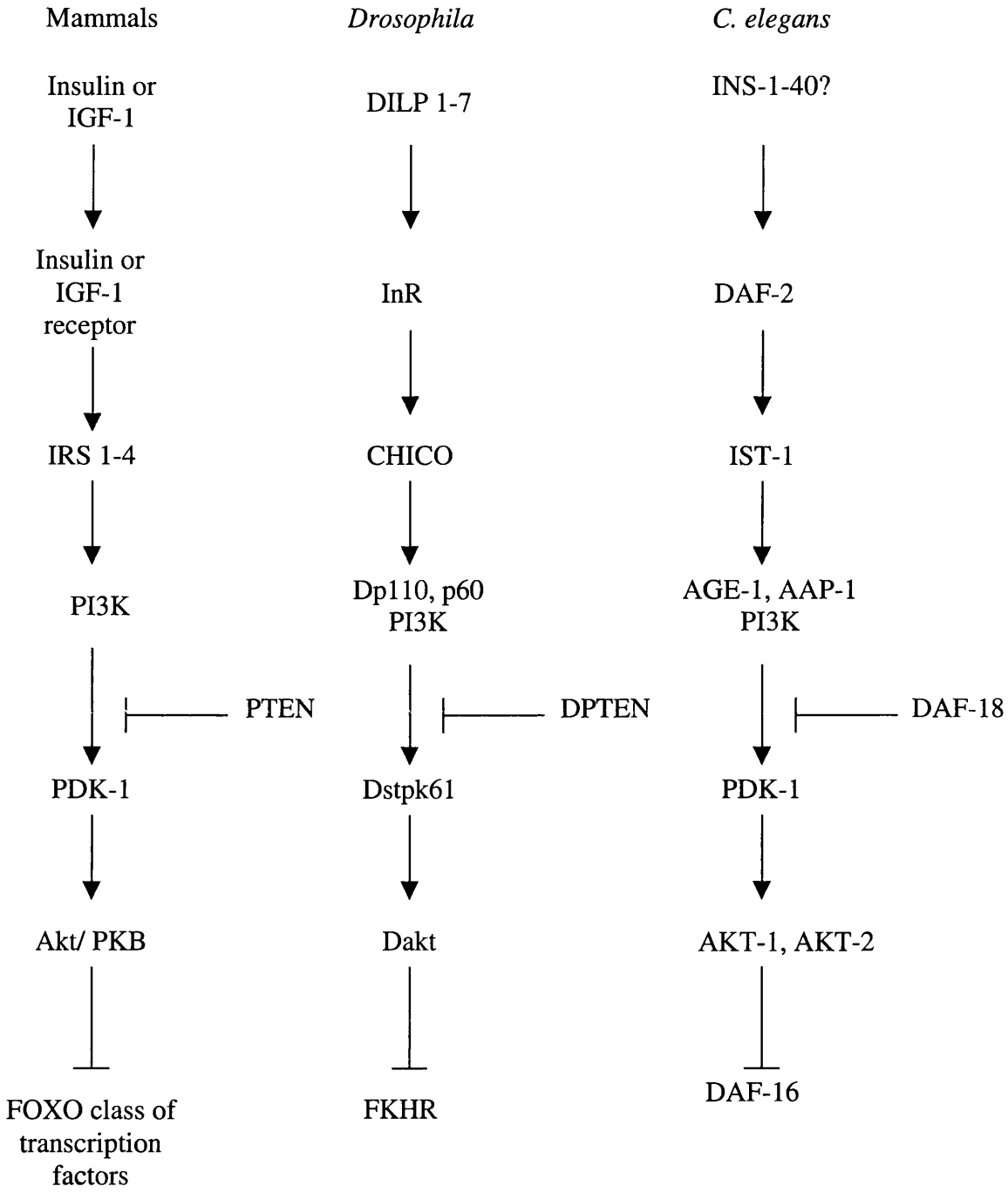
Disruption of IIS can also increase lifespan in *Drosophila.* As in *C. elegans*, *Drosophila* IIS has been highly conserved relative to mammalian insulin and IGF-1 signalling (Figure A.9). The *Drosophila* insulin/IGF-1 receptor homologue INR shows 39% identity in its extracellular domain to the corresponding regions in the mammalian IR and IGF-1R (Fernandez *et al* 1995). Strong homozygous *InR* mutations are lethal due to their effects on cell proliferation and hence epidermal and nervous system development (Fernandez *et al* 1995; Chen *et al* 1996a). However, certain heteroallelic *InR* mutants are able to develop to adulthood, but are up to 50% smaller than normal, due to both reduced cell number and cell size (Chen *et al* 1996a). Significantly, females of the heteroallelic mutant InR^{p5545}/InR^{E19} show lifespan increases of up to 85%, while males display reduced late age-specific mortality (Tatar *et al* 2001). Thus, mutation of the insulin/ IGF-1 receptor homologue extends lifespan in *Drosophila* as it does in *C. elegans.*

Mutation of *chico*, the *Drosophila* homologue of mammalian *IRS-1-4*, results in a similar effect on body size to *InR* mutations, implying that the growth-regulatory element of InR action occurs via CHICO (Böhni *et al* 1999). In addition, $chico^1$ homozygote females have lifespans up to 148% that of wild type (Clancy *et al* 2001). Like *C. elegans daf-2* mutants, *Drosophila InR* and *chico* mutants store more fat (Bohni *et al* 1999; Tatar *et al* 2001), and have elevated SOD levels (Tatar *et al* 2001; Clancy *et al* 2001), suggesting that oxidative stress resistance may underlie the Age phenotype. Since there are sex differences in effects of both these mutations on lifespan, they are considered further in Chapter 2. As yet, no other IIS mutations have been found to extend *Drosophila* lifespan, although this could in part be due to the fact that several of the homozygous IIS *Drosophila* mutants are lethal, and a lifespan effect may not be seen in heterozygotes.

Seven insulin-like peptides (*dilp1-7*) have been identified in *Drosophila*, and are predicted to be of a similar structure to mammalian insulin. Four *dilp* genes are expressed in neurosecretory cells in the larval and adult brain (Brogiolo *et al* 2001; Cao & Brown 2001; Rulifson *et al* 2002), consistent with the neurosecretory nature of IIS in *C. elegans* (Apfeld & Kenyon 1998). Ablation of brain neurons producing DILPS results

in delayed development, reduced growth and dysregulation of carbohydrate metabolism (Rulifson *et al* 2002). Effects on lifespan have yet to be determined.

Figure A.9: Summary of the PI3K branch of insulin/IGF-1 signalling in mammals and IIS in *C. elegans* and *Drosophila* (adapted from (Garofalo 2002))



Role of the growth hormone-IGF-1 axis in mammalian lifespan

In a significant parallel to mutant studies in *C. elegans* and *Drosophila*, IGF-1R heterozygous knockout mouse lifespan is extended by up to 33% (Holzenberger *et al* 2002). Such mice have normal body size, fertility, metabolism and feeding. However, they do display increased resistance to oxidative stress. Potentially linked to this is the finding that mutation of $p66^{shc}$ also extends mouse lifespan (Migliaccio *et al* 1999). $p66^{shc}$ is activated by tyrosine phosphorylation downstream of IGF-1, and acts on the MAPK pathway to regulate cellular response to oxidative stress. $p66^{shc}$ is underphosphorylated in IGF-1R heterozygous knockout mice, suggesting that the increased lifespan of this mutant may be due to effects on $p66^{shc}$ activity and hence stress resistance (Holzenberger *et al* 2002). Interestingly, the effects of IGF-1R heterozygous knockouts are sex-specific, a finding considered further in Chapter 2.

Most other mouse work to date has involved two dwarf mutants (for critical review see (Carter *et al* 2002)). Ames dwarf mice are mutant for the gene Prophet of Pit-1 (*Prop-1*), which is required for correct differentiation of the pituitary during embryogenesis. As a result, these mice have reduced pituitary function, resulting in growth hormone (GH), prolactin (PRL) and thyroid-stimulating hormone (TSH) deficiencies. As adults, Ames dwarves have approximately 1/3 the body mass of wild type, are infertile and are juvenile in appearance. The mutation also results in lifespan increases of 64% and 49% in females and males respectively (Brown-Borg *et al* 1996; considered further in Chapter 2). A similar lifespan effect is seen in the Snell dwarf mouse, which also has reduced pituitary function, due to mutation of the pituitary-specific transcription factor 1 (*Pit-1*) gene, and which shows an increase in mean lifespan of 42% (Flurkey *et al* 2001).

The effects on lifespan of the Ames and Snell dwarf mutations are likely to be mediated through loss of GH secretion rather than reduced PRL and/or TSH secretion, since the Laron dwarf, a GH receptor mutant, is also long-lived (Coschigano *et al* 2000). Since GH is a key stimulator of IGF-1 (reviewed in (Buul-Offers 1983)), and since IGF-1R heterozygous knockout mice are long-lived, it seems likely that the longevity of the dwarf mice is due to down-regulation of IGF-1 signalling. This is implied by the fact Laron dwarves also display abnormal glucose metabolism and insulin levels. Moreover,

while Ames dwarf mice are no less likely to develop tumours than wild-type mice, dwarf mouse tumours take longer to develop. Interestingly, peroxide levels are decreased in the livers of dwarf animals relative to controls, while dwarfs have higher levels of catalase (Brown-Borg *et al* 1998). Thus, as in the IGF-1R heterozygous knockout mice described above, increased resistance to oxidative damage may play a role in the increased lifespan of the dwarf mutants.

A.4.6 Relationship between IIS, body size and lifespan

A trait that often accompanies an increase in lifespan following a particular treatment is reduced body size, with some examples provided below.

- *chico*^{1} and *InR Drosophila* mutants have reduced body size and extended lifespan (Clancy *et al* 2001; Tatar *et al* 2001).
- Mouse lines selected for reduced body size are long-lived (Roberts 1961; Eklund & Bradford 1977; Miller *et al* 2000).
- Ames and Snell mutant mice are long-lived, while having approximately 30% the body mass of normal mice (for review see (Bartke 2000)).
- Rodents maintained under conditions of dietary restriction from early life are small, and show marked increases in lifespan.
- Lower body weight in mice at six months of age is a good predictor of increased lifespan (Miller *et al* 2000).
- A study of long-lived euthymic BALB/c-nu mice found that smaller individuals were more likely to live longer than larger ones throughout the life-history, up until very advanced ages (Piantanelli *et al* 2001).
- Increasing lifespan with decreasing body size has been reported for breeds of domestic dog (Michell 1999).
- The human equivalent of the *Prop-1* mutation that in mice leads to the Ames dwarf phenotype also results in dwarfism in humans. Limited data suggest that the lifespan of these people might be longer than average (Krzisnik *et al* 1999).

Within a species, therefore, smaller body size often correlates with greater longevity. However, it is possible that the two traits are simply correlated effects of a common mechanism, since reduced body size and increased lifespan phenotypes can be uncoupled in many cases. For example, rodents transferred to conditions of dietary restriction during adulthood, i.e. after reaching full size, are still long-lived. Similarly, while *chico*^{1} homozygote flies are small and long-lived, heterozygotes have normal body size but are still long-lived (although to a lesser extent than homozygotes) (Clancy *et al* 2001; Tu *et al* 2002).

In *C. elegans*, no correlation was found between body size and lifespan in a range of wild isolates, hence body size does not appear to affect wild-type lifespan (McCulloch & Gems 2003a). However, a positive correlation was seen between the two traits in *daf-2* mutants, suggesting that these two traits may co-vary in response to IIS. Interestingly, mutation of *daf-2* resulted in increased body size relative to wild type, suggesting that in *C. elegans* IIS acts to reduce body size, contrary to its effect on body size in mammals and *Drosophila* (McCulloch & Gems 2003a).

A.4.7 Gonadal signalling

Evolutionary theory predicts that where resources are limited, there will be a trade-off between allocation of those resources between reproduction and somatic maintenance (Williams 1966). Evidence for such a trade-off in flies has been seen in several instances (see introduction to Chapter 4). In *C. elegans*, adult hermaphrodite lifespan is increased by ~60% upon removal of the germline precursor cells in the L1 larva by laser ablation or mutation (Arantes-Oliveira *et al* 2002; Hsin & Kenyon 1999). However, removal of the whole gonad (germline and somatic gonad precursors) has no effect on lifespan (Hsin & Kenyon 1993). Thus, rather than there being a trade-off involving allocation of limited resources between reproduction and somatic maintenance in this species, there would appear to be a lifespan-reducing signal from the germline, and an equal and opposite lifespan-enhancing signal from the somatic gonad (Hsin & Kenyon 1993). These findings form a major topic in this work and are discussed in more detail in Chapter 4.

A.5 Interpretation of epistasis analyses

Much previous work characterising the genetics of dauer larva formation and lifespan regulation in *C. elegans* used epistasis analysis. Epistasis occurs when the effect of mutation of one locus is masked by mutation at a second locus, or when non-additive effects are seen upon mutation of two loci (Avery & Wasserman 1992; Huang & Sternberg 1995; Gems *et al* 2002). However, a number of potential pitfalls relating to interpretation of such data can be identified (Gems *et al* 2002). Some of these complications are outlined here.

The complete suppression of the phenotype of a mutation by mutation of another gene producing an opposite phenotype is consistent with the latter gene acting after (or downstream of) the former. However, in studies using mutations that result in similar effects, it is not always clear how to interpret the results when enhancement is seen. An enhancement of phenotype following combination of two null alleles strongly suggests that the two genes act separately to regulate the trait, since the mutant phenotype due to both mechanisms is presumably maximised in each single mutant. However, if reduction-of-function rather than null alleles are used, enhancement of a phenotype can mean (a) that the two genes act in different pathways to regulate the same trait or (b) that the two genes act within the same pathway, and that attenuation of the function of both is having a larger effect on the downstream target than each mutation alone (Figure A.10). Unfortunately, such complications are unavoidable in some cases where use of null alleles is not possible, a situation encountered in several instances in this work.

A further possible pitfall occurs during interpretation of suppression data. If the phenotype produced upon mutation of genes a and b is suppressed by mutation c in both cases, this could mean that a and b act within the same pathway. However, it is also possible that a and b act in via the same mechanism, but in different pathways that converge on gene c (Figure A.11). Alternatively, a and b may act via separate mechanisms, but converge on the same gene, c. Shared suppression therefore does not equal shared mechanism.

Another potential difficulty with interpretation of interactions data includes multiple (pleiotropic) gene effects. A gene could regulate an effect via more than one

mechanism. For example, such a complication could apply to the pleiotropic phenotypes associated with long-lived class 2, but not class 1 *daf-2(rf)* mutations.

The final point of relevance to the present study is the possibility that in a mutant genetic background gene interactions might be seen that would not normally occur in wild type. All the above factors are taken into consideration when interpreting data presented in this work.

Figure A.10: Two possible interpretations of enhancement of an *rf* mutant phenotype by addition of a second *rf* mutation

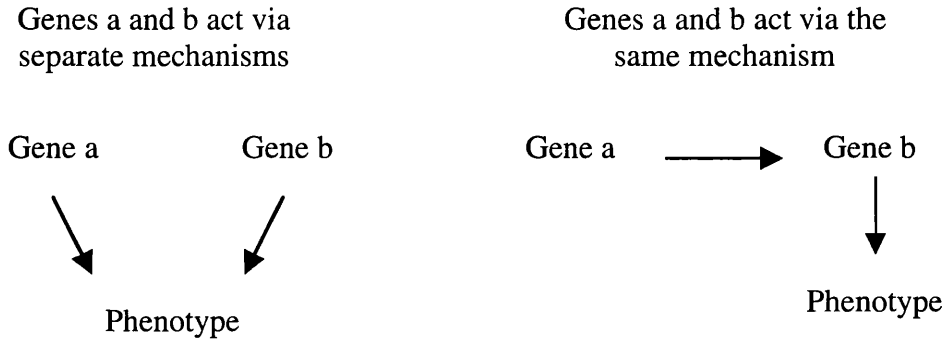
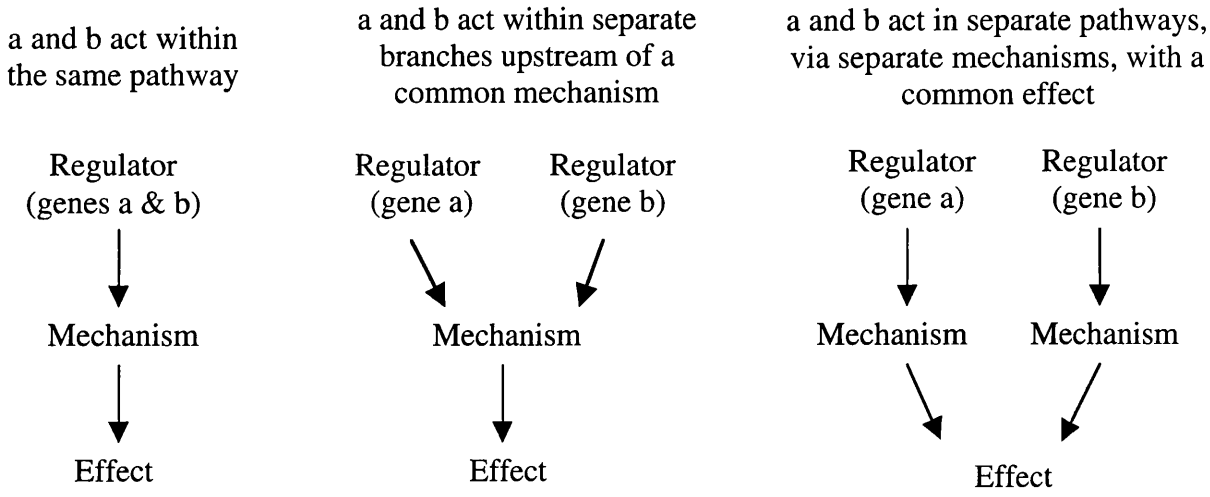


Figure A.11: Three possible interpretations of common suppression of separate mutations by a third mutation (adapted from (Gems *et al* 2002))



Section B: Main Materials and Methods

Contents

NB: For details of specific methods used of relevance only to a particular chapter, please refer to the Materials and Methods section in that chapter.

B.1 Solutions used

Compositions of all solutions utilised in this work are given in an alphabetical list in the Appendix Section E.1.

B.2 Origins of stocks

Unless otherwise stated, stocks were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota, St. Paul, US. Strains obtained from the CGC were stored at -70°C in a glycerol solution (Brenner 1974) and thawed as required. Multiple mutant strains were constructed from these stocks using the methodology described in the relevant chapters.

B.3 Wild-type strain employed

A number of wild isolates of *C. elegans* have been collected from around the globe and are held at the CGC. The isolate commonly used world-wide as the laboratory standard is N2 (isolated in Bristol, UK by E.C. Dougherty), which has been separated into a number of sub-cultures over the years. Previous work found the male stock CGCM to most closely approximate wild type with respect to lifespan (Gems & Riddle 2000a), hence this strain of N2 was employed as wild type throughout. Unless specifically stated, all stocks were back-crossed to N2 CGCM at least 3 times. This reduced the likelihood of the presence of any secondary mutations in the mutant strains and standardised the genetic background before any comparative analyses.

B.4 Routine maintenance of stocks

Animals were viewed using a Leica MZ8 dissecting microscope. Routine stock maintenance was in 60mm diameter Petri dishes, on nematode growth medium (NGM) agar streaked with *E. coli* OP50 as a food source (Brenner 1974). Since OP50 is a uracil-requiring strain, the limited amount of uracil in the medium prevented over-growth of the bacterial lawn, which would otherwise obscure the animals. Plate-to-plate transfer was effected by gently sticking animals to a small amount of OP50 on the end of a piece of thin platinum wire, which was first sterilised in a Bunsen burner flame and allowed to cool. Once lowered onto a fresh agar plate, worms were allowed to crawl free from the

OP50. Agar plates were kept inside sealed boxes within constant-temperature incubators or rooms. The temperature at which strains were raised prior to an experiment varied according to the nature of the strains in question, and is stated for each experiment in the Materials and Methods section within the relevant chapter. Temperature variations of approximately ±0.5°C were typical for the incubators/ rooms used.

B.5 Generation of males

Although spontaneous X0 males are rare in *C. elegans*, a higher frequency of male progeny can be produced by heat shocking, which increases the incidence of X chromosome non-disjunction. Heat shocking involved placing hermaphrodites of the final larval stage at 30°C for ~six hours. Once males had been generated in this way, stocks could be actively maintained in the laboratory by setting up plates of L4 males and hermaphrodites in a ratio of 2:1 respectively. ~50% of out-cross progeny from such stocks were male, inheriting one X chromosome from the hermaphrodite and none from the male.

Males of certain strains (e.g. severe recessive *unc* mutants) cannot mate, hence it was not possible to maintain male stocks of such strains. Males of such strains were generated by backcrossing (see Materials and Methods of relevant chapters).

B.6 Axenisation of stocks

It was important to ensure that strains used for any lifespan and dauer formation analyses were free from any non-OP50 bacterial contaminants, since these could affect both survival and dauer formation results. If bacterial contamination was detected in a sub-culture, stocks were axenised using one of the following two methods:

Axenisation when large numbers of worms are required

- Stocks were at a suitable stage for axenisation when a high proportion of gravid hermaphrodites were visible, since when lysed, these animals would give rise to a large number of eggs.

- 5ml of M9 were added to the agar plate holding the stock to be axenised and swirled around to wash gravid hermaphrodites from the surface. The M9 was then poured into a sterile 15ml centrifuge tube.
- 2ml of household bleach and 1ml 5M NaOH were added, and the tube inverted several times to mix the solutions well.
- After three minutes, the solution was centrifuged at 1000rpm in an ALC PK120R centrifuge for one minute and the supernatant drawn off, leaving an opaque pellet of lysed worms and eggs at the base of the tube.
- 10ml M9 were added to the tube, and the contents centrifuged at 1000rpm for one minute. Two further M9 washes were then carried out in this way.
- After the third M9 wash, all but 0.5ml of the supernatant was drawn off, and the remaining solution drawn up and down in a pipette tip a number of times to bring the eggs off the bottom of the tube.
- 150µl of the M9 containing the eggs was then placed as drops around a fresh lawn of OP50 on agar in a 60mm agar plate, and the eggs left to hatch overnight.
- The next day, newly hatched larvae which had crawled into the OP50 were removed and placed on a fresh culture plate, in order to avoid contamination with non-OP50 bacteria which might eventually grow up where the eggs had been placed.

Axenisation when smaller numbers of worms are sufficient

- Between 10 and 15 gravid hermaphrodites were transferred to the edge of a fresh agar plate streaked with OP50.
- A drop of 12% NaOCl in 1.5M NaOH just sufficient in size to cover the animals was added. This solution was sufficient to lyse the worms but was unable to penetrate the eggs. Eggs were left to hatch overnight.
- The next day, newly hatched larvae which had crawled into the OP50 were removed and placed on a fresh agar plate, in order to avoid contamination with non-OP50 bacteria which might eventually grow up where the adult worms had originally been placed.

Avoidance of fungal contaminants

Unlike bacterial contaminants, moulds were unlikely to affect survival data as long as growth was not prolific. If mould began to grow on an agar plate or on the surface of liquid culture, worms were transferred to a fresh plate/ well and left to crawl/ thrash around for several hours to detach any fungal spores. They were then transferred to another fresh plate/ well, at which point most fungal contaminants had been left behind.

B.7 Lifespan analyses

For reasons described in Chapter 1, it was necessary to maintain male *C. elegans* in isolation at all times when conducting lifespan experiments. However, owing to the active searching behaviour of solitary males and their subsequent crawling up the Petri dish walls, it was not possible to maintain males on plates since a high proportion died prematurely from desiccation (see Chapter 1).

All lifespan experiments involving males were therefore carried out in liquid culture, with hermaphrodites being cultured in identical conditions for comparability. Animals of both sexes were raised on plates at the temperature stated in the relevant chapter, and were transferred at L4 to 96-well microtitre plates (one worm per well). Each well contained 50µl OP50 suspended in S medium (Brenner 1974) at a concentration of $1\text{-}5x10^{9}$ cells ml^{-1}, which was deemed to be the most suitable concentration to obtain normal lifespan and brood size (Klass 1977) (see Chapter 1). Transfers were effected using platinum wire with a hooked end, which was first used to gently swirl the liquid medium, dislodging the animal from the base of the well. The wire was then placed underneath the floating worm and raised so the animal fell over the hook. The hook was then transferred as quickly as possible to the fresh well, in which the worm floated free.

Animals were examined every other day whenever possible, and the number of senescent deaths recorded. Animals that had died as a result of non-senescent causes such as internal hatching of eggs, extrusion of the gonad or accidental damage during transfer were censored from the survival analysis, as were animals in wells which had become contaminated with non-OP50 bacteria. Censored animals were included in the

data set up until the point of censoring in order to retain as much information as possible.

B.8 Axenic culture

For the Paraquat-resistance experiments described in Chapter 2 it was necessary to maintain animals in axenic medium, which contains no bacteria but instead is composed of a mixture of nutrients required for survival (peptone, yeast extract and bovine haemoglobin).

In order to reduce potentially confounding effects of axenic medium on development and brood size as much as possible during experiments, animals were raised on agar plates and transferred to axenic medium at L4. A transfer to fresh axenic medium was carried out approximately six hours later in order to leave behind as many bacteria as possible. Daily transfer of the worms to fresh axenic medium throughout the experiment then ensured that bacterial growth was not able to establish. Axenic medium was prepared as detailed in the Appendix Section E.1, and animals were cultured one-per-well in 96-well microtitre plates as described for monoxenic survival analyses above.

B.9 Statistics employed

Survival analyses were performed using the Kaplan-Meier method on censored data, and the significance of differences between survival curves calculated by a log rank test, which tests for homogeneity between groups. A log rank test was chosen rather than a Wilcoxon test because the former places more emphasis on later survival times, which are generally where differences between groups become apparent: the early sections of survival curves diverge less often. *P* values presented for survival curves analysed using the log rank test represent the probability of obtaining by chance alone a X^2 value greater than that computed if the survival functions are the same for all the groups (significance level $P<0.05$). Statistical software used was JMP v.3 (SAS Institute Inc.).

Maximum lifespan was recorded as the time point at which an animal was last scored as alive. Median rather than mean lifespans were calculated in all cases since typical age-specific death frequencies do not show a normal distribution. It was therefore

not possible to calculate standard errors for lifespan data. Instead 95% confidence intervals were generated around the median or mean of medians. Medians were considered to be significantly different when their 95% confidence intervals did not overlap. In order to obtain a mean of median lifespan values across replicates, and the 95% confidence limits around them, survival data were re-sampled 50,000 times using R software[1]. Medians presented in this work are either the re-sampled median of a single replicate or the mean of medians from a group of replicates. The R program was written by S. Pletcher. Lifespan differences between groups are therefore represented in two different ways: differences between median lifespan values, and significance values for differences between survival curves.

Mean percent dauer formation values were compared using a Student's *t* test (significance level $P \leq 0.05$), having first normalised the percentages. Percentages typically display a binomial distribution, and in such distributions the variance is a function of the mean. Transformation is required to remove this dependence so the mean and variance values are independent, as in normal distributions. Transformation was performed using the arcsine (angular) transformation, which calculates $\theta = \arcsin \sqrt{p}$.

B.10 DNA extraction, amplification and sequencing protocols

Regions sequenced and primer design

The ligand-binding domain of *daf-2* is composed of exons 6, 7, 8 and 9 while the kinase domain consists of exon 14. Primers were designed for these regions using Primer 3 software[2] and were BLAST[3] searched to check that there would be no cross-specificity. Due to the relatively small size of exons 6, 7 and 8, it was possible to design intronic primers to amplify each exon in its entirety. Exons 9 and 14 are larger, and it was necessary in this case to split each exon into 4 fragments (1a-2b) using intronic and exonic primers (Appendix E.2). 0.05 scale oligonucleotides were ordered either from MWG or from Sigma-Genosys Ltd.

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[1] www.r-project.org

[2] http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi [3]http://www.ncbi.nlm.nih.gov.BLAST/

Primer dilution

Upon receipt, primers were dissolved in MilliQ H_2O by adding a volume in µl equivalent to the mass of primer in µg. They were then further diluted 20:80 primer: MilliQ H_2O and were used at this concentration. All primers were stored at -20°C.

DNA extraction

Lysis buffer was prepared in sterile MilliQ water, autoclaved and stored at -20°C:

50mM KCl
10mM Tris (pH 8.3)
2.5mM $MgCl_2$
0.45% Nonidet P-40
0.45% Tween 20
0.01% gelatin

Just prior to use: proteinase K was added at 60µg per ml

8-10 worms were placed onto an OP50-free plate and allowed to crawl away from the bacteria transferred along with them. The worms were then added to 10µl of lysis buffer in the lid of a PCR tube. The lid was placed onto the PCR tube and the droplet spun to the bottom in a microcentrifuge (5 second spin). The tube was left at -70°C for ten minutes in order for lysis to occur, and then placed in a PCR machine using the following cycle:

60°C 1 hour
95°C 15 minutes
4°C hold
Samples were stored at -20°C until needed for PCR.

Polymerase Chain Reaction

The following were added to a 200µl PCR tube:

15µl 2x ReddyMix (AbGene) containing 1.5mM $MgCl_2$

11.5µl sterile dH_2O

2.5µl DNA

1µl of each primer

Samples were then cycled on a PCR machine as follows:

94°C 30 seconds (1 cycle)

94°C 30 seconds ⎫

55°C 30 seconds ⎬ (30 cycles)

72°C 40 seconds ⎭

4°C (hold)

Samples were stored at -20°C until needed.

Running samples on agarose gels

In order to verify that specific amplification had been achieved, a proportion of each sample was run on an agarose gel beside a 1Kb DNA ladder. 1% agarose was prepared in 1xTBE. 1µl ethidium bromide was added per 100µl gel. The gel was poured into the electrophoresis tank to the indicated level and allow to set, using the appropriate comb to create the required number of wells.

The gel was then loaded with:

10µl 1Kb DNA ladder (AbGene)

7µl PCR sample

For larger gels, a DNA ladder was loaded on each side of the samples to aid identification of band size should the gel warp. Gels were run at 100V for approximately 30 minutes, then the sample bands viewed using a UV trans-illuminator.

Cleaning PCR products

PCR products were made back up to 30µl using MilliQ H_2O, and split into two 15µl aliquots each in a 200µl PCR tube. 15µl of Microclean (Microzone Ltd) were added to each 15µl sample, mixed well and left at room temperature for 10 minutes.

Samples were microcentrifuged at 13000rpm for 10 minutes in an Eppendorf 5415D microcentrifuge. The PCR tubes were placed in a rack, which was inverted over tissue on a plate-carrying centrifuge rotor. The samples were gently centrifuged at 35G for 1 minute in order to draw off the supernatant, leaving a pellet of cDNA at the base of the tube. Tubes were re-spun if necessary, to remove all the supernatant. 150µl of 70% ethanol was added to the samples, which were then spun in the microcentrifuge at 13000rpm for 10 minutes. Again, samples were spun upside-down over tissue at 35G for 1 minute in order to remove supernatant. Samples were then dried without lids on a PCR machine at 65°C for five minutes.

Sequencing reaction

5.5µl of MilliQ H_2O was added to each dried sample. Enough stock mix was then prepared for all the samples, with the following volumes per sample:

5µl Better Buffer (Microzone Ltd)
1µl Big Dye Termination Mix (AbGene) (photosensitive)
2µl MilliQ H_2O

8µl of this mixture was added to each sample, along with 1.5µl of the relevant primer (see above). For each exon fragment, both DNA strands were sequenced by adding the reverse primer to one sample and the forward primer to the other.

Samples were then cycled in a PCR machine using the following program:

96°C 10 seconds ⎫
55°C 5 seconds ⎬ 25 cycles
60°C 4 minutes ⎭
4°C hold

80µl of 80% isopropanol was then added to each sample, mixed well and left at room temperature for 10 minutes. During this time, samples were transferred to large 1.7ml Eppendorf tubes.

Samples were spun at 13000rpm for 10 minutes, and the supernatant carefully poured off onto a piece of tissue. 150µl of 70% isopropanol were then added to each tube, followed by a further spin at 13000rpm for 10 minutes. The supernatant was poured off as before. Samples were then left to dry with lids off at 65°C on a hot-plate for 10 minutes, and stored at -20°C. Samples were prepared for sequencing by A. Smith (UCL Sequencing Technician). 10µl of high purity formamide was added to each tube, mixed well and heated at 65°C for five minutes. Samples were then transferred to 96-well plates, heated at 96°C for four minutes then cooled on ice for five minutes, after which time they were loaded into an ABI3100 capillary gel sequencer.

Sequence Analysis

Sequence data were downloaded and opened in ABI PRISM™ EditView ABI Automated DNA Sequence Viewer v 1.0.1 (Perkin Elmer). Nucleotide charts were converted into text format, and opened in EditSeq (DNASTAR©). Sequences obtained using reverse primers were reverse complemented so they could be aligned with both wild type and with the sequence obtained using the forward primer. The MegAlign program (DNASTAR©) was then used to align sequences for comparison. Resulting alignments were then checked for nucleotide differences from wild type occurring on both strands. If a potential mutation was detected, the position of that nucleotide was referred to on the original chart in order to verify that the reading for that nucleotide was

reliable (i.e. there was a narrow, tall peak on the chart). Sequence regions containing putative mutations were converted into amino acid sequences using the "Translate DNA" option in EditSeq, and the putative amino acid change identified.

B.11 Preparation of microscope slides

Several experiments involved viewing of animals under high power microscopy. To prevent damage, animals were placed on an agar pad on top of a microscope slide, prepared as follows. A tube of fresh, sterile 5% agar (containing no peptone) was melted and kept molten on a 65°C hotplate. One or two drops of the agar were placed using a sterile glass pipette onto a baked microscope slide. The slide was surrounded by two others, along the length of which had been placed a layer of tape. A fourth microscope slide was gently placed on top of the agar drop and allowed to rest on the outer slides. This resulted in the agar being flattened into a pad of a consistent thickness. After approximately 15 seconds the top slide was gently slid off, leaving a set pad of agar.

Section C: Results Chapters

Chapter 1
Establishing a suitable culture method for male survival analyses

Contents

1.0 Introduction

Most research into ageing using *C. elegans* to date has involved hermaphrodites. However, as described in the Main Introduction, previous work found that males are longer-lived than hermaphrodites (Gems & Riddle 2000b). This model organism therefore provides the opportunity to investigate the mechanisms underlying sex differences in ageing under controlled laboratory conditions, which is the aim of this project.

As well as allowing a comparison of the genetics of ageing in the two sexes, the use of males in survival analyses has certain practical advantages. For example, substantial numbers of hermaphrodites must often be excluded from survival analyses (censoring) due to internal hatching of eggs (matricide). This non-senescent mode of death occurs at low levels in wild type under replete nutritional levels but is seen more frequently in some mutant strains. In addition, if larvae have time to leave the cuticle of the dead parent before the worm is viewed, it becomes difficult to determine whether or not the death was senescent. It must therefore be decided in advance of the study how such deaths are to be recorded. For example, in this study matricide was only recorded if larvae could be seen within the old cuticle. Such arbitrary decisions will affect the accuracy of how deaths are recorded for hermaphrodite populations. Another inconvenience of maintaining hermaphrodites in a lifespan experiment is that during the first few days of adulthood, eggs are laid. As the resulting progeny grow up, there is a risk that they will be confused with the original parent, resulting in the inclusion of second-generation worms in survival data. Males, however, present fewer problems for ageing experiments in that they do not lay eggs, so there is no risk of the population under study being confused with second generation worms. They also do not experience non-senescent death from matricide. Determining whether a death is senescent is much more likely to be accurate in a population of males, since apart from contamination with non-OP50 bacteria, deaths are presumably almost always senescent.

If males show so many advantages over hermaphrodites for use in ageing studies, and if males may provide a novel genetic tool for ageing investigations, why have most of the *C. elegans* ageing studies to date been carried out in hermaphrodites? This is

likely to be for several reasons. Firstly, spontaneous males are only generated rarely in *C. elegans* populations (~0.2% of embryos), and males from mated stocks are soon out-competed by hermaphrodites. Male stocks must therefore be actively maintained, and males for longevity experiments must be picked from mixed-sex populations. In contrast, large numbers of hermaphrodites can easily be generated by self-fertilisation of homozygous strains.

Secondly, as described in the Main Introduction, the culture of males on plates is complicated by deleterious effects on lifespan of interactions with other worms. The solution to this problem would seem to be simply to maintain males in isolation, one per plate. However, males are much more active than hermaphrodites, spending less time foraging in the bacterial lawn and more time searching around the plate. In the absence of a pheromone cue from other individuals, males will often climb the walls of the Petri dish. Once on the dry plastic wall, it only takes a short time for the worm to desiccate and die, removing the individual from survival analysis as a censor.

Leaving is believed to be the result of adult male mate-searching behaviour, since adult hermaphrodites and juveniles of both sexes do not leave. Moreover, in the dioecious species *C. remanei*, where both sexes must search for a mate, both males and females leave (J. Lipton pers. com.). Adult males no longer leave when cultured with hermaphrodites, but still leave when cultured only with other males. The leaving behaviour of males appears to be regulated by the gonad, since removal of the germline using laser ablation or germline mutants altered leaving behaviour (J. Lipton pers. comm.). Screens for leaving-defective mutants have resulted in three mutants to date, one of which is known to be mutant for *unc-77*, resulting in hypersensitivity to serotonin. Moreover, males of the serotonin-deficient mutant *tph-1* were significantly less likely to leave than controls. It would therefore appear that serotonin plays a role in the modulation of male mate-searching (J. Lipton, pers. com).

Previous work in Missouri, USA using solitary male culture on agar plates had encountered high levels of leaving that had markedly reduced final sample sizes, although a large enough number of males did not leave in order for statistical analysis to be performed (D. Gems, pers. comm.). However, when pilot survival analyses were performed for the present work, it soon became apparent that solitary male culture on

agar plates would be unsuitable due to very high levels of leaving, sometimes up to 90% (Table 1.1). Why levels of male leaving were higher in London, UK than in Missouri, USA is unclear, but is presumably due to environmental variation between the two laboratories.

Table 1.1: Typical rates of leaving by solitary males from agar plates encountered in pilot studies.

Replicate	% males leaving in first 18 days	N*
1	85.4	75
2	76.0	125
3	94.0	50
4	74.0	100
5	67.5	200

* Initial sample size

One possible means of overcoming this problem would have been to increase the starting population, but this raised concerns regarding the skewing of the final data. For example, it was possible that males that did not leave were less active than those that did, perhaps because they were less healthy/ fertile or were behaving atypically. It was therefore clear that either this method had to be adapted or an alternative means of culturing males needed to be found. Therefore, before any further investigations into the *C. elegans* sex difference in lifespan could be performed, it was necessary to devise an effective means of culturing solitary males.

1.1 Materials, methods and results

1.1.1 Attempts at developing a suitable plate culture method for solitary males

Pilot lifespan studies were repeated for solitary males using a variety of altered culture conditions in an attempt to reduce the levels of leaving.

(1) Varying the pH of NGM agar

The standard pH of NGM agar plates is six, with buffering by potassium dihydrogen orthophosphate. Some way into the pilot studies it was discovered that the pH of laboratory stocks of this buffer had not been adjusted to six before use, which could have affected culture conditions and possibly male leaving behaviour. In case the pH of

the agar affected the tendency of males to leave, the pH of standard agar (normally pH 6) was adjusted over a range between five and seven, and the proportion of males leaving over the first six days assayed (Table 1.2). pH was adjusted by altering the pH of the KH_2PO_4 buffer.

Table 1.2: Percent leaving by solitary males on agar plates over a range of pH values

pH of agar	% males leaving in first 6 days	N*
5	79	100
5.7	77	100
6 (replicate 1)	51	100
6 (replicate 2)	85	100
6.3	92	100
7	70	100

* Initial sample size

The percentage of males leaving was high for all conditions and varied considerably between trials. Altering the pH of the agar was therefore deemed to be ineffective in preventing male leaving behaviour.

(2) Increasing agar volume in the Petri dishes

Because the cause of death of males that leave is desiccation on the plastic dish walls, attempts were made to reduce the amount of dry surface available to them to move over. This was effected by increasing the amount of agar added to each 35mm Petri dish, meaning that little or no dish wall was exposed around the edges. Proportions of male leaving were then assayed on these dishes (Table 1.3).

Table 1.3: Percent leaving by solitary males on filled and half-filled agar plates

Petri dish status	% males leaving in first 13 days	N*
Filled	46	100
Half-filled (control)	47.7	42
Half-filled (control)	60	50

*Initial sample size

Males in the second half-filled control replicate showed a higher level of leaving than males on filled plates, but since the other control replicate showed a level of leaving comparable to that on filled-plates, this was likely due to the variable nature of the behaviour rather than a significant effect. Males in the filled plates were still able to leave the agar by reaching up and attaching to the inside of the Petri dish lids, where

they desiccated. Even if filling the plates did result in a reduction in leaving, a censoring rate of 46% was still unacceptable for survival analyses both in terms of potential skewing of the results and the practicalities involved with setting up larger trials. This method was therefore deemed to be unsuitable for reducing male leaving behaviour.

(3) Entrapment of males using repellent rings

C. elegans actively avoids a range of chemicals at threshold concentrations. It was reasoned that if a suitable avoidance factor could be identified, males could be prevented from leaving by encircling them with a ring of such a chemical. Agar plates were therefore prepared with rings of such repellents at suitable concentrations streaked around the outside of a central bacterial lawn, a modification of a method previously used to identify osmotic avoidance mutants (Culotti & Russell 1978).

Repellents tried were 4M NaCl and 4.5M glucose, with dH_2O controls. Plates were initially screened every hour in order to view the response of males to the repellents. Upon encountering the ring of repellent, worms rapidly drew back and reversed their direction of movement. A concern, however, was that the avoidance factor would gradually diffuse through the agar until it was at too low a concentration to be effective. Indeed, it has been shown that it takes only 30 minutes for diffusion to reduce the NaCl molarity to one, at which concentration NaCl actually becomes attractive to *C. elegans* (Culotti & Russell 1978). For this reason, not only was a ring of repellent streaked around the edge of the agar, but the solution was also swirled around the plastic walls of the Petri dishes.

Rates of leaving were notably reduced on plates containing repellent, with only 10% of males leaving plates ringed with 4M NaCl over 48 hours, compared with 45% leaving on control plates. However, moisture on the treated plates encouraged the growth of mould to such an extent that they had to be abandoned. This was particularly true on the glucose-containing plates, which appeared to be very suitable for fungal growth. The unhealthy appearance of certain individuals also led to concerns that the chemicals may be having adverse effects on viability, which was clearly unacceptable for survival analyses. This method was therefore abandoned.

(4) Encouraging burrowing into the agar

When presented with a break in the agar, *C. elegans* actively burrow beneath the surface. It therefore seemed feasible that providing solitary males with such an opportunity might prevent the leaving behaviour by encouraging them to burrow downwards into the agar. There were two potential problems associated with this approach: (a) males may not encounter sufficient food compared with culture on the plate surface and (b) recovery of males from inside the agar for sub-culture to fresh plates would be difficult and time-consuming. It was therefore decided to perform this trial in 96-well microtitre plates, with each well containing one male on 50µl of agar pierced with a platinum wire and seeded with a drop of OP50. In this way, burrowed males would never be far from food and could be extracted from the small amount of agar more easily.

Although some of the males burrowed into the agar, a high proportion still left the wells. Many burrowed into the agar initially, but eventually left the agar and climbed the walls. It was also found to be impractical to find and extract burrowed males within a reasonable time. This method was therefore not suitable as an efficient means of culturing solitary males.

1.1.2 Culture of worms in liquid medium

Most published longevity studies to date have been performed using hermaphrodites on standard NGM agar plate culture. However, as described above, it proved extremely difficult and impractical to culture solitary males on agar plates. It was therefore decided that a more suitable means of male culture might be maintenance under liquid food, since surface tension would prevent worms from leaving the culture vessel.

Liquid culture protocol

Liquid culture of *C. elegans* is typically employed for large-scale growth of worm stocks in conical flasks. It is performed by suspending *E. coli* in S medium, which maintains bacteria in stationary phase, resulting in a constant, defined cell concentration (Sulston & Hodgkin 1988; Brenner 1974). This protocol was adapted for culture of solitary animals in 96-well microtitre plates (for details of solutions used see Appendix Section E.1). It was decided to culture hermaphrodites as well as males one per well since

overcrowding may occur more readily in the small wells, affecting the food concentration and possibly the lifespan of hermaphrodites if grouped.

Culture medium was prepared as follows, in a laminar-flow hood wherever possible:

- Two bottles of 200ml OP50 in minimal medium were incubated in a shaker at 37°C for 48 hours.
- The bacteria were spun down in an ultracentrifuge at 6000rpm, 4°C for 10 minutes. The supernatant was poured away and the bacterial pellet re-suspended in 150ml S medium at 4°C.
- Bacterial suspensions were gently shaken until all clumps of bacteria had dispersed and the cells were evenly distributed. Suspensions were then aliquotted into sterile 20ml glass screw-cap bottles using sterile disposable pipettes.
- The bacterial cell concentration of the batch was ascertained by performing serial dilutions in dH_2O, spreading a known volume of bacterial suspension onto an agar-filled Petri dish and estimating the number of bacteria per ml of medium. Acceptable concentrations were in the range $1\text{-}5x10^9$ cells ml^{-1}, the concentration range previously found to result in lifespans and brood sizes most similar to those seen on agar plates (Klass 1977).
- Test streaks were performed from each aliquot onto fresh, agar-filled 35mm Petri dishes. Dishes were left overnight at 37°C and at room temperature for at least 48 hours before being examined for growth of non-OP50 contaminants. Contaminated aliquots were discarded. Aliquots were stored at 4°C until use.
- For culture, 50µl of liquid medium were added to each well of a 96-well microtitre plate. This volume did not evaporate too quickly yet was small enough that oxygen was assumed to be able to diffuse through the depth of the liquid effectively (J.R. Vanfleteren, pers comm.).
- Microtitre plates were placed at the required temperature for approximately half an hour to allow the culture solution to attain the correct temperature. Animals from agar plates were then added to the microtitre plates one per well by sticking a worm

to a small amount of OP50 on a thin platinum wire. The wire was gently swirled in the liquid medium until the worm floated free.

Once the bacteria had settled inside the wells, they were clearly visible as a brown pellet at the base of the well. At first, worms added to the wells thrashed actively throughout the medium, but after a few hours were seen to rest amongst the bacteria on the bottom of the wells for the majority of the time, moving quite frequently. It was clear that worms had continuous access to ample amounts of food. Even if a worm was left in the same well for many days, the amount of bacteria visible was barely diminished, implying that food levels did not become limiting and worms were not starving. Supporting this observation was the fact that worms maintained in liquid culture did not adopt a starved appearance.

Transfer of animals was effected using a flamed and cooled platinum wire pick with a curved end, over which worms were hooked and lifted out of the medium. Pilot trials found that transfers were easier if worms were maintained in U-bottom, rather than V-bottom microtitre wells, since in V-bottom wells worms would rest in the narrow base of the well and were difficult to extract without damage.

Evaporation of the culture medium was found to become a factor after a few days inside the incubator, even when kept in a sealed box. Condensation was visible on the microtitre plate lids, and wells nearest the edge of the plates began to lose substantial proportions of their culture medium. Worms in such wells were extremely susceptible to desiccation and died prematurely as a result. For this reason, worms were never cultured in the outermost ring of wells in a microtitre plate. Instead, these wells were filled with autoclaved dH_2O, meaning that this outer "buffer zone" would start to evaporate before the culture wells. As an additional protection against evaporation, closed microtitre plates were wrapped in Parafilm and were always kept inside sealed boxes.

1.1.3 Establishing baseline responses to liquid culture

Effect of liquid culture on survival

A possible complication of using liquid rather than plate culture was that the overall concentration of food in liquid culture, although perfectly sufficient to maintain the worms, was probably less than that encountered by worms maintained on a dense lawn of OP50 on agar plates. Reduced food concentrations under several different conditions extend lifespan in wild-type *C. elegans* (Klass 1977; Hosono *et al* 1989). Because it was desirable to reproduce previous survival data from plates as closely as possible using the liquid culture method, it was decided to raise all worms on agar plates and transfer them to liquid culture at L4, the stage at which survival analyses began. In this way, any effects of liquid culture on larval development were avoided, with effects being limited to the adult phase of the life cycle.

Pilot lifespan studies (methodology described in Main Materials and Methods) indicated that for N2, both male and hermaphrodite survivals in liquid culture were comparable to those published previously on agar plates, although median and maximum lifespans were often slightly increased relative to agar plate culture. For example, while hermaphrodite median lifespan is typically 16 days at 22.5°C on agar plates, it ranged from 16 to 18 days in liquid culture at the same temperature. As in previous studies (Gems & Riddle 2000b), male survival was increased relative to that of hermaphrodites, but to variable extents. As noted previously, male median lifespan was particularly variable, sometimes being lower than that of hermaphrodites (Gems & Riddle 2000b). Male maximum lifespan, however, was almost always longer than hermaphrodite maximum lifespan, and male survival curves often displayed a "tail" indicating reduced mortality at later ages (e.g. Figure 1.1a). However, the degree of separation between male and hermaphrodite survival curves appeared to be less in liquid culture than previously recorded on agar plates (Gems & Riddle 2000b), and in some instances there was no significant overall difference between male and hermaphrodite survival (illustrated in Figure 1.1b below).

As experiments for the project were performed, it became clear that the variability in the extent of male longevity could be problematic. This was because in several trials where the dependence of intrinsic male longevity on certain genes was being determined, N2 males were not significantly longer lived than hermaphrodites. This resulted in an atypical wild-type baseline for increased male longevity with which to compare sex lifespan ratios of mutant strains.

The pilot survival curves shown in Figure 1.1 were obtained using a previously described transfer protocol for agar plate culture: that is, transferring hermaphrodites daily during egg-laying (to prevent confusion with second generation animals) and approximately weekly thereafter, while transferring males approximately weekly from Day 0. This had resulted in generally consistent greater male longevity (Gems & Riddle 2000b). It seemed possible, however, that the unequal treatment of the sexes with respect to transfer frequency could be affecting survival results in liquid culture. For example, it is possible that over time waste products build up in liquid culture wells which have detrimental effects on the worms. Alternatively, worms maintained in the same wells for longer periods may suffer from a degree of hypoxia that frequent transfers to fresh medium might alleviate. For this reason, pilot lifespan trials were repeated, this time transferring both males and hermaphrodites during hermaphrodite egg-lay, and both sexes approximately weekly thereafter (Figure 1.2 below). Care was taken to ensure males and hermaphrodites were always transferred on the same day.

It was found that by transferring both sexes at equal frequency resulted in greater separation of male and hermaphrodite survival, and gave more a consistent degree of difference between them. This meant that in trials where the underlying genetics of increased male longevity were being investigated there was now a more consistent wild-type survival sex ratio baseline for comparison. It was therefore decided to culture both sexes with daily transfer during hermaphrodite egg-lay for the remainder of the project. Since this effect only became apparent some way into the project, certain experiments were performed with unequal transfer of the sexes, and are highlighted in the relevant chapters. Male lifespan may therefore be slightly understated in such experiments.

Figure 1.1: Typical survival curves for N2 males (triangles) and hermaphrodites (squares) with unequal transfer of the two sexes showing (a) reduced male mortality at later ages, *P*= 0.19 and (b) very little difference between male and hermaphrodite survival *P*= 0.12 (22.5°C). *P*= probability that male and hermaphrodite survival differ by random chance (log rank test) (see Main Materials and Methods).

(a) (b)

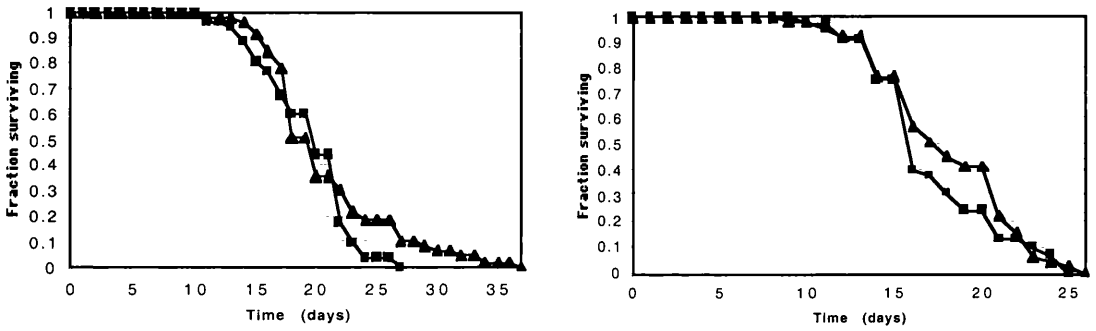
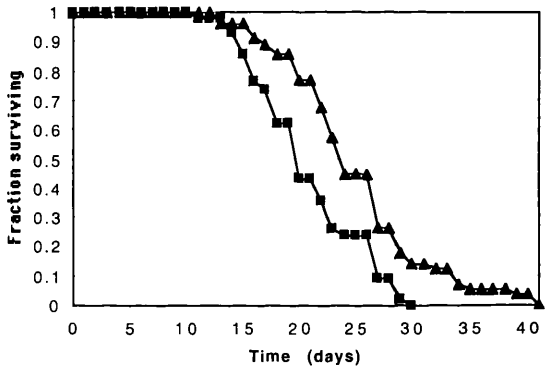


Figure 1.2: Typical survival curve for N2 males and hermaphrodites with equal transfer of both sexes (*P*= 0.0003) (20°C)



Effect of liquid culture method on brood size

Worms raised in liquid culture from eggs are usually longer and thinner and have significantly smaller brood sizes than those raised on plates (Klass 1977). As mentioned above, to minimise potential effects on fertility, a bacterial concentration of 1-5x10^9 cells ml^{-1} was used. In order to verify that the liquid culture method described above did not markedly affect brood size, counts of lifetime egg production were performed for N2 hermaphrodites raised on plates and transferred to standard liquid culture at L4 (Table 1.4). There was no significant difference in brood size between hermaphrodites cultured on plates and those maintained in liquid from L4 onwards (although note that the mean

brood size of the agar plate controls (260) was slightly lower than usual (~300)). By raising worms on plates until L4, it was therefore hoped that as little divergence as possible from results on plates culture had been achieved.

Table 1.4: Brood sizes of self-fertilised N2 hermaphrodites (raised on agar plates until L4, 20°C)

Culture method after L4	Mean brood size ± s.e.	N*	*P*†
60mm Petri dish- agar	260.7± 15.1	10	----
96-well microtitre plate- liquid (1)	266.2± 5.1	20	>0.1
96-well microtitre plate- liquid (2)	288.6± 15.0	9	>0.1

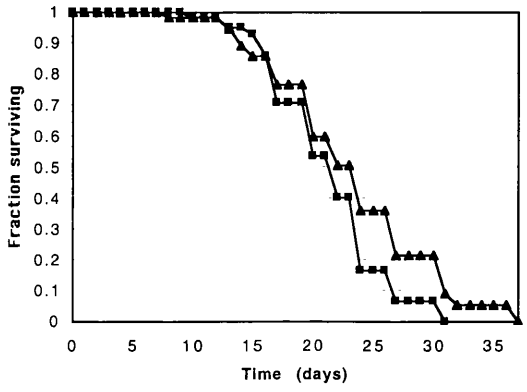
* Number of hermaphrodites scored. †Probability that experimental liquid culture mean differs from control mean on plates by random chance (Student's *t* test).

1.1.4 Are males able to mate in liquid culture?

Once it had been decided to employ liquid culture for all lifespan analyses involving males in this project, the question arose as to whether deleterious male-male interactions were still able to occur in liquid culture. If they did, males would have to be maintained one per well in 96-well microtitre plates. However, if mating were impeded by liquid culture, it might be possible to culture males grouped in 24-well flat-bottomed plates, which would facilitate ease of transfer considerably.

In order to test this, survival of solitary males in liquid culture was compared with that of males maintained in groups of ten (Figure 1.3). No significant effect of grouping on male survival was apparent (P= 0.09, log rank test). However, maximum lifespan was five days shorter in the grouped males, and there was a separation of the survival curves at latter stages, implying that there may be an effect of grouping despite the overall lack of statistical difference between the survival curves.

Figure 1.3: survival of N2 males in liquid culture when maintained in groups of ten (squares) or singly (triangles) (20°C).



It was therefore still possible that some interactions between males were still able to occur in liquid culture, which may have small but perhaps significant effects on survival. As a further measure of interactions in liquid culture, mating efficiency was determined using the mutant strain *fog-2(q71)* (Schedl & Kimble 1988). XX animals of this strain are unable to produce sperm and are phenotypically female, while sperm production by males is unaffected. In order to produce any viable progeny, therefore, *fog-2* females must out-cross with males. Ten males and five females of *fog-2(q71)* were placed in 0.5ml of *E. coli* in S medium per well of 24-well plates. Wells were scored for numbers of progeny over a 72-hour period. While in one replicate no progeny were produced, in a second replicate a number of eggs were laid by a single female. Thus, while mating is reduced by culture in liquid, it is not entirely eliminated, implying that male-male interactions may also still be possible. Due to this finding, and the possible effect of grouping on male lifespan obtained above, it was decided to culture males (and hermaphrodites for comparability) one-per-well in order to avoid any potential effects of male-male interactions on survival. Culturing animals singly had the added benefit in that it reduced censors due to contamination during survival analyses, since maintaining animals in groups leads to the risk that the entire group must be censored upon contamination of the medium.

1.2 Conclusions

Following several attempts to maintain solitary males on agar plates, the level of leaving was deemed to be too high. It was therefore decided to culture both males and hermaphrodites in liquid medium. Pilot trials found lifespans and brood sizes to be comparable to those seen on agar plates, although lifespan was often slightly increased in liquid culture in both sexes. Low levels of interactions between individuals were still able to occur in liquid culture, hence males (and hermaphrodites) were maintained one per well in 96-well microtitre plates. Transfer frequency in liquid culture affected male lifespan, with reduced transfer rates resulting in decreased male survival. Males were therefore transferred daily with hermaphrodites during egg-lay and weekly thereafter in order to maximise the wild-type sex difference in lifespan.

Chapter 2
Role of insulin/IGF-1-like signalling in increased male lifespan and dauer formation

Contents

2.0 Introduction

As described in the Main Introduction, *C. elegans* males are longer-lived than hermaphrodites when maintained in isolation to prevent deleterious interactions with other animals (Gems & Riddle 2000b) (see Chapter 1). As well as being longer-lived, males also form dauers more readily than hermaphrodites. This was first noted when almost all of the dauers forming during Daf-c mutant strain constructions were found to be male (Vowels & Thomas 1992). Later work using dauer pheromone at 25°C showed that wild-type males form dauers more readily than hermaphrodites, in this instance by ~20% (Ailion & Thomas 2000).

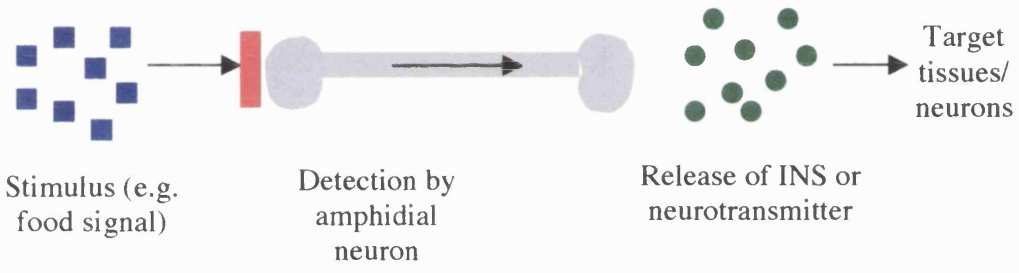
The increased wild-type male longevity and tendency to dauer formation suggest that there may be fundamental differences in neuroendocrine signalling between the sexes. Since insulin-/IGF-1-like signalling (IIS) is known to regulate both dauer formation and longevity, this pathway seemed a logical candidate for such sex-specific differences. As described in the Main Introduction, signalling to DAF-2, the *C. elegans* homologue of mammalian insulin and IGF-1 receptors, causes reduced activity of the forkhead transcription factor DAF-16, and results in development to adulthood and decreased lifespan. Thus, if IIS were intrinsically down-regulated in wild-type males relative to hermaphrodites, this would result both in increased male lifespan and an increased propensity to dauer formation.

The working hypothesis for the experiments described in this chapter is therefore that the increased male lifespan and tendency to form dauers is the result of constitutively down-regulated IIS in males relative to hermaphrodites. *C. elegans* males could potentially differ from hermaphrodites at one or more points in the IIS pathway (Figure 2.1).

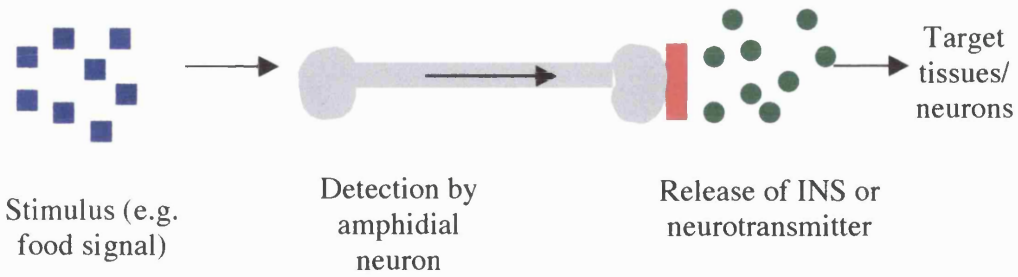
Figure 2.1: Potential points in IIS (red bar) at which males and hermaphrodites could differ.

(a) In the nature of the ligand produced. It could be that males produce different proportions of INS proteins compared with hermaphrodites, perhaps due to different neuronal architecture between the sexes. Alternatively, hermaphrodites may simply produce more INS ligand, perhaps because there are extra sources of INS (e.g. the hermaphrodite gonad).

(b) In the detection of the stimulus for IIS (such as a food signal) due to attenuated sensitivity of gustatory sensory neurons in males.



(c) In the response to the IIS stimulus due to decreased neurotransmission or neurosecretion of INS from the sensory neurons in males.

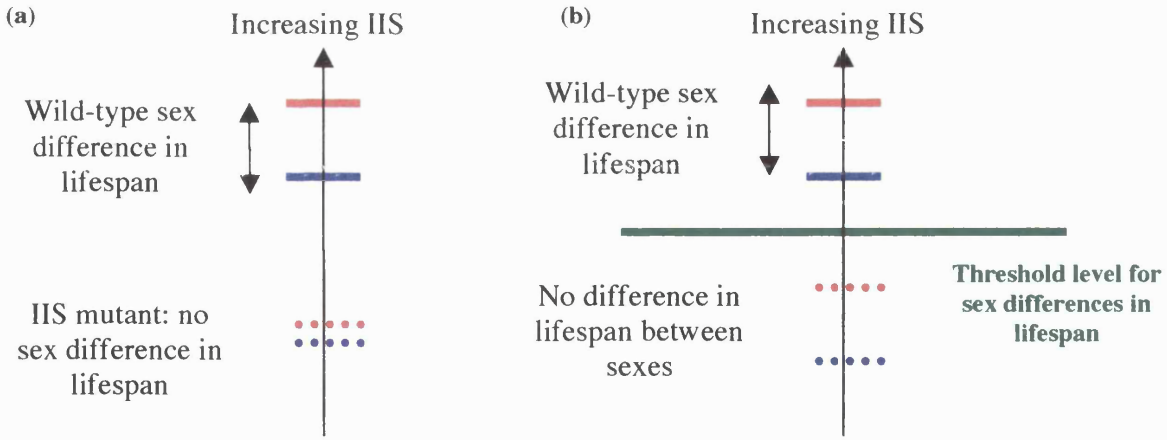


A prediction of the hypothesis that IIS is down-regulated in males relative to hermaphrodites is that increased male lifespan and dauer formation might no longer be apparent in IIS-defective genetic backgrounds, for example because:

(a) Levels of IIS in males and hermaphrodites are reduced to a similar baseline level, eliminating increased male lifespan and dauer formation (Figure 2.2a). In the absence of any other sex differences in lifespan regulation, lifespan of the two sexes would be the same.

(b) Levels of IIS in both sexes are reduced by the same proportional amount, but fall beneath a threshold below which sex differences in lifespan and dauer formation are no longer apparent (Figure 2.2b). This could be the case if subtle differences in IIS flux between the sexes were eliminated upon mutation.

Figure 2.2: Possible effects of IIS *rf* mutations on male (blue lines) and hermaphrodite (red lines) lifespan. (a) Mutation results in similar baseline levels of IIS between the sexes eliminating the sex difference; (b) IIS falls beneath a threshold level (green line) beneath which no sex difference in lifespan is seen. Solid lines= wild type; dotted lines= IIS *rf* mutant



Note, however, that a potential complication is the possibility of sex differences in the occurrence of pleiotropic effects associated with IIS mutations, which could suppress the Age phenotype differentially in the two sexes. It is for this reason that the effect of IIS mutations on the male bias to dauer formation would aid interpretation, since loss of the

male bias to dauer formation in IIS mutants would support an intrinsic involvement of IIS in the increased male lifespan. The sex bias to dauer formation was therefore determined in a number of IIS mutants. Since it is also possible that sex differences in longevity and dauer formation involve other pathways, such as TGF-β and/or cGMP signalling, lifespan and dauer formation were also determined for both sexes in several TGF-β and cGMP signalling mutants.

IIS mutant hermaphrodites show increased resistance to oxidative stress (Vanfleteren 1993; Larsen 1993), heat stress (Lithgow *et al* 1994; 1995) and UV irradiation (Murakami & Johnson 1996) relative to wild type. Thus, if IIS were reduced in males relative to hermaphrodites, it might also be expected that males would be more resistant than hermaphrodites to a variety of stresses. Experiments were therefore performed in order to determine (a) whether wild-type males were more resistant to stresses and (b) if so, whether this increased male resistance was attenuated in a *daf-16* mutant. Dependence of an increased male stress resistance on *daf-16* would provide further evidence that there are intrinsic sex differences in IIS.

Sequencing *daf-2(m41)*

daf-2 mutations can be grouped into two classes according to the presence or absence of a range of pleiotropic phenotypes at higher temperatures, implying a bi-functionality of the DAF-2 receptor (Gems *et al* 1998) (see Main Introduction Figure A.7). Previous work has advocated use of the *m41* allele as the canonical class 1 mutation of *daf-2*, while *e1370* displays typical class 2 traits (Gems *et al* 1998). It is for this reason these alleles were used as representative class 1 and class 2 alleles in this project.

To date, all *daf-2* alleles that have been sequenced have mutations in either the ~500 amino acid ligand-binding domain or the 275 amino acid kinase domain. Based on sequence analysis, it was previously suggested that class 1 mutations could be specific to the ligand-binding domain, while class 2 mutations could be located in the kinase or ligand-binding domains (Gems *et al* 1998). Class 2 lesions could affect DAF-2 functioning more significantly, and could result in the pleiotropic phenotypes seen in class 2 *daf-2* mutants. This would explain why previous work found a phenotypically

class 2 allele (*sa187*) to be the result of a mutation in a highly conserved cysteine in the ligand-binding domain (Gems *et al* 1998).

While previous sequencing of *e1370* found the lesion to be within the kinase domain of *daf-2* (Kimura *et al* 1997), no sequence had previously been published for *m41*. It was of interest to know where in the *daf-2* gene the *m41* mutation lies, since this may help to interpret the behaviour of *daf-2* mutant strains, for example in their interactions with *daf-12* (see Chapter 5) or in regulation of male and hermaphrodite lifespans (this chapter and Chapter 3). The kinase and ligand-binding domains of the *m41* allele of *daf-2* were therefore sequenced in order to determine whether the mutation lay within the ligand-binding domain as might be predicted.

2.1 Materials and Methods

2.1.1 Strains and stock maintenance

All alleles used were *rf* unless stated, and included: DR85 *daf-8(m85) I* (Riddle *et al* 1981), GR1307 *daf-16(mgDf50) I* (putative null allele) (Ogg *et al* 1997), DR1567 *daf-2(m577) III* (Gems *et al* 1998), DR1572 *daf-2(e1368) III* (Gems *et al* 1998), CB1370 *daf-2(e1370) III* (Riddle 1977), DR1564 *daf-2(m41) III* (Larsen *et al* 1995; Gems *et al* 1998), DR1359 *daf-4(m592) III* (Estevez *et al* 1993), DR40 *daf-1(m40) IV* (putative null allele) (Swanson & Riddle 1980), DR47 *daf-11(m47) V* (Vowels & Thomas 1992), AA86 *daf-12(rh61rh411) X* (putative null allele) (Antebi *et al* 1998), and JT709 *pdk-1(sa709) X* (Paradis *et al* 1999).

The *daf-16(mgDf50)*; *daf-1(m40)* strain was constructed using an *unc-75* marker closely linked to *daf-16* as follows. *daf-16(mgDf50)* males were crossed with *daf-1(m40)*; *unc-75(e950)* hermaphrodites. The F1 triple heterozygotes were cloned and self-fertilised at 15°C (phenotyping could not take place at this stage due to the maternal rescue displayed by *daf-1(m40)*). It was previously shown that *daf-16(0)*; *daf-1(m40)* animals form partial dauers (Vowels & Thomas 1992). F2 individuals were therefore cloned and self-fertilised at 25°C, and those animals segregating wild-type partial dauers were recovered and self-fertilised. Clones segregating no Unc progeny were sub-cultured as the putative *daf-16(mgDf50)*; *daf-1(m40)* strain. The genotype of this strain

was then confirmed as follows. *daf-16(mgDf50)* males were crossed with putative *daf-16(mgDf50); daf-1(m40)* hermaphrodites. The resulting *mgDf50; m40/+* progeny were self-fertilised at 15°C. F2 progeny were then self-fertilised at 25°C, and after 72 hours the offspring scored as either: partial dauers only (*mgDf50; m40*), adults only (*mgDf50*; +) or partial dauers and adults (*mgDf50, m40/+*). Animals were picked from plates containing only partial dauers and maintained as the putative double mutant. The partial dauer Daf-c phenotype was verified at 25°C, confirming the parental *daf-16(mgDf50); daf-1(m40)* genotype.

Males of all strains were generated by heat-shock and were actively maintained thereafter. Due to the high sensitivity of the TGF-β and cGMP strains to pheromone, it was necessary to sub-culture these strains at a lower density and to transfer frequently.

2.1.2 Survival analysis

All lifespan measurements were performed using liquid culture as described in Main Materials and Methods. Both sexes were transferred daily during hermaphrodite egg-lay and approximately weekly, and always concurrently, thereafter. Due to the ts Daf-c nature of the experimental strains, all animals were raised at 15°C and were shifted to the experimental temperature (22.5°C) at the L4 stage.

2.1.3 Measurement of the sex bias to dauer formation in wild type

(a) Pheromone method: This was adapted from previous work, which promoted wild-type dauer formation by culturing larvae on agar containing dauer pheromone (Golden & Riddle 1984a). Pheromone had already been extracted (D. Gems) as previously described (Golden & Riddle 1984a) and was being stored undiluted at -20°C. Dauer pheromone is extremely stable and therefore could be autoclaved before use.

Ideally, ~50% of animals would form dauers, resulting in adequate numbers of dauers and non-dauers for statistical analysis. Previous studies had used concentrations of pheromone between 20µl and 50µl pheromone/ ml agar (Golden & Riddle 1984a), so trials were performed within this range. Pheromone plates were prepared by adding the required volume of undiluted dauer pheromone preparation to a 35mm Petri dish and

adding 2ml of 1.7% NGM agar minus bactopeptone. Peptone was excluded from the medium so that the amount of bacteria supplied to the worms could be controlled, since the OP50 added would be unable to grow without a source of amino acids. The agar was gently swirled to mix in the dauer pheromone, and the plates dried at room temperature overnight. Once the agar had dried, 20µl of 1-5x10^9 cells ml^{-1} OP50 in S medium containing 2mg/ml streptomycin was added to each dish and was evenly spread over the agar surface using a flamed glass spreader. Inclusion of streptomycin in the food source further prevented bacterial growth, and ensured a constant pheromone to food ratio on all dishes.

L4 hermaphrodites and males were placed on standard NGM plates in a 1:2 ratio and kept at 15°C overnight. Self progeny laid during this time were discarded. The next day the mated adults were transferred to fresh 35mm Petri dishes containing pheromone, with one hermaphrodite and two males being placed in each dish. Animals were maintained at 25°C in order to promote dauer formation, and parents were transferred every 24 hours, leaving behind cohorts of progeny.

Plates were scored for dauers 44 hours post mid egg-lay (Ailion & Thomas 2000). Worms that had not formed dauers were counted and sexed. Any dauers/ L2ds that had formed were left to recover on standard NGM plates at 15°C and sexed.

(b) Starvation method: L4 mating stocks (four hermaphrodites and eight males) were set up on standard 60mm NGM Petri dishes and left at 25°C until populations were just starved. At this point, any dauers that had formed were placed onto fresh plates with *E. coli*, left at 15°C to recover and then sexed. Any animals on the starved plates that were L3 stage or above were counted and sexed. Plates were carefully checked to ensure that any hermaphrodites that had died due to internal hatching of eggs were included in the count, since levels of matricide increase under conditions of starvation. It should be noted that this method was only approximate in that it assumed that all animals L3+ were of the same generation as the dauer larvae. This assumption seemed reasonable, since the majority of other animals on the starved plates were L1 larvae from the next generation, which were ignored.

2.1.4 Measurement of the sex bias to dauer formation in Daf-c strains

Dauer formation in the Daf-c IIS, TGF-β and cGMP mutants tested could be induced by raising larvae at non-permissive temperatures. Experiments were performed for each Daf-c strain at the best temperature to give suitable proportions of dauers and non-dauers for statistical analysis. In several instances dauer formation varied considerably between replicates, since dauer formation is very sensitive to environmental fluctuations. For certain strains, therefore, data are presented that were gathered at different temperatures, as it was sometimes necessary to alter the temperature at which larvae were raised in order to obtain a suitable level of dauer formation. Note that the double mutant strain *daf-16(mgDf50)*; *daf-1(m40)* forms partial dauers as *daf-16(0)* partially suppresses the Daf-c phenotype of *daf-1(m40)* (Vowels & Thomas 1992). These larvae were paler than full dauers, were less radially constricted and continued to pump their pharynxes.

Mating stocks of L4 hermaphrodites and males in a ratio of 1:2 were left overnight at 15°C. The next day, adults were transferred to fresh plates with one hermaphrodite and two males per plate, and initial progeny (which were mainly self progeny) were discarded. Animals were shifted to the appropriate non-permissive temperature and were left to lay eggs for 24 hours. Parents were transferred to fresh plates every 24 hours, leaving behind cohorts of progeny for analysis. Dishes were scored 120 hours (15°C), 96 hours (20°C), 80 hours (22.5°C) or 44 hours (27°C) after mid egg-lay (Gems *et al* 1998; Ailion & Thomas 2000).

In all experiments described above, care was taken to ensure that results were not affected by dauers climbing the walls of the Petri dishes. It was a possible concern that there may be a sex difference in the rate of dauer leaving behaviour, but this seemed unlikely as pre-adult male and hermaphrodite animals have been shown to leave with equal tendency (J. Lipton, pers. com). In any case, in most trials, only a tiny proportion of dauers was lost in this way. As plates were checked daily, and since dauers are more resistant to desiccation than adults, it was often possible to "rescue" dauers from the walls by gently sticking them to OP50 on the end of a platinum wire and returning them to the agar surface.

2.1.5 Data analysis

For consistency, in advance of beginning these experiments it was decided to score L2d larvae as dauers throughout. Hence, "dauer formation" in all cases refers to dauer plus L2d formation.

For all strains, the number of cohorts derived from 24-hour egg-lays scored varied depending on the number of larvae already obtained. Some strains were more fertile than others, hence for the less fertile strains it was necessary to score over a longer time period in order to achieve sufficient numbers for data analysis. It was not possible to pool percent dauer formation results from trials performed at different times due to the inter-trial variation, which would result in too much variance to allow statistical analysis. Male and hermaphrodite dauer formation values were therefore only compared within single trials, hence several results for each strain are presented in Results.

The proportion of males forming dauers was divided by the proportion of hermaphrodites forming dauers to give a male: hermaphrodite dauer formation ratio. A ratio of one would indicate an equal tendency by the two sexes to form dauers, while ratios greater than one would suggest that males are more likely to form dauers than hermaphrodites. Similarly, ratios of less than one suggest that hermaphrodites form dauers more readily than males. Mean male and hermaphrodite dauer formation values were normalised using arcsine transformation and were compared using a Student's *t* test.

A potential problem when interpreting such data results from the fact that percent dauer formation has an upper limit. If 100% dauer formation is reached by one sex but not the other at a particular temperature, the male: hermaphrodite dauer formation ratio becomes less informative because the difference in the level of dauer formation by the two sexes may be underestimated. The only instance in which this effect became problematic was when comparing the sex bias to dauer/ partial dauer formation of *daf-1(m40)* and *daf-16(mgDf50); daf-1(m40).*

2.1.6 Evaluation of stress resistance

Resistance of males and hermaphrodites to oxidative stress was measured using the free-radical generator methyl viologen (Paraquat). Experiments were performed in axenic medium (see Main Materials and Methods), since it was not clear whether *E. coli* bacteria were able to metabolise Paraquat (J. Vanfleteren, pers. comm).

Mating stocks were raised on plates at 15°C, and at L4 males and hermaphrodites were placed one per-well in 96-well microtitre plates containing axenic medium, at 25°C. Preliminary trials found that transferring worms directly into a solution of Paraquat at the L4 stage disrupted the final moult. L4 larvae were therefore maintained in axenic medium minus Paraquat and transferred into 0.2μm filter-sterilised axenic medium containing Paraquat as adults (Paraquat concentrations stated in Results). Survival of these animals was then recorded, with transfer to fresh Paraquat-containing medium continuing daily until all animals were dead.

Measurements of thermal stress resistance were performed using an adaptation of a method previously devised (M. Muñoz, pers. com.). Hermaphrodites were mated with males in 1:2 ratios on NGM plates at 22.5°C overnight. Adults were then transferred to fresh plates and left for 24 hours. Sterile M9 buffer was used to wash adults and larvae from the plates, leaving cohorts of eggs. After one hour at 22.5°C the eggs had hatched, and the L1 larvae were transferred to fresh plates streaked with OP50 and were placed at 30°C for the stated time. Those animals not picked for heat stressing were left to grow at 20°C and were sexed at L4. Determination of this sex ratio allowed estimation of the sex ratio of the heat-stressed animals, since the majority of these never recovered and thus could not be sexed. After heat stress, animals were recovered 20°C. The sex of survivors was scored, and the predicted percent survival of each sex determined by reference to the sex ratio of the untreated portion of the population.

Resistance to ultraviolet (UV) irradiation was determined as follows. Animals of both sexes were selected at L4 and raised individually in liquid culture at 15°C until reaching day five of adulthood. They were then transferred to lid-less, agar-filled 35mm Petri dishes (minus OP50) and irradiated at 2000 J/m^2 in a UVP CL-1000 UV Crosslinker with 254nm bulbs. Irradiated animals were then maintained one per well in liquid culture at 22.5°C and their survival monitored until all were dead.

2.1.7 DAF-16::GFP localisation studies.

The strain employed in order to visualise DAF-16 cellular localisation was TJ356, which contains an integrated GFP reporter construct fused to the final amino acid of the DAF-16a2 protein (*IsDAF-16::GFP*- insertion position and copy number unknown) (Henderson & Johnson 2001). The insertion is in a wild-type genetic background, hence TJ356 is assumed to over-express *daf-16*, as implied by the fact that it is slow-growing, and forms 49% dauers at 25.5°C (Henderson & Johnson 2001). Males of this strain were generated by mating *IsDAF-16::GFP* hermaphrodites with N2 males, producing dominant heterozygote GFP animals. In order to ensure fair comparisons between the sexes, heterozygote GFP hermaphrodites resulting from these crosses were also used.

A roller (*rol-6* pRF4) mutation linked to the *IsDAF-16::GFP* fusion construct gave rise to a Rol phenotype and provided a dominant marker for strain constructions. The *daf-2(m577)*; *IsDAF-16::GFP* strain was constructed as follows (construction by M. Nanji). *daf-2(m577)* males were mated to the *IsDAF-16::GFP* strain, generating Rol worms heterozygous at both loci. These animals were self-fertilised at 25°C and Rol dauers picked and recovered at 15°C. These animals were then self-fertilised one-per-plate at 25°C and those giving rise to 100% dauer progeny were selected as the *daf-2(m577)*; *IsDAF-16::GFP* strain. Males of this strain were generated by mating *daf-2(m577)* males to *daf-2(m577)*; *IsDAF-16::GFP* hermaphrodites, resulting in males and hermaphrodites homozygous for recessive *daf-2(m577)* but heterozygous for dominant *IsDAF-16::GFP*.

Cellular localisation of DAF-16::GFP in response to a variety of stresses was determined. Response to starvation stress was measured by placing L4 animals into S medium minus OP50 at 15°C for the desired period of time. Animals were heat-stressed by placing L4 larvae into sealed agar plates streaked with OP50 and circulating them in a 35°C water bath for the desired period. Response to oxidative stress was observed by placing L4 animals into freshly prepared 20mM Paraquat in S medium containing OP50 at 15°C for the required period. Following each stress, animals were mounted in S medium plus $1x10^9$ cells ml^{-1} OP50 (or minus OP50 for starvation trials) on an agar pad on a slide. A maximum of 20 animals per slide minimised exposure of animals to stresses due to prolonged exposure to slide conditions.

Levels of nuclear localisation of DAF-16::GFP were determined using a Leica DM RXA2 microscope with a 20x objective. No anaesthetic was used for viewing the *IsDAF-16::GFP* strain since this could have affected DAF-16::GFP localisation patterns. Photographs were taken using a Hamamatsu Orca black-and-white camera, using identical exposure times within a set of comparisons. Care was taken to minimise the exposure of slides to U.V. light by closing the shutter in between viewings and photographs. Imaging software used was Openlab v 3.0.4, and images were edited in Adobe Photoshop v 7.0.

2.1.8 Sequencing of *daf-2(m41)*

Before sequencing, *daf-2(m41)* was phenotyped in order to confirm the strain. The proportion of dauers formed at different temperatures was assayed, as was the unusual maternal rescue of Daf-c noted previously (Gems *et al* 1998). The strain behaved true to type. All exons of the ligand-binding (exons 6, 7, 8 & 9) and tyrosine kinase (exon 14) domains were amplified from genomic DNA and sequenced (primers described in Appendix Section E.2). Details of the DNA extraction, amplification and sequencing protocols employed are provided in Main Materials and Methods.

2.2 Results

2.2.1 Dependence of increased male longevity on components of IIS

In order to determine whether the increased male lifespan was due to sex differences in IIS, male and hermaphrodite lifespans were measured for a range of IIS, TGF-β and cGMP mutants (Table 2.1, Figure 2.3). As found previously (Gems & Riddle 2000b), wild-type males were longer-lived than hermaphrodites, with significant differences between male and hermaphrodite medians and survival curves (Figure 2.3a). This was also the case for the null mutant *daf-12(rh61rh411)* (Figure 2.3g), hence the increased male lifespan is not dependent upon *daf-12*, the target gene of TGF-β and cGMP signalling (Riddle *et al* 1981). It is also interesting to note that *daf-12(rh61rh411)* increased both male and hermaphrodite lifespan (discussed in Chapter 5 Section 5.3.1).

In contrast, in *daf-16(mgDf50)* (Figure 2.3f) and all three class 1 *daf-2(rf)* mutants (Figure 2.3b-d) the increased male lifespan was absent, supporting the hypothesis that the increased longevity of wild-type males is the result of sex differences in IIS. However, males were still the longer-lived sex in the IIS mutant *pdk-1(sa709)* (Figure 2.3e).

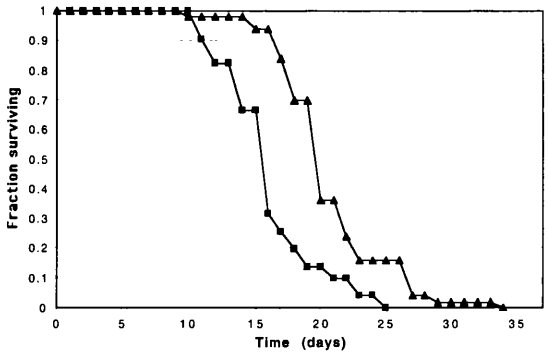
In agreement with the above finding that the male lifespan advantage is lost in a class 1 *daf-2* genetic background, results presented in Chapter 3 (Table 3.7) suggest that at 15°C (in the absence of pleiotropic phenotypes), class 2 *daf-2* males no longer display increased lifespan relative to hermaphrodites. Class 2 *daf-2* male and hermaphrodite lifespans were not compared here due to the potential confounding effects of the pleiotropic Unc phenotype at restrictive temperatures (see Chapter 3).

Note that in contrast to previous work on agar plates, *daf-1(m40)* and *daf-11(m47)* hermaphrodites (but not males) were long-lived relative to N2 controls. It is therefore possible that these mutations display an Age phenotype in liquid culture. However, N2 hermaphrodite control lifespan was uncharacteristically short in the present experiment, and one replicate was abandoned due to contamination, suggesting that the increased lifespans of *daf-1(m40)* and *daf-11(m47)* hermaphrodites may not be typical.

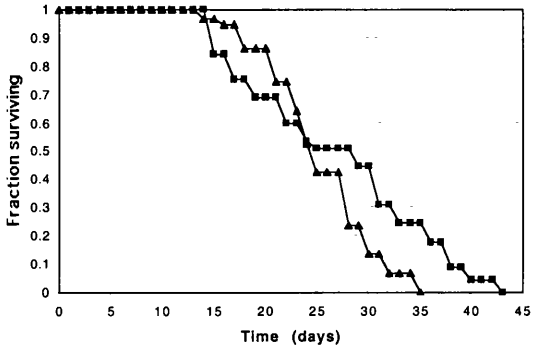
Males were shorter-lived than hermaphrodites in *daf-11(m47)* (Figure 2.3h) and showed no significant difference in survival from hermaphrodites in *daf-1(m40)* (Figure 2.3i). These results suggest a role for cGMP and TGF-β signalling in the regulation of sex-specific elements of lifespan. The above findings are all considered further in the Discussion.

Figure 2.3: Typical survival curves for males (triangles) and hermaphrodites (squares) of a range of IIS, TGF-β and cGMP mutants (22.5°C). *P* values are given in Table 2.1.

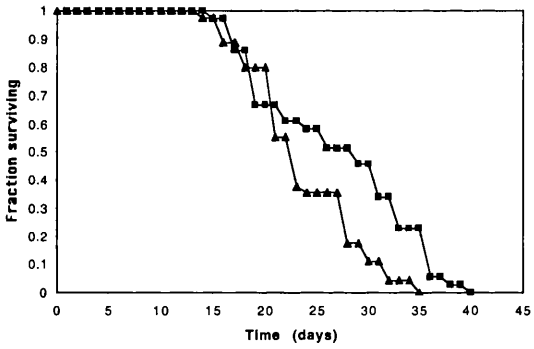
(a) N2



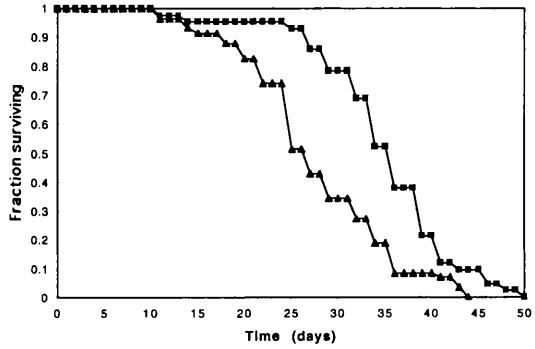
(b) *daf-2(e1368)*



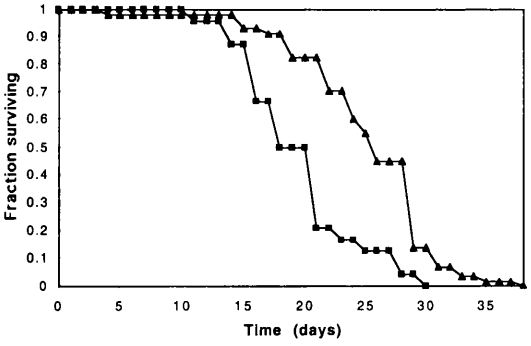
(c) *daf-2(m577)*



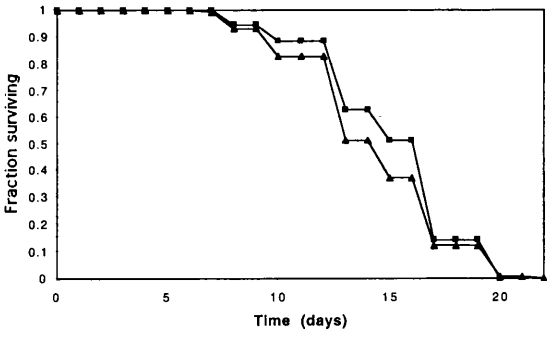
(d) *daf-2(m41)*



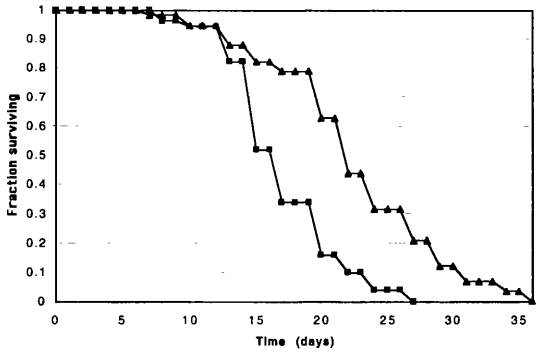
(e) *pdk-1(sa709)*



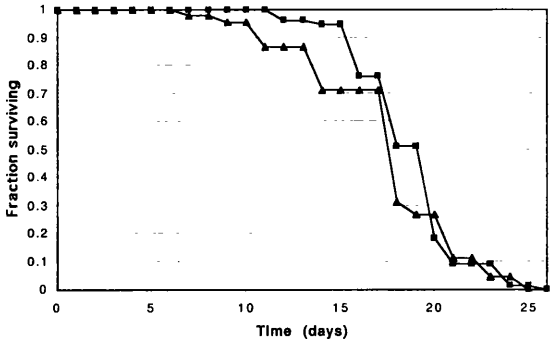
(f) *daf-16(mgDf50)*



(g) *daf-12(rh61rh411)*



(h) *daf-11(m47)*



(i) *daf-1(m40)*

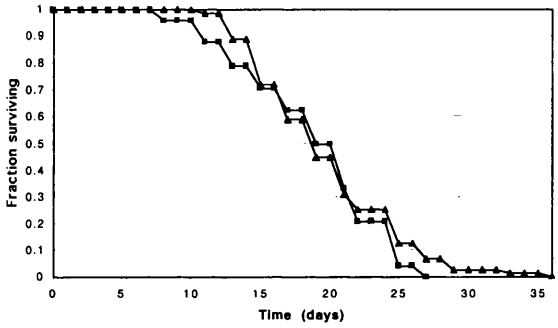


Table 2.1: Lifespans of males and hermaphrodites of a range of IIS, TGF-β and cGMP mutants (22.5°C)

	Male			Hermaphrodite					
Genotype	Median lifespan (days) ± 95% C.I.	Max. lifespan (days)	N*	Median lifespan (days) ± 95% C.I.	Max. lifespan (days)	N*	Ratio M:H median lifespan	Ratio M:H max. lifespan	*P*†
+#	20.0 (21.0, 20.0)	31.0	49 (55)	15.0 (15.0, 15.0)	22.0	49 (60)	1.33	1.41	<0.0001
daf-2(e1368)	24.0 (27.0, 23.5)	31.0	115 (120)	25.5 (29.3, 22.0)	39.0	88 (120)	0.94	0.79	0.066, 0.0024
daf-2(m577)	22.0 (23.5, 27.0)	31.0	102 (109)	27.0 (30.0, 23.0)	38.0	84 (110)	0.81	0.82	0.021, <0.0001
daf-2(m41)	25.0 (27.0, 23.0)	44.5	117 (120)	34.0 (36.0, 31.5)	46.5	80 (120)	0.74	0.96	<0.0001, 0.0038
pdk-1(sa709)	26.5 (28.0, 24.8)	37.5	110 (120)	22.0 (24.0, 20.0)	31.0	61 (95)	1.20	1.21	<0.0001, 0.021
daf-16(mgDf50)#	13.0 (15.0, 13.0)	20.0	60 (60)	15.0 (17.0, 13.0)	17.0	33 (60)	0.87	1.18	0.074
daf-1(m40)#	19.0 (21.0, 17.0)	33.0	71 (73)	20.0 (22.0, 17.0)	25.0	24 (60)‡	0.95	1.32	0.47
daf-11(m47)	18.0 (18.0, 18.0)	23.0	45 (45)	20.0 (20.0, 18.0)	24.0	76 (108)	0.90	0.96	0.021
daf-12(rh61rh411)#	22.0 (24.0, 22.0)	34.0	57 (60)	17.0 (17.0, 15.0)	24.0	50 (60)	1.29	1.42	<0.0001

*Number of senescent deaths (starting population). ‡Many worms censored due to internal hatching of eggs. †Probability that survival of males and hermaphrodites differ by random chance (log-rank test). Where two values are shown, these are comparisons from different trials. #Results from only one replicate due to contamination of second replicate.

2.2.2 Establishing the wild-type sex bias to dauer formation

(a) Dauer pheromone method

In agreement with previous work (Ailion & Thomas 2000), males were more likely to form dauers than hermaphrodites, with male to hermaphrodite dauer formation ratios (M:H DFRs) ranging from 1.12 to 1.38 (Table 2.2). The difference between male and hermaphrodite mean percent dauer formation was only statistically significant in one case due to the large amount of variation in levels of dauer formation between plates within each trial. Since increased male relative to hermaphrodite dauer formation was consistently seen in all replicates, it is likely that there is a central tendency to increased dauer formation in males.

Table 2.2: Percent dauer formation by males and hermaphrodites in the presence of a range of pheromone concentrations (25°C)

Amount of pheromone (µl)*	Mean % hermaphrodite dauer formation ± s.e.	Mean % male dauer formation ± s.e.	M:H DFR	N#	*P*†
20	29.9 ± 5.0	35.8 ± 8.9	1.20	369	>0.05
20	83.5 ± 5.7	96.7 ± 4.2	1.16	157	<0.05
30	83.2 ± 8.6	93.2 ± 4.3	1.12	204	>0.1
40	64.9 ± 9.6	89.5 ± 7.3	1.38	182	>0.1
50	73.8 ± 8.4	96.2 ± 3.8	1.30	213	= 0.1

*Per 2ml agar; #Total number of animals scored; †Probability that the proportion of dauer larvae formed by each sex differ by random chance (Student's *t* test on arcsine-transformed data).

(b) Starvation method

On average a greater proportion of N2 males (15.1%) formed dauers than hermaphrodites (7.5%) across trials, resulting in a mean M:H DFR of 2.0 (n= 1920). Again, however, due to the large amount of variation in dauer formation between trials this difference was not statistically significant (Student's *t* test $P>0.1$). These results further support the existence of a male bias to dauer formation, although it should be noted that this method gives only an approximate measure of the level of dauer formation.

2.2.3 Investigating sex bias to dauer formation in Daf-c mutants

In order to determine whether sex differences in levels of IIS are responsible for the increased male tendency to dauer formation, the sex bias to dauer formation was measured in a range of Daf-c mutants.

The sex bias to dauer formation is absent or reversed in IIS mutants

A male bias to dauer formation was not seen in any of the IIS mutants tested (Table 2.3). Two of the five strains (*daf-2(m41)* and *daf-2(e1370)*) showed no significant bias towards dauer formation by either sex. However, in three strains hermaphrodites were significantly more likely to form dauers than males, with M:H DFRs of less than one. Disruption of IIS therefore suppresses the male bias, and even results in a hermaphrodite bias to dauer formation.

Table 2.3: Male: hermaphrodite dauer formation ratios for a range of IIS mutants

Strain‡	Temperature at which larvae raised (°C)	% dauer formation ± s.e.		M:H DFR	N#	*P*†
		Hermaphrodites	Males			
daf-2(m577) (1)	22.5	21.6 ± 2.6	12.1 ± 1.6	0.56	1320	<0.01
daf-2(m577) (1)	22.5	16.5 ± 2.3	6.7 ± 1.8	0.41	1324	<0.002
daf-2(e1368) (1)	22.5	24.9 ± 2.3	9.9 ± 1.3	0.40	1416	<0.001
daf-2(e1368) (1)	22.5	26.1 ± 2.5	15.2 ± 1.3	0.58	3005	<0.001
daf-2(m41) (1)	20.0	5.8 ± 1.6	4.7 ± 1.1	0.80	1330	>0.1
daf-2(m41) (1)	20.0	20.3 ± 2.6	13.3 ± 3.7	0.66	444	>0.1
daf-2(m41) (1)	20.0	37.6 ± 5.1	37.3 ± 3.6	1.01	712	>0.1
daf-2(e1370) (2)	22.5	40.0 ± 5.4	31.9 ± 5.2	0.80	766	>0.1
daf-2(e1370) (2)	22.5	24.5 ± 6.9	31.6 ± 6.8	1.29	350	>0.1
pdk-1(sa709)	26.0	38.3 ± 8.3	30.5 ± 5.0	0.80	459	>0.1
pdk-1(sa709)	26.0	54.5 ± 8.1	28.7 ± 4.0	0.53	399	<0.02

‡ Class 1 *daf-2* alleles in order of increasing severity (allele class in parentheses); #Total number of animals scored; †Probability that proportions of dauers formed by males and hermaphrodites differ by random chance (Student's *t* test on normalised means following arcsine transformation).

The male bias to dauer formation is enhanced in TGF-β signalling mutants

Interestingly, the M:H DFRs for a range of ts Daf-c TGF-β mutants were higher than that seen for wild type in all cases, ranging from 1.34 to 9.64 (Table 2.4). The male bias to dauer formation was therefore enhanced in this class of Daf-c mutant. Note that where male dauer formation approached 100%, the extent of the male bias to dauer formation is likely to be understated (see Materials and Methods).

Table 2.4: Male: hermaphrodite dauer formation ratios for a range of TGF-β mutants

Strain	Temperature at which larvae raised (°C)	% dauer formation ± s.e.		M:H DFR	N#	*P*†
		Hermaphrodites	Males			
daf-4(m592)	22.5	28.3 ± 3.0	96.8 ± 1.0	3.42	942	<0.001
daf-4(m592)	22.5	32.8 ± 4.5	100 ± 0.0	3.10	744	<0.001
daf-4(m592)	22.5	72.9 ± 5.5	97.4 ± 2.0	1.34	767	<0.002
daf-1(m40)	20.0	9.8 ± 3.2	35.3 ± 9.9	3.60	1037	<0.05
daf-1(m40)	20.0	8.3 ± 3.4	94.5 ± 1.5	11.4	542	<0.001
daf-1(m40)	22.5	20.3 ± 3.5	99.3 ± 0.7	4.89	469	<0.001
daf-1(m40)	22.5	47.1 ± 2.1	99.4 ± 0.6	2.11	377	<0.001
daf-8(m85)	15.0	2.8 ± 1.0	14.4 ± 5.1	5.14	542	<0.01
daf-8(m85)	15.0	8.8 ± 6.2	76.6 ± 13.3	8.70	194	<0.02
daf-8(m85)	17.0	2.5 ± 1.3	24.1 ± 3.8	9.64	1031	<0.001

#Total number of animals scored; †Probability that proportions of dauers formed by males and hermaphrodites differ by random chance (Student's *t* test on normalised means following arcsine transformation).

The male bias to dauer formation is present in a cGMP signalling mutant

Males of the weak cGMP mutant *daf-11(m47)* also formed dauers more readily than hermaphrodites, with a M:H DFR of 1.67 (Table 2.5). Unfortunately, it was not possible to calculate a *P* value for the difference between *daf-11(m47)* male and hermaphrodite dauer formation due to the difficulty of maintaining male stocks of this strain because of high levels of male dauer formation, which meant that only one replicate could be performed. Although the extent of the male bias to dauer formation is similar to that found for N2 starvation dauers above, the fact that male stock maintenance was difficult suggests that the male bias to dauer formation may have been enhanced in this mutant, as it was in the TGF-β mutants.

Table 2.5: Male: hermaphrodite dauer formation ratios in a cGMP mutant

Strain	Temperature at which larvae raised (°C)	% dauer formation		M:H DFR	N#
		Hermaphrodites	Males		
daf-11(m47)	15.0	10.6	17.7	1.67	209

#Total number of animals scored

daf-16 *does not (fully) suppress the male bias to dauer formation*

In order to confirm that the wild-type male bias to dauer formation was dependent on IIS (as suggested by results in Table 2.3), a double IIS (*daf-16*)/ TGF-β (*daf-1*) mutant was constructed and its M:H partial DFR determined. If sex differences in IIS (and hence

DAF-16 activity) were responsible for the wild-type male bias to dauer formation, a prediction would be that there would be no male bias to dauer formation in a *daf-16(0)* genetic background. The partial DFR of *daf-16(mgDf50); daf-1(m40)* ranged from 2.80 to 3.75. The double mutant therefore displayed a significant male bias to dauer formation (Table 2.6).

Table 2.6: Male: hermaphrodite partial dauer formation ratios of *daf-16(mgDf50); daf-1(m40)*

Strain	Temperature at which larvae raised (°C)	% partial dauer/ dauer formation ± s.e.		M:H partial DFR	N#	*P*†
		Hermaphrodites	Males			
daf-16(mgDf50); daf-1(m40)‡	22.5	7.7 ± 1.5	28.9 ± 4.6	3.75	1767	<0.001
daf-16(mgDf50); daf-1(m40)	22.5	21.2 ± 2.3	64.3 ± 7.4	3.03	1239	<0.001
daf-16(mgDf50); daf-1(m40)	22.5	17.2 ± 1.8	48.2 ± 5.3	2.80	1829	<0.001
daf-1(m40)	22.5	47.1 ± 2.1	99.4 ± 0.6	2.11	377	<0.001

#Total number of animals scored. †Probability that proportions of dauers formed by males and hermaphrodites differ by random chance (Student's *t* test on normalised means following arcsine transformation). ‡Forms partial dauers- see Materials & Methods.

daf-16 therefore does not fully suppress the male bias to dauer formation of Daf-c mutants. However, it may reduce it. To test this, the M:H DFR and M:H partial DFR of *daf-1(m40)* and *daf-16(mgDf50); daf-1(m40)* were compared. However, a difficulty was that dauer formation by *daf-1(m40)* males at 22.5°C was consistently very close to 100%, potentially resulting in underestimation of the M:H DFR (see Materials and Methods above). It was therefore not possible to compare absolute M:H DFR values for *daf-1(m40)* and *daf-16(mgDf50); daf-1(m40)*, and attempts to perform the experiment at lower temperatures were unsuccessful because *daf-16(mgDf50); daf-1(m40)* formed very few partial dauers. However, as can be seen in Table 2.4, *daf-1(m40)* is capable of displaying a M:H DFR of up to 11 in some cases. Thus, while suppression of the *daf-1(m40)* male bias to dauer formation by *daf-16(0)* was not complete, data presented here do not exclude the possibility that there may be partial suppression.

2.2.4 Sex differences in stress resistance

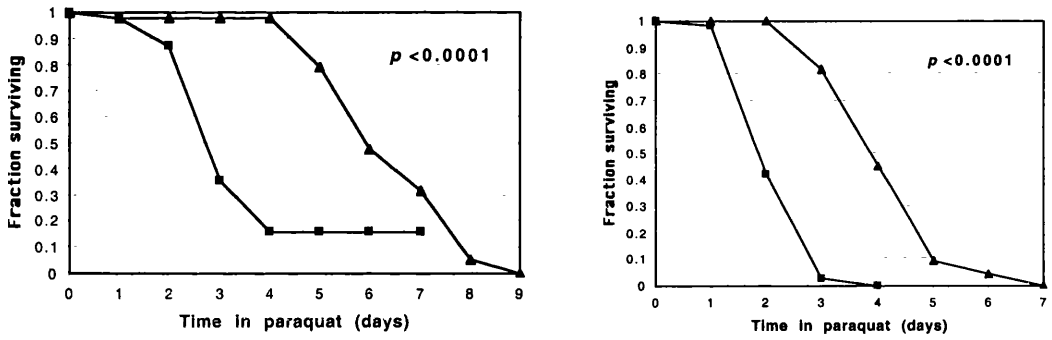
In order to determine whether males are more resistant to stress than hermaphrodites, relative resistance of the two sexes to oxidative, U.V. and heat stress was determined.

(a) Oxidative stress resistance

Resistance of males and hermaphrodites to oxidative stress was determined by exposure to the free radical-generator methyl viologen (Paraquat). Previous work found Paraquat concentrations of 10mM to result in a mean hermaphrodite lifespan of three days (Vanfleteren 1993). In the present study, therefore, initial trials employed Paraquat concentrations of 10mM and 20mM in order to determine the most suitable concentration for further experiments (Figure 2.4). Male survival was significantly longer than that of hermaphrodites at both concentrations ($P< 0.0001$), implying that males are better able to resist oxidative stress.

Figure 2.4: Survival of one-day-old N2 hermaphrodites (squares) and males (triangles) in (a) 10mM (n hermaphrodites=35, males= 38) and (b) 20mM (n hermaphrodites=48, males=44) Paraquat (25°C).

(a) (b)



However, a concern following the initial experiment was that it could be quite difficult to distinguish hermaphrodite senescent deaths from instances of internal hatching of eggs (matricide), since Paraquat treatment resulted in the death of many larvae inside the hermaphrodites. The lack of larval movement made it difficult to see instances of matricide, potentially leading to over-scoring of hermaphrodite deaths due to Paraquat. To exclude this possibility, the mitosis-inhibitor 5-fluoro-2'-deoxyuridine (FuDR) was added to the medium at a concentration of 50μM from the first day of adulthood, to prevent egg production. Again, survival was significantly greater in males (Figure 2.5), further suggesting that males are more resistant to oxidative stress.

However, worms of both sexes had a rather sickly appearance in FuDR, and contained numerous dark inclusions. Thus, although the FuDR had relieved the problem of confounding matricides, it did appear to be affecting the overall health of the worms. For this reason it was decided to abandon using FuDR and instead to measure Paraquat resistance in post-reproductive animals (Figure 2.6). Animals were therefore transferred to Paraquat-containing medium upon day five of adulthood. Once again, males were significantly more resistant to Paraquat than hermaphrodites ($P<0.0001$).

Figure 2.5: Survival in (a, b) 10mM and (c,d) 20mM Paraquat, with 50μM FuDR of one-day-old N2 hermaphrodites (squares: (a) n= 56; (b) n= 56; (c) n= 57; (d) n= 58) and males (triangles: (a) n= 58; (b) n= 58; (c) n= 57; (d) n= 58) (25°C).

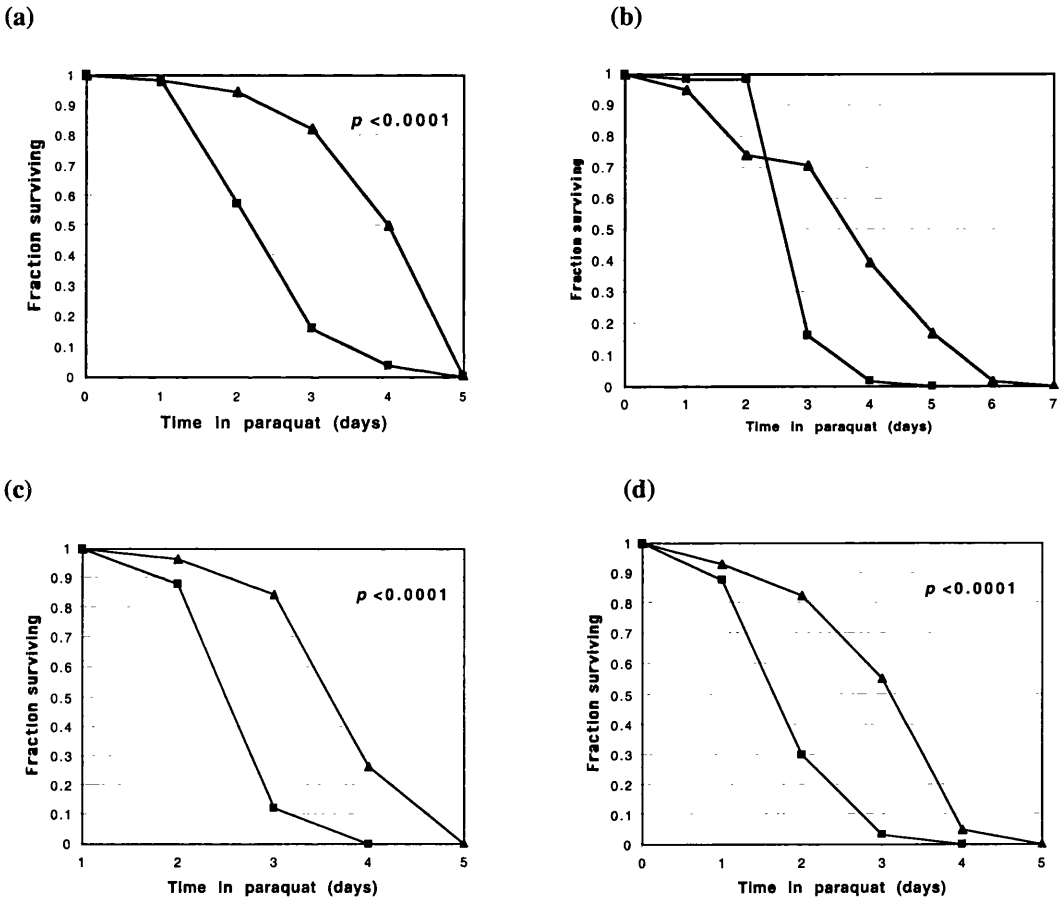
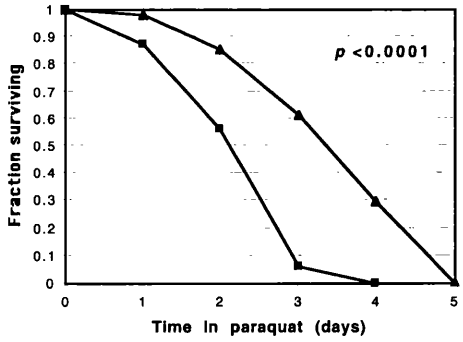


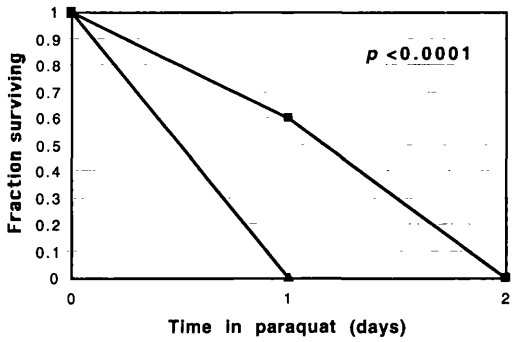
Figure 2.6: Survival in 20mM Paraquat of five-day-old N2 hermaphrodites (squares, n= 48) and males (triangles, n= 54) (25°C).



Overall, these results strongly imply that males are more resistant to oxidative stress than hermaphrodites. However, it should be noted that it is also possible that the increased male resistance to oxidative stress was simply a correlated result of slower male ageing, and hence younger biological age relative to hermaphrodites, which may not necessarily be caused by sex differences in IIS and/or DAF-16 activity. The relative resistance of the two sexes to Paraquat was therefore determined in a *daf-16(0)* genetic background (Figure 2.7). If the sex difference in response to oxidative stress with Paraquat were the result of differences in IIS, it might be expected that males would no longer demonstrate increased survival in this mutant background where IIS function is disrupted.

As expected, *daf-16(0)* animals were more susceptible to oxidative stress overall than N2 animals, with the survival of *daf-16(0)* in 20mM Paraquat being short compared with that of N2 under the same conditions (compare Figures 2.6 & 2.7). However, in this case *daf-16(0)* males were less resistant to oxidative stress than hermaphrodites (Figure 2.7, $P<0.0001$). These results indicate that the increased resistance of wild-type males relative to hermaphrodites to oxidative stress may be dependent on IIS. Moreover, the fact that hermaphrodites survived longer than males suggests that a factor other than *daf-16* promotes stress resistance in hermaphrodites. It should be noted, however, that due to contamination N2 control measurements were not performed at the same time as those for *daf-16(0)*, hence results should be interpreted cautiously. Despite several attempts to repeat this experiment, contamination with bacteria and excessive matricide of *daf-16(0)* hermaphrodites meant that trials had to be abandoned.

Figure 2.7: Survival of four-day-old *daf-16(mgDf50)* hermaphrodites (squares, n=51) and five-day-old males (triangles, n=38) in 20mM Paraquat (25°C)



(b) Heat stress

As a further measure of sex differences in stress resistance, relative resistance of males and hermaphrodites to heat stress was determined. Interestingly, it had been noted during screens for heat stress-resistant mutant hermaphrodites using this protocol that the majority animals recovering were wild-type males (M. Muñoz, pers. comm.), implying that males were more resistant to heat stress. Because heat stress resistance assays were performed using L1 larvae, the potential complication of sex-specific biological age noted for oxidative stress resistance trials above is eliminated.

A significantly higher proportion of males was consistently found to survive heat shock, with ratios of male: hermaphrodite recoveries ranging from 3.2 to 10.9 (Table 2.7). Males are therefore more resistant to heat stress (as L1 larvae) than hermaphrodites.

Table 2.7: Proportions of N2 males and hermaphrodites surviving a 48 hour 30°C heat shock

Sex ratio of starting population (those not stressed)		Number of L1 larvae stressed	% recoveries based on control sex ratio		Ratio of male: hermaphrodite recoveries	*P*†
Hermaphrodite	Male		Hermaphrodite	Male		
537	558	180	2.3	25.0	10.9	<0.01
537	558	160	6.8	21.7	3.2	
780	427	250	6.8	21.6	3.2	
499	474	250	3.1	21.3	6.9	

†Probability that mean male and hermaphrodite percent recoveries differ as a result of random chance (Student's *t* test on normalised values following arcsine transformation)

In order to determine whether the increased male resistance to heat stress was dependent upon *daf-16*, the experiment was repeated in a *daf-16(0)* genetic background (Table 2.8). As would be expected given the role of *daf-16* in response to heat stress, the null mutant *daf-16(mgDf50)* was more sensitive to heat stress than wild type, with almost no larvae recovering after a 48 hour 30°C heat shock. For this reason, this strain was subjected to heat stress for only 24 hours, after which time a measurable proportion of larvae were able to recover. Overall, there was no significant difference between mean recovery by *daf-16(0)* males and hermaphrodites. This suggests that increased male resistance to heat stress is dependent upon *daf-16*.

Table 2.8: Proportions of *daf-16(mgDf50)* males and hermaphrodites surviving a 24 hour 30°C heat shock

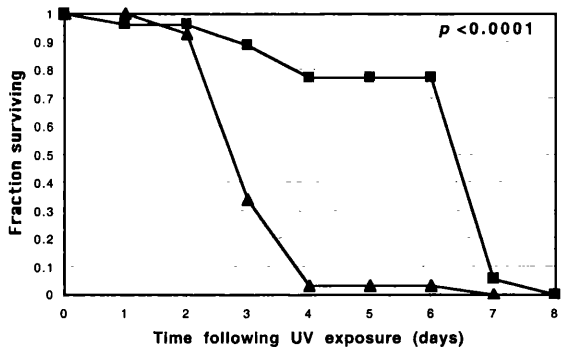
Sex ratio of starting population (those not stressed)		Number of L1 larvae stressed	% recoveries based on control sex ratio		Ratio of male: hermaphrodite recoveries	*P*†
Hermaphrodite	Male		Hermaphrodite	Male		
567	525	200	27.0	32.0	1.2	>0.1
471	498	130	15.8	10.4	0.7	
501	573	400	46.5	35.7	0.8	

†Probability that mean male and hermaphrodite percent recoveries differ as a result of random chance (Student's *t* test on normalised values)

(c) UV irradiation stress

As a final measure of sex differences in stress resistance, survival of five-day-old males and hermaphrodites after UV irradiation was determined as described in Materials and Methods above. Only one replicate was performed. As shown in Figure 2.8, in contrast to results for oxidative and heat stress resistance, hermaphrodites were strikingly more resistant to UV stress than males ($P<0.0001$). Since IIS mutants have been shown to be resistant to UV stress (Murakami & Johnson 1996), this preliminary result does not support the hypothesis that IIS is intrinsically down-regulated in males relative to hermaphrodites.

Figure 2.8: Survival of five-day-old N2 males (triangles, n=59) and hermaphrodites (squares, n=54) following UV irradiation (2000J/m^2).



2.2.5 Localisation of DAF-16::GFP in males and hermaphrodites

As described in the Main Introduction, IIS results in phosphorylation and subsequent cytosolic localisation and inactivation of the forkhead transcription factor DAF-16 (Paradis & Ruvkun 1998; Lee *et al* 2001; Lin *et al* 2001; Henderson & Johnson 2001). If IIS were down-regulated, and thus DAF-16 activity increased in males relative to hermaphrodites, it might be expected that there would be increased nuclear localisation of DAF-16 in males compared with hermaphrodites. Localisation patterns of DAF-16 protein in the two sexes were therefore compared using a green fluorescent protein (GFP) construct (described in Materials and Methods above).

Quantification of nuclear localisation levels in the DAF-16::GFP strain

The DAF-16a isoform is the major regulator of lifespan and dauer formation (Lee *et al* 2001). Work employing a *daf-16a*/GFP fusion gene has shown that expression of *daf-16a* is almost ubiquitous with the exception of the pharynx and much of the somatic gonad (Lee *et al* 2001). Localisation of the protein under non-stressed conditions is generalised within the cell, with DAF-16 occurring both in the cytosol and the nucleus, although there is more pronounced nuclear localisation in occasional neuronal, muscle or hypodermal cells (Lin *et al* 2001) (Figure 2.9).

To gain an understanding of the relative levels of nuclear localisation of DAF-16::GFP between different sexes/ treatments they were classified on a scale of 0-3 as

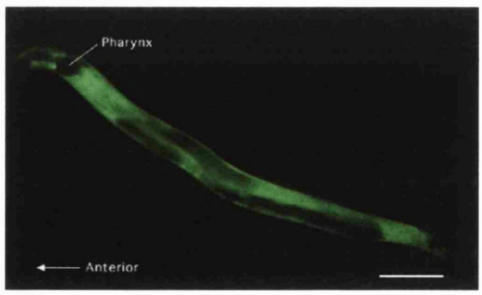
described below and as illustrated in Figure 2.10, since four levels of nuclear localisation could be clearly distinguished.

Level 0: Complete cytoplasmic localisation
Level 1: Some degree of nuclear localisation apparent
Level 2: Marked nuclear localisation
Level 3: Complete/ almost complete nuclear localisation

In this context "cytoplasmic" localisation refers to the localisation pattern mainly seen under standard conditions (that is, throughout both the cytosol and nuclei of individual cells), while "nuclear" localisation refers to accumulation of the protein almost exclusively in the nucleus. Worms displaying complete nuclear localisation no longer showed a diffuse DAF-16::GFP pattern but were spotted with foci of DAF-16::GFP where cell nuclei are found. The percentage of worms of a particular sex/ treatment displaying each level of nuclear localisation were then calculated, and compared with results for another sex/ treatment. For ease of sex identification, L4 animals were used.

Figure 2.9: (a) Photograph showing ubiquitous distribution of DAF-16::GFP protein in an otherwise wild-type L4 hermaphrodite, with the exception of the pharynx, under standard conditions. Scale bar= 150μm (b) Detail of head region of hermaphrodite showing largely ubiquitous localisation of DAF-16::GFP. Scale bar= 70μm

(a)



(b)

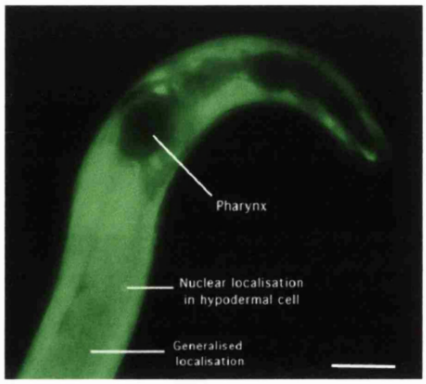
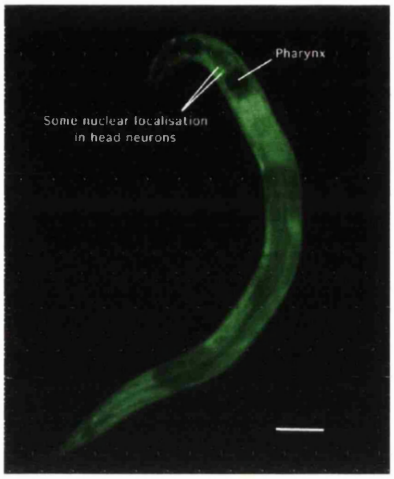
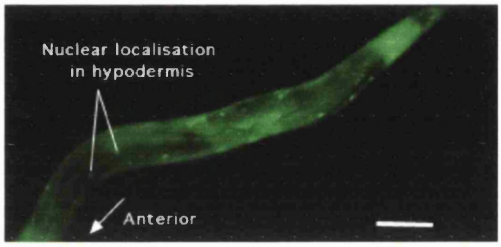


Figure 2.10: Photographs of typical animals (L4) displaying levels 0-3 of nuclear localisation of DAF-16::GFP (a-d) respectively.

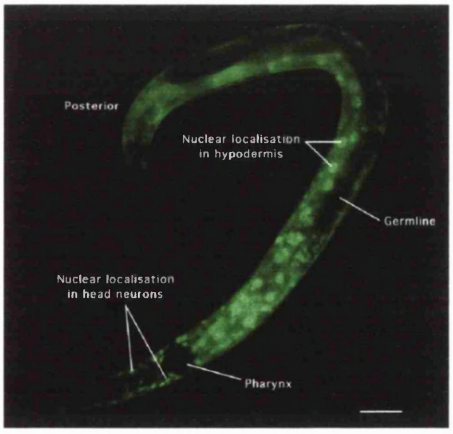
(a) Hermaphrodite: level 0
Generalised localisation. Scale bar= 90μm



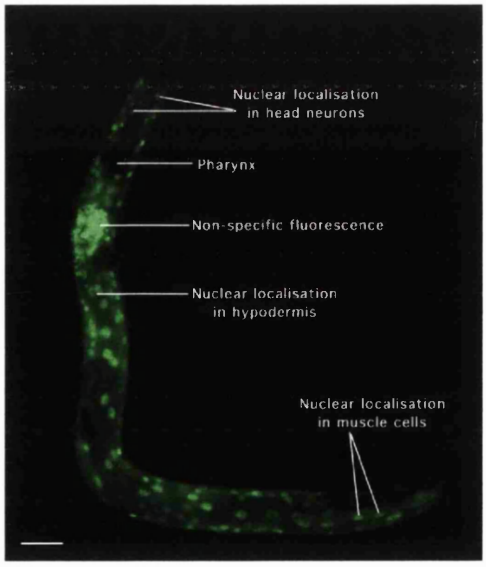
(b) Hermaphrodite: level 1
Some nuclear localisation. Scale bar= 90μm



(c) Male: level 2
Marked nuclear localisation.
Scale bar= 70μm



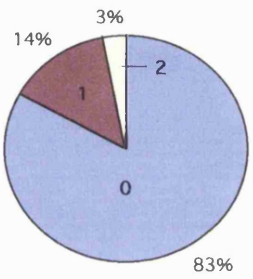
(d) Male: level 3
Almost complete nuclear localisation.
Scale bar= 70μm



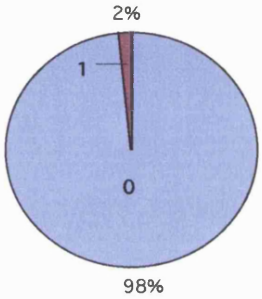
Males vs hermaphrodites: wild-type background under non-stressed conditions

Levels of nuclear localisation of DAF-16::GFP under replete food conditions at 15°C were determined for males and hermaphrodites in an otherwise wild-type genetic background (Figure 2.11).

Figure 2.11: Percentage of males and hermaphrodites showing each level of nuclear localisation of DAF-16::GFP under standard conditions (15°C)



Hermaphrodites, n=65



Males, n= 63

Contrary to expectations if IIS were reduced in males relative to hermaphrodites, levels of DAF-16::GFP nuclear localisation were slightly higher in hermaphrodites compared with males, suggesting that levels of IIS (or other negative regulators of DAF-16 activity) may be lower in hermaphrodites under these conditions. However, the difference between the percentage of animals displaying each level of nuclear localisation in males and hermaphrodites was not significant in any instance (Student's *t* test on normalised data, Appendix E.3). This finding does not support the working hypothesis of this chapter that IIS is intrinsically down-regulated in males. However, it remained possible that very small sex differences in DAF-16 localisation not visible using the GFP strain could result in marked sex differences in lifespan and dauer formation, and that sex differences in DAF-16::GFP nuclear localisation might only become apparent following stress.

Males vs hermaphrodites: wild-type genetic background under stress conditions

Data presented above imply that males are more resistant to oxidative and heat stress than hermaphrodites, and that this effect is dependent on *daf-16*. Previous work using

the TJ356 strain found a marked increase in nuclear localisation of DAF-16::GFP in hermaphrodites upon heat, oxidative and starvation stress (Henderson & Johnson 2001). If the increased male resistance to oxidative and heat stress were due to reduced IIS relative to hermaphrodites, more rapid nuclear localisation of DAF-16::GFP upon challenge with these stresses might be expected. DAF-16::GFP localisation was therefore measured in males and hermaphrodites following oxidative and heat stress. Full results are presented in Appendix Section E.3, and example results for each treatment displayed as pie charts below.

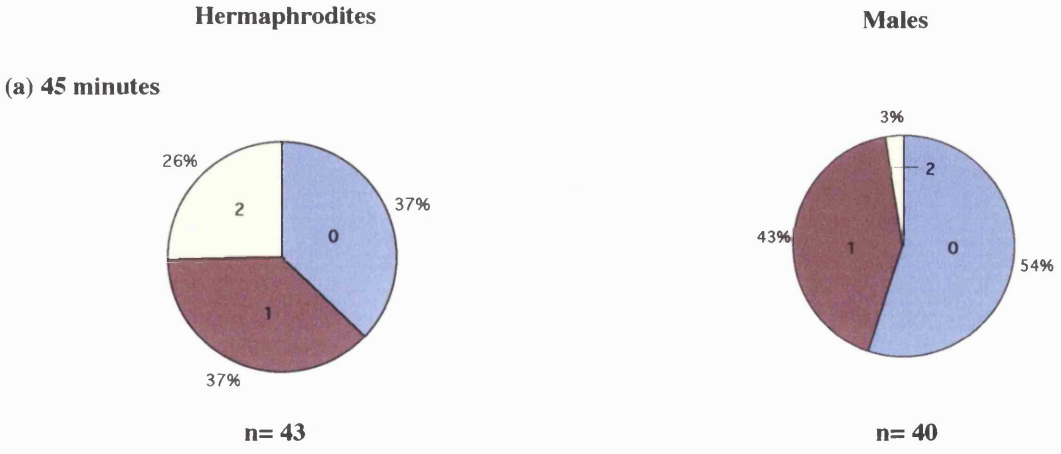
Nuclear localisation of DAF-16::GFP in response to starvation stress was determined by placing animals into S medium minus OP50 for various periods of time (Figure 2.12). Due to time constraints, experiments for each starvation time were not all performed on the same day, and there is clearly some variation between trials in that there is not always a general increase in nuclear localisation with increasing starvation time. However, within all starvation time data sets, hermaphrodites consistently displayed increased DAF-16::GFP nuclear localisation following starvation compared with males. Comparisons of male and hermaphrodite DAF-16::GFP nuclear localisation levels were mainly statistically insignificant, however (Appendix Section E.3), probably due to the large amount of variation between individual replicates for each treatment. Repeats of this trial using larger numbers of animals would most likely result in consistently significantly increased nuclear localisation in hermaphrodites in response to starvation stress. Thus, these results do not support the working hypothesis of this chapter that DAF-16 is constitutively up-regulated in males relative to hermaphrodites.

A similar experiment was then performed for males and hermaphrodites following various exposures to a 35°C heat shock (Figure 2.13). This stress resulted in rapid localisation of DAF-16::GFP to the nucleus in both sexes, with complete nuclear localisation being apparent after just 15 minutes. As seen following starvation stress above, nuclear localisation of DAF-16::GFP occurred more rapidly in hermaphrodites than in males in all cases. These results are therefore also inconsistent with the working hypothesis of this chapter. Again, however, differences in the percentage of males and

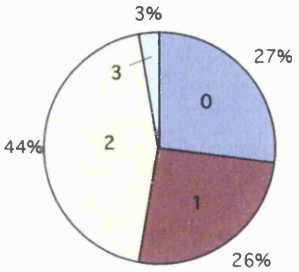
hermaphrodites displaying a particular level of nuclear localisation were often statistically indistinguishable due to inter-replicate variation (Appendix Section E.3).

In order to investigate the effect of oxidative stress on nuclear localisation of DAF-16::GFP in the two sexes, animals were exposed to 20mM Paraquat (Figure 2.14). Consistent with a previous study using the free radical generator juglone (Henderson & Johnson 2001), oxidative stress due to Paraquat administration resulted in nuclear localisation of DAF-16::GFP. Like the response to starvation, the response to Paraquat was not as marked as that seen following heat stress. As seen for the starvation trial above, the degree of nuclear localisation seen did not necessarily increase with extended exposure to oxidative stress. This could either be due to varying responses on different days, or perhaps because response to Paraquat in terms of DAF-16 localisation is maximal after shorter exposure times than those used. After both exposure times, however, once again nuclear localisation of DAF-16::GFP consistently occurred more rapidly in hermaphrodites than it did in males. As for the two previous trials, however, differences between the sexes were rarely significantly different due to high variation between replicates (Appendix Section E.3). However, the consistent trend towards increased nuclear localisation of DAF-16::GFP in hermaphrodites relative to males again suggests that DAF-16 activity increases in response to stresses more rapidly in hermaphrodites, and thus does not support the working hypothesis of the chapter.

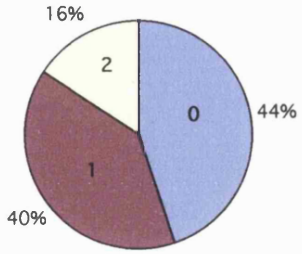
Figure 2.12: Percentage of L4 males and hermaphrodites showing each level of nuclear localisation of DAF-16::GFP following two periods of starvation (15°C).



(b) 60 minutes



n= 70

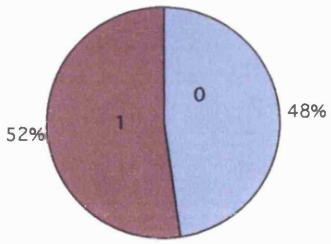


n= 58

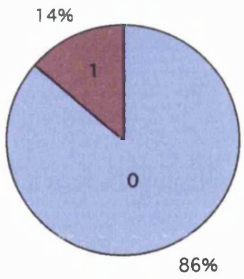
Figure 2.13: Percentage of L4 males and hermaphrodites showing each level of nuclear localisation of DAF-16::GFP following two heat shock times

Hermaphrodites **Males**

(a) 2.5 minutes

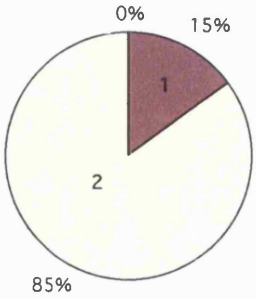


n= 40

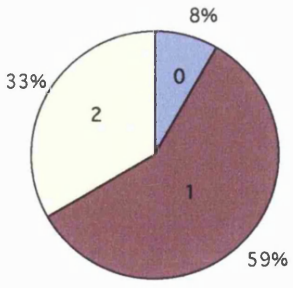


n= 51

(b) 10 minutes

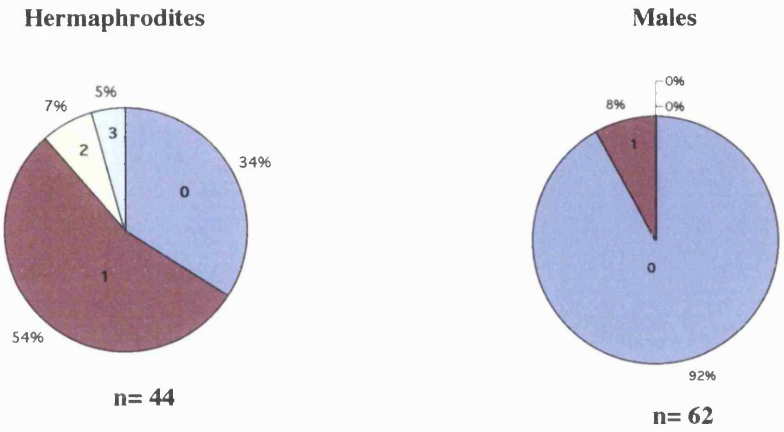


n= 40



n= 36

Figure 2.14: Percentage of L4 males and hermaphrodites showing each level of nuclear localisation of DAF-16::GFP following one hour's exposure to 20mM methyl viologen (15°C)

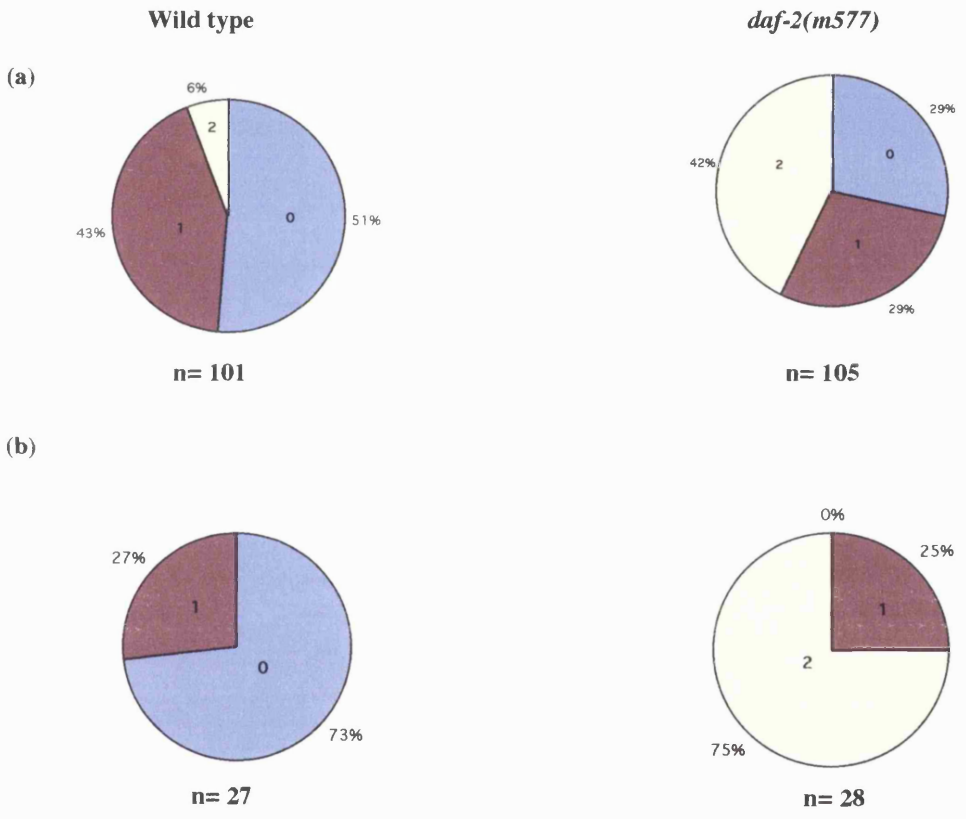


The finding that DAF-16::GFP localised to the nucleus faster in response to stress in hermaphrodites than males contradicts expectations based on data presented above. One possible explanation for this apparent discrepancy would be if a lower intrinsic level of IIS in males led to a reduced response to stress due to increased baseline stress defences. For example, males could have higher resting levels of heat shock proteins or antioxidant enzymes such as SOD. This could cause males to respond to stresses with nuclear accumulation of DAF-16 more slowly than hermaphrodites. If so, *daf-2(rf)* hermaphrodites might be expected to respond more slowly to stress in terms of DAF-16 activation than wild-type hermaphrodites. In order to test this possibility, the nuclear localisation of DAF-16::GFP in response to a 35°C heat stress was measured in both wild-type and *daf-2(m577)* genetic backgrounds. All animals were initially raised at 15°C, although in order to determine whether developmental temperature affected results, a subset of animals were shifted to 22.5°C at the L3 stage prior to heat shock as L4 larvae.

There was no difference between levels of nuclear localisation of DAF-16::GFP in *daf-2(m577)* and *daf-2*(+) animals at 15°C (data not shown). However, when hermaphrodites were shifted from either 15°C or 22.5°C to a heat stress of 35°C (Figure 2.15), the level of nuclear localisation of DAF-16::GFP was higher in a *daf-2(m577)* than an otherwise wild-type genetic background. Percentages of *daf-2(m577)*

animals displaying each level of nuclear localisation were significantly higher than that of controls in four of nine comparisons (Appendix E.3). This result does not support the above hypothesis that reduced levels of IIS lead to a slower DAF-16 response to stress, hence these DAF-16::GFP localisation results do contradict the hypothesis that IIS is reduced, and DAF-16 activity increased, in males.

Figure 2.15: Percentage of L4 hermaphrodites raised at (a) 15°C and (b) 22.5°C showing each level of nuclear localisation of DAF-16::GFP following five minutes of heat stress (35°C)



2.2.6 Sequencing of *daf-2(m41)*

With the exception of the class 1 allele *m41*, all other *daf-2* alleles employed in the present study had been sequenced (Kimura *et al* 1997; D. Patel, pers. comm). As described in the Introduction, it was of interest to determine the lesion in *daf-2(m41)* in order to better understand *daf-2* class-specific effects.

Sequencing was successfully performed for all exon fragments within the ligand binding and kinase domains of *daf-2(m41)* with the exception of exon 6, which did not amplify upon PCR following several attempts. This was probably due to the highly repetitive nature of intronic DNA before this exon, making effective primer design difficult. A G to A substitution was identified in the first ligand-binding domain of *daf-2(m41)* in exon 9 (Figure 2.16), resulting in a codon change from GAC to AAC. This lesion was identified in six sequencing reactions on genomic DNA from two separate PCR reactions (data not shown). No further mutations were identified in any of the fragments sequenced.

At the amino acid level, this change corresponded to a Gly-Glu substitution (G383E). This amino acid position is within the cysteine-rich region of the ligand-binding domain. The class 1 *daf-2(m41)* mutation therefore occurs in the extracellular ligand binding-encoding domain of the gene as found previously for other class 1 *daf-2* mutations sequenced to date (Kimura *et al* 1997; D. Patel, pers. comm (see Appendix E.4)). Such a change could result in a significant conformational change in this domain of the translated protein and thus presumably would disrupt ligand binding. Interestingly, this glycine is not conserved in the human insulin receptor, which carries a serine residue at that position (Yu & Larsen 2001). This lack of conservation may explain why *daf-2(m41)* mutants do not display any pleiotropic effects as do class 2 *daf-2* mutants, which are predicted to have more severe mutations, affecting wider aspects of DAF-2 function (Gems *et al* 1998).

daf-2(m41) was later sequenced using RT-PCR in order to confirm the mutation identified above and to check that there were no second site mutations present in exon 6, which had proved too difficult to amplify from genomic DNA. Sequencing of the entire cDNA sequence of *m41* confirmed the G383E mutation identified above, and found no other lesions (sequencing performed by D. Patel). This *m41* lesion was subsequently reported elsewhere (Yu & Larsen 2001), confirming the above results.

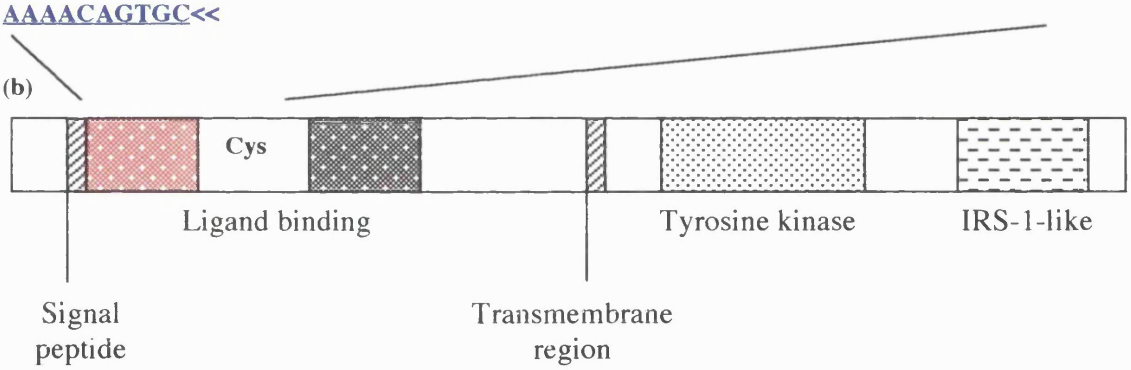
m41 is an unusual allele of *daf-2* in that it results in maternal rescue of the Daf-c phenotype (Gems *et al* 1998). *daf-2(m41)* oocytes of *daf-2+/m41* heterozygote hermaphrodites therefore appear to contain sufficient wild-type DAF-2 protein or *daf-2* mRNA to permit reproductive development at the restrictive temperature. *daf-2(m41)* is

also the only classical ts *daf-2* allele, in that unlike other *daf-2* alleles it displays no little lifespan increase at 15°C despite a relatively strong Daf-c phenotype at 22.5°C. It has been suggested that synthesis or function of DAF-2 in *daf-2(m41)* animals is thermolabile (Gems *et al* 1998). It is therefore possible that the glycine to glutamic acid change in *daf-2(m41)* may result in such an effect.

Figure 2.16: (a) *Position of the G to A nucleotide substitution in exon 9 of *daf-2(m41)*. Lower case: intron 8; upper case: exon 9 fragment 1; blue font: primer; (b) *daf-2* structure and location of region disrupted by the *m41* lesion (red) (adapted from (Kimura *et al* 1997))

(a)

>>tctcctgtttcaactgtctcca>>ctttttccatcaatttccattatttccagTGTGCCCACGTGGAGCTTGCGAAGAGGA
TAAAGGCGAATCAAAGTGTCATTATTTGGAGGAAAAGAATCAGGAACAAGGTGTCGAA
AGAGTTCAGAGTTGTTGGTCGAACACCACTTGCCAAAAGTCTTGTGCTTATGATCGTCT
TCTTCCAACGAAAGAAATCGG*ACCGGGATGTGATGCGAACGGCGATCGATGTCACGAT
CAATGCGTGGGCGGTTGTGAGCGTGTGAATGATGCGACAGCATGCCACGCGTGCAAGA
ATGTCTATCACAAGGGAAAGTGTATCGAAAAGTGTGATGCTCACCTGTACCTTCTCCTT
CAACGTCGTTGTGTGACCCGTGAGCAGTGTCTGCAGCTGAATCCGGT<<GCTCTCGAAC
AAAACAGTGC<<



2.3 Discussion

2.3.1 Sex differences in cellular localisation of DAF-16::GFP

The original working hypothesis of this chapter was that IIS is intrinsically down-regulated in males relative to hermaphrodites (Figure 2.1). A possible prediction of this is that removal of sex differences in IIS by mutation would result in loss of the increased male lifespan (Figure 2.2). Furthermore, males might be expected to display increased nuclear localisation of DAF-16 relative to hermaphrodites, as has been shown previously for *daf-2(rf)* mutant hermaphrodites relative to N2 (Henderson & Johnson 2001; Lin *et*

al 2001). This was tested using a strain containing an integrated *daf-16::gfp* construct (Henderson & Johnson 2001).

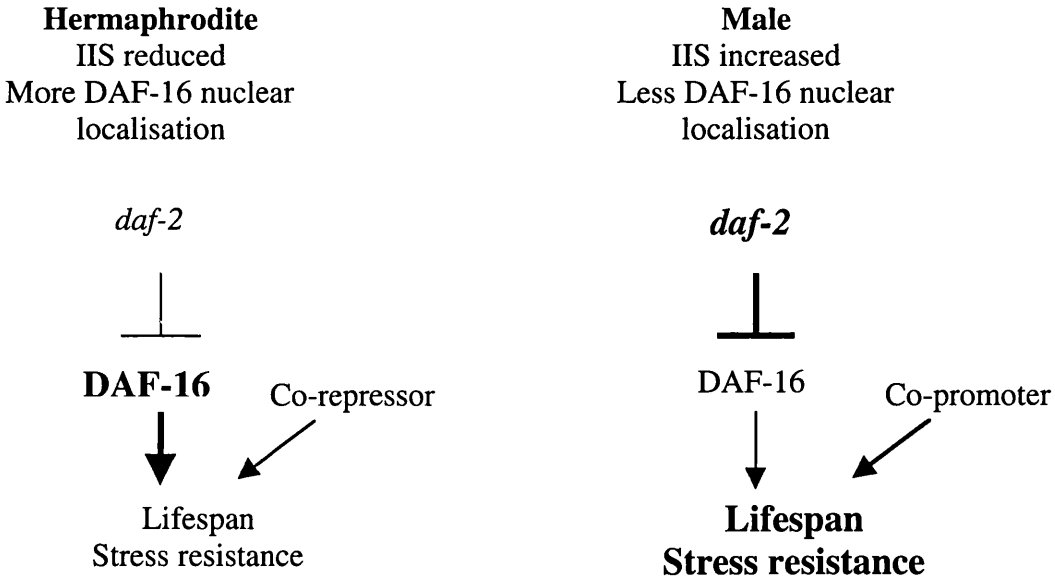
Measurement of DAF-16::GFP nuclear localisation in a wild-type genetic background under non-stress conditions found little difference between the sexes, although levels of nuclear localisation were slightly higher in hermaphrodites (Figure 2.11). Thus, if sex differences in DAF-16 activity were responsible for wild-type sex differences in lifespan, dauer formation or stress resistance, they were not readily visible using the DAF-16::GFP marker. However, it remained possible that under conditions of stress, which result in nuclear accumulation of DAF-16 (Henderson & Johnson 2001; Lin *et al* 2001), an increased male response may become apparent. However, contrary to expectations, nuclear localisation of DAF-16::GFP occurred more readily in hermaphrodites than males in response to oxidative, heat and starvation stresses (Figures 2.12-2.14).

Although these results appear not to support an intrinsic down-regulation of IIS in males, it remained possible that reduced IIS in males results in reduced DAF-16 nuclear localisation following stress due to constitutively higher levels of stress defences. If so, a similarly reduced response might be expected in *daf-2* mutant hermaphrodites relative to *daf-2*(+) controls. However, following heat stress, but not under control conditions at 15°C, the *daf-2(m577)* mutant showed markedly increased DAF-16::GFP nuclear localisation relative to *daf-2*(+) controls (Figure 2.15). Thus, an intrinsic reduction in IIS does not result in a reduced stress response in terms of DAF-16 localisation. Note, however, that a possible complication would be if overexpression of DAF-16 by the *IsDAF-16::GFP* strain resulted in increased stress resistance, making comparison of wild-type and *daf-2* mutants invalid. It would therefore be interesting to measure stress resistance of the *IsDAF-16::GFP* strain relative to wild type.

These results suggest that IIS upstream of the cellular localisation of DAF-16 may be down-regulated in hermaphrodites relative to males, the opposite scenario to that suggested by the working hypothesis. However, the increased stress resistance and lifespan of males were both dependent upon *daf-16*, strongly suggesting that DAF-16 activity is higher in males. How might these findings be reconciled? If the results obtained with the DAF-16::GFP strain are representative of the wild-type situation, they

suggest that although there may be constitutively down-regulated signalling from *daf-2* in hermaphrodites resulting in greater localisation of DAF-16 to the nucleus, DAF-16 activity within the nucleus (or a lifespan-promoting *daf-16*-dependent function) may be higher in males than hermaphrodites. For example, there may be sex differences in DAF-16 co-regulators, resulting in differences in transcriptional activity of target genes (Figure 2.17). Specifically, co-repressors and/or co-promoters of the activity of DAF-16 that promotes lifespan may be down- and up-regulated, respectively, in males relative to hermaphrodites. Thus, a new hypothesis is that sex differences in DAF-16 activity may still account for the increased male dauer formation, lifespan and stress resistance phenotypes, but at the level of DAF-16 transcriptional activity rather than DAF-16 cellular localisation.

Figure 2.17: Sex differences in DAF-16 activity may occur at the level of both cellular localisation and transcriptional co-regulation.



However, another possibility is that the results of the DAF-16::GFP experiments do not reflect differences in IIS in the wild-type. The transgene insertion in the *IsDAF-16::GFP* strain used is likely to include multiple copies and hence result in over-expression of *daf-16*, as implied by the Daf-c phenotype of this strain at 25.5°C (Henderson & Johnson 2001). If so, intrinsically reduced IIS in males relative to hermaphrodites could result in

higher DAF-16 activity in males due to its nuclear localisation, as proposed in the original working hypothesis. There are therefore two hypotheses which could explain the results obtained in this chapter, hence both are considered below in relation to lifespan, dauer formation and stress resistance data.

2.3.2 Intrinsic longevity of wild-type males is absent in *daf-16(0)* mutants

Increased male lifespan was no longer seen in the null mutant *daf-16(mgDf50)* (Table 2.1, Figure 2.3f). Overall, *daf-16(mgDf50)* hermaphrodite median lifespan was slightly higher than that of males, while *daf-16(mgDf50)* male maximum lifespan was only 18% longer than that of hermaphrodites, compared with 41% in wild type. This dependence of the increased male longevity on *daf-16* is consistent with the previous finding that *daf-16(m26)* male median and maximum lifespans were shorter than and equal to those of hermaphrodites, respectively (Gems & Riddle 2000b). It should be noted, however, that N2 male median lifespan was shorter than that of hermaphrodites in (Gems & Riddle 2000b), hence the *daf-16(m26)* result should be interpreted cautiously. Even so, *daf-16(m26)* was also found to suppress the male-specific increase in lifespan due to *unc-32* and *unc-4* mutations (Gems & Riddle 2000b; see Chapter 3).

Together these results imply a role for *daf-16* in regulation of sex differences in lifespan: specifically that DAF-16 activity is increased in males relative to hermaphrodites, with removal of DAF-16 function by null mutation resulting in a loss of this sex difference. As discussed in Section 2.3.1, this could be due to reduced IIS (the original working hypothesis) or increased co-activation of DAF-16 activity (the new working hypothesis) in males. If the latter possibility applies, one possibility is that sex differences in gonadal signalling play a role in regulating DAF-16 activity, since a *daf-16*-dependent germline signal has been identified in hermaphrodites (Hsin & Kenyon 1999; see Chapter 4). Interestingly, there appear to be intrinsic sex differences in regulation of lifespan by germline signalling, with ablation of the germline precursors resulting in a smaller lifespan increase in males under certain conditions (see Chapter 4). Thus, DAF-16 activity may be suppressed by the germline to a smaller extent in males than hermaphrodites. How germline signalling may affect DAF-16 activity is unclear, since although laser ablation of the germline does result in a certain amount of DAF-16

nuclear localisation (specifically in the young adult intestine) (Lin *et al* 2001), this effect is not nearly as marked as that seen in lifespan-extending *daf-2* mutants (Lin *et al* 2001; Henderson & Johnson 2001). It is therefore possible that the hermaphrodite germline signal suppresses DAF-16 activity largely by acting as a co-repressor, as illustrated in Figure 2.17.

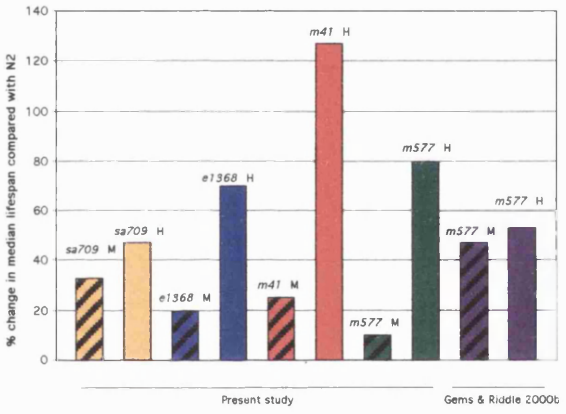
2.3.3 Intrinsic longevity of wild-type males is absent in *daf-2(rf)* mutants

Increased male lifespan was no longer apparent in three class 1 *daf-2* mutants (Table 2.1, Figure 2.3), a finding apparently consistent with the original working hypothesis that IIS is down-regulated in males relative to hermaphrodites. However, as described in Section 2.3.1 above, the DAF-16::GFP localisation studies suggest the opposite: that IIS may be intrinsically down-regulated in hermaphrodites. Moreover, the finding that males remained the longer-lived sex in the IIS mutant *pdk-1(sa709)* also suggests that intrinsic sex differences in IIS do not underlie the wild-type male longevity bias (Table 2.1, Figure 2.3e). However, alternative reasons for this apparent exception are: (a) *sa709* is too weak a mutation to remove the sex difference in IIS (supported by the smaller effect of *pdk-1(sa709)* on hermaphrodite lifespan compared with the *daf-2* mutations) or (b) redundancy of *pdk-1*: PI3K signalling directly to *akt-1* and *akt-2* (see Introduction Figure A.8) may by-pass the requirement for this gene in increased male lifespan. This would be consistent with the finding that inhibition of *pdk-1* by RNAi does not result in nuclear localisation of DAF-16::GFP, while RNAi inhibition of *daf-2* and *akt-1/akt-2* does (Henderson & Johnson 2001). The significance of the endurance of the male longevity bias in *pdk-1(sa709)* is therefore unclear.

Loss of the male longevity bias in the class 1 *daf-2* mutants occurred because the effects of these mutations on lifespan were markedly smaller in males than hermaphrodites (Figure 2.18), resulting in *daf-2* mutant hermaphrodites being longer-lived than males. Note that the larger effect of *daf-2* mutations on hermaphrodite lifespan was at least in part due to the fact that control N2 hermaphrodite lifespan in this experiment was slightly shorter than typically seen under these conditions. Even so, male median lifespans were only slightly increased in all three *daf-2* mutants in the present study (Figure 2.18), and male maximum lifespan was the same as that of wild

type for two of the *daf-2* mutants (Table 2.1). This contrasts with similar magnitudes of lifespan increase in males and hermaphrodites due to *daf-2(m577)* in a previous study on agar plates (Gems & Riddle 2000b). Moreover, the median lifespan of *daf-2(m577)* males at 22.5°C in the present study (22 days) was markedly lower than that of those at 20°C in (Gems & Riddle 2000b) (34 days), despite the increased penetrance of the *daf-2(rf)* Age phenotype at higher temperatures. Note also that class 2 *daf-2* mutant males were consistently longer-lived than hermaphrodites at 22.5°C in all experiments in this project (see Chapters 3 & 5), although pleiotropic effects of these mutations makes interpretation difficult (see Chapter 3).

Figure 2.18: Effect of a *pdk-1* and three class 1 *daf-2(rf)* mutations on male (striped bars) and hermaphrodite (plain bars) median lifespans (22.5°C). (Gems & Riddle 2000b)- agar plates at 20°C.



The shortevity of the class 1 *daf-2(rf)* males is inconsistent with the working hypothesis of this chapter, which, arguably, would predict that while male lifespan might be increased by *daf-2* mutations to a smaller extent than hermaphrodite lifespan (due to an intrinsic down-regulation of *daf-2* function in males), attenuation of IIS to a similar level/ removal of sex differences in IIS flux would result in *daf-2* mutant males and hermaphrodites having similar lifespans (Figure 2.2). The short lifespans of class 1 *daf-2(rf)* males are therefore difficult to interpret. One possibility is that under certain conditions (such as liquid culture) there are sex differences in production/ function of the INS ligands involved in IIS. Since the class 1 *daf-2* mutations used above all affect the ligand-binding domain of DAF-2 (see Appendix Section E.4), ligand binding to those DAF-2 regions affected by the mutations may play a larger role in IIS in hermaphrodites

than males under conditions of the present study. Such mutations would therefore disrupt IIS, and hence extend lifespan, more in hermaphrodites than males. Note that this would invalidate the model presented in Figure 2.2, which suggests that *daf-2(rf)* mutations either reduce IIS in males and hermaphrodites to the same level, or that IIS mutations may cause IIS to fall beneath a threshold level, eliminating sex differences in flux.

Alternatively, pleiotropic effects may have reduced class 1 *daf-2(rf)* male lifespan, perhaps as a result of liquid culture. Perhaps class 1 *daf-2(rf)* males are more frail (subject to more random effects on survival) and experience higher levels of age-independent mortality than hermaphrodites. Such an effect has previously been described in studies of *Drosophila* mutant for *chico*, the homologue of mammalian IRS-1-4. As described in the Main Introduction, homozygous mutant females are dwarf and long-lived, while ch^1/+ females display normal body size, and increased lifespan to an extent intermediate between that of ch^1/ch^1 and wild type (Tu *et al* 2002; Clancy *et al* 2001). ch^1/+ males are slightly (Clancy *et al* 2001) or markedly (Tu *et al* 2002) longer-lived than wild type, since $chico^1$ also slows the demographic rate of ageing in males (Tu *et al* 2002). However, ch^1/ch^1 males are not long-lived, and in some instances are shorter-lived than wild type (Tu *et al* 2002; Clancy *et al* 2001). This is because while the demographic rate of ageing in ch^1/ch^1 males is slowed, age-independent mortality and frailty are increased, reducing lifespan at least as much as the slowing of the ageing rate increases it (Tu *et al* 2002). It has been suggested that this is the result of pleiotropic effects associated with disrupted development, which may affect males more than the larger females (Tu *et al* 2002). In a similar manner to $chico^1$, heteroallelic mutation of *Drosophila InR* results in dwarfism and a marked increase in female lifespan, compared with high early mortality and reduced mortality only at late ages in males (Tatar *et al* 2001). Again, therefore, disruption of IIS may increase male age-independent mortality and frailty.

It is unclear whether the reduced size of *C. elegans* males relative to hermaphrodites would make them more frail and susceptible to age-independent mortality upon loss of IIS as proposed for ch^1/ch^1 male *Drosophila*. Unlike in *Drosophila* IIS mutants, *C. elegans daf-2* mutants are not dwarf, but rather are slightly

larger than wild type (McCulloch & Gems 2003). Thus, if male age-independent mortality and frailty are increased by disruption of IIS in *C. elegans* it is not clear by what mechanism this would occur. However, the fact that the *daf-2* mutant male Age phenotype is reduced in liquid culture relative to agar plates suggests that liquid culture could increase male frailty and age-independent mortality. Whether or not this is the case under any culture conditions remains to be determined. Large-scale mortality rate analysis comparing IIS mutant males and hermaphrodites would allow determination of any sex differences in age-independent mortality and frailty.

In an interesting parallel to the *C. elegans* and *Drosophila* findings considered above, studies of mice demonstrate sex differences in response to mutations affecting the IGF-1/ growth hormone (somatotrophic) axis. Firstly, mutation of the *Prop-1* gene, which is involved with pituitary development and hence growth hormone production, increases lifespan by 64% in females compared with just 49% in males (Brown-Borg *et al* 1996). Moreover, plasma insulin levels are normal in *Prop-1* mutant females but are reduced in males (see (Bartke 2000)). Growth hormone is a promoter of IGF-1, hence the effects of *Prop-1* mutations on lifespan may reflect down-regulation of IGF-1 due to reduced GH production (see (Hsieh *et al* 2002)). Support for this is provided by the fact that *Igf1r* heterozygote knockout mice are long-lived (Holzenberger *et al* 2002). Interestingly, as in *Prop-1* mutants, the increase in mean lifespan in $Igf1r^{+/-}$ mutant females (33%) is markedly greater than that in males (16%). Moreover, abnormal regulation of blood glucose is only seen in male $Igf1r^{+/-}$ mutants (Holzenberger *et al* 2002), while growth deficits due to $Igf1r^{+/-}$ mutation in many organs and tissues, including bone, muscle, brain and particularly adipose tissue are more severe in males (Holzenberger *et al* 2001).

Thus, male-specific pleiotropic effects may reduce the increase in male lifespan due to mutation of *Prop-1* and *Igf1r*, perhaps because these males are more frail or experience more age-independent mortality, like *Drosophila* ch^1/ch^1 males. For example, it has been suggested that the reduced glucose tolerance seen only in male $Igf1r^{+/-}$ mutants may have led to diabetic symptoms and hence masked a potentially greater increase in male lifespan due to the mutation (Holzenberger *et al* 2002). Potentially, such effects could be due to intrinsic sex differences in IGF-1 signalling (Holzenberger

et al 2002), the existence of which is supported by the fact that levels of plasma IGF-1 are higher in females than males in most primates (including humans), and higher in males than females in non-primate mammals (for review see (Gatford *et al* 1998)). Such sex differences in IGF-1 signalling could be mediated by demonstrated sex differences in GH secretion patterns (Mauras *et al* 1996, Gatford *et al* 1998), or differential regulation of local IGF-1 production/ activity by androgens and/or oestrogens in the two sexes (Holzenberger *et al* 2001; 2000, Gatford *et al* 1998). Although expensive, it would be interesting to determine whether IGF-1 mutant male mice display increased rates of age-independent mortality and frailty relative to females, as seen in *Drosophila chico*^{1} mutants.

If intrinsic sex differences in IIS/ IGF-1 signalling do underlie sex differences in response to IIS/ IGF-1 signalling mutations, it is unclear whether this would have any bearing on wild-type sex differences in lifespan. Results for *C. elegans* presented above suggest that the increased wild-type male lifespan may be due to intrinsic up-regulation of DAF-16 activity, but it is unclear whether this is at the level of cellular localisation in response to IIS (due to intrinsic sex differences in IIS levels) or in the level of co-regulators of DAF-16 activity downstream of IIS, similar to the promotion of IGF-1 activity in target tissues by androgens in mammals (see (Holzenberger *et al* 2001)). Further studies would be required before any role(s) of IIS/ IGF-1 signalling in sex differences in lifespan can be understood.

2.3.4 Effect of TGF-β/cGMP mutations on male longevity bias

daf-12, the target gene of TGF-β and cGMP signalling, is not required for the increased lifespan of males, since males were still the longer-lived sex in the null mutant *daf-12(rh61rh411)* (Table 2.1, Figure 2.3g). This was confirmed in subsequent studies using the mutants *daf-12(rh61rh411)* (Chapter 4, Tables 4.1 & 4.3) and *daf-12(m20)* (Chapter 5, Table 5.1). Even so, the findings that (a) there was no significant difference between male and hermaphrodite survival in the TGF-β mutant *daf-1(m40)*, and (b) that hermaphrodites were the longer-lived sex in the cGMP mutant *daf-11(m47)*, suggest that elements of TGF-β and cGMP signalling upstream of *daf-12* may be involved in sex differences in lifespan. However, while *daf-1(m40)* male and hermaphrodite median

lifespans were not statistically distinguishable, the maximum lifespan of males was 32% higher than that of hermaphrodites, implying that increased male survival was still apparent here, but only at later ages, as is often the case for N2 (see Chapter 1). Unfortunately, hermaphrodite sample sizes were small, especially for *daf-1(m40)* as a large proportion were censored due to the high incidence of matricide in this strain. This is typical of TGF-β mutant hermaphrodites due to their egg-laying defective phenotype (Trent *et al* 1983). Repeats of male and hermaphrodite TGF-β and cGMP survival analyses would be necessary before any conclusions can be drawn. Including the mitosis inhibitor FuDR in the medium would reduce instances of matricide and should allow larger sample sizes to be obtained.

Note that unexpectedly, median lifespans of *daf-1(m40)* and *daf-11(m47)* hermaphrodites (both 20 days) were significantly different from that of wild type (15 days), while previous work on agar plates found TGF-β/cGMP mutant hermaphrodites not to be long-lived (Kenyon *et al* 1993; Larsen *et al* 1995). It seems likely that because the median lifespan of N2 hermaphrodites was uncharacteristically short in this trial, a larger difference between N2 and *daf-1(m40)/ daf-11(m47)* median lifespans was apparent than might be expected. Further experiments investigating the effects of TGF-β and cGMP mutations on hermaphrodite lifespans under different conditions are required before these findings can be interpreted.

2.3.5 Increased tendency of males to form dauers

Results presented above confirmed the previous finding that wild-type males form dauers more readily than hermaphrodites (Ailion & Thomas 2000), although male and hermaphrodite dauer formation values were not consistently significantly different due to high variation between replicates (Table 2.2). In a range of IIS mutants, however, the male bias to dauer formation was no longer seen (Table 2.3). In two of the five IIS mutant strains (*daf-2(m41)* and *daf-2(e1370)*) there was no significant difference between the sexes in the tendency to form dauers, while in the other three (*daf-2(e1368)*, *daf-2(m577)* and *pdk-1(sa709)*), hermaphrodites were significantly more likely to form dauers than males. These results are in agreement with the previous casual observation that there is no male bias to dauer formation in the IIS mutants *unc-31(rf)* or *unc-64(rf)*

(Ailion & Thomas 2000). They also parallel the finding that hermaphrodites were the longer-lived sex in three class 1 *daf-2* mutants above (Table 2.1). However, in this instance, *pdk-1(sa709)* resulted in loss of the male bias to dauer formation, while it did not result in loss of the male longevity bias. Possibly, therefore, loss of both the increased male dauer formation and lifespans in class 1 *daf-2* mutants is coincidental and does not reflect underlying regulation of these traits by a common mechanism as proposed in the Introduction.

The fact that the male bias to dauer formation is lost or even reversed in IIS mutants suggests that IIS may be intrinsically down-regulated in males relative to hermaphrodites, consistent with the original working hypothesis of this chapter. However, as described in Section 2.3.1, DAF-16::GFP localisation studies suggest that IIS may in fact be slightly down-regulated in wild-type hermaphrodites relative to males, the opposite effect to that predicted. Possible explanations for the loss of the male bias to dauer formation in IIS mutants depend upon whether or not interpretation of the DAF-16::GFP results is correct.

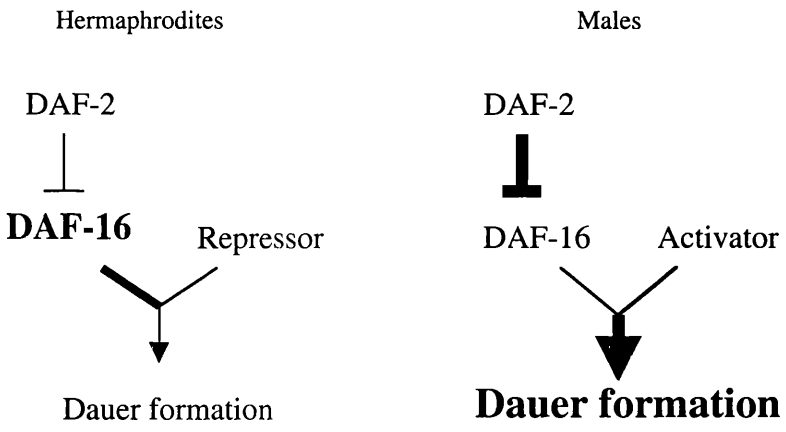
As a first scenario, consider that the DAF-16::GFP results do reflect the wild-type situation, and hence that IIS levels are reduced in hermaphrodites, but that DAF-16 activity is higher in males due to sex differences in DAF-16 co-regulation, proposed as a new hypothesis in Section 2.3.1. If so, this suggests that loss of the male bias to dauer formation in IIS mutants does not reflect removal of an intrinsically reduced level of IIS in males, but may simply reflect a larger effect of IIS mutations on dauer formation in hermaphrodites than males (as seen for lifespan in Section 2.3.3 above). Possibly, this is due to differential ligand use by the two sexes as proposed for lifespan above. However, since the dauer formation experiments were performed on agar plates, and since male lifespan was increased by *daf-2(m577)* to the same extent as hermaphrodite lifespan on agar plates in a previous study (Gems & Riddle 2000b), this explanation seems unlikely. Moreover, the male bias to dauer formation was also lost in the class 2 *daf-2* mutant *e1370*, (which carries a mutation in the *daf-2* region encoding the kinase domain (see Appendix E.4)) and in *pdk-1(sa709)*.

An alternative explanation is that IIS mutations eliminate subtle sex differences in DAF-16 activity that are only detectable when DAF-16 activity is low. As described

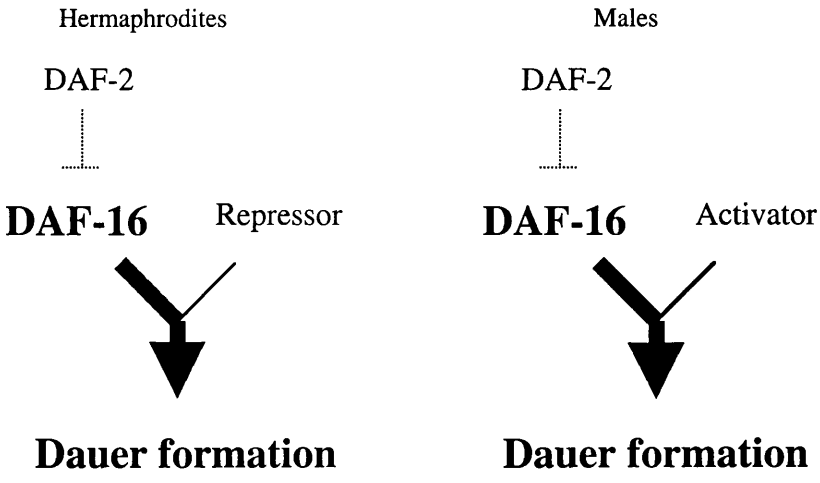
in Section 2.3.1 above, lifespan and stress resistance data strongly suggest that DAF-16 activity is higher in males relative to hermaphrodites. Assuming that the DAF-16::GFP data are representative, this would occur at the level of DAF-16 co-regulation rather than DAF-16 cellular localisation regulated by IIS. If so, the loss of the male bias to dauer formation in IIS mutants suggests that general up-regulation of DAF-16 activity by attenuation of DAF-2 signalling results in removal of subtle sex differences in DAF-16 activity (Figure 2.19). Thus, co-factors regulating DAF-16 activity may only exert an influence when DAF-16 activity is low.

Figure 2.19: Model for loss of male bias to dauer formation in IIS mutants assuming the DAF-16::GFP data do represent the wild-type situation. Sex differences in co-activators/ suppressors of DAF-16 only result in a male bias to dauer formation when DAF-16 activity is relatively low.

(a) *daf-2*(+). Repressors and activators make a large contribution to regulation of DAF-16 activity.



(b) *daf-2(rf)*. Repressors and activators make little contribution to regulation of DAF-16 activity.



The second scenario is that the DAF-16::GFP data are not representative of the natural situation and that IIS is not intrinsically down-regulated in hermaphrodites. If so, it remains possible that IIS levels are reduced in males, as proposed in the original working hypothesis, and that IIS mutations eliminate this sex difference as illustrated in Figure 2.2.

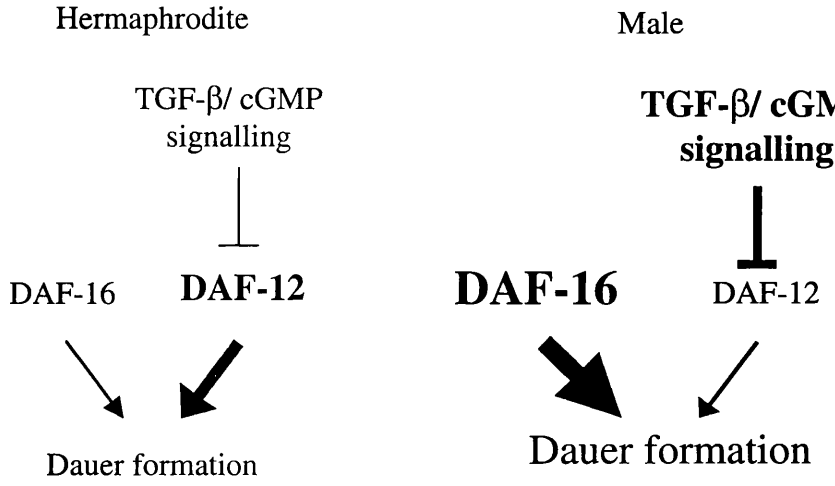
Whichever of the two hypotheses (if either) may apply to sex differences in DAF-16 activity, the situation is complicated by the finding that the male bias to dauer formation was markedly stronger in TGF-β mutants than in wild type (Table 2.4). In agreement with this, while non-crowded N2 males form no dauers at 20°C, males of the TGF-β mutant *unc-3(rf)* frequently form dauers at that temperature (Ailion & Thomas 2000). This effect was also noted during routine sub-culture of TGF-β and cGMP mutant male stocks for this study. The fact that the male bias to dauer formation was strengthened in these mutants implies that in wild type, sex differences in TGF-β signalling suppress dauer formation in males relative to hermaphrodites.

Differential levels of both DAF-16 activity and TGF-β/ cGMP signalling may therefore combine to produce the overall wild-type male bias to dauer formation. A hypothetical model for how this may occur is presented below (Figure 2.20). Here, DAF-16 activity is up-regulated in males relative to hermaphrodites (for one of the reasons proposed above), while TGF-β and/ or cGMP signalling is reduced in hermaphrodites, resulting in increased DAF-12 signalling. However, the increase in DAF-16 activity in males is greater than the reduction of TGF-β signalling in hermaphrodites, resulting in an overall increased tendency to form dauers by males. This would explain why reduction of TGF-β/ cGMP signalling removes the element of signalling that pushes hermaphrodites more strongly towards dauer formation, and reveals further the increased DAF-16 activity in males, resulting in an even stronger bias to male dauer formation than seen in wild type.

This model is complicated by the fact that the Daf-c phenotype of *daf-1(m40)* is partially suppressed by *daf-16(0)* mutations (Vowels & Thomas 1992), suggesting that a part of TGF-β signalling down-regulates DAF-16 activity to suppress dauer formation. It has been suggested that TGF-β signalling SMAD proteins (such as *daf-3* and *daf-8*) may interact with DAF-16, forming complexes on gene promoters (Ogg *et al* 1997). Based on

the model in Figure 2.20, if such an interaction were to exist, suppression of DAF-16 by TGF-β signalling would be stronger in males than hermaphrodites due to the higher levels of TGF-β signalling relative to hermaphrodites. This would further explain why mutation of TGF-β components increased the male bias to dauer formation, since removal of sex differences in TGF-β signalling would not only remove the sex difference in TGF-β signalling promoting hermaphrodite dauer formation, it would also remove the increased synergistic suppression of DAF-16 activity in males.

Figure 2.20: Model of interaction between DAF-16 and TGF-β signalling in regulation of the sex difference in dauer formation. DAF-16 activity is intrinsically up-regulated in males due to either reduced IIS or increased co-activation of DAF-16.



However, a problem with the above model is that when male: hermaphrodite partial dauer formation was measured for a *daf-16(mgDf50); daf-1(m40)* mutant, a male bias to (partial) dauer formation was still evident, whereas it might be expected that removal of sex differences in IIS and TGF-β signalling would result in no sex bias to dauer formation. Unfortunately, it was not possible to rule out a slight reduction in the *daf-1(m40)* male bias to dauer formation by *daf-16(0)* since male dauer formation by the *daf-1(m40)* control was maximised, which may have led to an underestimation of the M:H DFR. It therefore remains possible that the strong male bias is partially attributable to increased DAF-16 activity in males. However, since all sex differences in DAF-16 activity and TGF-β signalling had presumably been removed in this double mutant, the

male bias to partial dauer formation must have been due to sex differences in another element regulating dauer formation. Potentially, sex differences in *daf-12* expression/ activity may underlie the residual male bias to dauer formation. Specifically, results would imply that in males, dauer formation-promoting DAF-12b activity may be up-regulated relative to DAF-12a activity, which promotes reproductive development (see Chapter 5). That this is possible is supported by results in Chapter 5, which suggest that *daf-12* functions differently to regulate lifespan in the two sexes.

2.3.6 Sex differences in stress resistance

Data presented in Section 2.2.4 indicate that males are more resistant to certain stresses than hermaphrodites. Males showed increased survival/ recovery rates relative to hermaphrodites after both treatment with Paraquat (Figures 2.4-2.6) and heat shock (Table 2.7). In light of this, it is interesting to note that expression of *sod-2*, encoding the antioxidant enzyme Mn-superoxide dismutase, is up-regulated between 5.7 and 13.7 times in wild-type males relative to hermaphrodites (Stanford Microarray Database), although this is not the case for the four other *sod* genes.

Interestingly, when oxidative and heat stress trials were repeated in a *daf-16(0)* genetic background, increased male stress resistance was no longer seen, suggesting that *daf-16* is required for the male component of the stress response. This is consistent with there being increased DAF-16 activity in males relative to hermaphrodites, as discussed in Section 2.3.1 above. However, in *C. elegans*, *sod-3* but not *sod-2* expression is regulated by DAF-16 (Honda & Honda 1999), suggesting that the up-regulation of *sod-2* in males relative to hermaphrodites suggested by the Stanford Microarray Database may not reflect an intrinsic up-regulation of DAF-16 activity in males. Possibly, therefore, factors other than DAF-16 activity also contribute to the increased male stress resistance. An interesting alternative is that while DAF-16 does not regulate *sod-2* expression in hermaphrodites, it may do so in males, perhaps due to sex differences in co-regulation of DAF-16 activity.

In contrast to the increased male resistance to oxidative and heat stress, hermaphrodites survived significantly longer than males after UV irradiation. Why males should be more resistant to oxidative and heat stress, but not to UV stress, is

unclear. Conceivably, the methodology used may have resulted in the smaller males receiving equivalently more irradiation due to their increased surface area: volume ratio compared with hermaphrodites. Alternatively, it could be that response to UV stress is regulated at least in large part by a different mechanism from that regulating response to oxidative and heat stress. In this respect, previous work has found that while oxidative, heat and starvation stress result in localisation of DAF-16::GFP to the nucleus, UV irradiation does not (Henderson & Johnson 2001). In addition, while four DAF-16 over-expressing GFP strains were markedly more resistant to heat stress than controls, they displayed no increase in resistance to UV stress (Henderson & Johnson 2001). However, the fact that *daf-2* mutants show resistance to UV stress (Murakami & Johnson 1996) suggests that the dose of UV irradiation used by (Henderson & Johnson 2001) may have been insufficient to affect DAF-16 localisation. Since only one preliminary replicate was performed in the present study, further repeats would be desirable.

2.4 Conclusions

- Levels of nuclear localisation of DAF-16::GFP were slightly higher in hermaphrodites, and DAF-16::GFP localised to the nucleus faster following stress in hermaphrodites than in males, suggesting that IIS is lower in hermaphrodites.
- Males were both longer-lived and more resistant to oxidative and heat stress than hermaphrodites, in a *daf-16*-dependent manner. It therefore seems likely that increased DAF-16 activity in males underlies these male traits. However, because levels of DAF-16::GFP nuclear localisation were higher in hermaphrodites than males, and because the reliability of the DAF-16::GFP data is not certain, it is unclear whether sex differences in DAF-16 activity occur at the level of DAF-16 transcriptional activity (perhaps due to differential co-factor production) or IIS-regulated DAF-16 cellular localisation.
- Class 1 *daf-2* mutations only slightly increased male lifespan in liquid culture. However, it is unclear whether IIS plays a role in the regulation of the greater lifespan of wild-type males, since (a) DAF-16::GFP localisation studies suggest that

if anything, IIS is down-regulated in hermaphrodites and (b) male survival may have been pleiotropically reduced by *daf-2(rf)* in liquid culture.

- IIS mutations removed the male bias to dauer formation. This suggests that (a) IIS is down-regulated in males relative to hermaphrodites as proposed in the original working hypothesis or (b) that IIS mutations remove the male bias to dauer formation by overriding subtle sex differences in DAF-16 activity that are only apparent when DAF-16 activity is low.
- TGF-β mutations enhanced the male bias to dauer formation. TGF-β signalling may therefore be down-regulated in hermaphrodites relative to males. The overall sex bias to dauer formation could therefore be the result of a balance between levels of TGF-β signalling and DAF-16 activity in each sex.
- There was still a male bias to dauer formation in a *daf-16(0)*; *daf-1(0)* mutant. Thus, even with sex differences in DAF-16 activity and TGF-β signalling presumably removed, there is still a sex bias to dauer formation. This suggests that the sex bias to dauer formation involves another component, perhaps DAF-12 activity.

2.5 Future directions

To assess whether the inferences drawn from the DAF-16::GFP strain were correct, the experiments could be repeated using a DAF-16::GFP construct with a low copy number in a *daf-16(0)* background. It could be that the over-expression of *daf-16* in the GFP strain masks the sex differences in IIS proposed to occur in wild-type animals, and that the faster response of hermaphrodite DAF-16::GFP to stresses reflects a different underlying stress response mechanism.

Class 1 *daf-2* mutations extended male lifespan to a smaller extent than previously seen on agar plates. To investigate this further, it would be necessary to repeat survival analyses for males and hermaphrodites of a range of IIS mutants. This should be performed in liquid culture and on agar plates (perhaps in a non-Age *unc* genetic background to prevent male leaving), in order to determine whether culture conditions result in different responses by the two sexes to the mutations.

Survival analyses presented above suggested that in liquid culture, TGF-β and cGMP mutant hermaphrodites may be longer-lived than wild type, in contrast to previous studies on agar plates. Due to the high levels of matricide displayed by hermaphrodites of these mutants, sample sizes in these trials were small. It would therefore be desirable to repeat survival analyses for these mutants in the presence of the mitosis-inhibitor FuDR or in a mutant background such as *fer-15(rf)*, in order to prevent matricide from reducing sample size. Control wild-type hermaphrodite survival was also unusually low, hence a repeat trial would be beneficial.

Results in this chapter suggest that sex differences in DAF-16 activity and TGF-β signalling may underlie wild-type sex differences in dauer formation, although the fact that a male bias to dauer formation remained in a *daf-16(mgDf50)*; *daf-1(m40)* mutant implied that other factors may also be involved. However, due to consistent saturation of male dauer formation in the *daf-1(m40)* control at 22.5°C, the possibility could not be excluded that *daf-16(0)* suppressed the male bias to dauer formation to any extent, since the male bias to dauer formation by *daf-1(m40)* may have been understated. It was not possible to repeat this experiment at a lower temperature because the *daf-16(mgDf50)*; *daf-1(m40)* double mutant forms few partial dauers below 22.5°C. As an alternative, measurement of the sex bias to partial transient dauer formation in a *daf-16(0)* mutant compared with N2 (by raising animals in the presence of pheromone) might provide useful additional data.

Given that males were more resistant to oxidative and heat stress, it might have been expected that they would be more resistant to UV stress, which results in DNA damage in a similar manner to free radical action. Only one UV stress replicate was performed in the present study. It would therefore be beneficial to repeat the UV stress resistance trial having controlled UV dosage for the different surface area: volume ratios of males and hermaphrodites. This could determine whether hermaphrodites are truly more resistant to UV stress than males or whether the effect observed above was unrepresentative.

Chapter 3
Sex-specific effects of *unc* mutations on lifespan

Contents

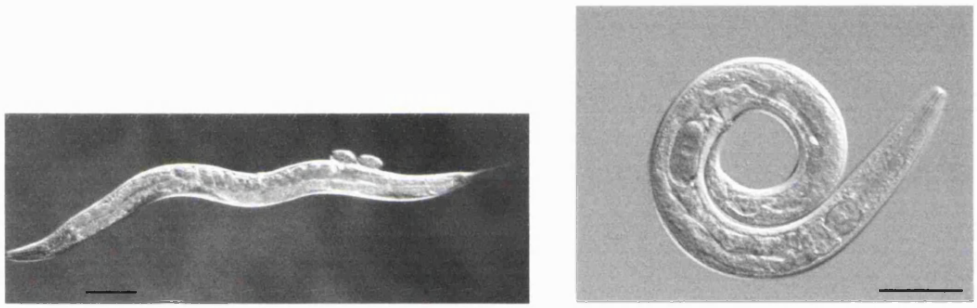
3.0 Introduction

The *unc* class of mutations

Uncoordinated (*unc*) mutations affect motility, with mutant phenotypes ranging from mild (e.g. an inability to reverse) to complete paralysis (Brenner 1974) (Figure 3.1). A number of *unc* mutants also display slow pharyngeal pumping, and males often have reduced or no ability to mate. *unc* mutations can be assigned to two overlapping categories: neuronal (which disrupt an element of neuronal development) and muscle (which cause defects in muscle structure or function).

Figure 3.1: DIC images of (a) wild-type young adult hermaphrodite (with eggs) and (b) an older *unc-32(e189)* hermaphrodite. This neuronal *unc* mutation results in a coiling phenotype. Scale bars= 100μm.

(a) (b)



Sex-specific effects of *unc* mutations on lifespan

Previous work on agar plates found that solitary male lifespan was extended by a range of *unc* mutations, while hermaphrodite lifespan was mainly unaffected (Gems & Riddle 2000b). The extent to which male lifespan was increased was found to correlate negatively with the mating efficiency (ME) of the *unc* strain. From this it was inferred that some element of wild-type male behaviour usually reduces lifespan, and that this behaviour is increasingly inhibited the more severe the *unc* mutation (Gems & Riddle 2000b). However, mutation of certain *unc* genes, such as *unc-64* and *unc-31* also increases hermaphrodite lifespan (Gems & Riddle 2000b; Ailion *et al* 1999), suggesting that the mechanism of male lifespan increase by *unc* mutations may not be entirely behavioural.

Even so, the effect of *unc* mutations is always more marked in males. Of the nine *unc* mutations studied in (Gems & Riddle 2000b), the three resulting in the largest increases in male median lifespan were: *unc-13(e51)* (ME0) (80%), *unc-4(e120)* (ME1) (50%) and *unc-32(e189)* (ME0) (60%). This contrasted with negligible effects on hermaphrodite median lifespan. Since all three of these mutations are of the neuronal class, it seemed possible that the mechanism of lifespan increase by neuronal *unc* mutations could be disruption of a neuroendocrine component of lifespan regulation. If so, the increased male lifespan response to *unc* mutations could reflect underlying sex differences in neuroendocrine function.

A working hypothesis of this chapter was therefore that lifespan-extending *unc* mutations do so by disrupting neurosecretion. To investigate this possibility, lifespan was measured in males and hermaphrodites of neuronal and muscle *unc* mutants. If neurosecretory defects were at least in part responsible for the increased male longevity of *unc* mutants, then only neuronal, and not muscle *unc* mutations should result in increased male (and perhaps hermaphrodite) lifespan.

If neuronal *unc* mutations do act to increase lifespan via their effects on neuronal secretion, disruption of which element(s) of secretion could be responsible? One candidate is insulin-/IGF-1-like signalling (IIS), a neuroendocrine pathway known to regulate lifespan and dauer formation (see Main Introduction and Chapter 2). Previous work has shown that *daf-2*, the gene encoding the *C. elegans* homologue of mammalian insulin and IGF-1 receptors, is required in specific neuronal subsets for reproductive development and wild-type lifespan (Apfeld & Kenyon 1998) and that *ins* ligand genes are expressed in sensory neurons (Pierce *et al* 2001). It therefore seemed possible that disruption of IIS by neuronal *unc* mutations could increase lifespan in a manner similar to *daf-2* mutations. Consistent with such a possibility, the increases in male lifespan due to *unc-32(e189)* and *unc-4(e120)* are dependent on *daf-16*, the forkhead transcription factor that is the ultimate target of IIS (Gems & Riddle 2000b). In addition, mutation of *unc-64* and *unc-31* not only increases lifespan but also enhances dauer formation at high temperatures, and both these mutant phenotypes are dependent upon *daf-16* (Ailion *et al* 1999; Ailion & Thomas 2000). The neuronal mutant *unc-32(e189)* was therefore selected to investigate this further using epistasis analysis.

Role of the class 2 *daf-2* pleiotropic Unc phenotype in male longevity

As described in the Main Introduction (Figure A.7), *daf-2* alleles can be grouped into two classes according to their mutant phenotypes (Gems *et al* 1998). Both sexes of class 2 *daf-2* mutants display a range of pleiotropic effects at restrictive temperatures, including reduced motility (Unc). Males of such mutants are extremely long-lived relative to hermaphrodites at restrictive temperatures. Since most *unc* mutations enhance male, but not hermaphrodite lifespan, it seemed possible that the extreme longevity of these males could be the result of their pleiotropic Unc phenotype. If so, it might be expected that *unc* mutations would not increase class 2 *daf-2(rf)* male lifespan further at restrictive temperatures. However, at permissive temperatures where no pleiotropic Unc phenotype is apparent, *unc* mutations might be expected to increase class 2 *daf-2(rf)* male lifespan to a similar extent as in a wild-type background. Conversely, the prediction would be that *unc* mutations would increase the lifespan of class 1 *daf-2(rf)* males (which show no ts pleiotropic Unc phenotype) to a similar extent as wild-type lifespan at all temperatures. In order to investigate this, the effect on male and hermaphrodite lifespan of *unc-32(e189)* at permissive and restrictive temperatures was determined.

The effect of *unc-32(e189)* on *daf-2* hermaphrodite lifespan was also of interest, because a previous study had suggested that there might be an interaction between *daf-2* and *unc-32* with respect to lifespan (Mair & Gems 1998). Specifically, *unc-32(e189)* had been found to enhance the lifespan of class 1 and class 2 *daf-2* mutant, but not wild-type hermaphrodites, at both permissive and restrictive temperatures (Mair & Gems 1998). It therefore seemed possible that attenuation of IIS resulted in a hermaphrodite lifespan response to *unc* mutations, suggesting that sex differences in IIS may underlie the differential response of males and hermaphrodites to *unc* mutations.

3.1 Materials and Methods

3.1.1 Strains employed

All alleles used were reduction-of-function unless specifically stated, and included: GR1307 *daf-16(mgDf50) I* (putative null allele) (Ogg *et al* 1997), GA20 *daf-16(m26) I* (putative null allele) (Riddle *et al* 1981), CB1370 *daf-2(e1370) III* (Riddle 1977), DR1564 *daf-2(m41) III* (Larsen *et al* 1995; Gems *et al* 1998), DR40 *daf-1(m40) IV* (Swanson & Riddle 1980) and AA86 *daf-12(rh61rh411) X* (putative null allele) (Antebi *et al* 1998). *unc* mutants employed were: GA2 *unc-13(e51) I* (putative null allele), CB120 *unc-4(e120) II* (putative null allele), CB189 *unc-32(e189) III*, CB306 *unc-50(e306) III*, CB66 *unc-22(e66) IV*, CB205 *unc-26(e205) IV* and CB155 *unc-27(e155) X*. References for *unc* mutant strains are provided in Table 3.1. Unfortunately it was only possible to include two muscle *unc* mutants in the trial due to the high incidence of deleterious pleiotropic effects associated with most muscle *unc* strains, which would have confounded lifespan measurements.

The *unc-32(e189)*; *daf-12(rh61rh411)* double mutant strain was constructed as follows. *daf-12(rh61rh411)* males were mated to *unc-32(e189)* hermaphrodites, generating *unc-32(e189)*/+; *daf-12(rh61rh411)*/+ hermaphrodite out-cross progeny. These were self-fertilised and Unc hermaphrodite progeny selected. These animals were all homozygous for *unc-32(e189)*, and at the *daf-12* locus were either +/+, *rh61rh411*/+, or *rh61rh411*/ *rh61rh411*. These were self-fertilised at 25°C and left to starve. Those giving rise to no dauers were selected as the double homozygous mutant strain.

3.1.2 Survival Analyses

All survival analyses were performed in liquid culture as described in Main Materials and Methods. Due to the ts Daf-c nature of many of the strains, animals were raised at 15°C and at L4 were shifted to the experimental temperature stated. At least two replicates were performed for each strain unless specifically stated.

3.1.3 Dauer formation assays

Strains were raised on NGM plates at 15°C. At the L4 stage, hermaphrodites were placed one-per-plate at the stated temperature. After 24 hours, hermaphrodites were transferred to fresh plates leaving behind cohorts of progeny. Cohorts were scored for dauer/ L2d formation at fixed points (20°C: 96 hours, 22.5°C: 80 hours, 27°C: 44 hours) following mid-egg-lay as previously described (Gems *et al* 1998; Ailion & Thomas 2000). The proportions of dauers/ L2ds and L3+ animals were then calculated. For analysis, animals scored as L2d were pooled with those scored as dauer.

3.1.4 DAF-16::GFP studies

These were performed as described in Chapter 2 Section 2.1.7, using the same *IsDAF-16::GFP* strain (TJ356) (Henderson & Johnson 2001). The *unc-32(e189)*; *IsDAF-16::GFP* strain was constructed as follows. *unc*/+ males were generated by mating wild-type males with *unc* hermaphrodites. The *unc*/+ males were then mated with *IsDAF-16::GFP* hermaphrodites. 50% of out-cross progeny were heterozygous *unc*, and all were heterozygous *IsDAF-16::GFP*. Out-cross progeny were cloned and selfed. 1/4 of the resulting progeny were homozygous *unc*, and 3/4 of these were Rol (2/3 heterozygous and 1/3 homozygous). Unc; Rol animals were cloned and selfed, and those giving rise to all-Rol progeny selected. Males of this strain were generated by mating males heterozygous for *unc-32(e189)* to *unc-32(e189)*; *IsDAF-16::GFP* hermaphrodites and selecting Unc Rol progeny for viewing. These were homozygous for recessive *unc-32* and heterozygous for dominant *IsDAF-16::GFP*. For comparability, hermaphrodites heterozygous for *IsDAF-16::GFP* were also employed.

3.2 Results

3.2.1 Effect of neuronal and muscle *unc* mutations on lifespan

To investigate the mechanism of lifespan extension in *unc* males, lifespans of both sexes of five neuronal and two muscle *unc* mutants were compared (Table 3.1). Preliminary trials had indicated that there were possible interactions between lifespan and liquid culture in certain *unc* mutants, since slight increases in *unc-4(e120)* and *unc-32(e189)*

hermaphrodite lifespan had been recorded in liquid culture but not on agar plates (data not shown). In case culture conditions had an effect on results, one trial was performed on agar plates and another in liquid culture (Tables 3.2a & b, respectively).

It was not possible to run wild-type controls on agar plates due to the leaving behaviour of solitary males. The lifespans of *unc* mutants of both sexes on plates were therefore compared with those of wild-type animals in the liquid culture trial, since N2 lifespan in liquid culture had previously been demonstrated to be similar to those seen on plates (see Chapter 1). Note, however, that wild-type lifespans are sometimes slightly longer in liquid culture than on plates, hence comparing *unc* mutant survival on plates with N2 survival in liquid culture may result in a slight underestimation of lifespan extension. Survival analyses were performed at 20°C in order to provide comparability with previous work using *unc* mutants (Gems & Riddle 2000b). Unfortunately, lifespan measurements of *unc-22(e66)* on agar plates had to be abandoned due to microbial contamination, hence data for this mutant are only available in liquid culture.

Muscle unc *mutations*

unc-27(e155) hermaphrodites on agar plates were significantly shorter-lived than N2 controls, (Table 3.2a). However, comparing *unc* lifespans from plates with N2 lifespans in liquid culture may slightly understate the lifespans of *unc* mutants relative to N2 (see above). In liquid culture, however, *unc-27(e155)* and *unc-22(e66)* hermaphrodites were slightly longer-lived than N2 (Table 3.2b)

In males, *unc-27(e155)* slightly increased lifespan both on agar plates and in liquid culture. However, *unc-22(e66)* male survival was decreased in liquid culture. Overall, therefore, the muscle *unc* mutations had slight but sometimes significant effects on male and hermaphrodite lifespan, with lifespan being increased in three instances and decreased in two.

Neuronal unc *mutations*

The only neuronal *unc* mutation to increase hermaphrodite median lifespan on agar plates was *unc-32(e189)*, by 28% (Table 3.2a, Figure 3.2a). In two other neuronal *unc* mutants on agar plates (*unc-4(e120)*, *unc-26(e205)*), only hermaphrodite maximum

lifespan was increased, although overall survival was significantly longer than that of N2 (Figure 3.2a). The one neuronal *unc* mutation that did not significantly affect hermaphrodite survival on agar plates was *unc-13(e51)*, while *unc-50(e306)* significantly reduced hermaphrodite lifespan (Table 3.2a).

In liquid culture, neuronal *unc* hermaphrodite survival was significantly higher than that of N2 for all mutants except *unc-26(e205)* and *unc-50(e306)*. Again, maximum rather than median hermaphrodite lifespans were typically increased, except for *unc-4(e120)*, which showed a robust increase in median lifespan (75%) (Table 3.2b, Figure 3.2b). This increase in *unc-4* mutant hermaphrodite survival was much greater than that seen on plates. Since *unc-4(e120)* does not increase male lifespan in liquid culture any more than it does on plates, this indicates that there is an interaction between *unc-4*, liquid culture and the hermaphrodite gender.

Male survival on agar plates was significantly longer than that of N2 for all neuronal *unc* mutants except for *unc-26(e205)* (Table 3.2a). In all cases except *unc-26(e205)*, median lifespan was robustly increased, and the increases in maximum lifespan were markedly larger than any seen in hermaphrodites. For example, *unc-32(e189)* extended male median and maximum lifespans by 70% and 78%, compared with just 28% and 19%, respectively, for hermaphrodites (Figure 3.2a).

In liquid culture male survival was significantly increased relative to that of N2 for all neuronal *unc* mutants (Table 3.2b). Furthermore, the magnitude of increase was greater than that seen on agar plates (with the exception of *unc-4(e120)*). It would therefore appear that maintaining males in liquid culture enhances the longevity response to neuronal *unc* mutations. As seen on agar plates, the effects of neuronal *unc* mutations on male median and maximum lifespans were much greater than in hermaphrodites.

Table 3.1: Nature of the neuronal and muscle *unc* mutations employed for survival analysis

Genotype	Neuronal or muscle class?	Mating efficiency*	Phenotype	Protein characterisation	Previous findings with respect to lifespan
unc-4(e120) II[a]	N	1	Moves forward well; unable to back	Homeoprotein expressed in VA motorneurons	Male: increase in median & max. lifespan; Hermaphrodite: increase in max. lifespan[c]
unc-13(e51) I[a]	N	0	Paralysed. Pharyngeal movement irregular.	Required for synaptic vesicle docking	Male: increase in median & max. lifespan; Hermaphrodite: *e51* increase in max. lifespan only[c], all other alleles sig. increase in survival[g]
unc-22(e66)[a]	M	0	Twitching during normal movement.	Interacts with and regulates myosin	---
unc-26(e205) IV[a]	N	0	Slow growth, Unc and small	Synaptojanin homologue required for synaptic vesicle recycling	Significant increase in hermaphrodite mean lifespan[d]
unc-27(e155) X[a]	M	2	Sluggish, poor backing, slightly dumpy. Abnormal body muscle.	----	----
unc-32(e189) III[a]	N	0	Severe Unc. Coiler.	Subunit of vacuolar ATPase, expressed in cholinergic neurons	Significant increase in male median and maximum lifespan[c]
unc-50(e306) III[b]	N	1	Kinker Unc. Levamisole resistant.	Novel protein possibly required for assembly of AChR[e]	No effect on male or hermaphrodite lifespan[f]

[a](Brenner 1974); [b](Lewis *et al.* 1980); *Mating efficiency as determined previously (Hodgkin 1983); [c](Gems & Riddle 2000b); [d](Lakowski & Hekimi 1998); [e](Hengartner *et al* 1992); [f](Arantes-Oliveira *et al* 2002); [g](Munoz & Riddle 2002).

Table 3.2a: Effect of neuronal and muscle *unc* mutations on male and hermaphrodite lifespan (agar plate culture, 20°C)

Genotype (neuronal/ muscle)	Males					Hermaphrodites				
	Median lifespan (days) ± 95% C.I	Max. lifespan (days)	% effect on N2 median & max. lifespan#	N*	*P*†	Median lifespan (days) ± 95% C.I	Max. lifespan (days)	% effect on N2 median & max. lifespan#	N*	*P*†
+	NA	NA	-	-	-	NA	NA	-	-	-
unc-4(e120) N	39.0 (48.0, 29.0)	63.0	+95; +133	35 (100)	<0.0001	20.0 (26.0, 17.0)	43.0	± 0; +59	25 (80)‡	0.007
unc-13(e51) N	24.0 (25.0, 24.0)	39.0	+20; +44	81 (82)	0.0005	17.0 (25.0, 13.5)	32.0	-15; +19	22 (72)‡	0.803
unc-26(e205) N	19.0 (22.0, 19.0)	35.0	-5 ; +30	28 (31)[1]	0.393	17.0 (20.0, 17.0)	22.0	-15; +10	57 (90)	0.023
unc-32(e189) N	34.0 (36.0, 32.0)	48.0	+70; +78	75 (100)	<0.0001	25.5 (25.0, 20.0)	32.0	+28; +19	48 (120)	<0.0001
unc-50(e306) N	16.0 (19.0, 11.0)	27.0	-20; ±0	26 (53)	0.051	16.0 (16.0, 16.0)	21.5	-20; -22	133 (180)	0.003
unc-27(e155) M	21.0 (24.0, 16.0)	37.5	+5; +39	54 (100)	0.028	16.5 (19.5, 16.5)	19.0	-18; -5	93 (120)	<0.0001

#Compared with wild type lifespan from liquid culture trial (Table 3.2b); *Number of senescent deaths (starting population); [1]Results from one replicate only due to contamination of second replicate; ‡High proportion of censors due to matricide; †Probability that *unc* mutant and wild-type survival differ by random chance (log rank test), using wild-type survival data from Table 3.2b.

Table 3.2b: Effect of neuronal and muscle *unc* mutations on male and hermaphrodite lifespan (liquid culture, 20°C)

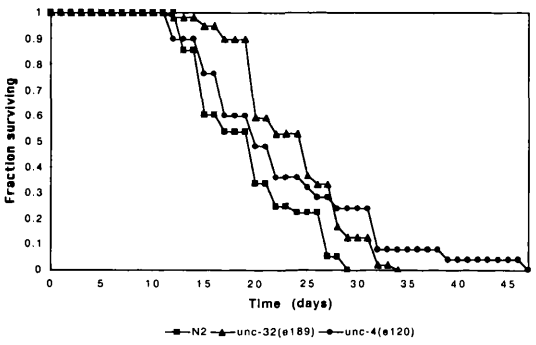
Genotype (neuronal/ muscle)	Males					Hermaphrodites				
	Median lifespan (days) ± 95% C.I	Max. lifespan (days)	% effect on N2 median & max. lifespan	N*	*P*†	Median lifespan (days) ± 95% C.I	Max. lifespan (days)	% effect on N2 median & max. lifespan	N*	*P*†
+	20.0 (22.0, 20.0)	27.0	-	55 (60)	-	20.0 (20.0, 15.0)	27.0	-	44 (60)	-
unc-4(e120) N	37.5 (41.5, 34.0)	64.5	+88; +140	77 (88)	<0.0001	35.0 (35.0, 30.0)	44.0	+75; +63	31 (60)	<0.0001
unc-13(e51) N	47.0 (51.0, 42.0)	70.0	+135; +159	32 (57)	<0.0001	18.0 (24.0, 16.0)	39.0	-10; +44	32 (60)	0.03
unc-26(e205) N	27.0 (34.0, 22.0)	50.0	+35; +85	23 (24)‡	0.0001	18.0 (21.0, 16.0)	23.0	-10; -15	25 (60)	0.45
unc-32(e189) N	42.0 (44.5, 37.5)	67.5	+110; +150	95 (110)	<0.0001	21.0 (25.0, 18.0)	38.0	+5; +41	50 (60)	0.008
unc-50(e306) N	21.0 (25.0, 18.0)	33.5	+5; +24	37 (40)	0.05	21.0 (21.0, 17.0)	28.0	+5; +4	28 (60)	0.11
unc-22(e66) M	16.0 (18.0, 15.0)	23.0	-20; -15	31 (44)	0.002	24.0 (30.0, 18.5)	35.0	+20; +30	26 (60)	0.001
unc-27(e155) M	22.5 (24.0, 21.0)	35.5	+13; +31	98 (120)	0.0009	25.0 (28.0, 25.0)	30.0	+25; +11	45 (60)	<0.0001

*Number of senescent deaths (starting population); ‡Results from one replicate only due to contamination of second replicate; †Probability that *unc* mutant and wild-type survival differ by random chance (log rank test).

To summarise, the largest increases in lifespan were seen in neuronal *unc* mutants, with the exception of *unc-50(e306)* which only extended lifespan by a small amount. Although hermaphrodite lifespan was increased by neuronal *unc* mutations in several instances, this effect was smaller than in males. Overall, liquid culture generally slightly enhanced the lifespan increase due to *unc* mutations compared with agar plates. The increase in male lifespan due to *unc* mutations would not appear to be due to suppression of deleterious male behaviours, since two of the mutations that are ME0 or ME1 (*unc-22(e66)* and *unc-50(e306)*, respectively), resulted in no lifespan increase.

Figure 3.2a: Typical effect of two neuronal *unc* mutations on (a) hermaphrodite and (b) male survival on agar plates (20°C). *P*<0.0001 in all cases.

(a) Hermaphrodites



(b) Males

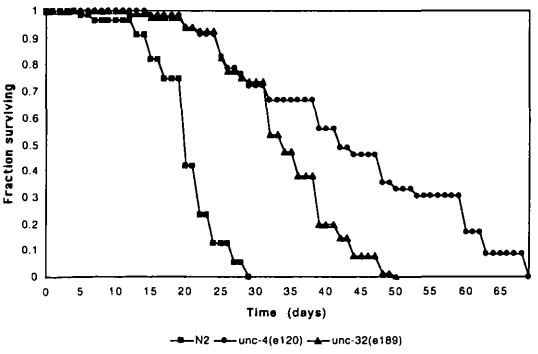
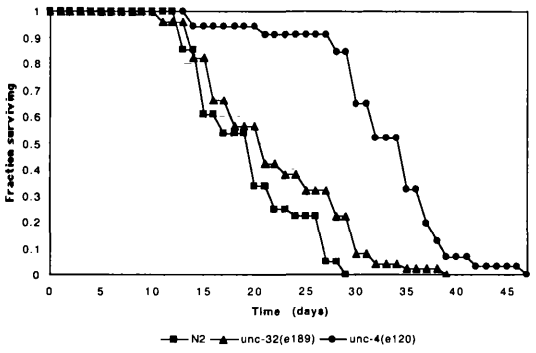
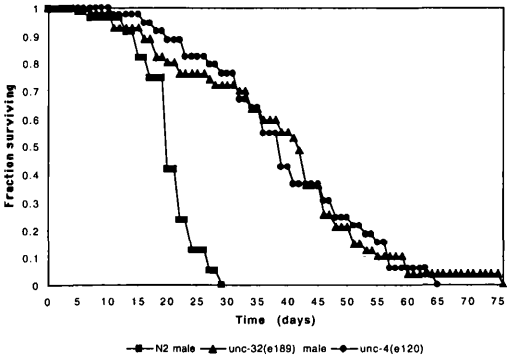


Figure 3.2b: Typical effect of two neuronal *unc* mutations on (a) hermaphrodite and (b) male survival in liquid culture (20°C). *P*<0.0001 in all cases.

(a) Hermaphrodites



(b) Males



3.2.2 Interaction of *unc-32* with IIS and TGF-β signalling in dauer formation

As described in the Introduction, previous work has found that certain long-lived *unc* mutations result in a high temperature-induced dauer formation (Hid) phenotype at temperatures above 25°C, for example *unc-31(e928)* and *unc-64(e246)* (Ailion & Thomas 2000). The Hid and Age phenotypes of *unc-31* and *unc-64* mutants are dependent upon *daf-16*, suggesting that these genes may affect IIS (Ailion *et al* 1999; Ailion & Thomas 2000). Since *unc-32(e189)* extends male lifespan in a *daf-16*-dependent manner (Gems & Riddle 2000b), it was of interest to determine whether *unc-32(e189)* also displayed a Hid phenotype, and if so, whether it was dependent upon *daf-16*. *unc-32(e189)* does not form dauers at 20°C or 22.5°C (Table 3.6 below), nor does it at 25°C (unquantified observation). However, at 27°C *unc-32(e189)* was Hid, forming over 90% dauers (Table 3.3). As previously reported, N2 formed some dauers at 27°C (Ailion & Thomas 2000).

Table 3.3: Dauer/ L2d formation by N2 and *unc-32(e189)* at 27°C.

Genotype	% dauer/ L2d formation	N*	*P*†
+	34.4 ± 12.5	1278	----
unc-32(e189)	91.9 ±2.9	1686	<0.01

*Number of progeny scored; †Probability that mean percent dauer/L2d formation of N2 and *unc-32(e189)* differ by random chance (Student's *t* test, normalised data).

However, *unc-32(e189)* no longer resulted in significantly higher dauer formation than N2 in a *daf-16(m26)* genetic background (Table 3.4), hence the Hid phenotype of *unc-32(e189)* is dependent on *daf-16* activity. This is consistent with *unc-32* disrupting IIS, as seen previously for *unc-31* and *unc-64* (Ailion & Thomas 2000).

Table 3.4: Dauer/ L2d formation by N2, *unc-32(e189)* and *daf-16(m26)*; *unc-32(e189)* at 27°C.

Genotype	% dauer/ L2d formation ± s.e.	N*	*P*†
+	7.8 ± 1.3	2116	----
unc-32(e189)	38.6 ± 4.8	899	<0.01 (a)
daf-16(m26); unc-32(e189)#	12.6 ± 2.7	1084	<0.01 (b), >0.1 (c)

*Number of progeny scored; †Probability that mean percent dauer/L2d formation of (a) N2 and *unc-32(e189)*, (b) *daf-16(m26); unc-32(e189)* and *unc-32(e189)* and (c) *unc-32(e189); daf-16(m26)* and N2 differ by random chance (Student's *t* test, normalised data). #Partial dauers.

The Hid phenotype of *unc-32(e189)* was also suppressed by the null mutation *daf-12(rh61rh411)* (Table 3.5). This is consistent with *unc-32(e189)* affecting TGF-β/cGMP signalling.

Table 3.5: Dauer/ L2d formation by N2, *unc-32(e189)* and *unc-32(e189); daf-12(rh61rh411)* at 27°C.

Genotype	% dauer/ L2d formation ± s.e.	N*	*P*†
+	1.9 ± 0.6	1237	----
unc-32(e189)	31.1 ± 5.6	445	<0.01 (a)
unc-32(e189); daf-12(rh61rh411)	0.0 ± 0.0	530	<0.01 (b), <0.01 (c)

*Number of progeny scored; †Probability that mean percent dauer/L2d formation of (a) N2 and *unc-32(e189)*, (b) *unc-32(e189); daf-12(rh61rh411)* and *unc-32(e189)* and (c) *unc-32(e189); daf-12(rh61rh411)* and N2 differ by random chance (Student's *t* test, normalised data).

Potentially, therefore, *unc-32* may affect both IIS and TGF-β/ cGMP signalling to regulate dauer formation. However, since *daf-12(0)* mutants are completely Daf-d, (implying that *daf-12* is absolutely required for dauer formation, as proposed in (Ailion & Thomas 2000)), it is not possible to conclude that *unc-32(e189)* affects cGMP/ TGF-β signalling using epistasis analysis with *daf-12*, since suppression of *unc-32(e189)* Hid phenotype by *daf-12(0)* may simply reflect the downstream position of *daf-12* relative to all dauer formation pathways.

In order to investigate the relationship between *unc-32*, IIS and TGF-β signalling further, therefore, the effects of *unc-32(e189)* on dauer formation by *rf* IIS mutants *daf-2(m41)* (class 1) and *daf-2(e1370)* (class 2) at 20°C and 22.5°C and the TGF-β nonsense mutant *daf-1(m40)* at 22.5°C were determined. A lack of enhancement of the Daf-c phenotypes of any of these mutants by *unc-32(e189)* would suggest that the two mutations promote dauer formation via the same mechanism.

At 20°C, *unc-32(e189)* significantly increased the proportion of dauers/ L2ds formed by *daf-2(m41)* but not by *daf-2(e1370)* (Table 3.6, Figure 3.3), and at 22.5°C, significantly increased dauer/ L2d formation by both *daf-2(m41)* and *daf-2(e1370)*. Because it was not possible to use a null allele of *daf-2* due to its constitutive arrest phenotype, the finding that *unc-32(e189)* enhanced the Daf-c phenotype of *daf-2(rf)* mutants could imply (a) that *unc-32* acts in the same pathway as *daf-2*, and that mutation of both components has a larger effect than mutation of each alone, or (b) that *unc-32* and *daf-2* act in separate pathways (but perhaps via a common mechanism such as *daf-*

16), and that mutation of both genes results in a synergistic effect on the Daf-c phenotype (see Main Introduction, Section A.5).

In contrast, *unc-32(e189)* had no effect on dauer formation by the TGF-β mutant *daf-1(m40)* at 22.5°C (Table 3.6, Figure 3.3). This strongly implies that *unc-32* acts in the same pathway as *daf-1* to regulate dauer formation. Thus, enhancement of *daf-2* mutant Daf-c by *unc-32(e189)* could represent synergy between IIS and TGF-β mutations, respectively, rather than further disruption of IIS by *unc-32(e189).*

Figure 3.3: Effect of *unc-32(e189)* on mean percent dauer formation by *daf-2(m41)* (blue), *daf-2(e1370)* (red) and *daf-1(m40)* (green). Significant effects are denoted as a star.

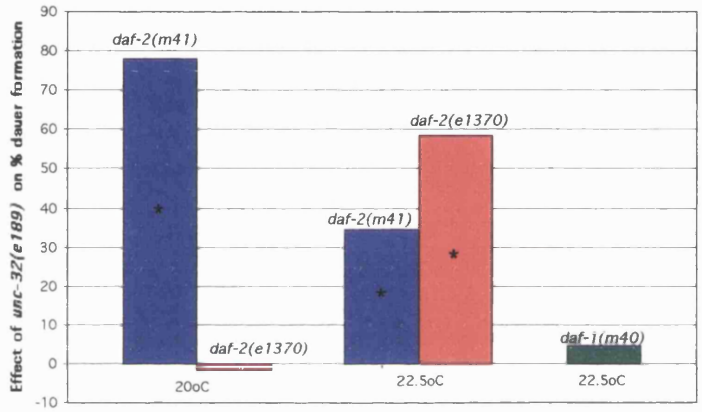


Table 3.6: Effect of *unc-32(e189)* on dauer/ L2d formation by both classes of *daf-2* mutant at 20°C and 22.5°C and by *daf-1(m40)* at 22.5°C.

	20°C						22.5°C					
Genotype	Mean % dauer/ L2d formation ± s.e.		N*		*P*†		Mean % dauer/ L2d formation ± s.e.		N*		*P*†	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
+	0.0 ± 0.0	ND	2262	ND	----	----	0.0 ± 0.0	ND	1723	ND	----	----
unc-32(e189)	0.0 ± 0.0	0.0 ± 0.0	1902	2678	NS	ND	0.0 ± 0.0	0.0 ± 0.0	1391	2325	NS	ND
daf-2(m41)	2.9 ± 1.2	1.8 ± 0.6	806	2398	----	----	50.5 ± 8.8	80.3 ± 7.5	674	707	----	----
unc-32(e189) daf-2(m41)	82.7 ± 7.3	78.4 ± 2.3	915	1449	<0.01	<0.01	100.0 ± 0.0	99.9 ± 0.1	721	1366	<0.01	<0.01
daf-2(e1370)	1.0 ± 0.4	14.1 ± 6.7	948	1218	----	----	61.8 ± 4.3	16.9 ± 2.3	744	516	----	----
unc-32(e189) daf-2(e1370)	0.2 ± 0.2	11.5 ± 5.1	388	1092	NS	NS	99.8 ± 0.2	95.7 ± 1.7	865	401	<0.01	<0.01
daf-1(m40)	ND	ND	----	----	----	----	63.1 ± 4.3	ND	1537	----	----	----
unc-32(e189); daf-1(m40)	ND	ND	----	----	----	----	67.8 ± 5.3	ND	1423	----	NS	----

*Total number of progeny scored. †Probability that means of percent dauer/ L2d formation by a strain with and without *unc-32(e189)* differ by random chance (Student's *t* test, normalised data); NS= not significant; ND= not determined

3.2.3 Interaction of *unc-32(e189)* with *daf-2* in lifespan regulation

To investigate whether the extreme longevity of class 2 *daf-2* males at restrictive temperatures was due to the pleiotropic Unc phenotype, and to investigate the effect of neuronal *unc* mutations on *daf-2* mutant hermaphrodite lifespan, the effects of *unc-32(e189)* on class 1 (*m41*) and class 2 (*e1370*) *daf-2* male and hermaphrodite survival were determined at a restrictive (22.5°C) and a permissive (15°C) temperature (Table 3.7).

In this trial there was no significant difference between N2 male and hermaphrodite median and maximum lifespans at 15°C (Table 3.7). This is unusual and does not reflect the typical relationship between the sexes at this temperature. At 22.5°C, while there was no significant difference between male and hermaphrodite median lifespans, male maximum lifespan was 5.5 days longer. A probable explanation for these observations is that this experiment was performed before it was discovered that in liquid culture, daily transfer of males as well as hermaphrodites during the period of egg lay resulted in a greater lifespan difference between the sexes (Chapter 1 Section 1.1.3). This should not be of consequence in interpreting these results since male and hermaphrodite survivals are not being compared, but the survivals of mutants strains within each sex.

Effect of unc-32(e189) *on* daf-2(rf) *male lifespan*

Consistent with previous experiments at 20°C (Gems & Riddle 2000b), wild-type male survival was markedly increased by *unc-32(e189)*, at both 22.5°C and 15°C (Table 3.7). This effect was larger at 15°C (+115%) than it was at 22.5°C (+52%) (Figure 3.4a[1]), as seen previously (Fletcher & Gems 1998). The effect of *e189* on *daf-2(m41)* male lifespan at both temperatures was similar to that seen in a wild-type background (Figure 3.5a), hence no interaction between class 1 *daf-2* signalling and *unc-32* with respect to male lifespan regulation was apparent.

However, while class 2 *daf-2(e1370)* male median lifespan was enhanced by *e189* to a similar extent as seen in an *m41* or wild-type genetic background at 15°C

[1] Note that the representative survival curves may be for individual replicates rather than pooled data, hence actual values may differ between survival curves and Table 3.7.

(~110%), at 22.5°C class 2 *daf-2(e1370)* male median lifespan was only increased by 5% (Figure 3.6a), compared with 35% and 52% in class 1 *daf-2(m41)* and wild type respectively. The fact that the effect of *unc-32(e189)* on class 2 *daf-2* male lifespan was smaller at the restrictive than the permissive temperature supports the working hypothesis that the extreme longevity of class 2 *daf-2* mutant males at restrictive temperatures is at least in part due to the pleiotropic Unc phenotype (i.e. that the mechanisms of lifespan increase by class 2 *daf-2* and *unc-32* mutations overlap). However, *daf-2(e1370)* male maximum lifespan was increased by *unc-32(e189)* at 22.5°C to a similar extent (+56%) as were *daf-2(m41)* and N2 maximum lifespans (55% and 48% respectively), a finding that is not consistent with the working hypothesis.

Survival analyses were again performed for the above strains at 22.5°C (but not 15°C) as controls for a different experiment (Chapter 5, Table 5.2), with relevant data reproduced in Table 3.8 below. Again, both male and hermaphrodite lifespans were increased by *unc-32(e189)*, and the effect was greater in males. Also as seen previously, class 1 *daf-2(m41)* male survival was significantly increased by *unc-32(e189)*. However, unlike in the above trial, *unc-32(e189)* resulted in a larger increase in *daf-2(e1370)* male lifespan (+83%) than it did in wild-type (+57%) or class 1 *daf-2* (+76%) male lifespan. Although in this case 15°C controls are not available, these results are inconsistent with the hypothesis that the extreme longevity of class 2 *daf-2(rf)* males at restrictive temperatures is due to the Unc phenotype. These results are considered further in the Discussion.

Effect of unc-32(e189) *on* daf-2 *mutant hermaphrodite lifespan*

In contrast to the previous work (Gems & Riddle 2000b), and in agreement with results presented in Tables 3.2a & b above, wild-type hermaphrodite survival was slightly but significantly increased by *unc-32(e189)* at both temperatures (Figure 3.4b). In particular, the increase in maximum hermaphrodite lifespan at 15°C was notable (Table 3.7). However, as found in the experiment described previously, the effect of *unc-32(e189)* on hermaphrodite survival was smaller than in males, at both temperatures (Table 3.7).

The slight increase in class 1 *daf-2(m41)* hermaphrodite lifespan at 15°C due to *unc-32(e189)* was similar to that seen in a wild-type background (Table 3.7, Figure

3.5b). However, no effect of *e189* on *daf-2(m41)* hermaphrodite survival was seen at 22.5°C (Table 3.7, Figure 3.5b). This is consistent with results from the experiment presented in Chapter 5 and part-reproduced in Table 3.8 below, and suggests that *unc-32(e189)* may (slightly) extend hermaphrodite lifespan via the same mechanism as *daf-2(m41)*. Moreover, the effect of *daf-2(m41)* on hermaphrodite median lifespan was reduced in an *unc-32(e189)* (48%) compared with a wild-type (73%) genetic background, further suggesting that the mechanisms of lifespan increase due to these two mutations may overlap.

As seen in a class 1 *daf-2(rf)* genetic background, the effect of *unc-32(e189)* on class 2 *daf-2(e1370)* hermaphrodite survival at 15°C was similar to that seen in wild type (Table 3.7, Figure 3.6b). However, unlike in a class 1 *daf-2(rf)* or wild-type genetic background, *unc-32(e189)* markedly extended class 2 *daf-2(e1370)* hermaphrodite median lifespan at 22.5°C (Tables 3.7 & 3.8, Figure 3.6b). Likewise, the effect of *daf-2(e1370)* on hermaphrodite median lifespan was greater in an *unc-32(e189)* genetic background than in a wild-type genetic background at 22.5°C (Tables 3.7 & 3.8). These findings suggest that there is a super-additive or synergistic interaction between *unc-32* and class 2 *daf-2* signalling with respect to longevity.

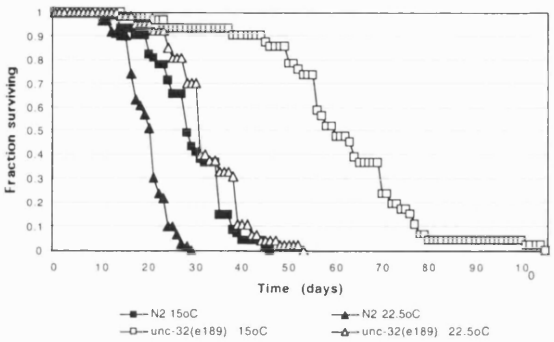
Table 3.7: Effect of *unc-32(e189)* on *daf-2* survival at permissive and restrictive temperatures.

Genotype/ sex	Median lifespan (days) ± 95% C.I.		% change median lifespan with *unc-32*		Maximum lifespan (days)		% change maximum lifespan with *unc-32*		N*		*P*†	
	15°C	22.5°C	15°C	22.5°C	15°C	22.5°C	15°C	22.5°C	15°C	22.5°C	15°C	22.5°C
+ H	32.0 (35.5, 28.5)	18.5 (20.0, 17.0)	-	-	44.0	23.5	-	-	55 (80)	64 (120)	-	-
+ M	28.3 (31.5, 26.0)	18.5 (19.5, 18.0)	-	-	44.0	29.0	-	-	70 (80)	147 (156)	-	-
unc-32(e189) H	36.5 (42.0, 31.0)	21.3 (23.5, 18.0)	+14	+15	64.5	26.0	+47	+11	79 (100)	153 (190)	0.0006	0.005; <0.0001
unc-32(e189) M	61.0 (67.5, 56.0)	28.2 (32.3, 26.5)	+115	+52	100.0	43.0	+127	+48	65 (76)	125 (160)	<0.0001	<0.0001
daf-2(m41) H	35.0 (38.5, 32.5)	32.0 (33.3, 30.5)	-	-	43.5	47.0	-	-	46 (80)	145 (231)	-	-
daf-2(m41) M	37.2 (40.3, 33.8)	30.8 (32.3, 29.0)	-	-	52.0	44.0	-	-	100 (120)	223 (261)	-	-
daf-2(e1370) H	55.5 (60.5, 50.0)	31.0 (27.3, 37.3)	-	-	79.0	50.0	-	-	51 (80)	99 (264)	-	-
daf-2(e1370) M	44.5 (49.8, 41.5)	65.0 (70.5, 58.8)	-	-	74.5	88.0	-	-	65 (76)	214 (285)	-	-
unc-32(e189) daf-2(m41) H	40.0 (46.0, 33.5)	31.5 (32.5, 29.5)	+25	-2	59.0	49.0	+36	+4	46 (60)	114 (161)	0.011	NS
unc-32(e189) daf-2(m41) M	77.0 (81.0, 74.0)	41.5 (46.3, 37.5)	+107	+35	102.0	68.0	+96	+55	36 (60)	101 (139)	<0.0001	<0.0001
unc-32(e189) daf-2(e1370) H	64.0 (67.5, 59.5)	46.0 (50.0, 42.0)	+15	+48	97.0	106.0	+23	+112	72 (90)	110 (159)	0.001	<0.0001
unc-32(e189) daf-2(e1370) M	92.0 (112.5, 80.0)	68.0 (79.0, 62.0)	+107	+5	146.0	116.0	+96	+56	68 (100)	66 (196)	<0.0001	<0.0001

*Number of senescent deaths (starting population); †Probability that survival curves of a strain with and without *unc-32(e189)* differ by random chance (log rank test). Multiple values represent results from separate trials giving different *P* values.

Figure 3.4: Typical effect of *unc-32(e189)* on wild-type (a) male and (b) hermaphrodite survival at 15°C (squares) and 22.5°C (triangles). Filled shapes N2, open shapes *unc-32(e189)*. $P < 0.0001$ in all cases (log rank test)

(a) Males



(b) Hermaphrodites

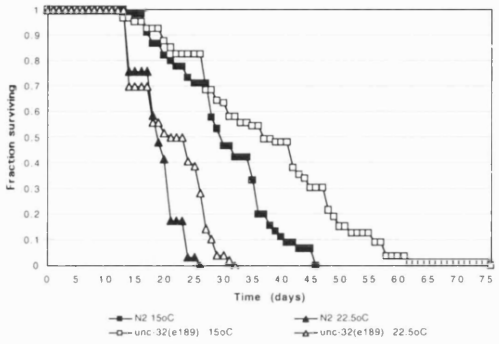
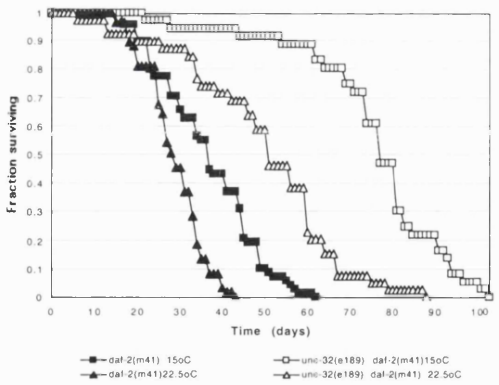


Figure 3.5: Typical effect of *unc-32(e189)* on *daf-2(m41)* (a) male and (b) hermaphrodite survival at 15°C (squares) and 22.5°C (triangles). Filled shapes *daf-2(m41)*, open shapes *unc-32(e189) daf-2(m41)*. $P < 0.0001$ in all cases except hermaphrodites at 22.5°C (not significant) (log rank test)

(a) Males



(b) Hermaphrodites

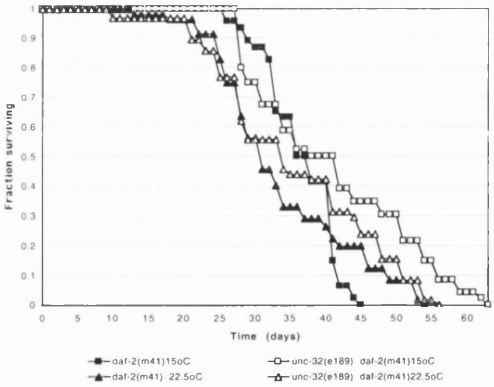
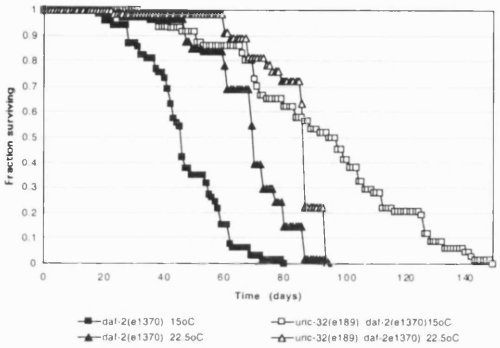


Figure 3.6: Typical effect of *unc-32(e189)* on *daf-2(e1370)* (a) male and (b) hermaphrodite survival at 15°C (squares) and 22.5°C (triangles). Filled shapes *daf-2(e1370)*, open shapes *unc-32(e189) daf-2(e1370)*. *P* values are presented in Table 3.3. Note the premature mortality displayed by class 2 *daf-2* hermaphrodites at 22.5°C, implied by the reduced ratio of median: maximum lifespan.

(a) Males



(b) Hermaphrodites

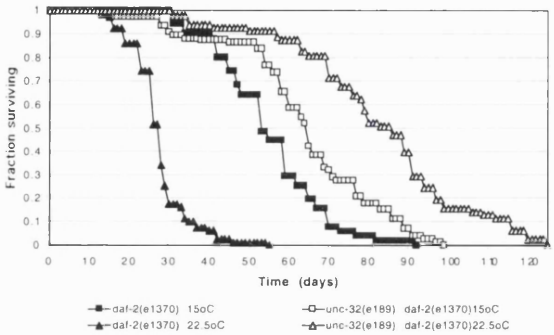


Table 3.8: Effect of *unc-32(e189)* on *daf-2* survival (22.5°C) (part-reproduced from Chapter 5 Table 5.2)

Genotype/ sex	Median lifespan (days) ± 95% C.I.	% effect on median of *unc-32(e189)*	Maximum lifespan (days)	N*	*P*†
+ H	17.7 (19.0, 16.5)	----	23.0	165 (200)	----
+ M	19.8 (21.3, 18.3)	----	29.0	135 (211)	----
unc-32(e189) H	22.0 (24.0, 20.3)	+24	25.0	57 (80)	<0.0001
unc-32(e189) M	31.0 (35.5, 27.3)	+57	45.0	45 (80)	<0.0001
daf-2(m41) H	31.8 (36.0, 28.8)	----	51.5	44 (80)	----
daf-2(m41) M	29.0 (32.0, 26.5)	----	41.5	59 (80)	----
unc-32(e189) daf-2(m41) H	32.8 (39.5, 28.0)	+3	52.5	48 (62)	0.21
unc-32(e189) daf-2(m41) M	51.0 (60.0, 46.0)	+76	83.0	39 (43)	<0.0001
daf-2(e1370) H	29.3 (32.0, 27.3)	----	45.0	124 (332)	----
daf-2(e1370) M	53.0 (56.0, 50.8)	----	105.0	209 (262)	----
unc-32(e189) daf-2(e1370) H	84.5 (90.0, 76.3)	+188	119.0	77 (90)	<0.0001
unc-32(e189) daf-2(e1370) M	97.0 (107.8, 85.8)	+83	130.0	51 (100)	<0.0001

*Number of senescent deaths (starting population); †Probability that survival curves of a strain with and without *unc-32(e189)* differ by random chance (log rank test)

3.2.4 Effect of *unc-32(e189)* on localisation patterns of DAF-16::GFP

The fact that the increase in male lifespan due to *unc-32(e189)* and the Hid phenotype of *unc-32(e189)* are dependent upon *daf-16* (Gems & Riddle 2000b) (Section 3.2.2), suggests that mutation of *unc-32* results in up-regulation of DAF-16 activity. As described in the Main Introduction (Section A.4.5), DAF-16 is active when de-phosphorylated in the nucleus, and is inactive when phosphorylated in the cytosol. Since male lifespan is increased by neuronal *unc* mutations to a greater extent than hermaphrodite lifespan, if *unc-32(e189)* extends lifespan by increasing DAF-16 activity, predictions might be that (a) nuclear localisation of DAF-16 is higher in *unc-32(e189)* males than wild-type males and hermaphrodites or *unc-32(e189)* hermaphrodites and (b) there may be a slight increase in nuclear localisation of DAF-16 in *unc-32(e189)* hermaphrodites compared with wild-type hermaphrodites.

To investigate this, levels of nuclear localisation of DAF-16::GFP were measured for *unc-32(e189)* males and hermaphrodites, using the same GFP strain and DAF-16::GFP nuclear localisation scale as described in Chapter 2 Section 2.2.5. Preliminary investigations revealed no difference between levels of nuclear localisation of DAF-16::GFP in *unc-32(e189)* and N2 for either sex (raised at 15°C where the effects of *unc-32(e189)* on male lifespan are greatest, data not shown). Thus, if *unc-32(e189)* does affect DAF-16::GFP cellular localisation under standard conditions, it does not do so to a level that can be visualised using this method, despite a dramatic increase in male lifespan due to *unc-32(e189).*

However, if *unc-32(e189)* results in increased DAF-16 activity, it seemed possible that increased nuclear localisation of DAF-16::GFP might occur following heat/ oxidative stress in *unc-32(e189)* males to such an extent that it may be visible using the GFP marker. Levels of DAF-16::GFP nuclear localisation were therefore measured for *unc-32(e189)* males and hermaphrodites following a 35°C heat shock. No differences were apparent between levels of DAF-16::GFP nuclear localisation displayed by wild-type and *unc-32(e189)* hermaphrodites after either period of heat shock (Figure 3.7[2], Appendix E.5). However, there was a markedly higher incidence of nuclear localisation of DAF-16::GFP in *unc-32(e189)* males relative to wild-type males after both five and

[2] Pie charts illustrate results for five minute heat stress only- see Appendix E.5 for remaining data.

ten minutes of heat shock, although these differences were only significant in two out of seven instances due to wide variation between replicates (Figure 3.8, Appendix E.5). It would therefore appear that attenuation of *unc-32* function reveals an increased male response in terms of DAF-16 nuclear localisation under conditions of stress. Conceivably, this is because levels of DAF-16 nuclear localisation are increased in males both by the *unc-32(e189)* mutation and by heat stress.

Note that these results are in contrast to those presented in Chapter 2 Section 2.2.5, where stress resulted in consistently higher levels of DAF-16::GFP nuclear localisation in N2 hermaphrodites relative to males. It was therefore suggested that any sex differences in DAF-16 activity may occur at the level of transcriptional response to DAF-16 rather than in DAF-16 cellular localisation. Results presented here, however, are consistent with the original working hypothesis of Chapter 2: that IIS is down-regulated (or DAF-16 activity in terms of nuclear localisation up-regulated) in males.

Figure 3.7: Effect of a five minute 35°C heat shock on cellular localisation of DAF-16::GFP in hermaphrodites (raised at 15°C)

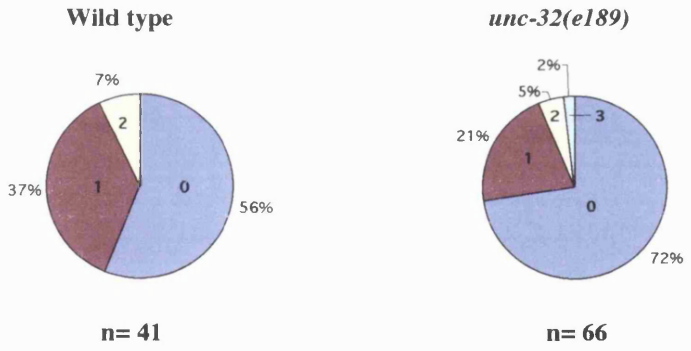
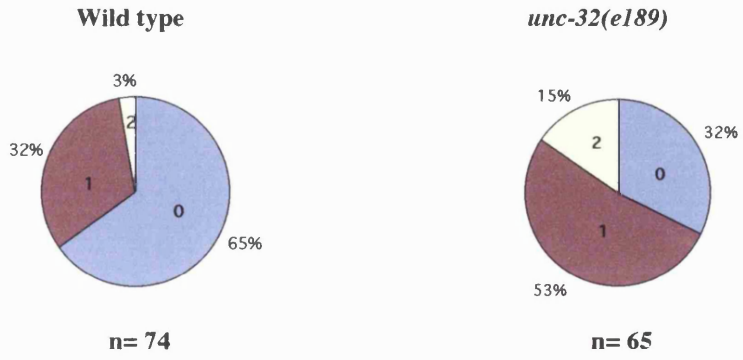


Figure 3.8: Effect of a five minute 35°C heat shock on cellular localisation of DAF-16::GFP in males (raised at 15°C)



3.3 Discussion

3.3.1 Mechanism of lifespan extension by neuronal *unc* mutations

Previous work found that most *unc* mutations tested increased male, but not hermaphrodite lifespan (Gems & Riddle 2000b). Since the degree of male lifespan extension appeared to correlate with the severity of the *unc* mutant motility defect, the increased lifespan of *unc* mutant males was attributed to the inhibition of deleterious male behaviours. However, the fact that certain *unc* mutations also extend hermaphrodite lifespan (Gems & Riddle 2000b; Ailion *et al* 1999) implies that at least part of the mechanism of lifespan extension by *unc* mutations could be due to disruption of neuronal secretion rather than behavioural effects. Lifespan was therefore determined for a range of neuronal and muscle *unc* mutants. If disruption of neurosecretion were responsible for the increased lifespan of *unc* mutants, no effect of muscle *unc* mutations on lifespan would be expected.

Although it was only possible to use two muscle *unc* mutations due to a high incidence of pleiotropic effects in others, they both had small or negligible effects on lifespan in both sexes, resulting in both increased and decreased survival. In contrast, the overall effect of neuronal *unc* mutations was to increase lifespan in both sexes, although *unc-50(e306)* only had a small effect (Tables 3.2a & b). Mating efficiency did not influence the extent of lifespan extension, since male median lifespan in liquid culture was reduced by 20% in *unc-22(e66)* but increased by 110% in *unc-32(e189)*, and both mutants are ME0. This implies that the effect of *unc* mutations on male lifespan is not simply the result of inhibition of certain behaviours as originally proposed (Gems & Riddle 2000b).

Neuronal *unc* mutations extended hermaphrodite lifespan in several cases (Table 3.2, Figure 3.2). However, lifespan was consistently increased to a much greater extent in males than hermaphrodites. Males are therefore potentiated in some way to respond to *unc* mutations, perhaps because they are in a particular neuroendocrine state. In order to understand how disruption of neuronal function by the neuronal *unc* mutations above could affect lifespan of either sex, it is necessary to consider the wild-type functions of the genes and their mutant phenotypes, which are described below.

unc-4 encodes a homeoprotein required for the correct wiring of interneurons to the VA motor neurons during development. The putative null allele *e120* used here causes VA motorneurons to form connections appropriate for VB motorneurons, resulting in an inability to move backwards (White *et al* 1992). However, mutation of *unc-4* also results in decreased numbers of synaptic vesicles, with reduced abundance of the vesicular proteins UNC-17, choline acetyltransferase, synaptotagmin, synaptobrevin and RAB-3 (Lickteig *et al* 2001). This not only occurs in the VA motorneurons, but also in the other classes of cholinergic ventral nerve cord motor neuron and associated ganglia where *unc-4* is expressed (Miller & Niemeyer 1995; Pflugrad *et al* 1997).

UNC-13 interacts with the protein syntaxin, which along with synaptobrevin and SNAP-25 constitutes the SNARE complex. This complex is required for priming of synaptic vesicles before their fusion with the plasma membrane. In order for the SNARE complex to form, syntaxin must be in an open configuration. Binding of UNC-13 to the amino terminus of syntaxin may cause it to adopt this open configuration, allowing synaptic vesicle fusion to take place (S. Nurrish, pers. comm.). Mutation of *unc-13* results in complete paralysis and reduced pharyngeal pumping, presumably due to diminished effectiveness of signalling across cholinergic neuromuscular junctions.

unc-26 encodes a homologue of mammalian synaptojanin, a protein required for synaptic vesicle recycling (see (Harris *et al* 2000)). *unc-26* mutants are small, tend to coil and move in a "jerky" manner. At the ultrastructural level these animals display defective budding, uncoating, recovery and tethering of synaptic vesicles in synaptic termini, which results in a depletion of vesicles in the synapses and a build-up of clathrin-coated vesicles (Harris *et al* 2000). Such mutants have disrupted cholinergic and GABA neurotransmission (see (Harris *et al* 2000)).

unc-32 encodes one of a family of four vacuolar ATPase α subunits. Vacuolar ATPases are ATP-dependent proton pumps required for cell compartment acidification. Mammalian vacuolar ATPases have also been shown to be required for neurotransmitter trafficking, with the proton gradients established allowing import of neurotransmitters such as acetylcholine (ACh) (see (Pujol *et al* 2001)). *e189* is a mutation in the *unc-32* B transcript, which localises to synaptic vesicles within cholinergic neurons (Pujol *et al* 2001). In *unc-32(e189)* mutants, synaptic vesicles exhibit a distinctive, abnormal

morphology (Nelson *et al* 2000). *In situ* hybridisation analysis reveals expression in the intestine, gonad and a number of head and motor neurons (Pujol *et al* 2001).

Long-lived neuronal *unc* mutants not employed in this study include *unc-31(rf)* and *unc-64(rf)* (Ailion *et al* 1999; Ailion & Thomas 2000; Gems & Riddle 2000b). *unc-31* encodes Ca^{2+}-dependent activator protein for secretion (CAPS), required for regulation of exocytosis of dense-core vesicles (Ann *et al* 1997). Using a *lacZ* reporter, *unc-31* expression has been detected throughout the nervous system (see (Ann *et al* 1997)), hence it is not clear where *unc-31* expression is specifically required to regulate lifespan and dauer formation. *unc-64* encodes syntaxin, which forms part of the SNARE complex, and is thought to interact with UNC-13 to effect vesicle priming.

Of all the neuronal *unc* mutants employed in this study, the only one not to result in a marked increase in male lifespan was *unc-50(e306)*. *unc-50* encodes a novel protein that is known to be required for ACh receptor function/ assembly (Hengartner *et al* 1992). Thus, unlike the other *unc* genes described here, *unc-50* is likely to encode a post-synaptic protein, the significance of which is considered further below.

Disruption of cholinergic signalling?

It is interesting to note that all the above neuronal *unc* mutations disrupt cholinergic signalling to some extent, due to defective secretory vesicle formation, docking, priming, exocytosis or recycling. Cholinergic signalling is effected using the excitatory neurotransmitter ACh, and in vertebrates functions within sympathetic and parasympathetic ganglia, the CNS, the adrenal medulla and neuromuscular junctions. There are two types of mammalian ACh receptor: nicotinic and muscarinic, homologues of which have been identified in *C. elegans* and *Drosophila* (Tissenbaum *et al* 2000).

In nematodes, ACh is required for somatic muscle stimulation, but also occurs in sensory neurons such as amphidial chemosensory and olfactory neurons (see (Walker *et al* 2000)). Interestingly, sensory cilium function mutants show increased lifespan (Apfeld & Kenyon 1999), and *daf-2* activity in certain neuronal subsets is sufficient to rescue the Daf-c phenotype of *daf-2(rf)* mutants (Apfeld & Kenyon 1998). It could therefore be that disruption of cholinergic signalling interferes with neuroendocrine IIS, having a similar effect to a weak *daf-2(rf)* mutation. If so, neuronal *unc* mutants would

be less able to transduce IIS-stimulatory environmental signals to the target tissues. Evidence for an involvement of IIS comes from the fact that mutation of *unc-31* and *unc-64* results in *daf-16*-dependent dauer formation (27°C) and lifespan extension (Ailion *et al* 1999; Ailion & Thomas 2000). Likewise, the male-specific lifespan increase due to *unc-32(e189)* and *unc-4(e120)* mutants is *daf-16*-dependent (Gems & Riddle 2000b), as was the *unc-32(e189)* Hid phenotype above (Table 3.4). It is also interesting to note that in males *unc-32(e189)* increased nuclear localisation of DAF-16::GFP following heat stress (Figure 3.8), further suggesting an interaction between neuronal *unc* mutations and IIS.

Interestingly, previous work has shown that manipulation of cholinergic signalling can affect dauer formation and recovery: muscarinic agonists promote dauer recovery, while the muscarinic antagonist atropine inhibits it (Tissenbaum *et al* 2000). This confirms the role of cholinergic signalling in transduction of environmental signals that regulate dauer formation. Significantly, the promotion of dauer recovery by muscarinic agonists was not seen in *daf-2* or *age-1* mutants, implying that the effects of manipulation of cholinergic signalling act through IIS. It is known that muscarinic agonists promote release of insulin from the pancreatic β cells in mammals (Miller 1981; Hawdon & Schad 1993), and it has been suggested that those cells secreting insulin-like hormone in *C. elegans* are similarly regulated by cholinergic inputs (Tissenbaum *et al* 2000). A role for cholinergic signalling in developmental regulation was also suggested for the parasitic nematode *Ancylostoma caninum*, since treatment with muscarinic agonists resulted in resumption of development from an arrested state, while the cholinergic antagonist atropine prevented it (Tissenbaum *et al* 2000). Thus, muscarinic regulation of development may be a general feature of nematodes.

Disruption of peptide neurosecretion?

Neuronal *unc* mutations could therefore reduce cholinergic neurotransmission, and thus inhibit the transduction of the sensation of the stimulus (presumably food) to the cells in which insulin-like ligand is produced. However, it is also possible that it is the disruption of peptide, rather than neurotransmitter secretion in these *unc* mutants which leads to increased lifespan. Cholinergic signalling is widespread throughout the body, and the

association of disrupted cholinergic signalling with the longevity of these neuronal *unc* mutants may be indirect. In support of this, it is interesting to note that *unc-50* encodes a post-synaptic protein involved with cholinergic signalling, and, when mutated, results in a much smaller increase in male lifespan than the other neuronal *unc* mutants tested.

Neurosecretion of various molecules such as peptide hormones is effected via dense core vesicles. Unlike neurotransmission vesicles, dense core vesicles are not docked and primed at active zones ready for fast release in response to Ca^{2+} influx. Instead, movement of these vesicles to the pre-synaptic membrane only occurs after the diffusion of Ca^{2+} into the body of the nerve terminal. It is possible that neuronal *unc* mutations increase lifespan by disrupting this element of neuronal function, for example by inhibiting the release of the insulin-like ligand(s). In support of this possibility, *unc-31*, mutation of which results in a *daf-16*-dependent Age phenotype (Ailion *et al* 1999), encodes Ca^{2+}-dependent activator protein for secretion (CAPS), which is required for neuronal peptide secretion by regulating exocytosis of dense-core vesicles (Ann *et al* 1997).

This theory would be consistent with the finding that all *ins* genes (except *ins-11*) are expressed by the amphidial sensory neurons ASI and ASJ (Pierce *et al* 2001), which are known to regulate dauer formation (Bargmann & Horvitz 1991). INS proteins may therefore be released directly in response to the external stimulus, without the need for cholinergic signalling.

It is possible that neuronal *unc* mutations increase lifespan by disrupting neuronal secretion of both neurotransmitter and peptide hormone. For example, disruption of neurosecretion could result in reduced INS release from sensory neurons, while decreased cholinergic signalling could interfere with modulatory neuron function involved with INS release from other cells. Expression patterns of *ins* promoter-driven GFP show that *ins* genes are not only expressed in sensory amphidial neurons, but that expression also occurs in various other neurons including NSM and those of the circumpharyngeal nerve ring, as well as intestinal, hypodermal, pharyngeal and vulval cells (Pierce *et al* 2001). Release of INS protein from such cells could require cholinergic signalling-mediated stimulation and modulation of neurosecretion.

Work in *Drosophila* suggests that the effect of *unc* mutations on *C. elegans* lifespan may have wider significance. Mutation of the *methuselah* (*mth*) gene results in increased fly lifespan and stress resistance (Lin *et al* 1998). The G protein-coupled receptor encoded by *mth* is required for neurotransmitter exocytosis at the neuromuscular junction. Release of neurotransmitter is therefore reduced by ~50% in *mth* mutants, which have fewer exocytotic vesicles (Song *et al* 2002). Thus, it seems possible that reductions in neurotransmission and/or neuroendocrine function can directly affect lifespan, perhaps through positive effects on stress resistance. It would be of interest to see whether neuronal *unc* mutants of *C. elegans* show increased resistance to stresses in a similar manner to *mth* flies. That they may do so is suggested by the finding above that in males, *unc-32(e189)* results in increased nuclear localisation of DAF-16::GFP in response to heat stress (Figure 3.8).

Note that the fact that in some cases slight increases in lifespan were also seen in muscle *unc* mutants of both sexes implies that the effect of *unc* mutations on lifespan may not be entirely neurosecretory. It may be, for example, that the reduced pharyngeal pumping rate exhibited by many of these mutants results in slight lifespan increases due to dietary restriction. This might explain the enhanced lifespan responses seen to many *unc* mutations in liquid compared with plate culture, where food concentration is reduced. Alternatively, inhibition of movement by *unc* mutations may lead to reduced production of harmful by-products of metabolism such as reactive oxygen species (Gems & Riddle 2000b).

3.3.2 Interaction of *unc-32* with IIS and TGF-β signalling in dauer formation

Neuronal *unc* mutations may therefore extend lifespan by disrupting IIS and so up-regulating DAF-16 activity. If so, such mutations might also be expected to affect dauer formation. Consistent with this, previous work found that the neuronal *unc* mutants *unc-64(rf)* and *unc-31(rf)* display *daf-16*-dependent Age and high temperature-induced dauer formation (Hid) phenotypes (Ailion *et al* 1999; Ailion & Thomas 2000).

The neuronal mutant *unc-32(e189)* was selected to investigate this further. Like *unc-31* and *unc-64* mutants, *unc-32(e189)* displayed a Hid phenotype at 27°C, which

was completely suppressed by *daf-16(m26)* (Tables 3.3 & 3.4). This parallels the earlier finding that the increased lifespan of *unc-32(e189)* males is dependent upon *daf-16* (Gems & Riddle 2000b). *unc-32(e189)* may therefore promote dauer formation and lifespan by up-regulating DAF-16 activity. Potentially, this could be as a component of IIS (acting in neurons required for transduction of environmental signals/ production of INS ligands) or via a separate DAF-16-regulatory mechanism.

The Hid phenotype of *unc-32(e189)* was also dependent on *daf-12* (Table 3.5), suggesting that *unc-32* may affect cGMP/ TGF-β signalling. However, absolutely no dauers at all formed in a *daf-12(0)* genetic background at 27°C, compared with low levels of partial dauer formation in a *daf-16(0)* genetic background at that temperature. This is consistent with the previous suggestion that *daf-12* is absolutely required for dauer formation (Ailion & Thomas 2000). It is therefore unclear whether mutation of *unc-32* affects TGF-β/ cGMP signalling and thereby *daf-12*, or whether the *daf-12*-dependence of the *unc-32(e189)* Hid phenotype merely reflects suppression of dauer formation downstream of all dauer-regulatory pathways, including IIS. In order to clarify this, the effects of *unc-32(e189)* on dauer formation by the two classes of *daf-2* mutant and by the TGF-β mutant *daf-1(m40)* were measured.

unc-32(e189) had no effect on dauer formation by *daf-1(m40)* (Table 3.6). This strongly implies that *daf-1* and *unc-32* act via the same mechanism to regulate dauer formation. *unc-32* may therefore be a component of TGF-β signalling, as found previously for *unc-3* (Ailion & Thomas 2000). *unc-3* encodes a transcription factor expressed specifically in the ASI sensory neuron (Prasad *et al* 1998), which is believed to be the origin of TGF-β signalling (Schackwitz *et al* 1996). Mutation of *unc-32* may similarly disrupt TGF-β signalling in source neurons and promote dauer formation through up-regulation of DAF-12 activity (Figure 3.9a).

In contrast, *unc-32(e189)* markedly enhanced dauer formation by both classes of *daf-2* mutant (Table 3.6). Because it was not possible to use *daf-2* null mutants, it is unclear whether this enhancement was the result of (a) combined effects on DAF-16 activity of *unc-32* and *daf-2* mutations, with both genes acting in a common pathway (Figure 3.9b) or (b) enhancement of dauer formation through disruption of IIS due to *daf-2(rf)* and disruption of another dauer formation pathway (possibly TGF-β signalling)

due to *unc-32(e189)* (Figure 3.9c). For example, a previous study found that while the Daf-c mutants *daf-2(e1370)* (IIS) and *daf-1(m40)* (TGF-β) formed 5% and 1% dauers at 20°C, respectively, the *daf-2(e1370)*; *daf-1(m40)* double mutant formed 100% dauers (Ogg *et al* 1997).

The fact that *unc-32* is expressed widely among neurons makes it possible that *unc-32(e189)* disrupts both IIS and TGF-β signalling. However, since the Hid phenotype of *unc-32(e189)* is completely dependent on *daf-16* (Table 3.4), any element of the *unc-32(e189)* Hid phenotype due to disruption of TGF-β signalling may result from effects of TGF-β signalling on DAF-16 activity rather than DAF-12 activity (Figure 3.10a). That this is possible is suggested by DAF-16::GFP localisation studies, which demonstrate that mutation of the TGF-β signalling gene *daf-7* results in nuclear localisation and hence activation of DAF-16 at the L2d stage during the dauer decision (Lee *et al* 2001). In addition, the Daf-c phenotype of *daf-1(m40)* is partially suppressed by mutation of *daf-16* (Vowels & Thomas 1992; see Chapter 2). It has been suggested that TGF-β signalling SMAD proteins such as DAF-3 and DAF-8 may interact with DAF-16 to regulate gene transcription (Ogg *et al* 1997).

Alternatively, the DAF-16-dependence of *unc-32(e189)* Hid may suggest that both DAF-16 and DAF-12 activity are required for dauer formation in this instance due to co-regulation of common target genes (Figure 3.10b), as suggested for the Age phenotype of class 1 *daf-2* mutant hermaphrodites (Gems *et al* 1998; see Chapter 5 Section 5.3.3).

Figure 3.9: Models for role of *unc-32* in dauer formation. (a) *daf-1* and *unc-32* act in the same pathway. (b,c) Possible interactions between *daf-2* and *unc-32*.

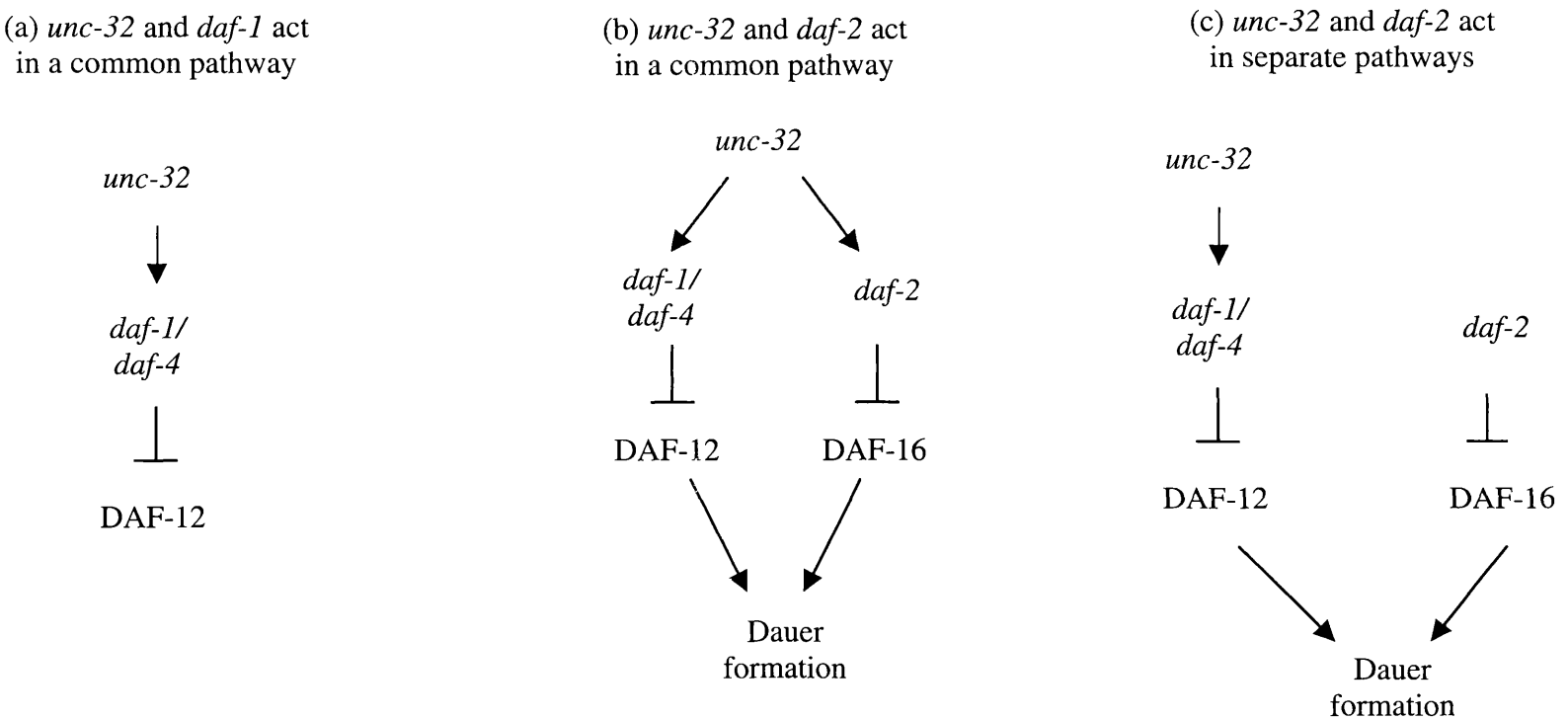
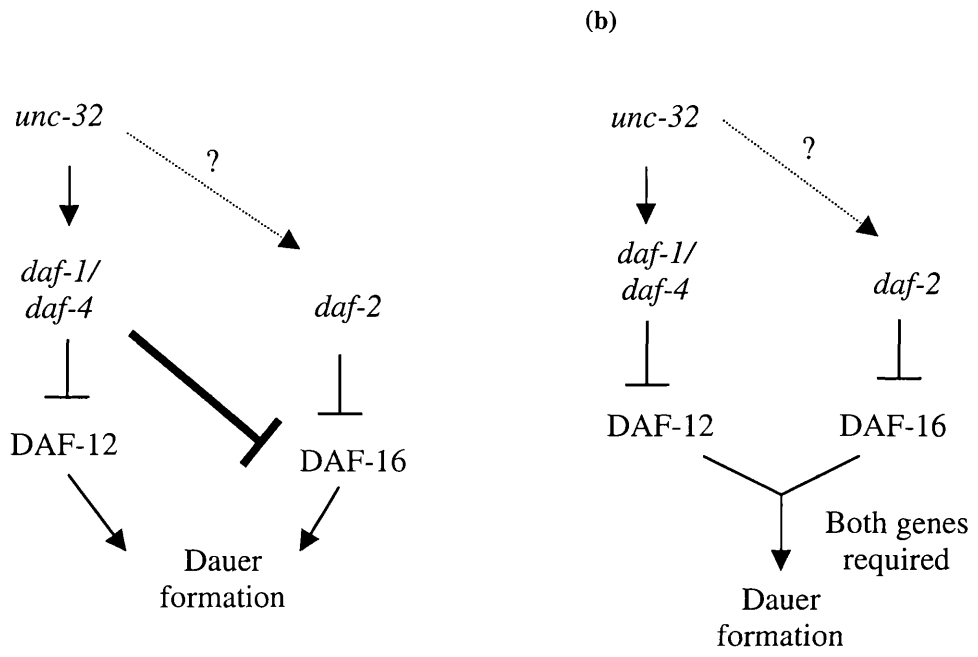


Figure 3.10: Model explaining the *daf-16*-dependence of *unc-32(e189)* Hid phenotype. (a) Disruption of TGF-β signalling by *unc-32(e189)* may largely up-regulate DAF-16 activity; (b) *daf-12* and *daf-16* may both be required for dauer formation by *unc-32(e189)*



3.3.3 Mechanism of sex differences in lifespan extension by neuronal *unc* mutations

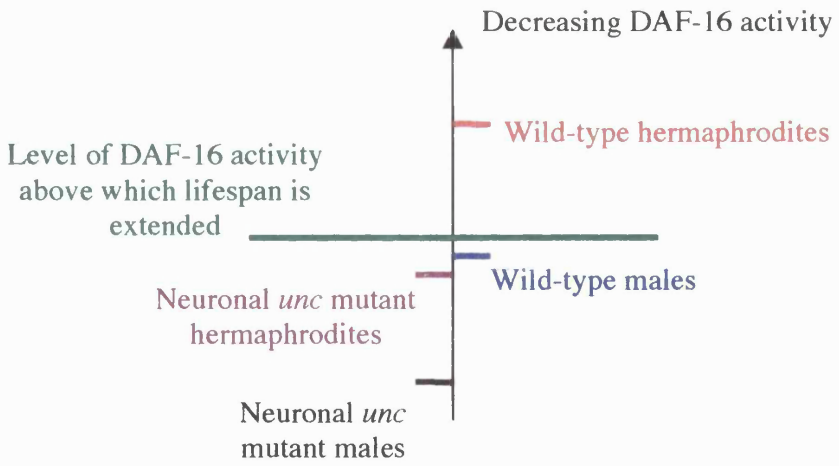
The effect of neuronal *unc* mutations on lifespan is consistently greater in males than in hermaphrodites (Gems & Riddle 2000b) (Table 3.2). As described above, it is possible that at least part of the mechanism of lifespan increase by neuronal *unc* mutations is attenuation of IIS, with defective neuronal signalling in response to environmental signals resulting in increased DAF-16 activity and hence increased lifespan. If this were the case, why is the effect greater in males? One possibility is that there are intrinsic differences in DAF-16 activity between the sexes. The involvement of DAF-16 activity in the wild-type male longevity bias is implied by the fact that the male lifespan advantage is absent in *daf-16(0)* mutants (Gems & Riddle 2000b; see Chapter 2 Section 2.3.2).

If there were a threshold level of DAF-16 activity above which increases in lifespan occur, and if DAF-16 activity were intrinsically higher in wild-type males than hermaphrodites, neuronal *unc* mutations may increase DAF-16 activity further above this threshold in males, resulting in increased lifespan. In hermaphrodites, however, up-

regulation of DAF-16 activity due to neuronal *unc* mutations could occur to a certain level without affecting lifespan, since the wild-type level of DAF-16 activity would be lower to begin with (Figure 3.11). Moreover, if liquid culture affects DAF-16 activity (perhaps by down-regulating IIS) this might explain why the effects of neuronal *unc* mutations on lifespan were generally larger in liquid culture than on agar plates.

Such a model might appear to contradict that already proposed in Chapter 2 to explain the potential loss of male lifespan advantage in *daf-2(rf)* genetic backgrounds, since reduction of IIS due to *daf-2(rf)* was predicted to eliminate the sex difference in lifespan (Chapter 2 Figure 2.2). Speculatively, the effect of neuronal *unc* mutations on DAF-16 activity may be weaker than the effect of *daf-2* mutations, meaning that sex differences in levels of DAF-16 activity could persist.

Figure 3.11: Hypothetical scenario where DAF-16 activity is intrinsically up-regulated in males and where there is a threshold level of DAF-16 activity above which lifespan is extended (green line). In this instance the neuronal *unc* mutation would result in a slight increase in hermaphrodite lifespan.



According to the model in Figure 3.11, only under conditions of up-regulated DAF-16 activity (for example in a *daf-2* mutant) would *unc-32(e189)* be able to increase hermaphrodite lifespan. Consistent with this, class 2 *daf-2(e1370)* hermaphrodite lifespan was enhanced by *unc-32(e189)* at the restrictive temperature (Tables 3.7 & 3.8, Figure 3.6b). However, in the present experiment class 1 *daf-2(m41)* hermaphrodite lifespan at 22.5°C was not enhanced by *unc-32(e189)* (Tables 3.7 & 3.8). If *unc-*

32(e189) does increase lifespan by up-regulation of DAF-16 activity, this finding suggests that DAF-16 activity was already maximised in *daf-2(m41)*, such that *unc-32(e189)* could not increase it further. This seems unlikely, however, given that *unc-32(e189)* increased *daf-2(e1370)* hermaphrodite lifespan, and male lifespan in both classes of *daf-2* mutant. It is therefore possible that elements other than or in addition to sex differences in DAF-16 activity may underlie the differential male and hermaphrodite lifespan responses to *unc-32(e189)*. This is considered further in Chapter 5 Section 5.3.5, where an involvement of *daf-12* is implied.

Unlike most other neuronal *unc* mutations studied to date, both *unc-31(rf)* and *unc-64(rf)* increase hermaphrodite lifespan to a large extent (Ailion *et al* 1999; Gems & Riddle 2000b). In addition, a range of *unc-13* mutations markedly extend hermaphrodite lifespan (Munoz & Riddle 2002). Thus, neuroendocrine function may be so disrupted in these mutants that hermaphrodites as well as males display lifespan extension. In this respect it is interesting to note that *unc-13*, *unc-31* and *unc-64* are all required for efficient functioning of syntaxin, a fundamental component of SNARE complexes during synaptic vesicle priming, docking and fusion. *unc-4(e120)* also resulted in a large increase in hermaphrodite lifespan in the present study, but only in liquid culture (Table 3.2b, Figure 3.2). *e120* is a putative null allele, hence the effect of this mutation on neurosecretion may be severe. Potentially, slight attenuation of IIS due to reduced food concentration in liquid culture may have acted together with *unc-4(e120)* to up-regulate DAF-16 activity in hermaphrodites sufficiently to extend lifespan.

3.3.4 Cold-sensitivity of male lifespan response to *unc-32(e189)*

An interesting feature of the lifespan response of males to *unc-32(e189)* was that it is cold-sensitive: *unc-32(e189)* increased male median lifespan by 52% at 22.5°C, compared with 115% at 15°C (Table 3.7). This was also the case in both classes of *daf-2* male mutant. Although the magnitude of the weak effect of *unc-32(e189)* on hermaphrodite median lifespan did not differ between the two temperatures, hermaphrodite maximum lifespan was increased to a greater extent at 15°C than at 22.5°C. Thus, the lifespan response to *unc-32(e189)* is cold-sensitive, an effect which has been noted previously (Fletcher & Gems 1998). This finding contrasts with most

other ts Age phenotypes, which typically get stronger as temperature increases, as seen for *daf-2(rf)*.

If neuronal *unc* mutations such as *unc-32(e189)* up-regulate DAF-16 activity as proposed above, why might the effect on lifespan be more marked at lower temperatures? Potentially, neuronal *unc* mutations disrupt the temperature-sensing apparatus, resulting in inappropriate reduction of IIS at cold temperatures. Thus, while the ts Daf-c phenotypes of IIS mutants suggest that IIS decreases as temperature increases, this regulatory aspect would appear to be dysfunctional in mutants such as *unc-32(e189)*. However, the enhancement of *daf-2(e1370)* Daf-c at 22.5°C but not 20°C argues against this. Alternatively, if higher temperatures reduce IIS in wild type, (as implied by the increased penetrance of mutant Daf-c phenotypes at higher temperatures), DAF-16 activity will be increased at higher temperatures. Thus, if *unc-32(e189)* also extends lifespan through up-regulation of DAF-16 activity, the effect of *unc-32(e189)* on lifespan would be expected to become smaller as temperature increases. However, this is inconsistent with the fact that hermaphrodite lifespan is increased by *unc-32(e189)* in a *daf-2(e1370)* genetic background, where DAF-16 activity is up-regulated. The reason for the cold-sensitivity of the *unc-32(e189)* Age phenotype is therefore unclear and would require further investigation.

3.3.5 Pleiotropic Unc phenotype and the extreme longevity of class 2 *daf-2* males

As described in the Introduction, it seemed possible that the extreme longevity of class 2 *daf-2* males at restrictive temperatures could be due to the pleiotropic Unc phenotype. How the pleiotropic Unc phenotype is induced in class 2 *daf-2* mutants at restrictive temperatures is unclear. Since insulin-related proteins may regulate neuronal survival and differentiation in vertebrates and invertebrates (Smit *et al* 1998), severe reduction of IIS in source neurons may result in generalised reduction of cell activity, including neurotransmission. If the extreme longevity of class 2 *daf-2* males at restrictive temperatures were due to the pleiotropic Unc phenotype, it might be expected that *unc-32(e189)* would extend class 1 but not class 2 *daf-2* male lifespan at the restrictive temperature. However, at a permissive temperature, where no pleiotropic class 2

phenotypes are seen, *unc-32(e189)* might be expected to extend both class 1 and class 2 *daf-2* male lifespan.

A significant increase in male survival was seen in both classes of *daf-2* mutant at both temperatures upon addition of *unc-32(e189)* (Table 3.7). However, the effect of *e189* on class 2 *daf-2(rf)* male median lifespan was much greater at 15°C (+107%) than at 22.5°C (+5%), with most of the increase in survival at 22.5°C occurring late-on during the time course of the survival trial. This suggests that the pleiotropic Unc phenotype of the class 2 *daf-2* mutant males is at least in part responsible for the extreme longevity of such animals at restrictive temperatures. A potential complication was that the effect of *unc-32(e189)* on wild-type male lifespan was cold-sensitive, implying that a similar effect was occurring in a class 2 *daf-2* mutant background. However, the effect of *unc-32(e189)* on wild-type median male lifespan at 22.5°C was larger than its effect on class 2 *daf-2(rf)* male median lifespan at the same temperature, supporting the hypothesis to some extent.

However, the above results were contradicted by a subsequent study in which *unc-32(e189)* enhanced class 2 *daf-2(rf)* male median lifespan more than wild-type and class 1 *daf-2(rf)* male median lifespan at the restrictive temperature (Table 3.8). This implies that if the pleiotropic Unc phenotype of class 2 *daf-2* at restrictive temperatures is responsible for the extreme longevity of males, it acts via a different mechanism from *unc-32(e189)*. However, although class 2 *daf-2* mutant male median lifespan was markedly increased by *unc-32(e189)*, maximum lifespan was only increased by 24%, compared with a doubling of maximum lifespan seen due to *unc-32(e189)* in a class 1 *daf-2* mutant background. In addition, the median lifespan of the *daf-2(e1370)* males was reduced in this trial relative to that presented in Table 3.7. It is therefore possible that the effect of *unc-32(e189)* on class 2 *daf-2* mutant male is slightly over-represented here. These results are therefore inconclusive, and a further repeat of this experiment would be necessary in order to determine whether the extreme longevity of class 2 *daf-2* mutant males is the result of the temperature-sensitive pleiotropic Unc phenotype.

3.4 Conclusions

- Hermaphrodite as well as male lifespan can be extended by neuronal *unc* mutations, while muscle *unc* mutations either have much smaller/ no effects on lifespan in either sex. These findings imply that inhibition of deleterious male behaviours is not responsible for the increased lifespan of *unc* males.
- Neuronal *unc* mutations may extend lifespan by disrupting neurotransmission and/ or neuroendocrine function. The *daf-16*-dependence of the Hid and Age phenotypes of several neuronal *unc* mutants suggests that IIS may be the neuroendocrine component affected.
- The *daf-2(rf)* Daf-c phenotype is enhanced by *unc-32(e189)*. This could reflect (a) combined up-regulation of DAF-16 activity, with *daf-2* and *unc-32* acting in the same pathway or (b) disruption of IIS (by *daf-2(rf)*) and TGF-β signalling (by *unc-32(e189)*).
- The *daf-1(m40)* Daf-c phenotype was not enhanced by *unc-32(e189)*. This suggests that these two genes regulate dauer formation via a common mechanism.
- Disrupting neuronal function affects lifespan to a greater extent in males than hermaphrodites, suggesting sex differences in one or more neuroendocrine components. Sex differences in DAF-16 activity are likely to be involved, but additional elements (potentially DAF-12 activity) may also play a role.
- It is not possible to conclude whether the extreme longevity of class 2 *daf-2* males at restrictive temperatures is a result of the pleiotropic Unc phenotype, since data from separate trials are contradictory. Further experiments are therefore necessary.

3.5 Future directions

A logical continuation of these studies would to be catalogue in greater detail those neuronal *unc* mutations which affect male (and possibly hermaphrodite) lifespan. Combining these *unc* mutations with mutations in components of IIS would clarify which, if any, Age *unc* mutations exert their effects through IIS. Ideally, null alleles

should be employed to answer more definitively whether two genes are acting in a common pathway, an option that was not possible in the case of *daf-2* and *unc-32* above.

Results presented above suggest the possibility that cholinergic signalling is involved with lifespan regulation via IIS, a suggestion that is supported by the finding that muscarinic agonists and antagonists can influence dauer recovery. However, it is also possible that other classes of neuronal signalling are involved. Studies with a range of drugs acting as agonists or antagonists of a range of *C. elegans* neurotransmitters (such as gamma-aminobutytic acid, serotonin, dopamine and glutamate), or with strains mutant for specific neurotransmitter function could determine whether any of these drugs/ mutants mimic the Age or Hid phenotypes of some of the neuronal *unc* mutants investigated above.

The possibility remains that lifespan extension due to neuronal *unc* mutations is the result of disrupted neurosecretion rather than neurotransmission, perhaps due to attenuated release of insulin-like ligand(s) from neurons. In order to distinguish between disruption of neurotransmission and neurosecretion as the mechanism of lifespan increase, a range of pre-synaptic and post-synaptic neuronal *unc* mutants could be studied. If neurosecretion rather than neurotransmission were involved, the expectation would be that post-synaptic mutations would not result in increased lifespan. Such a finding is predicted based on the fact that the post-synaptic neuronal *unc* mutation *unc-50(e306)* resulted in a much smaller increase in lifespan than the other neuronal *unc* mutations tested.

If neuronal *unc* mutations do extend male (and in some cases hermaphrodite) lifespan by disrupting IIS, it could be possible to exploit this trait to clarify which neuronal subsets are required for effective IIS. Many neuronal *unc* genes involved with lifespan regulation are expressed almost ubiquitously, hence it remains unclear where gene activity is required to regulate lifespan. By measuring lifespan extension in animals with a range of ablated neurons (either through mutation or laser microsurgery), it should be possible to identify such neurons: there will be no lifespan extension due to neuronal *unc* mutations in animals with the relevant neurons ablated. Alternatively, mosaic analysis and targeted gene expression could be employed in order to see where *unc* expression is required to restore normal lifespan.

Chapter 4
Role of gonadal signalling in the regulation of male lifespan

Contents

4.0 Introduction

Relationship between fertility and longevity

It has long been suggested in evolutionary biology that there may be a trade-off between reproduction and lifespan. This could be because acts such as mating may be physically damaging (Chapman *et al* 1995; Gems & Riddle 1996), or because germline and somatic tissues are in competition for limited resources (Williams 1966). Production of gametes (particularly oocytes), gestation (in viviparous organisms) and development of sexual ornaments and behaviours are costly in terms of energetic investment. The disposable soma theory of ageing postulates that as a result of this, fewer limited resources than necessary are allocated to somatic maintenance and repair, resulting in senescence and, eventually, death (Kirkwood 1977). However, although there are a number of instances demonstrating a trade-off between reproductive and somatic investment in animals, it remains unclear whether this is due to direct demands on limited resources, or whether it is the result of signalling that regulates reproductive and somatic investment in opposite ways (Leroi 2001).

A link between reproduction and longevity potentially has a bearing on sex differences in lifespan. This is because males and females/ hermaphrodites often differ in their lifetime reproductive strategies and in their investment in the process of reproduction itself. This chapter therefore investigates whether there are any differences in the reproductive regulation of ageing between male and hermaphrodite *C. elegans*, and begins with some background on what is known to date concerning links between reproduction and lifespan in *C. elegans* and other animals.

Mammals

The relationship between longevity and reproduction in mammals is poorly understood, in large part due to the complexity of mammalian systems, which makes it difficult to identify those reproduction-associated components that act to regulate lifespan. Castration has been shown to extend male lifespan in a number of mammalian species, including a marsupial mouse (Diamond 1982), cats (Hamilton 1965), dogs (Bronson 1981) and even humans (Hamilton & Mestler 1969). These effects are likely to be

mediated largely by changes in male hormones, however, and probably do not reflect differential resource allocation. For example, in humans, testosterone modulates cholesterol levels and leads to a higher male incidence of cardiovascular disease (Holden 1987).

Interestingly, long-lived Ames and Snell dwarf mutant mice (which have disrupted pituitary function) are also infertile. Female lifespan is increased to a greater extent by these mutations than is male lifespan, and the degree of hypogonadism is more marked in females than males. However, it has been suggested that anabolic sex hormones may increase metabolic rate and hence reduce lifespan (Bartke 2000). Again, therefore, these findings do not necessarily suggest differential allocation of resources between reproduction and somatic maintenance but rather effects of sex hormones on other factors. Further studies are therefore required in these complex mammalian systems before the relevance of reproductive trade-offs can be determined.

Insects

There is much evidence that trade-offs between reproduction and longevity exist in insects. For example, selection for long-lived *Drosophila* lines has consistently resulted in a decrease in early fecundity e.g. (Rose 1984; Luckinbill *et al* 1984; Partridge *et al* 1999), and in cockroaches there is a significant correlation between decreased brood size and increased longevity (Corley & Moore 1999). Moreover, a marked pulse of mortality is observed in *Drosophila* females a certain time after reproduction, which has been proposed to be due to costs of egg production (Sgrò & Partridge 1999). However, it has yet to be definitively demonstrated that such relationships between reproduction and lifespan are the result of differential resource allocation rather than other mechanisms such as the presence or absence of reproductive signals regulating the rate of ageing. In support of the former, work has shown that when female Mediterranean fruit flies (*Ceratitis capitata*) are raised in conditions of deprived protein, lifespan is reduced by 27%, compared with only 6% in males. Potentially, there is greater diversion by females of proteins into egg production and away from somatic repair and maintenance (Müller *et al* 1997). Presumably, the cost to somatic maintenance of reduced protein intake in

males is therefore not as great, as less protein is required to produce accessory fluids than to produce yolk.

Studies of *Drosophila* mutants have also provided some evidence in support of the disposable soma theory. For example, female flies with a mutation in the female-germline-specific transcription factor gene *ovo* are long-lived (Sgrò & Partridge 1999). Disruption of *ovo* prevents vitellogenesis by interrupting oogenesis, meaning that ovo^{D} mutants do not invest any energy in costly yolk production.

Drosophila mutant for *chico*, which encodes a homologue of mammalian insulin receptor substrates 1-4 (Bohni *et al* 1999), are also long-lived (Clancy *et al* 2001). Interestingly, $chico^{1}$ mutant females are sterile when homozygous and show reduced fertility when heterozygous, with lifespan increased more in homozygotes than heterozygotes. That fertility is involved with regulation of lifespan is implied by the fact that when $chico^{1}$ heterozygote fertility is reduced to zero by combining it with the ovo^{D1} mutation, there is no additional increase in lifespan, implying that ovo^{D1} and $chico^{1}$ act to extend lifespan via the same mechanism. However, $chico^{1}$ heterozygotes are longer-lived than the ovo^{D1} mutant. Potentially, reducing *chico* expression allows effective reallocation of resources that had been released from investment in reproduction by mutation of *ovo*. Thus, although oogenesis is completely interrupted in ovo^{D1} mutants, the resources need to be re-allocated by regulatory components before the full effect on lifespan is seen (Clancy *et al* 2001). As in females, $chico^{1}$ heterozygote male lifespan is increased. Thus, dynamic allocation of limited resources between the germline and somatic tissues can also occur in males in this species.

Further evidence for a link between fertility and lifespan in *D. melanogaster* comes from work involving strains mutant for InR, the homologue of the mammalian insulin and IGF-1 receptors. Transheterozygote InR^{p5545}/InR^{E19} females are sterile, have reduced mortality rates and mean lifespan increases of up to 85% (Tatar *et al* 2001). InR^{p5545}/InR^{E19} males have a level of fertility 35% that of wild type (estimated by proportion of fertilised eggs produced by females mated with InR^{p5545}/InR^{E19} males), but show no increase in mean lifespan, although they do show late-life mortality rate reduction. Potentially, production of male gametes and accessory fluids could require less investment of resources than does reproduction in females, although there could

simply be negative pleiotropic effects of the mutation that are specific to males, as suggested by the premature mortality of males InR^{p5545}/InR^{E19} but not females (Tatar *et al* 2001).

C. elegans

Investigations into a possible trade-off between lifespan and fertility in *C. elegans* have provided variable results. Laser ablation of gonadal precursor cells (resulting in adult animals without somatic gonad structures or germline) does not result in any increase in lifespan (Kenyon *et al* 1993; Hsin & Kenyon 1999). In addition, treatment of adult worms with the mitosis-inhibitor 5-fluoro-2'-deoxyuridine (FuDR), and hence prevention of cell division in the germline, does not lead to greater longevity. Feminising mutations that cause sperm production defects and hence infertility, such as *fog-1, fog-2, fog-3* (feminisation of germline) and *fem-3,* (feminisation) also fail to increase lifespan (Kenyon *et al* 1993; Gems & Riddle 2000; Arantes-Oliveira *et al* 2002). Thus, blocking reproduction *per se* does not increase lifespan in *C. elegans*.

Interestingly, certain long-lived *daf-2* mutants have reduced fertility. For example, *daf-2(e1391)* has a lifespan 179% that of wild type at 22.5°C and a brood size reduced by 60%. However, brood size and longevity do not correlate in all long-lived class 2 *daf-2* mutants, with some having normal fertility (Gems *et al* 1998). Reduced fertility in some long-lived *daf-2* mutants may therefore only be a correlated phenotype, or at most only part of the reason for the lifespan increase.

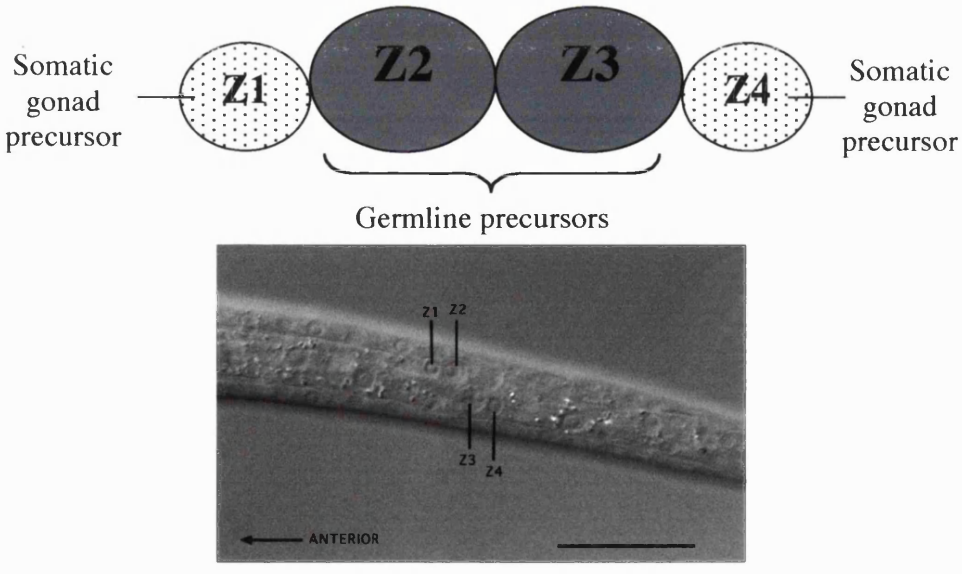
One of the largest energetic costs to *C. elegans* hermaphrodites during reproduction is likely to be the production of the vitellogenic proteins that nourish the developing embryo inside the egg. These proteins are synthesised in the intestine, secreted across the intestinal wall into the body cavity, and from there taken up by the gonad (Kimble & Sharrock 1983). Vitellogenin production is not regulated by the gonad, since hermaphrodites with their entire gonad removed following laser ablation still contain much yolk protein in the body cavity (Kimble & Sharrock 1983). Thus, potentially, the cost of yolk production is a permanent one and does not depend upon the presence or absence of eggs. The disposable soma model therefore might not be

applicable to *C. elegans*, which could explain why removal of reproduction *per se* does not increase *C. elegans* hermaphrodite lifespan.

Gonadal signalling and lifespan in *C. elegans*

Despite the above examples of evidence against a role for reproduction *per se* in the regulation of lifespan in *C. elegans*, there is evidence that signalling from the gonad may play a role. The development of the *C. elegans* gonad begins with four gonadal precursor cells in the L1 larva, Z1-Z4 (Figure 4.1). The outermost cells, Z1 and Z4, are slightly smaller than the inner pair and divide to give rise to the somatic gonad structures, including the uterus and spermathecae. The developing hermaphrodite somatic gonad is led into two U shaped-arms by a distal tip cell (DTC) at the end of each arm. In males, only one such arm develops, led by two DTCs. The inner pair of cells of the primordial gonad, Z2 and Z3, give rise to stem cells which divide to produce the germ cells- spermatozoa and oocytes in hermaphrodites and spermatozoa only in males. Cell division changes from mitotic to meiotic in the distal to proximal direction along the gonad arm.

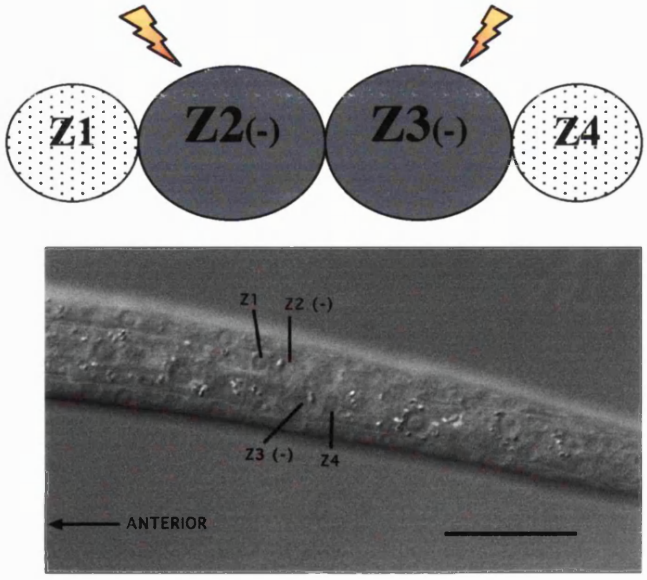
Figure 4.1: Fates of gonadal precursor cells, and DIC image of intact early L1 gonad. Scale bar= 50μm



Ablation of Z2 and Z3 in the L1 larva using a laser microbeam results in adult worms with an intact somatic gonad but lacking any germline (Figure 4.2). Interestingly, this results in up to a 60% increase in mean adult hermaphrodite lifespan (Hsin & Kenyon 1999). Perhaps significantly, these animals also display increased resistance to heat and oxidative stress, as well as increased expression of the antioxidant-encoding gene *sod-3* (Arantes-Oliveira *et al* 2002). Germline-ablated worms also show increased body size (~46%), although this appears to be independent of the lifespan effect (Patel *et al* 2002).

Lifespan extension by germline ablation was found to be completely dependent upon *daf-12* and *daf-16* activity (Hsin & Kenyon 1999). Increased body size due to germline ablation, however, is independent of *daf-16* (Patel *et al* 2002). Because lifespan extension by germline ablation was still apparent and actually enhanced in *daf-2(rf)* mutants, it was concluded that *daf-2* and germline signalling act via separate mechanisms to regulate lifespan (Hsin & Kenyon 1999) (considered further in the Discussion).

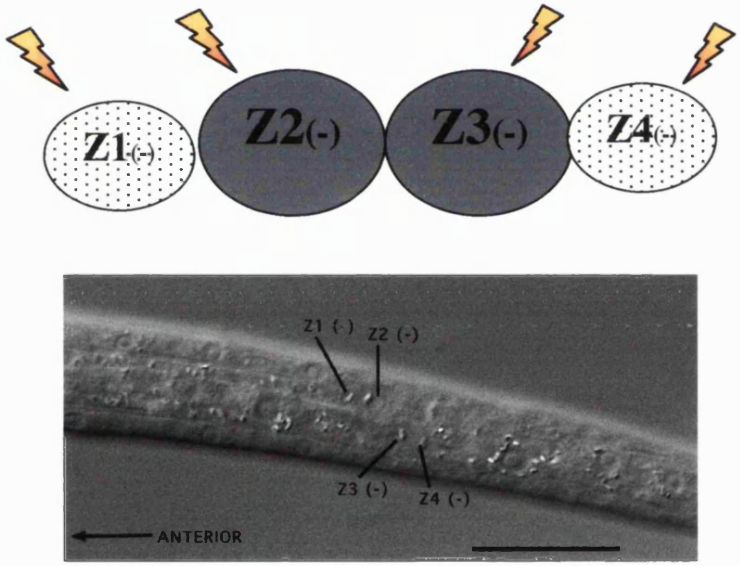
Figure 4.2: Effect of germline ablation (Z2/Z3(-)) on wild-type hermaphrodite lifespan, and DIC image of early L1 gonad with germline precursors ablated. Note blistering effect on nucleus due to laser microbeam. Scale bar= 50μm



Hermaphrodite mean lifespan increased by ~60%
Increased body size

As stated above, ablation of the entire adult gonad by removal of all four L1 precursor cells does not affect hermaphrodite lifespan[1] (Kenyon *et al* 1993; Hsin & Kenyon 1999) (Figure 4.3). Since removal of reproduction *per se* does not extend lifespan, one interpretation of this finding is that the somatic gonad gives rise to an equal and opposite lifespan regulatory signal to that from the germline (Hsin & Kenyon 1999). In contrast, body size is increased by whole-gonad ablation as it is by germline ablation, implying that unlike lifespan, body size is only regulated by germline signalling (Patel *et al* 2002). The putative somatic gonad lifespan signal was found to be at least in part dependent on *daf-2*, since whole-gonad ablation in *daf-2(rf)* hermaphrodites resulted in lifespan extension similar to or slightly less than that seen upon germline ablation.

Figure 4.3: Effect of whole-gonad ablation (Z1-Z4(-)) on wild-type hermaphrodite lifespan, and DIC image of early L1 gonad with germline and somatic gonad precursors ablated. Scale bar= 50μm



No effect on hermaphrodite lifespan
Body size increased as in Z2/Z3(-)

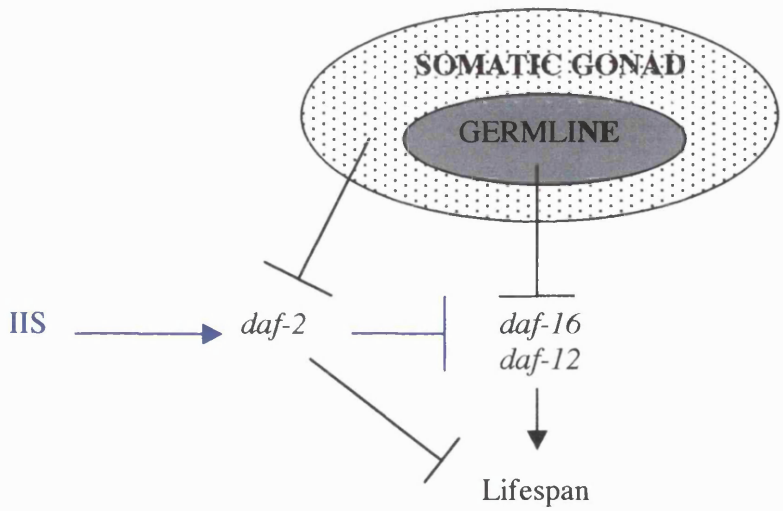
When whole-gonad ablations were performed in *daf-16* and *daf-12* mutant genetic backgrounds, a significant reduction in hermaphrodite lifespan was seen (Hsin &

[1] It was not possible to investigate the effect on lifespan of loss of the somatic gonad alone since the germline is dependent upon the somatic structures for its maintenance. According to the working model (Figure 4.4) removal of the somatic gonad precursors alone would (hypothetically) reduce lifespan.

Kenyon 1999). This was interpreted as implying that *daf-12* and *daf-16* are not required for somatic gonad signalling, since if they were, removal of the somatic gonad in these mutant backgrounds (in which the germline signal is absent) would, as in wild-type, not affect lifespan.

The model proposed by (Hsin & Kenyon 1999) for gonadal regulation of longevity in *C. elegans* hermaphrodites is therefore as illustrated in Figure 4.4 below, with a *daf-16*- and *daf-12*-dependent lifespan-reducing signal from the germline being counteracted by a *daf-2*-dependent lifespan-enhancing signal from the somatic gonad. It is assumed that somatic gonad signalling suppresses rather than enhances *daf-2* signalling, since *daf-2(rf)* mutations increase lifespan (Kenyon *et al* 1993). Note that this model is based on epistasis data and that dependence of the germline signal on *daf-16* and *daf-12* does not necessarily mean they act downstream of the signal, but could simply be required for its transduction. A separate element of *daf-2* signalling is pictured in blue, representing a somatic gonad-independent *daf-2* function that acts via *daf-16*, as identified from studies of long-lived mutants.

Figure 4.4: Model for gonadal regulation of lifespan in hermaphrodite *C. elegans* (adapted from (Hsin & Kenyon 1999)).



In *C. elegans*, therefore, gonadal laser ablation experiments suggest that rather than a trade-off between reproduction and somatic maintenance involving differential

allocation of resources, there is regulation of lifespan by molecular signals from the gonad (discussed in (Leroi 2001)).

Effect of gonad ablations on male lifespan

Since gonadal signalling regulates hermaphrodite lifespan, and since males are intrinsically longer-lived than hermaphrodites (Gems & Riddle 2000b), it seemed possible that sex differences in gonadal signalling may contribute to sex differences in lifespan. Not only do males and hermaphrodites differ in the structure and function of the gonad, they have different reproductive strategies: males produce sperm throughout life, while hermaphrodites (in the absence of mating with males) no longer reproduce after a few days. Potentially, a decreased germline signal in wild-type males relative to hermaphrodites could contribute to the increased male longevity. Survival analyses were therefore performed for germline and whole-gonad ablated N2 males and hermaphrodites.

Since the hermaphrodite germline signal is dependent on *daf-12* (Hsin & Kenyon 1999), it was also possible that differential regulation of *daf-12* expression could result in different levels of gonadal signalling between the sexes. This was suggested by a preliminary observation suggesting that *daf-12::gfp* somatic gonadal expression was lower in males than hermaphrodites (A. Antebi, pers. comm.; Chapter 5 Section 5.3.7), and by sex differences in lifespan regulation by *daf-12* (see Chapter 5). To investigate this further, germline and whole-gonad ablations were performed in *daf-12(0)* males and hermaphrodites.

Germline proliferation mutations and lifespan in *C. elegans*

As well as germline laser ablation, a number of mutants with defects in early germline proliferation (which hence produce neither oocytes nor sperm) show increased hermaphrodite lifespan, including *glp-1, glp-4* (germline proliferation), *gld-1*, (germline differentiation abnormal), *pgl-1* (p granule abnormality) and *mes-1* (maternal effect sterile) mutants (Ng *et al* 2001; Arantes-Oliveira *et al* 2002). Thus, while hermaphrodites sterile due to lack of sperm production only (such as *fem-3* mutants) are not long-lived (Kenyon *et al* 1993; Arantes-Oliveira *et al* 2002), prevention of all

germline activity early in development can result in increased lifespan. Interestingly, the lifespan extension due to *glp-1* and *mes-1* mutations was found to be dependent on *daf-16* (using RNAi feeding) and on the presence of intact somatic gonad precursor cells (using laser ablation), similar to the hermaphrodite response to germline ablation (Hsin & Kenyon 1999; Arantes-Oliveira *et al* 2002). In order to complement investigations into the effect of gonadal ablations on male lifespan, the effect of one such germline mutation (*mes-1(bn7ts)*) on male lifespan was determined.

Interaction of *unc* with germline mutations in lifespan regulation

As described in Chapter 3, neuronal *unc* mutations extend male lifespan while having little effect on hermaphrodite lifespan (Gems & Riddle 2000b). Previous work using hermaphrodite germline mutants had suggested that there could be an interaction between neuronal *unc* mutations and germline mutations with respect to lifespan regulation, since germline mutations linked to *unc* markers showed much more robust increases in lifespan than did non-*unc*-linked germline mutations (Ng *et al* 2001). This suggests that loss of the germline might cause hermaphrodites to exhibit increased lifespan upon mutation of neuronal *unc* genes that normally have little effect on hermaphrodite lifespan. It therefore seemed possible that differences between germline signalling in the sexes may regulate the sex difference in response to neuronal *unc* mutations. In order to investigate this, lifespan increases due to *mes-1(bn7ts)* were compared in both sexes, with and without the *unc-32(e189)* mutation.

4.1 Materials and methods

4.1.1 Strain maintenance and constructions

Strains used in this chapter included: CB189 *unc-32(e189) III* (Brenner 1974), AA86 *daf-12(rh61rh411) X* (Antebi *et al* 1998) and SS149 *mes-1(bn7ts) X* (Strome *et al* 1995). *mes-1(bn7ts)* is temperature-sensitive maternal effect sterile, with 70% of progeny of animals raised at 25°C having no germline and growing into sterile adults. However, animals raised at 15°C are almost all fertile and can be cultured normally. The sterile phenotype is the result of defective divisions of P_2 and P_3 and of the transformation of P_4

from a germline precursor into a muscle precursor. *mes-1* mutants therefore have no germline precursors, and contain up to 20 extra muscle cells instead of germ cells (Strome *et al* 1995). Unlike mutations in other *mes* genes (such as *mes-2*, *mes-3*, *mes-4* and *mes-6*), which affect mainly hermaphrodites due to their sensitivity to X chromosome dosage (Garvin *et al* 1998), *mes-1* is unlikely to display sex-specificity in its effects due to its more general role in germline development. Consistent with this, a selection of *mes-1(bn7ts)* males examined under high power microscopy contained no sperm in their gonads (data not shown).

The *unc-32(e189) III*; *mes-1(bn7ts) X* double mutant was constructed as follows. Heterozygote *e189*/+ males were crossed with *mes-1(bn7ts)* hermaphrodites at 15°C, and a number of the resulting hermaphrodite progeny cloned. Individuals with an Unc phenotype were selected; these were either +/+, +/*bn7* or *bn7/bn7* at the *mes-1* locus. Individual Unc animals were left to lay eggs at 25°C, and those producing 70% sterile progeny were picked as putative double homozygous mutants.

Males of SS149 were readily generated by heat shock and showed normal mating efficiency at 15°C. *unc-32(e189)*; *mes-1(bn7ts)* males were generated as follows. *mes-1(bn7ts)* males were mated with *unc-32(e189)*; *mes-1(bn7ts)* hermaphrodites at 15°C. Males resulting from this cross were heterozygous *unc-32(e189)*, homozygous *mes-1(bn7ts)*. These males were back-crossed to the original *unc-32(e189)*; *mes-1(bn7ts)* strain at 25°C, and Unc progeny selected for survival analysis, ~70% of which were sterile.

4.1.2 Laser ablations

Mating stocks of approximately 20 hermaphrodites and 40 males were left at 20°C overnight, and self progeny discarded. An hour before L1 larvae were required for ablation, the mated adults and any larvae were washed off the plates with sterile M9 buffer. Eggs adhered to the bacteria and remained on the plates. After one hour at 22.5°C, many eggs had hatched into L1 larvae. 2ml of M9 were added to the plate and gently swirled around to lift the larvae from the surface. A 10µl sterile glass capillary tube was flamed and pulled in half to form a very narrow tip. This was attached to some tubing and used to carefully mouth-pipette up the animals, with as little buffer as

possible. The L1 larvae collected were deposited into a small drop of 0.5-0.7% phenoxypropanol anaesthetic in M9 on the inside of a sterile Petri dish lid and left for approximately five minutes. After several minutes the anaesthetised larvae were collected in a fresh capillary tube using as little liquid as possible, and were placed onto the centre of an agar pad on a slide (see Main Materials and Methods). A baked coverslip was then placed carefully over the droplet containing the larvae, and the slide was ready for viewing. Intact controls were anaesthetised and placed on slides for the duration of the experimental ablations to ensure equal treatment.

Ablations were performed using a Micropoint dual nitrogen dye laser (beam wavelength 440nm) linked via fibre optics to a Zeiss Axioplan 2 microscope. Photographs were taken using a Hamamatsu ORCA-ER digital camera. A laser-safe filter cube was fitted to the filter wheel of the microscope, which prevented scattered laser light from reaching the eyes.

Before beginning the ablations, a "map" showing the relative position of all the larvae on the slide was drawn so they could be located more easily under high power and so larvae that had undergone ablation could be marked off. Ablations were performed using the x100 objective under oil immersion. The beam was targeted to the correct cells using a "sight" in the form of an eyepiece graticule, and the laser fired by pressing a foot pedal. The focus and intensity of the laser were first tested by focusing onto the plane of the coverslip and firing the laser to burn a small, neat hole in the glass. The power of the laser could be adjusted using a sliding, graduated neutral density filter.

The four gonadal precursor cells were located and the sight positioned over the nucleus of the first cell to be ablated. The laser was fired until the nucleus was seen to "bubble" and contract into a small spot (illustrated in Figures 4.2 & 4.3 above). For larger nuclei, it was sometimes necessary to move the laser over the surface and fire again in order to ensure that ablation was successful: nuclei that received insufficient energy from the laser were still capable of undergoing a limited number of divisions. Ablations were carried out as quickly as possible, since it was not desirable to leave the larvae in phenoxypropanol for longer than approximately 30 minutes, after which time it became damaging to the animals. In addition, the anaesthetic began to wear off after this

time, with larval movement making ablation impossible. Slides were therefore never prepared containing more than approximately 40 larvae.

Once all the larvae on the slide had been ablated, the coverslip was carefully slid off the agar, leaving the larvae on the pad. A small drop of sterile M9 was then added to the pad, and a fresh capillary tube used to draw the larvae up and deposit them on a fresh agar plate streaked with OP50. This was repeated for non-ablated controls. The larvae were then left to grow at 20°C. At the L4 stage, they were sexed and transferred to liquid culture as described in Main Materials and Methods. Lifespan measurements were performed at 22.5°C, with both sexes transferred equally. Four replicates were performed for each treatment for both sexes of each genotype.

4.1.3 Survival analysis for germline mutants

mes-1 mutants were raised at 15°C for sub-culture and 25°C for experiments due to the ts nature of *mes-1(bn7)*. Survival analyses were performed at either 22.5°C or 25°C as stated in Results. By the young adult stage the sterility phenotype was apparent in ~70% of hermaphrodites as a clear patch where the developing eggs were normally seen. However it was not possible to tell under the dissecting microscope whether a *mes-1(bn7ts)* male raised at 25°C was sterile or not. For this reason, L4 males and hermaphrodites were selected randomly from the stock plate, and both sterile and non-sterile individuals included in the survival analysis. It was assumed that the increased longevity of sterile hermaphrodites would still be apparent even though ~30% of the population had some degree of fertility, and that it would therefore be possible to ascertain whether male longevity was affected by this germline mutation. L4 animals were transferred to liquid culture under OP50 in S medium as described in Main Materials and Methods, with both sexes transferred equally. At least two replicates were performed for each genotype of each sex unless specifically stated.

4.2 Results

NB: ablation experiments were performed in collaboration with Adam Antebi and Veerle Rottiers, at the Max-Planck-Institut für Molekulare Genetik, Berlin. All aspects of practical work relating to these experiments were shared equally between DM and VR.

4.2.1 Effect of gonad ablations on N2 male and hermaphrodite lifespan

In order to investigate whether there were sex differences in the gonadal regulation of lifespan, the effects of germline and whole-gonad ablations on N2 male and hermaphrodite survival were determined. Data presented here are the pooled results for all four replicates; results for individual replicates can be found in Appendix E.6.

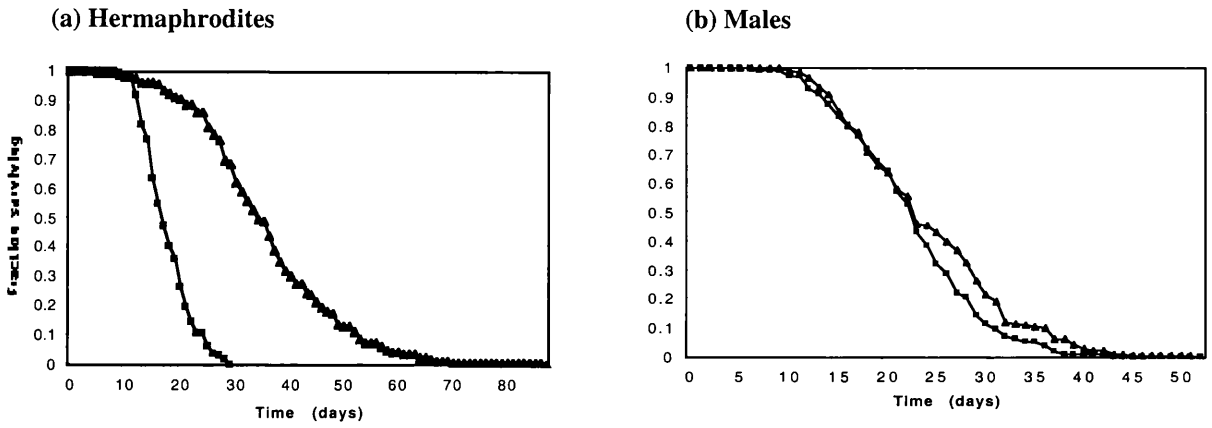
A significant increase in hermaphrodite survival upon germline ablation ($P<0.0001$, Figure 4.5a) confirmed previous work (Hsin & Kenyon 1999). However, in the present study, median hermaphrodite lifespan was increased to a greater extent (109%, Table 4.1) than was mean lifespan in (Hsin & Kenyon 1999) (~60%). Interestingly, unlike in hermaphrodites, male median lifespan was unaffected by germline ablation, although maximum lifespan was increased by 12% (Table 4.1). Male survival was not significantly affected by germline ablation in three of the four replicates. (Appendix E.6 Table 6.1a-d). However, overall, Z2/Z3(-) male survival was significantly different from that of intact controls, presumably reflecting the late-life separation between germline-ablated and intact survival curves ($P= 0.0037$, Figure 4.5b). Note, however, that the effect of germline ablation on male lifespan was not nearly as marked as that seen in hermaphrodites.

Table 4.1: Effect of germline ablation on N2 survival (22.5°C)

Treatment/ sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	16.8 (17.9, 16.0)	----	26.8	----	145 (251)	----
Z2/Z3(-) H	35.0 (36.8, 32.5)	+109	62.0	+113	161 (203)	<0.0001
Intact M	22.8 (23.8, 21.5)	----	38.0	----	245 (273)	----
Z2/Z3(-) M	23.1 (25.0, 21.4)	+2	42.5	+12	208 (225)	0.0037

*N= number senescent deaths (initial sample size) †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

Figure 4.5: Effect of germline ablation on (a) hermaphrodite and (b) male N2 survival (22.5°C). Squares: intact control; triangles: Z2/Z3(-).



In contrast to (Hsin & Kenyon 1999), whole gonad ablation extended hermaphrodite lifespan compared with intact controls, both overall (P<0.0001, Figure 4.6a) and in all four replicates (Appendix E.6 Table 6.2a-d). Although hermaphrodite median lifespan was increased by whole-gonad ablation (Table 4.2), the increase (64%) was less than that following germline ablation (109%). Thus, whole-gonad ablation incompletely suppressed the increase in hermaphrodite lifespan due to germline ablation. This implies that the strength of the somatic gonad signal was reduced in this experiment relative to the previous study (Hsin & Kenyon 1999).

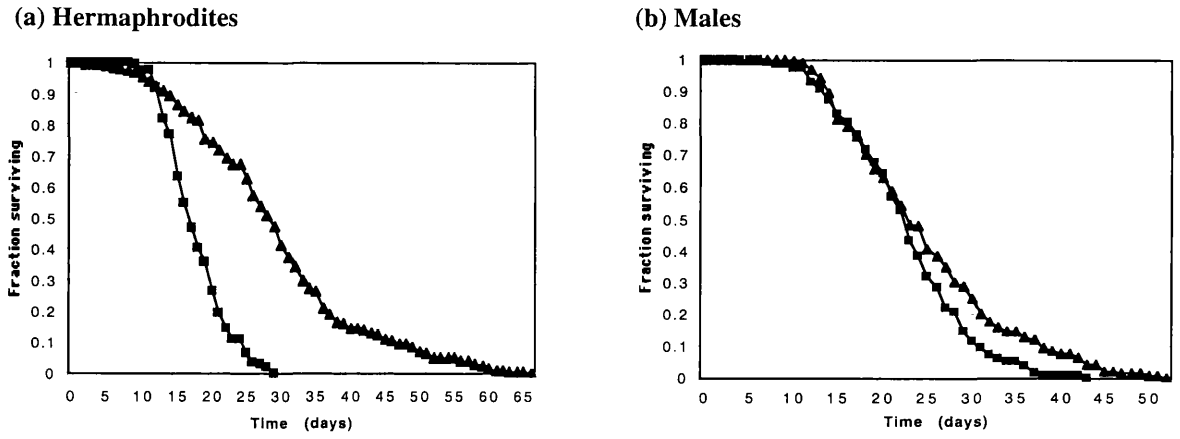
Overall, male survival was also significantly increased by whole-gonad ablation (P= 0.0003, Figure 4.6b), although it was unaffected in two out of four individual replicates (Appendix E.6 Table 6.2a-d). Again, male median lifespan was unchanged (Table 4.2), with the difference in survival only becoming apparent late in time. Where significant increases in male survival were seen, they were to a much lesser extent than in hermaphrodites.

Table 4.2: Effect of whole gonad ablation on N2 survival (22.5°C)

Treatment/ sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	P†
Intact H	16.8 (17.9, 16.0)	----	26.8	----	145 (251)	----
Z1-Z4(-) H	27.5 (29.3, 26.0)	+64	57.25	+114	246 (288)	<0.0001
Intact M	22.8 (23.8, 21.5)	----	38.0	----	245 (273)	----
Z1-Z4(-) M	23.8 (25.8, 22.0)	+4	42.8	+13	216 (226)	0.0003

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

Figure 4.6: Effect of whole-gonad ablation on (a) hermaphrodite and (b) male N2 survival (22.5°C). Squares: intact control; triangles: Z1-Z4(-).



4.2.2 Effect of gonad ablations on *daf-12(0)* male and hermaphrodite lifespan

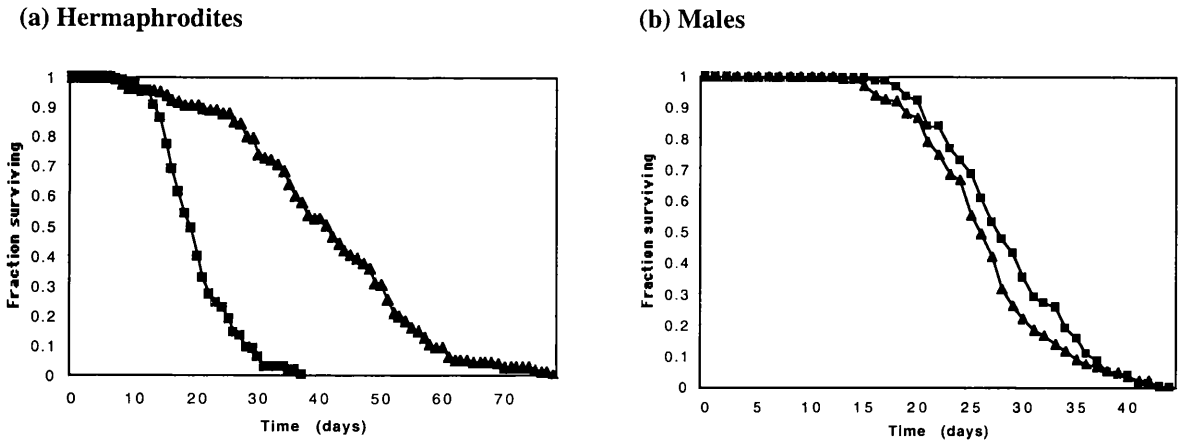
Alongside the above experiment, survival was also determined for males and hermaphrodites of the putative null mutant *daf-12(rh61rh411)*. Unexpectedly, results contradicted (Hsin & Kenyon 1999) in that hermaphrodite survival was still significantly increased upon germline ablation in a *daf-12(0)* background ($P<0.0001$, Figure 4.7a). The magnitude of this lifespan extension was similar to that seen above in a wild-type background (Table 4.3). In contrast, *daf-12(0)* male median lifespan was unchanged by germline ablation in three replicates and slightly decreased in one (Appendix E.6 Table 6.3a-d). Overall *daf-12(0)* male survival was slightly but significantly decreased by germline ablation ($P=0.019$, Figure 4.7b), although median and maximum lifespans were unaffected (Table 4.3).

Table 4.3: Effect of germline ablation on *daf-12(rh61rh411)* survival (22.5°C)

Treatment/ sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	18.8 (19.8, 17.8)	----	33.5	----	291 (284)	----
Z2/Z3(-) H	39.9 (42.9, 36.9)	+113	69.8	+108	236 (308)	<0.0001
Intact M	27.0 (28.3, 25.3)	----	39.8	----	238 (281)	----
Z2/Z3(-) M	26.3 (27.5, 25.0)	-3	39.0	-2	154 (220)	0.019

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

Figure 4.7: Effect of germline ablation on (a) hermaphrodite and (b) male *daf-12(rh61rh411)* survival (22.5°C). Squares: intact control; triangles: Z2/Z3(-).



As seen in a wild-type genetic background, *daf-12(0)* hermaphrodite survival was significantly increased by whole-gonad ablation ($P<0.0001$, Figure 4.8a), with *daf-12(0)* median lifespan increased to a greater extent (155%, Table 4.4) than in *daf-12*(+)(64%). Interestingly, however, the increase in *daf-12(0)* median lifespan upon whole gonad ablation was greater than that upon germline ablation alone. This finding implies that the effect of the somatic gonad signal on hermaphrodite lifespan was reversed (i.e. negative) in *daf-12(0)* compared with *daf-12*(+), since removal of the somatic gonad in *daf-12(0)* enhanced lifespan extension due to germline ablation rather than suppressing it.

In contrast, *daf-12(0)* male survival was unaffected by whole-gonad ablation in three of four replicates, but slightly increased in one (Appendix E.6 Table 6.4a-d). Overall, whole-gonad ablation slightly but significantly increased *daf-12(0)* male survival ($P= 0.0074$, Figure 4.8b) and maximum lifespan, but did not affect median lifespan (Table 4.4).

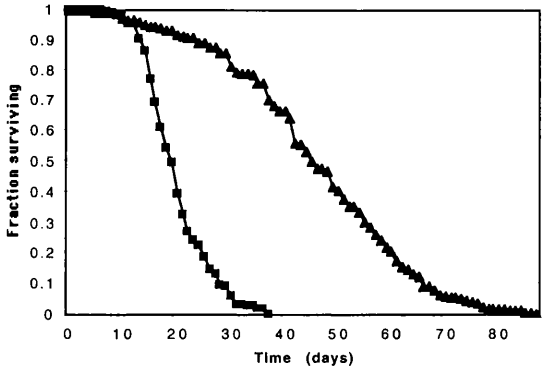
Table 4.4: Effect of whole-gonad ablation on *daf-12(rh61rh411)* survival (22.5°C)

Treatment/ sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	18.8 (19.8, 17.8)	----	33.5	----	291 (284)	----
Z1-Z4(-) H	47.8 (50.1, 44.8)	+155	89.8	+168	249 (278)	<0.0001
Intact M	27.0 (28.3, 25.3)	----	39.8	----	238 (281)	----
Z1-Z4(-) M	29.3 (31.0, 27.4)	+8	47.5	+19	188 (198)	0.0074

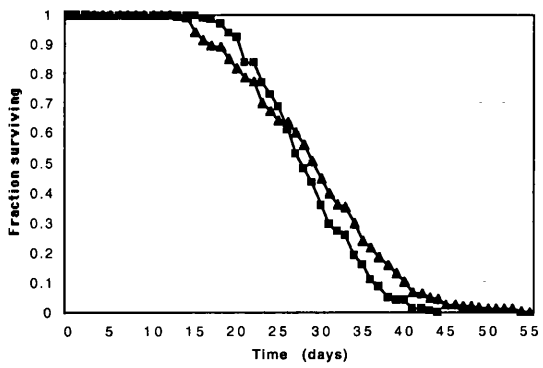
*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

Figure 4.8: Effect of whole-gonad ablation on (a) hermaphrodite and (b) male *daf-12(rh61rh411)* survival (22.5°C). Squares: intact control; triangles: Z1-Z4(-).

(a) Hermaphrodites



(b) Males



It is interesting to note that *daf-12(rh61rh411)* increased the lifespans of both sexes, a finding discussed more fully in Chapter 5 Section 5.3.1.

4.2.3 Effect of *mes-1(bn7ts)* on male and hermaphrodite lifespan

Before presenting the effects of *mes-1(bn7ts)* on male and hermaphrodite lifespan, it should be noted that unexpectedly, control N2 hermaphrodites were significantly longer-lived than N2 males in liquid culture at 25°C (Table 4.5, Figure 4.9a). Three repeat survival analyses for N2 confirmed that males were not the longer-lived sex under these conditions (Figure 4.9b-d). This reversal in the sex bias to lifespan was due to hermaphrodite lifespan being markedly greater in the present experiment than would be expected based on previous experiments on agar plates at 25°C. It would therefore appear that there is an interaction between temperature, liquid culture and sex differences in lifespan, a concept considered further in the Discussion.

In agreement with previous work (Arantes-Oliveira *et al* 2002; Ng *et al* 2001), *mes-1(bn7ts)* resulted in a significant increase in hermaphrodite survival (Figure 4.10a), with median and maximum lifespans increased by 29% and 52% respectively (Table 4.5; Figure 4.11). Male survival was also significantly increased by *mes-1(bn7ts)* (Figure 4.10b), but to a smaller extent than hermaphrodite survival, with median and maximum lifespans both increased by 20% (Table 4.5; Figure 4.11). However, the increase in male lifespan due to *mes-1(bn7ts)* was markedly greater than that seen following germline

ablation above, since both median and maximum lifespan were extended in this case, compared with a 12% increase in maximum lifespan only following germline laser ablation. This was despite the fact that ~30% of animals (of both sexes) included in the survival analysis were non-sterile. As explained above, this was because it was not possible to determine which of the *mes-1(bn7ts)* males raised at 25°C were sterile.

Figure 4.9: Survival curves for N2 males (triangles) and hermaphrodites (squares) at 25°C in liquid culture. (a) Present experiment (Table 4.5); (b) Males: n=96, hermaphrodites: n=67; (c) Males: n=47, hermaphrodites: n=40; (d) Males: n=48, hermaphrodites: n=29

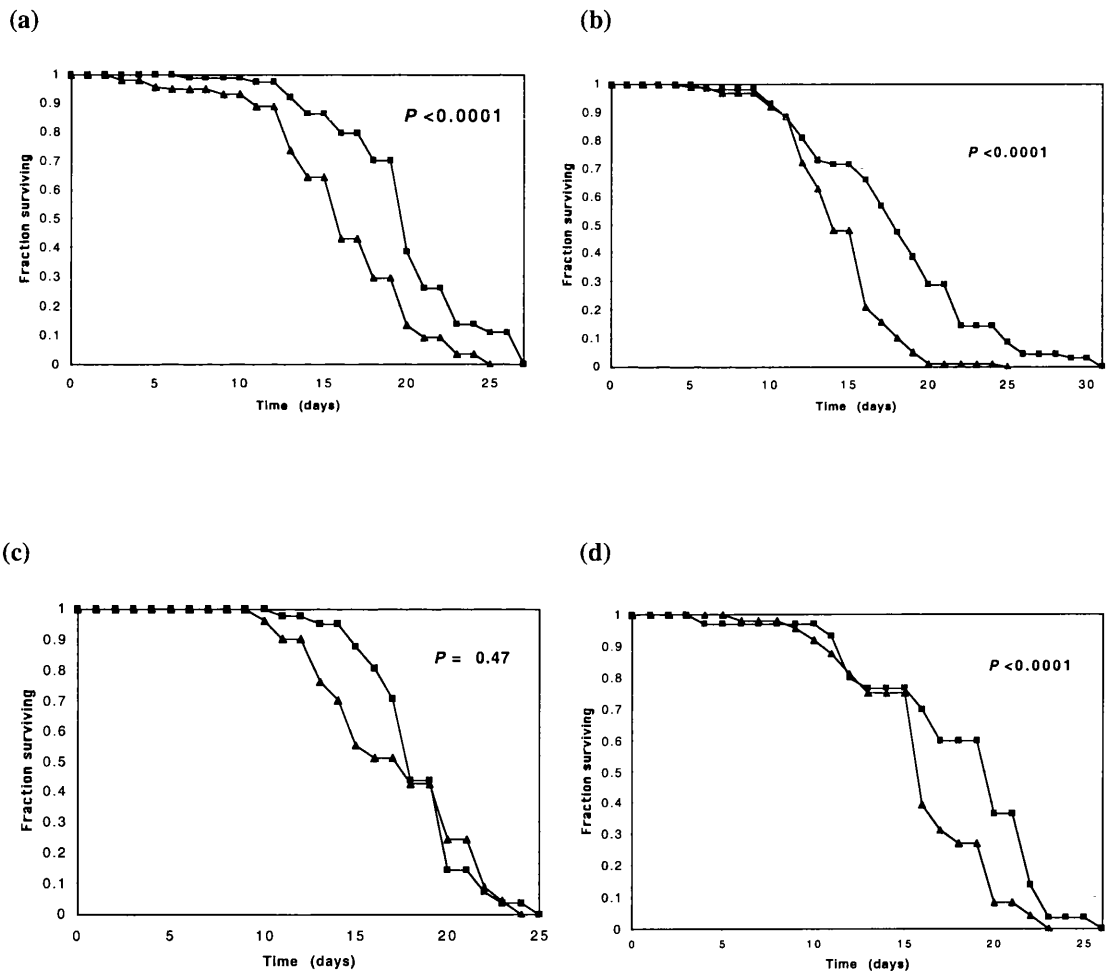


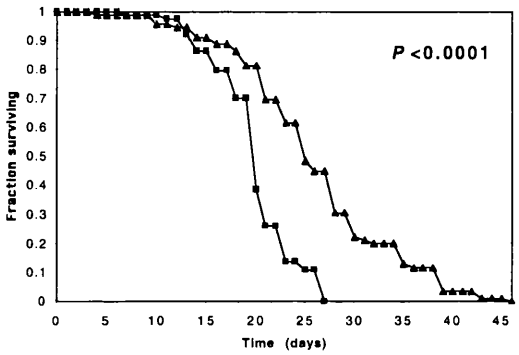
Table 4.5: Effect of *mes-1(bn7ts)* on male and hermaphrodite lifespan (25°C)

Genotype/ sex	Median lifespan (days) ± 95% C.I.	% change in median lifespan relative to N2	Maximum lifespan (days)	% change in max. lifespan relative to N2	N*	*P*†
+ H	20.0 (20.5, 19.0)	----	25.0	----	73 (111)	----
mes-1(bn7ts) H	25.8 (28.0, 24.0)	+29	38.0	+52	85 (114)	<0.0001
+ M	16.0 (17.5, 15.0)	----	23.0	----	118 (120)	----
mes-1(bn7ts) M	19.2 (20.3, 18.2)	+20	30.0	+20	164 (167)	<0.0001

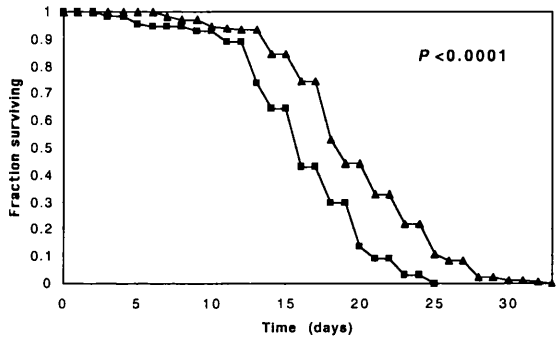
*Number of senescent deaths (starting population); †Probability that *mes-1(bn7ts)* survival differs from that of wild type by random chance (log rank test).

Figure 4.10: Survival curves for N2 (squares) and *mes-1(bn7ts)* (triangles) in (a) hermaphrodites and (b) males (25°C)

(a)



(b)



Due to the unexpected hermaphrodite longevity bias seen in N2 controls at 25°C described above, the effect of *mes-1(bn7ts)* on male and hermaphrodite lifespan was also determined at 22.5°C (Table 4.6). As noted for many N2 survival analyses at this temperature, in this instance male and hermaphrodite median lifespans were the same, although there was a tail on the male survival curve and male maximum lifespan was ten days longer than that of hermaphrodites (Table 4.6; Figure 4.12).

mes-1(bn7ts) extended hermaphrodite median and maximum lifespans by 31% and 123% respectively at 22.5°C (Table 4.6, Figures 4.11 & 4.12). This increase in hermaphrodite median lifespan due to *mes-1(bn7ts)* was markedly less than that seen upon germline laser ablation at the same temperature (109%). This is probably at least in part because a proportion of the hermaphrodites included in the survival analysis were not sterile for the reason described above. Consistent with this, the increase in

hermaphrodite maximum lifespan due to *mes-1(bn7ts)* (123%) was comparable with that seen following germline ablation above (113%).

Male median lifespan was unaffected by *mes-1(bn7ts)* at 22.5°C, while maximum lifespan was decreased by 29% (Table 4.6, Figures 4.11 & 4.12). These results are consistent with the results following germline laser ablation at the same temperature above, where only male maximum lifespan was slightly increased. Therefore, in addition to the laser ablation experiments, these results further suggest that there are sex differences in germline signalling.

Table 4.6: Effect of *mes-1(bn7ts)* on male and hermaphrodite lifespan (22.5°C)

Genotype/ sex	Median lifespan (days) ± 95% C.I.	% change in median lifespan relative to N2	Maximum lifespan (days)	% change in max. lifespan relative to N2	N*	*P*†
+ H	16.0 (18.0, 16.0)	----	21.0	----	46 (54)	----
mes-1(bn7ts) H	21.0 (22.5, 18.5)	+31	47.0	+123	75 (120)	<0.0001
+ M	16.0 (18.0, 16.0)	----	31.0	----	60 (60)	----
mes-1(bn7ts) M	17.0 (20.0, 15.0)	+6	22.0	-29	39 (41)	0.78

*Number of senescent deaths (starting population); †Probability that *mes-1* mutant survival differs from that of wild type by random chance (log rank test).

Figure 4.11: Effect of *mes-1(bn7ts)* on hermaphrodite (red) and male (blue) median (no pattern) and maximum (striped bars) lifespans at 25°C and 22.5°C. Significant effects on median lifespan are denoted as stars.

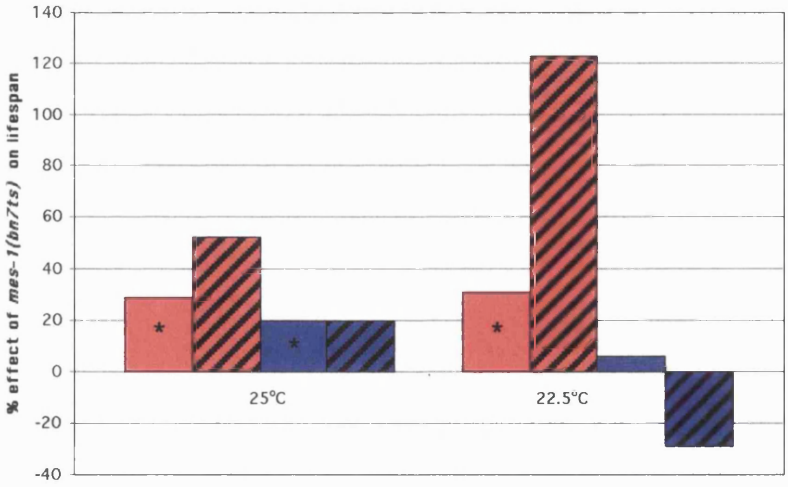
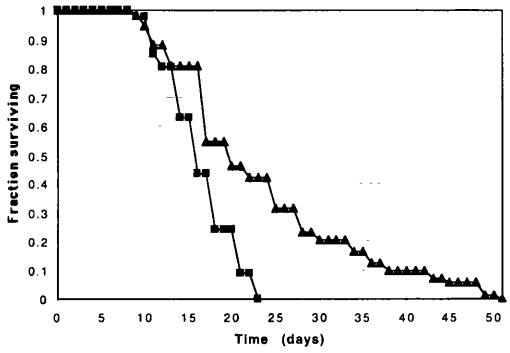
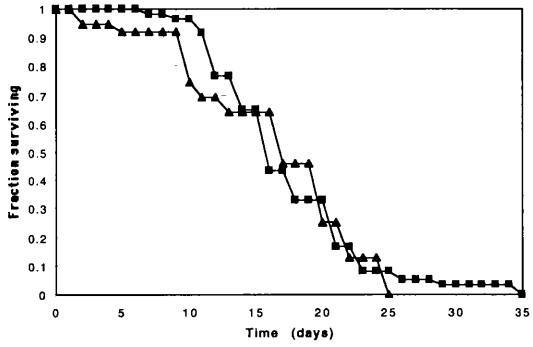


Figure 4.12: Survival curves for N2 (squares) and *mes-1(bn7ts)* (triangles) in (a) hermaphrodites and (b) males (22.5°C) *P* values are provided in Table 4.6.

(a) Hermaphrodites



(b) Males



4.2.4 Interaction of *unc* with germline signalling in lifespan regulation

As described in the Introduction, a working hypothesis of this chapter was that sex differences in germline signalling may underlie the sex differences in lifespan response to neuronal *unc* mutations. In order to investigate this, male and hermaphrodite *mes-1(bn7ts)* lifespans in an *unc-32(e189)* genetic background were determined concurrently with those presented above in a wild-type genetic background.

The increase in hermaphrodite *mes-1(bn7ts)* median lifespan due to *unc-32(e189)* (58%) was much greater than that seen in N2 hermaphrodites (25%) (Table 4.7; Figures 4.13 & 4.14). This supports the hypothesis that the germline regulates the hermaphrodite lifespan response to *unc-32(e189)*. The effect of *mes-1(bn7ts)* on hermaphrodite lifespan was also much more marked in *unc-32(e189)* than in wild-type (Table 4.7; Figure 4.13). Thus, *mes-1(bn7ts)* and *unc-32(e189)* act together (either via the same or separate mechanisms) to enhance hermaphrodite lifespan.

In contrast, male *mes-1(bn7ts)* survival was increased to a lesser extent by *unc-32(e189)* than was N2 male survival (Table 4.7; Figures 4.13 & 4.14). Moreover, there was no effect of *mes-1(bn7ts)* on *unc-32(e189)* male lifespan (Table 4.7; Figures 4.13) (although conceivably this could be because a smaller proportion of the randomly selected males were sterile than in an *unc-32*(+) genetic background). Potentially, this could be due to pleiotropic effects resulting in reduced viability of *unc-32(e189)*; *mes-1(bn7ts)* males, which could partially mask the effect of these two mutations on lifespan.

Alternatively, *unc-32(e189)* and *mes-1(bn7ts)* may affect a common mechanism to extend male lifespan.

Figure 4.13: Effect of *unc-32(e189)* on N2 (closed shapes) and *mes-1(bn7ts)* (open shapes) survival (25°C). *P* values are given in Table 4.7.

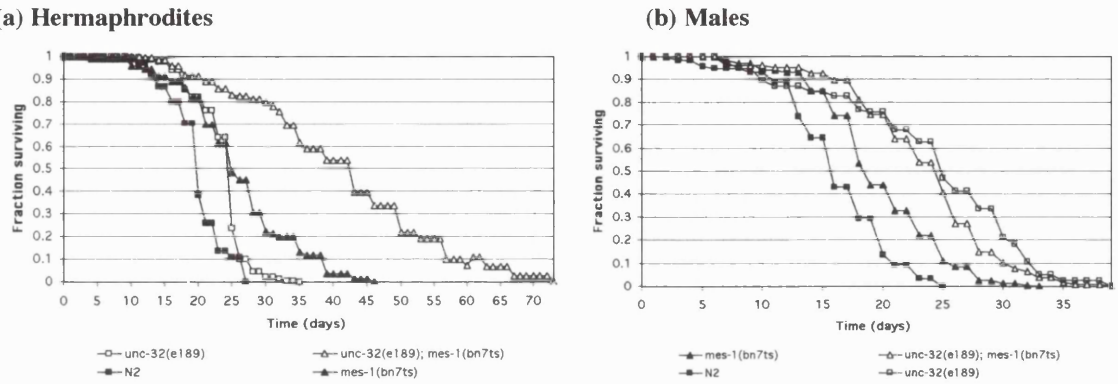


Figure 4.14: Effect of *unc-32(e189)* on male (M) and hermaphrodite (H) median (no pattern) and maximum (striped bars) lifespans in wild-type and *mes-1(bn7ts)* genetic backgrounds (25°C). All effects are significant.

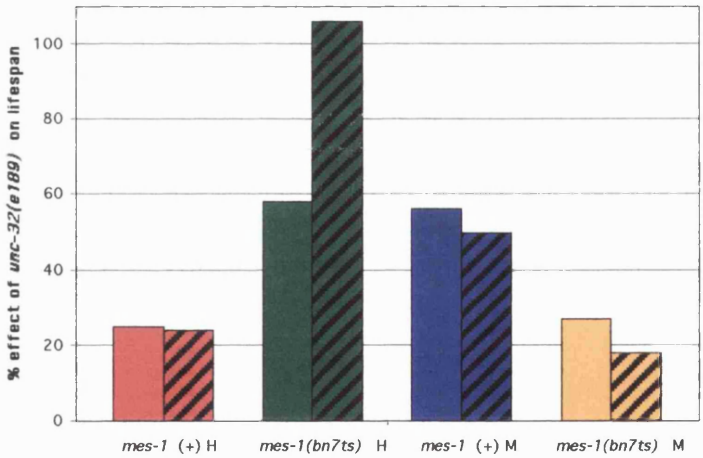


Table 4.7: Effect of *unc-32(e189)* on male and hermaphrodite lifespans in wild-type and *mes-1(bn7ts)* genetic backgrounds (25°C).

Genotype/ sex	Median lifespan (days) ± 95% C.I.	% effect on median lifespan of		Maximum lifespan (days)	% effect on max. lifespan of		N*	*P#*	*P†*
		bn7	*e189*		*bn7*	*e189*			
+ H	20.0 (20.5, 19.0)	----	----	25.0	----	----	73 (111)	----	----
mes-1(bn7ts) H	25.8 (28.0, 24.0)	+29	----	38.0	+52	----	85 (114)	<0.0001	----
+ M	16.0 (17.5, 15.0)	----	----	23.0	----	----	118 (120)	----	----
mes-1(bn7ts) M	19.2 (20.3, 18.2)	+20	----	30.0	+20	----	164 (167)	<0.0001	----
unc-32(e189) H	25.0 (25.0, 24.3)	----	+25	31.0	----	+24	171 (180)	----	<0.0001
unc-32(e189); mes-1(bn7ts) H	40.7 (45.3, 37.0)	+63	+58	64.0	+106	+68	131 (164)	<0.0001	<0.0001
unc-32(e189) M	25.0 (26.5, 23.0)	----	+56	34.5	----	+50	114 (118)	----	<0.0001
unc-32(e189); mes-1(bn7ts) M	24.3 (25.5, 23.0)	-3	+27	35.5	-3	+18	106 (109)	0.045	<0.0001

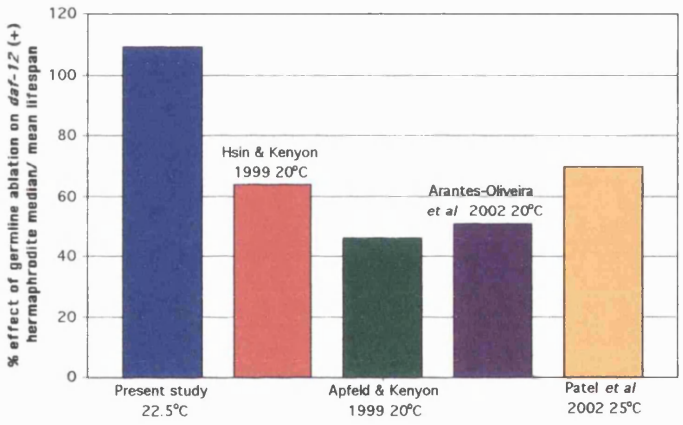
*Number of senescent deaths (starting population); #Probability that *mes-1(bn7ts)* survival differs from that of *mes-1*(+) in the same genetic background by random chance (log rank test); †Probability that *unc-32(e189)* survival differs from that of *unc-32*(+) in the same genetic background by random chance (log rank test).

4.3 Discussion

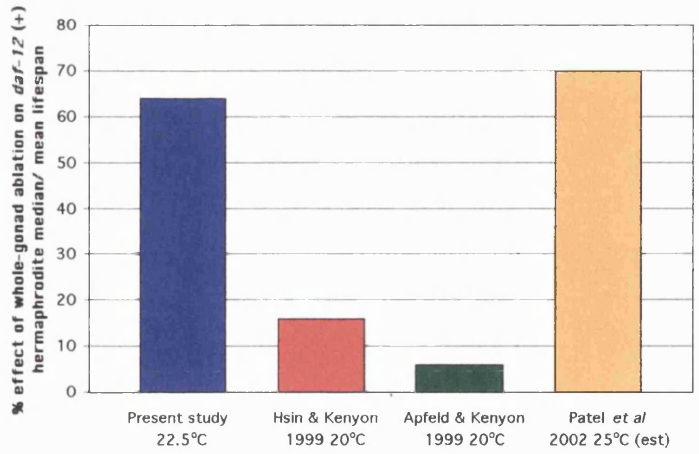
In order to aid interpretation, the reader may find it helpful to repeatedly refer to Figure 4.15 below, which summarises the effects of germline and whole-gonad ablation on wild-type and *daf-12(0)* hermaphrodite and male lifespans presented in this section, and compares them with relevant results from previous studies where available.

Figure 4.15 Summary of laser ablation results. Note that all studies were performed on agar plates with the exception of the present one, which employed liquid culture.

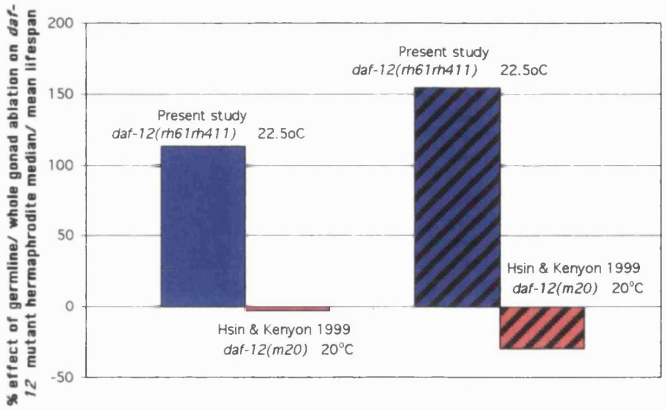
(a) Effect of germline ablation on *daf-12*(+) (*unc-50(e306)* in the case of (Arantes-Oliveira *et al* 2002)) hermaphrodite median/ mean lifespan



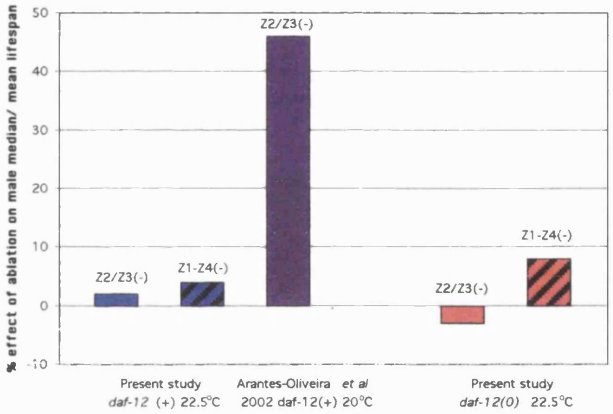
(b) Effect of whole-gonad ablation on *daf-12*(+) hermaphrodite median/ mean lifespan



(c) Effect of germline (no pattern) and whole-gonad (striped bars) ablation on *daf-12(0)* hermaphrodite median/ mean lifespan



(d) Effect of germline and whole gonad ablation on N2 (*unc-50(e306)* in the case of Arantes-Oliveira *et al* 2002)) and *daf-12(0)* male median/ mean lifespan



4.3.1 Wild-type hermaphrodite response to germline ablation

Germline laser ablation significantly increased wild-type hermaphrodite survival, as seen previously (Hsin & Kenyon 1999). However, the increase in median lifespan in the present study (109%) was markedly greater than seen previously ((Hsin & Kenyon 1999; Apfeld & Kenyon 1999; Arantes-Oliveira *et al* 2002; Patel *et al* 2002) (Figure 4.15a). This is consistent with germline signalling being stronger in hermaphrodites of the present study. However, since intact control lifespan was not increased, this seems unlikely unless the strength of germline signalling did not result in a proportional increase in lifespan. Alternatively, there may have been an interaction between the effect

of germline ablation and another lifespan regulatory component under conditions of the present study.

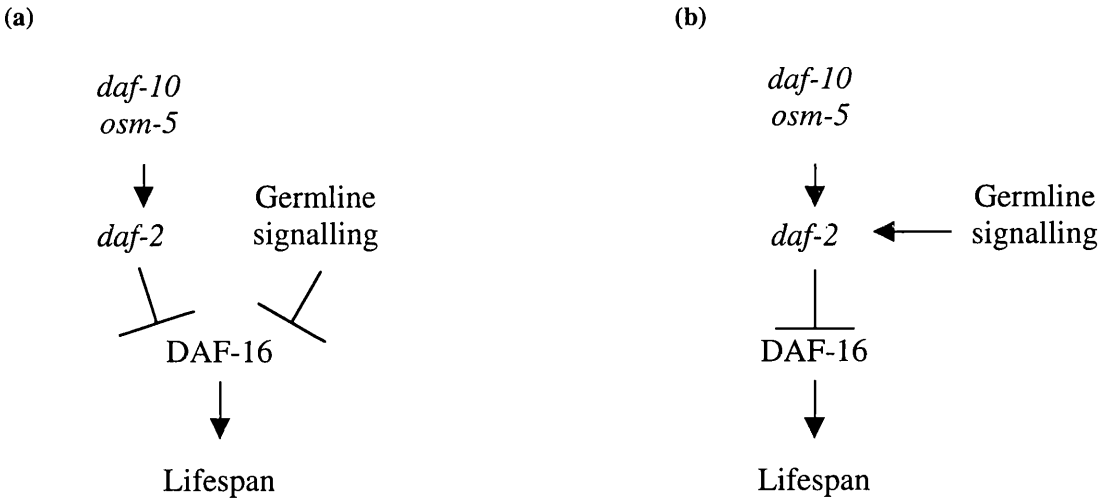
A larger increase in N2 hermaphrodite lifespan upon germline ablation as seen above is similar to those previously reported in certain mutants. Firstly, germline ablation in the long-lived sensory cilium mutants *daf-10(m79)* and *osm-5(p813)* increased mean lifespan by 76% and 64% respectively, compared with just 46% in wild type (Apfeld & Kenyon 1999). Sensory cilia are located at the dendritic endings of sensory neurons that detect environmental signals (White *et al* 1986; Perkins *et al* 1986; Peckol *et al* 1999). *daf-10* and *osm-5* mutants feed normally and have wild-type brood sizes, suggesting that the Age phenotype results from defects in sensory perception rather than reduced food intake (Apfeld & Kenyon 1999). The increased longevity of these mutants is suppressed by *daf-16(0)*, hence neurons expressing these genes are candidates for detection of an environmental signal that regulates *daf-2* signalling and thus influences DAF-16 activity (Apfeld & Kenyon 1999). Secondly, increased lifespan responses to germline ablation were seen in certain *daf-2(rf)* mutants (Hsin & Kenyon 1999). Specifically, increases in *daf-2* mutant hermaphrodite mean lifespan were 98% (*m596*) and 107% (*e1368*) compared with ~60% in a *daf-2*(+) genetic background. These data suggest that a sensory neuron-*daf-2* signalling pathway may regulate either the strength of the germline signal or the lifespan response to it.

The fact that in the present study, the N2 hermaphrodite lifespan response to germline ablation was enhanced in a similar manner to that of the mutants described above suggests the possibility that element(s) of the culture conditions used in the present study mimicked the effects of these mutations. *daf-2* is a component of insulin-/IGF-1-like signalling (IIS), which speculatively may respond at least in part to nutritional cues, in a similar manner to insulin signalling in mammals. Some IIS mutants are also ts in their effects on lifespan (Larsen *et al* 1995; Gems *et al* 1998). Possibly, differences in temperature and/or the food signal in the present study may be responsible for the increased magnitude of response to germline ablation relative to studies using other culture conditions, such as (Hsin & Kenyon 1999). For example, the decreased bacterial concentration in liquid culture relative to agar plates may reduce signalling

from sensory neurons expressing *daf-10* and *osm-5*, which in turn could reduce *daf-2* signalling.

How might attenuation of IIS increase the effect of germline ablation on lifespan? Firstly, there may be synergy between *daf-10/ osm-5/ daf-2* mutations and germline ablation with respect to lifespan, which would perhaps be due to common up-regulation of DAF-16 activity, since both mechanisms of lifespan increase are suppressed by *daf-16(0)* (Kenyon *et al* 1993; Apfeld & Kenyon 1999; Hsin & Kenyon 1999) (Figure 4.16a). Alternatively, germline signalling might normally promote DAF-2 activity (Figure 4.16b). Since the *daf-2* mutations used in (Hsin & Kenyon 1999) were not null, and any potential reduction in *daf-2* signalling due to culture conditions in the present study is presumably not maximised, removal of the germline may therefore reduce DAF-2 activity (or increase DAF-16 activity) further.

Figure 4.16: Two possible relationships between *daf-2* and germline signalling, based on results from (Apfeld & Kenyon 1999) and (Hsin & Kenyon 1999). (a) *daf-2* signalling and germline signalling are separate but act on a common target; (b) Germline signalling acts to promote a lifespan-regulatory component of *daf-2* function, suppressing DAF-16 activity.



Action in a common pathway as illustrated in Figure 4.16b was discounted by (Hsin & Kenyon 1999) due to the fact that *daf-2(rf)* animals with a second mutation in another gene of the PI3K branch of IIS, *age-1*, are not longer-lived than *daf-2* mutants alone (Dorman *et al* 1995), suggesting that mutation of *daf-2* maximises the effect on DAF-16 activity and lifespan. However, it remains possible that a separate element of *daf-2*

signalling, (i.e.) not the PI3K branch, is involved with germline signalling, in which case the synergistic lifespan effect of germline ablation in *daf-2* mutants could be the result of increased attenuation of a common pathway.

4.3.2 Wild-type hermaphrodite response to whole-gonad ablation

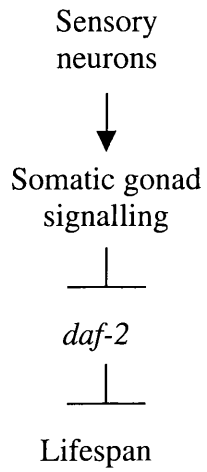
In contrast to (Hsin & Kenyon 1999), where whole-gonad ablation had no effect on hermaphrodite lifespan, in the present study whole-gonad ablation enhanced adult hermaphrodite survival, with median hermaphrodite lifespan being increased by an average of 64% (Figure 4.15b). Thus, removal of the somatic gonad failed to fully suppress the lifespan increase due to germline ablation, implying either that any somatic gonad signal was weaker in this study than in (Hsin & Kenyon 1999) or that elements that act to promote lifespan together with somatic gonad signalling were down-regulated.

Increased wild-type hermaphrodite lifespan following whole-gonad ablation was also reported (on agar plates) by (Patel *et al* 2002) (Figure 4.15b). In this case, whole-gonad ablation extended hermaphrodite lifespan to the same extent as did germline ablation, implying that the somatic gonad signal was completely absent under the conditions used. While experiments in (Hsin & Kenyon 1999) were performed at 20°C, they were performed at 25°C in (Patel *et al* 2002). It is therefore possible that increased temperature results in attenuation of the somatic gonad signal, consistent with the finding in the present study that the somatic gonad signal was reduced at 22.5°C relative to results in (Hsin & Kenyon 1999) at 20°C. Further studies would be required to determine whether the lifespan response of hermaphrodites to whole-gonad ablation varies according to temperature, or other environmental inputs. It is interesting to note that (Patel *et al* 2002) concluded that there was no regulation of body size by the somatic gonad, since both germline and whole-gonad ablation resulted in comparable increases in body size. However, if the effect of somatic gonad signalling on lifespan was in this case not apparent, it is possible that a similar situation may have applied to body size, and that under different conditions (such as lower temperatures), loss of somatic gonad signalling may suppress the increase in body size seen upon germline ablation.

Interestingly, reduction of the somatic gonad signal has also been noted in certain mutants. Firstly, lifespans of the sensory cilium mutants *daf-10(m79)* and *osm-5(p813)* on agar plates were increased by whole-gonad ablation to a similar extent as germline ablation (Apfeld & Kenyon 1999). Secondly, whole-gonad ablation in a range of *daf-2* mutants (particularly *daf-2(e1370)*) extended lifespan to varying extents (Hsin & Kenyon 1999). These results suggest that somatic gonad signalling requires *daf-2* signalling, as well as sensory neurons expressing *daf-10* and *osm-5*.

If *daf-2* signalling and somatic gonad signalling act within the same pathway, it is unclear whether the somatic gonad signal would act upstream or downstream of *daf-2*. Potentially, signalling from sensory neurons could up-regulate *daf-2* activity, which could in turn promote somatic gonad signalling and enhance lifespan. This scenario seems unlikely, however, since mutation of *daf-2* consistently acts to increase, rather than decrease lifespan. Alternatively, sensory neurons could signal to the somatic gonad, which could in turn down-regulate *daf-2* activity (Figure 4.17). Such a mode of action of *daf-2* might seem unlikely, given that sensory neurons are a major site of *ins* gene expression (Pierce *et al* 2001). Moreover, neurons are a major site of *daf-2* and *age-1* expression (Apfeld & Kenyon 1998; Wolkov *et al* 2000). Thus, either somatic gonad signalling feeds back to specific neuronal subsets to suppress activity of DAF-2 (as suggested in (Wolkow *et al* 2000)), or somatic gonad signalling may regulate ligand(s) which are produced by other tissue types, possibly by the somatic gonad itself.

Figure 4.17: Possible interaction of somatic gonad signalling with *daf-2* and sensory neuron signalling based on results from (Apfeld & Kenyon 1999) and (Hsin & Kenyon 1999). Somatic gonad signalling suppresses *daf-2* activity and promotes lifespan



Potentially, disruption of *daf-2* function prevents differential modulation of *daf-2* signalling by the somatic gonad, and somatic gonad signalling would effectively be constitutively on. Thus, removal of the somatic gonad would have little effect on lifespan, and the suppressive effect of whole gonad ablation on the lifespan increase due to germline ablation would be reduced. Thus, in a similar manner to that proposed for response to germline ablation above, conditions in the present study (such as liquid culture or high temperature) may simulate the effects of *daf-10/ osm-5/ daf-2* mutations, resulting in a reduced response to whole-gonad ablation.

The potential involvement of *daf-2* in somatic gonad signalling might suggest the involvement of *daf-16*, since no *daf-16*-independent outputs of *daf-2* have yet been identified. (Hsin & Kenyon 1999) concluded that the somatic gonad signal was not dependent upon *daf-16*, since whole-gonad ablation in a *daf-16(0)* genetic background resulted in a decrease in lifespan. This implied that a lifespan-extending somatic gonad signalling was still active in these animals, since while they were unable to respond to germline ablation (due to the *daf-16*-dependence of the germline signal) lifespan was still reduced upon loss of the positive somatic gonad signal. However, the reduction in *daf-16(0)* hermaphrodite mean lifespan upon whole-gonad ablation was only 18%, compared with a 60% increase in N2 mean lifespan upon germline ablation. If somatic gonad signalling and germline signalling are equal but opposite as proposed, it might be expected that loss of somatic gonad signalling in animals unable to respond to germline signalling (i.e. *daf-16(0)* animals) would result in a decrease in lifespan of comparable magnitude to the increase in lifespan upon germline ablation. Somatic gonad signalling may therefore be in part dependent upon *daf-16*.

Similar reasoning can be applied to the role of *daf-12* in somatic gonad signalling. As in the case of *daf-16*, the decrease in *daf-12(0)* mean lifespan upon whole gonad ablation (29%) was markedly lower than the 60% increase in wild-type lifespan due to germline ablation. In addition, other work on agar plates has shown that the somatic gonad signal requires *daf-9*, the gene that regulates production of the unidentified DAF-12 ligand (Gerisch *et al* 2002). This finding further suggests that *daf-12* does play a role in the somatic gonad signal, although it was also suggested that somatic gonad signalling could involve an as yet unidentified NHR, the ligand for which

is also metabolised by DAF-9 (Gerisch *et al* 2002). Further evidence for a role of *daf-12* in somatic gonad signalling is presented in Section 4.3.4 below.

Note, however, that it remains possible that the smaller reduction in *daf-16(0)* and *daf-12(0)* lifespan upon whole-gonad ablation relative to the increase in N2 lifespan upon germline ablation may be due to complex effects on other lifespan-regulatory components in these genetic backgrounds, and that *daf-16* and *daf-12* are not involved in somatic gonad signalling.

4.3.3 *daf-12(0)* hermaphrodite response to germline ablation

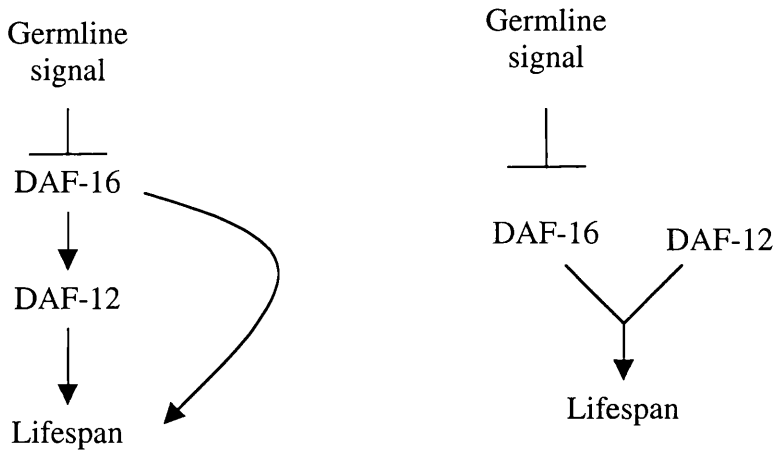
Previous work found the lifespan-reducing germline signal in hermaphrodites to be dependent on both *daf-12* and *daf-9* (Hsin & Kenyon 1999; Gerisch *et al* 2002). It was suggested that germline signalling might suppress DAF-9 activity in the somatic gonad, preventing an element of DAF-12 transcriptional activity required to promote lifespan. Removal of the germline would therefore increase DAF-9 and DAF-12 activity and increase lifespan (Gerisch *et al* 2002).

In contrast to the original finding, germline ablation significantly increased *daf-12(rh61rh411)* hermaphrodite survival, with an average increase in median lifespan similar to that seen in a wild-type background (Figure 4.15c). Thus, germline signalling did not require *daf-12* under the conditions of the above experiment. It is therefore possible that culture conditions, such as liquid culture or temperature, resulted in loss of *daf-12*-dependence of the germline signal in the present study.

A variable *daf-12*-dependence of germline signalling suggests either that *daf-12* does not act downstream of germline signalling, but acts in parallel to modulate it, or that germline signalling only acts via *daf-12* under certain conditions. Potentially, DAF-16 and DAF-12 regulate expression of common target genes, with DAF-12 transcriptional activity required for a lifespan increase upon up-regulation of DAF-16 activity only under certain conditions (Figure 4.18). That this is possible is suggested by the fact that *daf-12* is required for at least part of the Age phenotype of class 1 *daf-2* mutants (Larsen *et al* 1995; Gems *et al* 1998; see Chapter 5 Section 5.3.3). Speculatively, under the conditions of the present experiment, DAF-16 activity was sufficiently high due to germline ablation and culture conditions that the requirement for

daf-12 was by-passed. A variable requirement for *daf-12* may reflect an ability to alter reproductive strategy according to environment.

Figure 4.18: Speculative models for the variable *daf-12*-requirement of the germline lifespan-regulatory signal. *daf-12* modulates germline signalling only under certain conditions (a) downstream of or (b) in parallel to germline signalling.

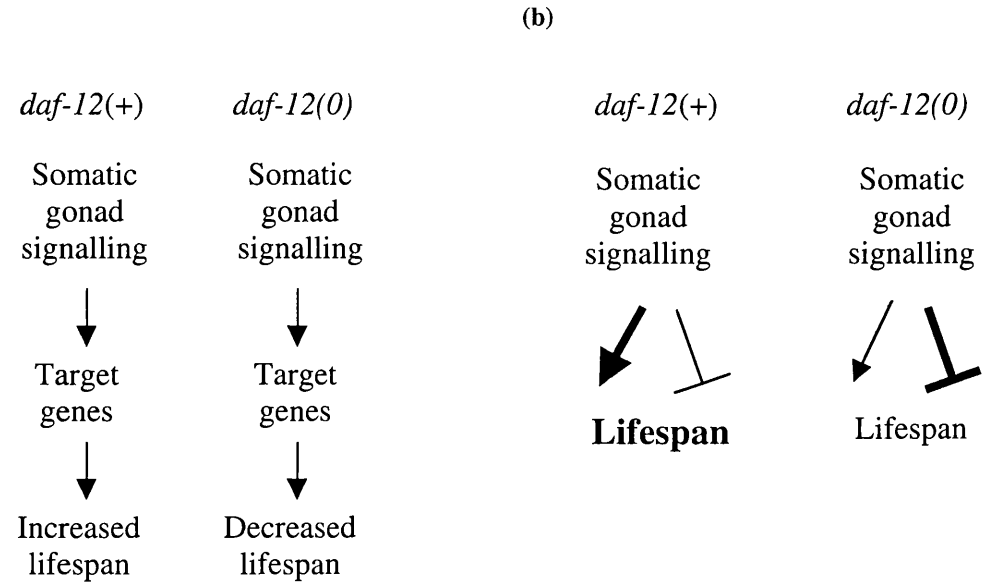


4.3.4 *daf-12(0)* hermaphrodite response to whole-gonad ablation

While (Hsin & Kenyon 1999) reported a decrease in *daf-12(0)* hermaphrodite lifespan upon whole-gonad ablation, in this study an increase was apparent (Figure 4.15c), as seen here also in N2. Median hermaphrodite lifespan was increased by whole-gonad ablation in *daf-12(0)* to a much larger extent than in N2 (Tables 4.2 & 4.4). In fact, the increase in *daf-12(0)* median lifespan upon whole-gonad ablation was greater than that seen upon germline ablation in the same genetic background (Tables 4.3 & 4.4). This is consistent with the effect of somatic gonad signalling on lifespan being reversed (i.e. acting to reduce lifespan) in *daf-12(0)*, since not only did removal of the somatic gonad fail to suppress the lifespan increase due to germline ablation, it enhanced it. This could be due to a reversal of the mode of action of downstream targets of somatic gonad signalling (Figure 4.19a). Stimulation of nuclear hormone receptors such as DAF-12 with ligand often results in transcription of target genes, while absence of ligand results in no gene transcription (see (Glass & Rosenfeld 2000)). The fact that somatic gonad signalling no longer increases lifespan in the absence of *daf-12* suggests that DAF-12 activity results in transcription of genes required for the lifespan-enhancing somatic

gonad signal. Alternatively, *daf-12(0)* may reveal a separate somatic gonad signal that reduces lifespan, but is normally masked by a lifespan-enhancing signal (Figure 4.19b).

Figure 4.19: Possible ways in which *daf-12* could affect the direction of lifespan regulation by the somatic gonad. (a) In the absence of *daf-12* signalling, target genes of somatic gonad signalling reverse their effects; (b) There are two opposite effects of somatic gonad signalling on lifespan, with the positive effect being stronger. In the absence of *daf-12*, the negative effect on lifespan becomes stronger.



The results described here demonstrate the importance of standardising treatments and environmental conditions as much as possible during lifespan analysis. It is not clear what the wild-type scenario with respect to gonadal regulation of hermaphrodite lifespan is, since natural conditions are likely to differ greatly from those provided in the laboratory, either in liquid culture or on agar plates. It is possible that the variable responses to gonadal ablation under different conditions revealed here represent outcomes of a plastic process evolved to respond to a fluctuating environment, which might be expected in the patchy soil environment of *C. elegans*.

4.3.5 Wild-type male response to germline ablation

N2 male lifespan was unaffected by germline ablation in three of four replicates, although overall it was slightly increased (Figure 4.15d). This result implies that either (a) there is a greatly reduced lifespan-reducing signal produced by the germline in intact

wild-type males relative to hermaphrodites, (b) the germline signal is not transduced into a lifespan response as readily in males or (c) modulators of germline signalling differ between the sexes. This finding suggests that sex differences in gonadal signalling may be involved with the sex differences in lifespan seen in wild-type *C. elegans*. However, the fact that germline-ablated hermaphrodites live longer than intact males implies that attenuated response to germline signalling in males relative to hermaphrodites is unlikely to be the only mechanism underlying the sex difference, since if it were, germline-ablated hermaphrodites would be expected to have a similar lifespan to intact males. Instead, the results obtained imply that once germline signalling is removed, hermaphrodites are intrinsically longer-lived than males.

Interestingly, work on agar plates at 20°C has found male mean lifespan to be significantly extended by germline ablation (46%), and to a similar extent as hermaphrodite lifespan (51%) (Arantes-Oliveira *et al* 2002) (Figure 4.15d). Thus, under certain conditions, males respond to germline ablation as do hermaphrodites. This implies that the strength of the germline signal and/or its transduction/ modulation can be the same in both sexes under certain conditions. An element of the experimental conditions that differed between the present work and (Arantes-Oliveira *et al* 2002) therefore resulted in attenuation of the male response to germline ablation. As proposed for hermaphrodites above, therefore, liquid culture and/ or increased temperature may have acted to alter the response to germline ablation.

However, the effect of culture conditions in the present study on response to germline ablation was opposite in males compared with hermaphrodites, since while the increase in hermaphrodite lifespan due to germline ablation in the present study was larger relative to (Arantes-Oliveira *et al* 2002), the male lifespan response was attenuated. Why this might be is unclear. Possibly, the strength of the male germline signal at source was reduced in the present experiment. For example, reduced food concentration or increased temperature may have reduced germline proliferation specifically in males. Alternatively, strain differences could have resulted in the different magnitudes of male response to germline ablation between the two experiments. (Arantes-Oliveira *et al* 2002) used the mutant *unc-50(e306)* for lifespan analysis because the Unc phenotype prevented the leaving behaviour of males on agar plates. As

described in Chapter 3, *unc-50* mutants have disrupted post-synaptic cholinergic neurotransmission and a slightly increased lifespan, hence potentially this mutant could have had altered levels of acetylcholine-mediated signalling relative to N2.

It is also possible that while the strength of the germline signal was comparable in males in the present experiment and (Arantes-Oliveira *et al* 2002), the extent of lifespan regulation by interacting components may have been altered. For example, results in other chapters of this thesis suggest that there may be sex differences in DAF-16 and DAF-12 activity. If these genes interact with germline signalling to regulate lifespan (as discussed above), and if culture conditions affect their activity, it is possible that ablation of the germline would have different effects in males and hermaphrodites under different conditions.

4.3.6 Wild-type male response to whole-gonad ablation

N2 male lifespan was largely unaffected, but slightly increased by whole-gonad ablation (Figure 4.15d). The fact that male lifespan was increased by the same small amount following both germline and whole-gonad ablation implies that in males, either there is no lifespan-enhancing somatic gonad signal, or if there is, it is not transduced. If there were a somatic gonad signal, some suppression of the small increase in lifespan upon germline ablation would be expected, as in hermaphrodites. A lack of effect of somatic gonad ablation on male lifespan could be due to a lack of the somatic gonad signal at source, or, since somatic gonad signalling appears to require *daf-2* (see Figure 4.17 above), a constitutive down-regulation of *daf-2* signalling in males. This latter possibility seems unlikely, however, given that DAF-16::GFP localisation experiments suggested that IIS may in fact be constitutively down-regulated in hermaphrodites (see Chapter 2 Section 2.3.1).

Why might the somatic gonad signal be attenuated in males? As described in Section 4.3.4 above, null mutation of *daf-12* resulted in a reversal in hermaphrodite response to somatic-gonad ablation. Expression of *daf-12* may be reduced in male somatic gonad relative to hermaphrodites, and *daf-12* does not appear to regulate male lifespan in the same manner as it does in hermaphrodites (see Chapter 5). Potentially,

therefore, sex differences in *daf-12* expression may underlie the sex difference in response to somatic gonad ablation.

4.3.7 *daf-12(0)* male response to germline and whole-gonad ablation

daf-12(0) male survival was also largely unaffected by germline ablation. This is in contrast to *daf-12(0)* hermaphrodites, where germline ablation resulted in a large increase in lifespan. No germline signal would therefore appear to be present in *daf-12(rh61rh411)* males (Figure 4.15d). This is consistent with the finding above that there was little or no lifespan-reducing germline signal in wild-type males.

Similarly, *daf-12(0)* male survival was largely unaffected by whole-gonad ablation, although overall it was slightly increased (Figure 4.15d). This effect was not nearly as marked as in hermaphrodites, however. Consistent with results for N2 above, therefore, somatic gonad signalling appears to influence lifespan much less in males than in hermaphrodites.

Germline mutant studies

4.3.8 Wild-type hermaphrodite response to *mes-1(bn7ts)*

To compliment the laser ablation experiments, survival analyses were performed for males and hermaphrodites of the germline mutant *mes-1(bn7ts)* at 25°C. Unexpectedly, N2 control hermaphrodite survival was significantly higher than that of males under the conditions used (Table 4.5). Subsequent experiments confirmed that males are not the longer-lived sex in liquid culture at 25°C. What environmental factors could contribute to such variation in the sex difference in lifespan? Since hermaphrodite lifespan on agar plates, but not in liquid, is reduced at 25°C relative to 22.5°C, high temperature alone is clearly not responsible. Liquid culture does result in a slight increase in hermaphrodite lifespan relative to plates, but at lower temperatures males are still the longer-lived sex, hence liquid culture alone is not the cause. High temperature and liquid culture may therefore interact to regulate hermaphrodite lifespan relative to that of males. A candidate for the mediation of these effects is IIS, since not only does IIS regulate lifespan (via *daf-16*), it is likely to be a temperature-sensitive process that may respond

to nutritional status. Evidence for sex differences in DAF-16 activity was presented in Chapter 2.

mes-1(bn7ts) extended hermaphrodite median and maximum lifespans by 29% and 52%, respectively at 25°C. However, due to the unusually long lifespan of N2 hermaphrodites at that temperature, the experiment was repeated at 22.5°C. At this temperature, *mes-1(bn7ts)* increased hermaphrodite median and maximum lifespans by 31% and 123%, respectively, (i.e. to a greater extent than at 25°C). This increase in hermaphrodite median lifespan at 22.5°C was smaller than that following germline laser ablation at the same temperature (109%). However, it is likely that median lifespan was reduced in *mes-1(bn7ts)* by inclusion of those animals that were not sterile (for the reason explained above). Consistent with this, the increase in maximum hermaphrodite lifespan by *mes-1(bn7ts)* at 22.5°C was similar to that following germline ablation at the same temperature. The fact that hermaphrodite lifespan was increased more by *mes-1(bn7ts)* at 22.5°C than at 25°C suggests that (a) *mes-1(bn7ts)* results in premature mortality at 25°C; (b) there were more non-sterile individuals in the randomly selected hermaphrodite population at 25°C than at 22.5°C or (c) the mechanism of hermaphrodite lifespan increase due to liquid culture at 25°C and *mes-1(bn7ts)* was the same. Potentially, this could be through shared up-regulation of DAF-16.

4.3.9 Wild-type male response to *mes-1(bn7ts)*

As seen in hermaphrodites, male lifespan was also increased upon mutation of *mes-1* at 25°C, although to a lesser extent: median and maximum male lifespans were both increased by only 20%, compared with 29% and 52%, respectively in hermaphrodites. This result is in agreement with the implication of the laser ablation experiment: that the lifespan-reducing germline signal is reduced in males relative to hermaphrodites, However, the increase in male lifespan due to *mes-1(bn7ts)* was notably more marked than that due to germline laser ablation above. Thus, the magnitude of the male lifespan response to loss of the germline is variable, as suggested by the contrast between the laser ablation experiment presented above and that in (Arantes-Oliveira *et al* 2002).

At 22.5°C, male median lifespan was unaffected by *mes-1(bn7ts)*, while maximum lifespan was reduced by 29% (Table 4.6). Thus, as for germline ablation at

the same temperature (Table 4.1), the increase in lifespan upon losing the germline due to *mes-1(bn7ts)* was smaller in males than in hermaphrodites. The lack of male lifespan increase due to *mes-1(bn7ts)* at 22.5°C suggests that a germline signal was entirely absent/ not transduced in males at this temperature.

Why male lifespan was increased by *mes-1(bn7ts)* at 25°C and not at 22.5°C is unclear. Potentially, the randomly selected experimental population at 22.5°C may have contained too few sterile males for an effect on lifespan to be seen. The possibility also exists that *mes-1(bn7ts)* has weaker effects on germline proliferation in males than hermaphrodites at lower temperatures. Although the nature of the *mes-1(bn7ts)* mutation suggests that this would not be the case (see Materials and Methods), it would be desirable to verify the penetrance of the sterile phenotype in males relative to hermaphrodites at different temperatures using high power microscopy.

4.3.10 Germline signalling and hermaphrodite lifespan response to *unc-32(e189)*

Hermaphrodite lifespan is either unaffected or slightly increased by neuronal *unc* mutations, in contrast to marked increases in male lifespan (Gems & Riddle 2000b; Chapter 3). One possibility is that sex differences in gonadal signalling may underlie sex differences in lifespan response to neuronal *unc* mutations. This was tested using the neuronal mutation *unc-32(e189)* in combination with *mes-1(bn7ts)*. Consistent with the hypothesis, hermaphrodite median lifespan was increased to a much greater extent by *unc-32(e189)* in *mes-1(bn7ts)* than in *mes-1*(+) at 25°C. Similarly, the increase in wild-type hermaphrodite lifespan due to *mes-1(bn7ts)* was larger in *unc-32(e189)* compared with *unc-32*(+). *unc-32(e189)* and *mes-1(bn7ts)* therefore appear to act together to enhance hermaphrodite lifespan.

The mechanism of lifespan extension by neuronal *unc* mutations has yet to be identified. It was proposed in Chapter 3 that mutation of *unc-32* and other neuronal *unc* genes may result in attenuation of IIS and/ or up-regulation of DAF-16 activity. This is suggested by the finding that the Age and Hid phenotypes of *unc-32(e189)*, *unc-13(e928)* and *unc-64(e246)* are suppressed by null mutations of *daf-16* (Gems & Riddle 2000b; Chapter 3), and that *unc-32(e189)* results in nuclear accumulation of DAF-16 in males following heat stress (Chapter 3). Since the lifespan-reducing germline signal

requires *daf-16* (Hsin & Kenyon 1999), *unc-32(e189)* and *mes-1(bn7ts)* may therefore both extend hermaphrodite lifespan by up-regulating DAF-16 activity.

One possible explanation for the enhanced lifespan response of *mes-1(bn7ts)* hermaphrodites to *unc-32(e189)* is as follows. The putative positive effect of *unc-32(e189)* on DAF-16 activity may be too small to be apparent in terms of a lifespan response in a germline-intact hermaphrodite, perhaps because DAF-16 activity is below a threshold level for lifespan increase. Removal of germline signalling by *mes-1(bn7ts)* may result in up-regulation of DAF-16 activity to above this threshold, so up-regulation of DAF-16 activity due to *unc-32(e189)* is able to increase lifespan (Figure 4.20). In this model, *unc-32(e189)* is placed upstream of *daf-2* in IIS to regulate DAF-16 activity, although it remains possible that *unc-32* influences DAF-16 activity via another pathway.

It is also possible that the combined effect of *mes-1(bn7ts)* and *unc-32(e189)* on hermaphrodite lifespan is the result of each mutation disrupting different elements of a common pathway rather than acting in separate pathways to affect a common downstream target. Thus, if signalling from *unc-32*-expressing neurons were to promote germline signalling, reduction of neuronal function due to *unc-32(e189)* and of the germline signal due to *mes-1(bn7ts)* may result in an increase in DAF-16 activity above a threshold level required for lifespan increase (Figure 4.21).

However, the model proposed in Figure 4.20 conflicts with the finding in Chapters 3 & 5 (Tables 3.7 & 5.2, respectively) that *unc-32(e189)* failed to enhance the lifespan of *daf-2(m41)* hermaphrodites, despite the presumably high levels of DAF-16 activity in this mutant (see Chapter 3 Section 3.3.3). Results presented in Chapter 5 suggest that sex differences in DAF-12 activity may underlie sex differences in response to neuronal *unc* mutations rather than, or in addition to sex differences in DAF-16 activity (see Section 5.3.5). If so, it would suggest that DAF-12 activity was reduced in the *mes-1(bn7ts)* mutant. Why this might be is unclear: the interactions between germline signalling, *daf-16* and *daf-12* have yet to be clarified, hence it is possible that germline signalling normally promotes an element of DAF-12 activity.

Figure 4.20: One possible model for interaction between germline signalling and lifespan response to *unc-32(e189)* based on findings in this chapter and Chapter 3.

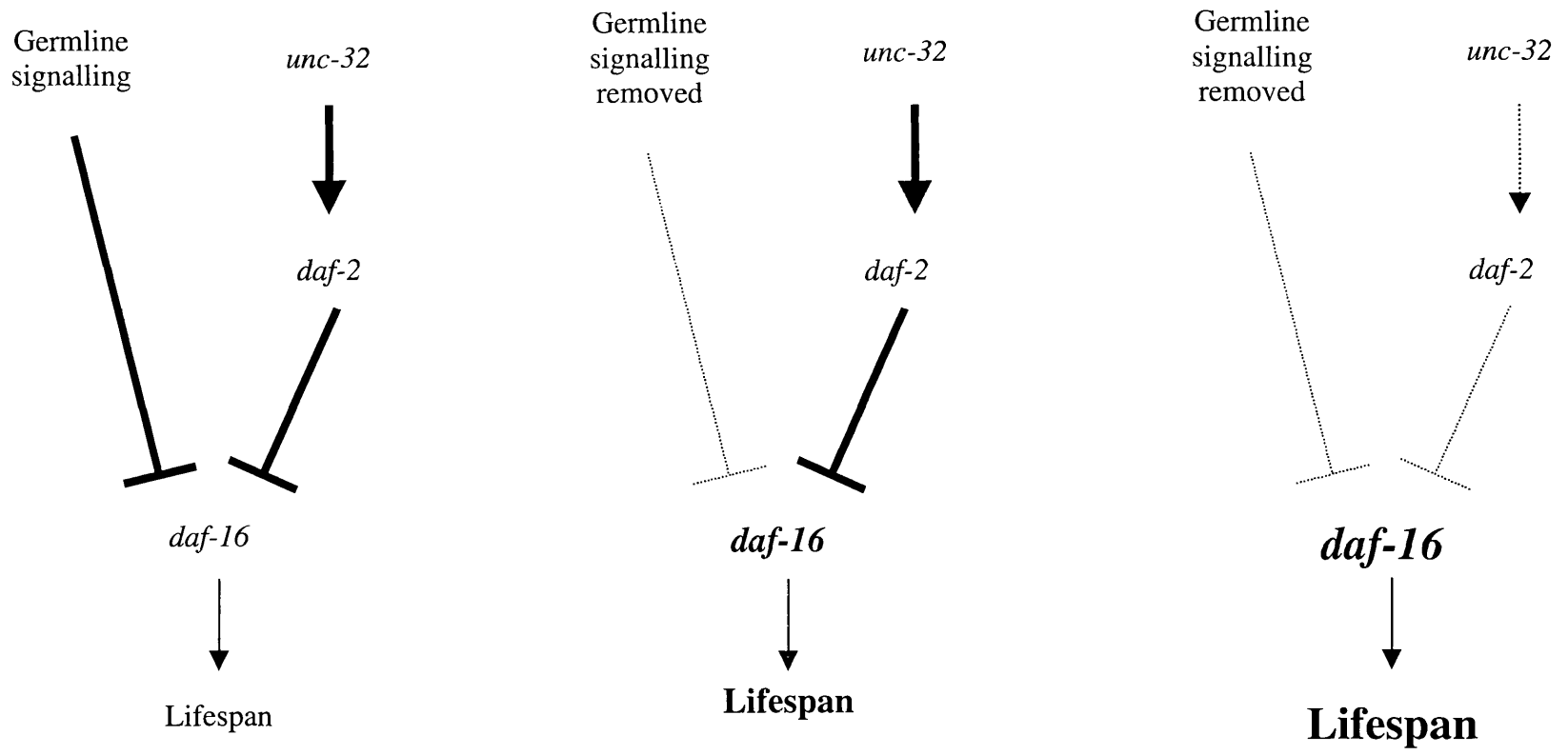
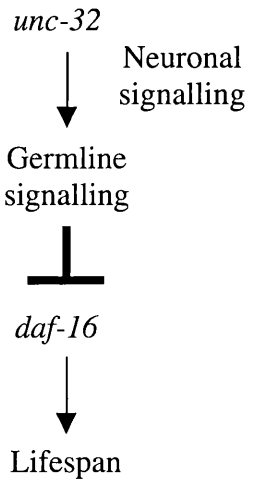


Figure 4.21: Alternative speculative model of lifespan enhancement by *unc-32(e189)* and *mes-1(bn7ts)* in hermaphrodites if *mes-1* and *unc-32* acted in a common pathway.



4.3.11 Germline signalling and male response to *unc-32(e189)*

In contrast to results for hermaphrodites, the effect of *unc-32(e189)* on male median and maximum lifespans at 25°C was reduced in a *mes-1(bn7ts)* compared with a *mes-1*(+) genetic background. Likewise, while *mes-1(bn7ts)* increased male median and maximum lifespans by 20% in an *unc-32*(+) genetic background, in *unc-32(e189)* it had no effect (Table 4.7)). This suggests that *mes-1* and *unc-32* may act at least in part via a common mechanism to regulate male lifespan, and that the effect on this mechanism is maximised in the double mutant.

Assuming that *unc-32* and germline signalling regulate DAF-16 activity (either in a common or separate pathways) as proposed above for hermaphrodites, why might attenuation of germline signalling in this case fail to enhance the lifespan increase due to *unc-32(e189)* as was seen in hermaphrodites above? As proposed in Chapter 2, DAF-16 activity may be up-regulated in wild-type males relative to hermaphrodites. If this were the case, levels of DAF-16 activity in males may be nearer to, or even above, the threshold for lifespan increase proposed above than in hermaphrodites. Thus, mutation of *unc-32* would result in up-regulation of DAF-16 activity to above the threshold level for lifespan increase more readily in males than hermaphrodites, visible as a larger effect of *unc-32(e189)* on male lifespan. Possibly, mutation of *mes-1*, and the subsequent

decrease in germline signalling, could have little additional effect on DAF-16 activity in males, resulting in no further lifespan increase.

It also remains possible that these results are unrepresentative due to the fact that an unknown proportion of males randomly selected were not sterile. Thus, the smaller effect of *mes-1(bn7ts)* on *unc-32(e189)* male lifespan may simply represent a lower proportion of sterile males compared with in an *unc-32*(+) genetic background. Repeat experiments with larger numbers to overcome this confounding factor would clarify whether this was the case. However, this would still not explain why the effect of *unc-32(e189)* on male lifespan was reduced in a *mes-1(bn7ts)* genetic background unless perhaps this double mutant displayed an element of premature mortality due to deleterious pleiotropic effects.

4.3.12 Mechanism of sex differences in gonadal signalling

Results presented above from laser ablation and germline mutant studies indicate that gonadal signalling regulates lifespan to a lesser extent in males than hermaphrodites. Differences in gonadal signalling between the sexes could be the result of a number of factors.

(a) In the magnitude of the source of the germline and/or somatic gonad signal. This seems unlikely in the case of germline signalling, which is thought to originate from mitotic divisions of germline stem cells (Arantes-Oliveira *et al* 2002), since males continue to produce sperm throughout adulthood and thus might be expected to show a similar if not larger increase in lifespan upon germline ablation. It remains possible, however, that male germline stem cells differ intrinsically from those of hermaphrodites and do not result in a lifespan-reducing mitotic signal. The source of the somatic gonad signal remains unclear.

(b) In the expression/ activity of genes required for the germline and/or somatic gonad signal. For example, *daf-9*, the gene encoding the cytochrome P450 responsible for production of the DAF-12 ligand is expressed in hermaphrodite, but not male somatic gonad (Gerisch *et al* 2002). It is also possible that *daf-12* expression is down-regulated in males relative to hermaphrodites (Chapter 5). This could result in

attenuation of transduction of a germline signal in males, and possibly attenuation of the somatic gonad signal.

(c) In levels of lifespan regulatory pathways that interact with gonadal signals. For example, it was suggested above that *daf-12* interacts with somatic gonad signalling to regulate lifespan. Evidence for sex differences in *daf-12* function is presented in Chapter 5. Alternatively, sex differences in IIS and/or DAF-16 activity may result in altered interactions with germline signalling in the two sexes.

4.3.13 Evolution of sex differences in gonadal signalling

As described in the Introduction, it seems unlikely that a trade-off between allocation of limited resources to the germline and the soma exists in *C. elegans*, since prevention of reproduction *per se* does not increase lifespan (discussed in (Leroi 2001)). Thus, the effects of germline and whole-gonad ablation on lifespan probably represent removal of lifespan-reducing and lifespan-enhancing signals, respectively (Hsin & Kenyon 1999). How these lifespan effects are mediated is unclear, but potentially it could be through regulation of DAF-16 activity and, speculatively, stress resistance. This possibility is supported by the fact that germline ablated hermaphrodites show increased resistance to oxidative and heat stress (Arantes-Oliveira *et al* 2002). By linking gonadal signalling to environmental conditions, a hermaphrodite could control its lifespan (and possibly rate of reproduction) according to nutritional and population status.

If so, why would males evolve reduced lifespan regulation by the gonad? Possibly, the potential skewing of male reproduction towards later ages (see Chapter 6) may require increased somatic maintenance early in life, which could be effected by reduced germline signalling. In addition, because males continue to produce sperm throughout adulthood, regulation of timing of reproduction is not necessary. In contrast, hermaphrodites must produce progeny quickly when conditions are suitable in order to achieve a competitive advantage for their offspring (Hodgkin & Barnes 1991).

4.4 Conclusions

- Germline laser ablation extended hermaphrodite lifespan as found previously, although the magnitude of this response was greater in the present study. This mimics the effect of germline ablation on lifespan of certain sensory cilium and *daf-2(rf)* mutants, suggesting that liquid culture may mimic certain effects of such mutations. Hermaphrodite lifespan was also extended by the germline mutation *mes-1(bn7ts)*.
- Germline ablation and *mes-1(bn7ts)* resulted in little or no increase in male lifespan. Germline signalling therefore played a much smaller role in the regulation of male than hermaphrodite lifespan under the conditions of this study.
- Germline-ablated hermaphrodites are longer-lived than wild-type males, hence differences in germline signalling are unlikely to be the sole mechanism underlying wild-type sex differences in longevity.
- The hermaphrodite germline signal was not dependent upon *daf-12* activity, in contrast to previous work. *daf-12* is therefore not always required for germline signalling.
- In contrast to one previous study, but in agreement with another, whole-gonad ablation extended hermaphrodite lifespan. Lifespan regulation by the somatic gonad signal may therefore vary in response to environmental conditions.
- The lifespan-enhancing effect of somatic gonad signalling was reversed in *daf-12(0)*, suggesting that *daf-12* plays a role in somatic gonad signalling.
- Removal of the germline greatly enhanced the increase in hermaphrodite lifespan due to *unc-32(e189)*, suggesting that fundamental differences in germline signalling between the sexes may determine the sex difference in response to *unc-32(e189)*.
- Hermaphrodites are consistently longer-lived than males in liquid culture, but not on plates at 25°C. There is therefore an interaction between culture conditions, hermaphroditism and lifespan.

4.5 Future directions

Results presented above demonstrate that culture conditions can result in marked changes in response to gonadal ablations. It would therefore be of interest to measure the magnitude of hermaphrodite lifespan increase following germline ablation under a range of carefully regulated environmental conditions in order to determine which element(s) of the environment modulate the gonadal lifespan signals.

Evidence presented in this chapter using *mes-1(bn7ts)* suggests that germline signalling blocks the hermaphrodite lifespan extension due to *unc-32(e189)*. This could be verified using laser ablation rather than mutation to remove the germline, which would discount any pleiotropic interactions that may have occurred between *unc-32(e189)* and *mes-1(bn7ts)*. It would then be interesting to confirm that hermaphrodite lifespan is increased to a greater extent by a range of other neuronal *unc* mutations following removal of the germline. Further characterisation of the mechanism of lifespan extension by neuronal *unc* mutations (see Chapter 3), and clarification of the involvement of *daf-2*, *daf-16* and *daf-12* in gonadal signalling would aid understanding of how the germline regulates response to these mutations.

Regulation of lifespan by gonadal signalling has been identified in a number of other nematode species (Hsin & Kenyon 1999; Patel *et al* 2002). It would be of interest to determine whether there are sex differences in this trait in such species, and whether any sex differences correlate with the reproductive strategy (e.g. androdioecious or dioecious) of the species (see Chapter 6).

Chapter 5
Role of *daf-12* in regulation of male and hermaphrodite lifespans

Contents

5.0 Introduction

daf-12 encodes a nuclear hormone receptor

As described in the Main Introduction, the cGMP and TGF-β signalling pathways converge on the gene *daf-12* (Riddle *et al* 1981; Vowels & Thomas 1992; Thomas *et al* 1993; Gottleib & Ruvkun 1994; Larsen *et al* 1995). *daf-12* encodes a 753 amino acid putative nuclear hormone receptor (NHR) (Yeh 1991; Antebi *et al* 2000) which, as is characteristic of NHRs, contains conserved DNA-binding and ligand-binding domains. DAF-12 is most closely related to the *C. elegans* receptors NHR-8 and NHR-48, and shows high homology with *Drosophila* DHR96 (Fisk & Thummel 1995) and vertebrate vitamin D and pregnane-X receptors (Antebi *et al* 2000; Snow & Larsen 2001). Nuclear receptors can bind a range of hormones, including steroids (e.g. oestrogen) and nonsteroidal ligands (e.g. thyroid hormones or fatty acids) (Glass & Rosenfeld 2000).

NHRs such as DAF-12 can both activate and inhibit gene transcription, with transcriptional activity being regulated via several mechanisms (reviewed in (Glass & Rosenfeld 2000)). For example, activation or repression of target gene transcription varies according to direct binding of NHRs to DNA response elements as homo- or hetero-dimers, or through NHR binding to other DNA-bound transcription factors. In addition, active repression of target genes can occur due to the presence or absence of ligand binding. Such differential activities of NHRs are regulated by a range of co-factors, many of which affect chromatin condensation. Many co-regulators are cell- or promoter-specific, allowing precise spatial and temporal regulation of transcription by NHRs.

Role of *daf-12* in dauer formation

daf-12 mRNA is detected at all developmental stages, but peaks in L2d larvae before dauer formation (Snow & Larsen 2000). Being ubiquitously expressed (Antebi *et al* 2000), *daf-12* is a candidate for the receipt and transduction of neuron subset-specific signals during development, resulting in an organism-wide developmental decision (Antebi *et al* 1998; Apfeld & Kenyon 1998; Antebi *et al* 2000).

Different mutations in *daf-12* result in either Daf-c or Daf-d phenotypes, implying that this gene has separable functions responsible for co-ordinating development either into a dauer or an L3 larva. *daf-12* mutations also result in a range of heterochronic defects during gonadal and extragonadal development (Riddle *et al* 1981; Antebi *et al* 1998). Generally, mutant phenotypes suggest that there are two main functions of *daf-12* with respect to dauer formation: *daf-12a* (required for reproductive development) and *daf-12b* (required for dauer larva development). Thus, Daf-d *daf-12* mutations reduce or eliminate *daf-12b* function (Antebi *et al* 1998). The two activities of *daf-12* are likely to reflect activation or repression of transcription of common targets according to the developmental program specified (Gerisch *et al* 2002). These distinct functions may arise from the production of different isoforms (of which there are four), communication with other loci or interaction of modular domains of the receptor (Antebi *et al* 2000).

The ligand for DAF-12 has yet to be identified, although it may be a steroid, since deficiency of cholesterol (the steroid precursor) results in similar phenotypes to Daf-c *daf-12* mutations (Gerisch *et al* 2002). A cytochrome P450, encoded by the gene *daf-9*, is likely to metabolise this ligand, with reduction-of-function mutations in *daf-9* resulting in Daf-c and heterochronic phenotypes that are suppressed by Daf-d *daf-12* mutations (Albert & Riddle 1988; Antebi *et al* 1998; Gerisch *et al* 2002; Jia et al 2002). Cytochrome P450 enzymes are a superfamily of monooxygenases (Hasemann *et al* 1995), which metabolise steroids, cholesterol, fatty acids, bile acids, arachidonates and xenobiotics. Like that of *daf-12*, expression of *daf-9* peaks at the L2 moult (Gerisch *et al* 2002). *daf-9* is expressed in IL1V/URAV neurons, hypodermis, syncitial epidermis and transiently in vulval blast cells (Gerisch *et al* 2002; Jia *et al* 2002). Interestingly, while strong *daf-9* expression is apparent in the hermaphrodite somatic gonad (specifically the spermathecae), no expression has been detected in male somatic gonad using a *daf-9*::GFP strain (Gerisch *et al* 2002), a finding discussed later in this chapter.

How might DAF-12 regulate two developmental programs? Potentially, it acts in a similar manner to many other nuclear hormone receptors: that is, stimulation by ligand results in gene expression while lack of ligand activation results in transcriptional repression of the same genes (Glass & Rosenfeld 2000). The fact that *daf-9* and *daf-12*

null mutants are Daf-c and Daf-d, respectively suggests that *daf-9* suppresses dauer development via *daf-12* (Gerisch *et al* 2002). Thus, signalling to DAF-12 may result in expression of genes required for reproductive development, while attenuation of the signal would repress these genes and lead to dauer formation. This would explain why mutations in the ligand-binding domain of DAF-12 result in Daf-c phenotypes (Antebi *et al* 1998; Gerisch *et al* 2002). Alternatively, there may be differential ligand production by DAF-9, resulting in different effects on DAF-12 function (Gerisch *et al* 2002). Separate DAF-12 activities could arise upon formation of different transcriptional complexes depending on ligand stimulation, with different co-regulators acting to modify transcriptional activity.

Those *daf-12* alleles resulting in a Daf-d phenotype completely suppress the Daf-c phenotype of both cGMP and TGF-β mutants (Gottleib & Ruvkun, 1994; Larsen *et al* 1995), suggesting that *daf-12* is the major, if not only, output of these dauer formation pathways. *daf-9* is also completely epistatic to TGF-β signalling (Jia *et al* 2002). However, *daf-12* shows complex, class-specific interactions with IIS with respect to dauer formation. While dauer larva morphogenesis and larval arrest in class 1 *daf-2* mutants are completely suppressed by the Daf-d nonsense mutation *daf-12(m20)*, in class 2 *daf-2* mutants dauer larva morphogenesis is suppressed but larval arrest is enhanced (Vowels & Thomas 1992; Larsen *et al* 1995; Gems *et al* 1998). *daf-9* is epistatic to *daf-16*, since *daf-16(0)*; *daf-9(rf)* double mutants are Daf-c (Gerisch *et al* 2002; Jia *et al* 2002). However, this epistasis is not complete, since *daf-16(0)*; *daf-9(rf)* mutants form partial rather than complete dauer larvae (Gerisch *et al* 2002; Jia *et al* 2002). These findings suggest that IIS acts in parallel to and upstream of *daf-9* and *daf-12* to regulate dauer formation. These findings are considered further in the Discussion.

Role of *daf-12* in hermaphrodite lifespan regulation

The role of *daf-12* in lifespan regulation appears to be complex, and is as yet unclear. All work investigating the role of *daf-12* mutations on lifespan to date has been performed in hermaphrodites. The Daf-d mutation *daf-12(m20)* slightly decreases hermaphrodite lifespan (Larsen *et al* 1995; Gems *et al* 1998). However, in a similar manner to its effects on dauer formation, *daf-12(m20)* partially suppresses class 1 *daf-2*

mutant Age, but enhances class 2 *daf-2* mutant Age at restrictive temperatures (Larsen *et al* 1995; Gems *et al* 1998).

The relationship between *daf-12* and *daf-9* in lifespan regulation appears to be complex, perhaps due to the separable activities of *daf-12*. In addition to their Daf-c and heterochronic phenotypes, some *daf-9(rf)* mutations result in slight increases in lifespan under certain conditions, an effect that is suppressed by *daf-12(m20)*, suggesting that differential ligand production downstream of *daf-9* regulates lifespan via its effects on DAF-12 activity (Jia *et al* 2002; Gerisch *et al* 2002). However, like *daf-12(m20)*, the *rf daf-9(rh50)* mutation partially suppresses the Age phenotype of class 1 *daf-2(e1368)*, but enhances the maximum lifespan of *daf-2(e1370)* at the restrictive temperature (Gerisch *et al* 2002). Thus, as for dauer formation, *daf-9/daf-12* signalling may act both downstream of and in parallel to IIS to regulate lifespan. *daf-12* and *daf-9* also play a role in regulation of lifespan via germline and somatic gonad signalling (Hsin & Kenyon 1999; Gerisch *et al* 2002; Chapter 4).

Mode of enhancement of class 2 *daf-2(rf)* hermaphrodite lifespan by *daf-12(m20)*

It is possible that the extension of class 2 *daf-2(rf)* hermaphrodite lifespan by *daf-12(m20)* is not a direct effect on ageing, but rather the result of suppression of premature mortality associated with class 2 *daf-2(rf)* hermaphrodites at restrictive temperatures (Gems *et al* 1998). This is consistent with the fact that enhancement of the class 2 *daf-2(rf)* hermaphrodite Age phenotype by *daf-12(m20)* is not seen at permissive temperatures, where no class 2 *daf-2(rf)* pleiotropic phenotypes are evident (Larsen *et al* 1995; Gems *et al* 1998). Moreover, *daf-12(m20)* suppresses the internal hatching phenotype of several class 2 *daf-2* mutants (although it slightly enhances late progeny production and motility defects) (Gems *et al* 1998). Alternatively, *daf-12(m20)* may enhance class 2 *daf-2(rf)* hermaphrodite lifespan directly, perhaps by interacting with common downstream target genes of class 2 *daf-2* signalling. The temperature-sensitivity of this effect may reflect a lifespan-regulatory component of *daf-12* function that only becomes apparent at higher temperatures.

In contrast to hermaphrodites, class 2 *daf-2(rf)* males at restrictive temperatures apparently do not exhibit premature mortality (Gems & Riddle 2000b). Thus, the effect

of *daf-12(m20)* on class 2 *daf-2(rf)* male lifespan could be used to investigate whether or not *daf-12(m20)* directly enhances the lifespan of class 2 *daf-2(rf)* hermaphrodites, assuming a similar mode of action of *daf-12* in the two sexes. If enhancement of class 2 *daf-2(rf)* hermaphrodite Age by *daf-12(m20)* were indirect, it might be expected that *daf-12(m20)* would have either no effect or even a suppressive effect on class 2 *daf-2(rf)* male lifespan, since males do not exhibit any premature mortality. If, however, class 2 *daf-2(rf)* hermaphrodite lifespan were directly enhanced by *daf-12(m20)*, it might be expected that a similar effect would be seen in males. It would also be predicted that class 1 *daf-2(rf)* male lifespan would be suppressed by *daf-12(m20)* to a similar extent as in hermaphrodites. The working hypothesis of this chapter is therefore that *daf-12(m20)* enhances the lifespan of class 2 *daf-2(rf)* hermaphrodites by suppressing premature mortality. To test this, the effect(s) of *daf-12(m20)* on class 1 (*m41*) and class 2 (*e1370*) *daf-2* mutant male lifespan were determined.

When considering the interactions between *daf-12* and the two classes of *daf-2* mutation, it is important to note that mutant interactions are not necessarily informative regarding wild-type gene function. Thus, while enhancement of class 2 *daf-2(rf)* hermaphrodite lifespan and early larval arrest by *daf-12(rf)* may reflect gene interactions that occur in wild-type animals, it may be an artefact associated with mutant alleles. It is therefore unclear whether mutant interactions described here and previously will necessarily be informative regarding wild-type *C. elegans* gene function.

Interactions between *unc-32*, *daf-2* and *daf-12*

As introduced in Chapter 3, the neuronal mutation *unc-32(e189)* displays *daf-16*-dependent Age and Hid phenotypes (Gems & Riddle 2000b; Chapter 3 Section 3.3.2). *unc-32* may therefore interact with IIS to regulate dauer formation, potentially as an upstream component within sensory neurons required to transmit environmental signals to the DAF-2 receptor.

It was found in Chapter 3 that while *unc-32(e189)* had no effect on class 1 *daf-2(rf)* hermaphrodite lifespan at 22.5°C, it markedly increased class 2 *daf-2(rf)* hermaphrodite lifespan at the same temperature. Like *daf-12* mutations, therefore, the effects of *unc-32(e189)* on *daf-2* mutant hermaphrodite lifespan were class-specific. In

males, however, *unc-32(e189)* extended both class 1 and class 2 *daf-2* lifespan at 22.5°C. In addition, the increase in hermaphrodite lifespan due *unc-32(e189)* was markedly increased in the germline mutant *mes-1(bn7ts)*, while in males this was not the case (Chapter 4). Since *daf-12* is involved with lifespan regulation through interactions with *daf-2* (see above) and germline signalling (see Chapter 4), it seemed possible that sex differences in *daf-12* activity may underlie these findings, at least in part. To further investigate these complex results, interactions between *unc-32(e189)*, *daf-12(m20)* both classes of *daf-2(rf)* were investigated in males and hermaphrodites at 22.5°C.

5.1 Methods

5.1.1 Maintenance of strains and strain construction

Strains used in this chapter were: DR1564 *daf-2(m41) III* (Larsen *et al* 1995; Gems *et al* 1998), CB1370 *daf-2(e1370) III* (Riddle 1977), DR20 *daf-12(m20) X* (Riddle *et al* 1981) and AA120 *dhIs26* DAF-12a::GFP (Antebi *et al* 2000) All strains were raised on agar plates at 15°C.

The GFP of AA120 was integrated in-frame in exon 1 of the *daf-12* gene, and the array integrated on chromosome I in a wild-type genetic background (Antebi *et al* 2000). *daf-12(m20)* is a nonsense mutation which has nearly normal gonadal and extragonadal development but is completely Daf-d (Antebi *et al* 1998, 2000; Snow & Larsen 2000).

Double *unc-32(e189)*; *daf-12(m20)* mutants were constructed as follows. *daf-12(m20)* males were mated to *unc-32(e189)* hermaphrodites, generating *e189*/+; *m20*/ + hermaphrodite out-cross progeny. These were self-fertilised and Unc hermaphrodite progeny selected. These animals were all homozygous for *unc-32(e189)*, and at the *daf-12* locus were either +/+, *m20*/+, or *m20*/ *m20*. Cloned animals were selfed at 25°C and left to starve. Those giving rise to no dauers were selected as the double homozygous mutant strain, and the presence of *daf-12(m20)* confirmed as follows. *daf-2(e1370)* males were mated with putative *unc-32(e189)*; *daf-12(m20)* hermaphrodites at 15°C, and the resulting heterozygous out-cross hermaphrodites self-fertilised at 25°C. Dauer progeny were selected; these were homozygous for *daf-2(e1370)* and either +/+ or *m20*/+ at the *daf-12* locus. These animals were recovered and self-fertilised one-per-

plate at 25°C. It was confirmed that two thirds of these clones gave rise to some *daf-2(e1370)*; *daf-12(m20)* L1/L2-arrested larvae as described previously (Gems *et al* 1998), confirming the presence of *daf-12(m20)* in the original putative double mutant strain.

Triple *unc-32(e189) daf-2(rf)*; *daf-12(m20)* mutants were constructed as follows. *daf-12(m20)* males were mated with *unc-32(e189) daf-2(rf)* hermaphrodites at 15°C. Resulting out-cross hermaphrodites (heterozygous at all three loci) were self-fertilised at 25°C. Unc dauer progeny were selected. These were homozygous for *unc-32(e189)* and *daf-2(rf)*, and at the *daf-12* locus were either *m20*/+ or +/+. The dauers were recovered and selfed at 15°C, and resulting progeny cloned and selfed at 25°C. Parents were transferred after 24 hours, and the cohort of offspring scored for dauer formation. For construction using the *m41* allele of *daf-2*, clones were selected that gave rise to Unc non-dauers. These were putative triple homozygous mutants, which were selected for sub-culture. It was verified that these animals gave rise to exclusively Unc non-dauer progeny at 25°C. For construction using the *e1370* allele of *daf-2*, clones were selected that gave rise to Unc early developmental arrests. These were putative triple homozygous mutants, which were selected for sub-culture. It was verified that these animals gave rise to Unc early arrests at 25°C compared with an *unc-32(e189) daf-2(e1370)* control which formed Unc dauers only.

To confirm that the *daf-12(m20)* mutation was present in the putative triple mutant strains, *daf-12* males were mated with putative triple mutant hermaphrodites at 15°C. Resulting hermaphrodite progeny were heterozygous for *daf-2(rf)* and *unc-32(e189)* and homozygous for *daf-12(m20)*. These were self-fertilised at 25°C, and the phenotypes of the 25% of progeny that were Unc (and due to linkage also *daf-2* homozygotes) were checked according to the *daf-2* allele used. Phenotypes were compared against controls which were generated as above but using wild-type rather than *daf-12(m20)* males for the original mating.

5.1.2 Generation of males

Stocks of all non-Unc mutant males were generated following heat-shock treatment of hermaphrodites, and maintained by routine sub-culture thereafter. Because *unc-32(e189)* males cannot mate, males of strains containing this mutation were generated by

backcrossing. N2 males were crossed with *unc-32(e189)* hermaphrodites, and the resulting heterozygous males back-crossed to *unc-32(e189)* hermaphrodites. Unc male progeny were then selected. Double mutant *unc-32(e189); daf-12(m20)* males were generated in the same manner, but beginning with *daf-12(m20)* males and *unc-32(e189); daf-12(m20)* hermaphrodites. Triple mutant *unc-32(e189) daf-2(rf); daf-12(m20)* males were generated as follows. *daf-2(rf); daf-12(m20)* males were mated with *unc-32(e189) daf-2(rf); daf-12(m20)* hermaphrodites at 15°C. Resulting males were heterozygous for *unc-32(e189)* and homozygous for *daf-2(rf)*. *daf-12* is X-linked, hence all males were hemizygous for *m20*. These males were crossed with *unc-32(e189) daf-2(rf); daf-12(m20)* hermaphrodites at 15°C, and Unc male progeny picked.

5.1.3 Lifespan analyses

Lifespan analyses were performed in liquid culture as described in Main Materials and Methods. Lifespan data for all strains were recorded at 22.5°C, a non-permissive temperature at which class 2 *daf-2* mutants display temperature-sensitive pleiotropic phenotypes. Where only one *P* value of the difference between survival of two strains is shown, this is the result of a log rank test on pooled replicates performed concurrently. Multiple *P* values represent results from replicates performed at separate times. At least two replicates were performed for each strain with a few noted exceptions.

5.1.4 Visualising *daf-12* expression using GFP

The AA120 DAF-12::GFP strain was raised at 25°C since expression was stronger at this temperature (A. Antebi, pers. comm.). Males were readily generated by heat shock and were able to mate normally, allowing maintenance of male stocks by normal sub-culture. When at the appropriate age for viewing, animals were placed onto an agar pad on a microscope slide and anaesthetised with 1mM levamisole before being covered with a coverslip. Identical exposure times were used when taking photographs of animals from within a set of comparisons and exposure of slides to U.V. light was minimised by closing the U.V. shutter between viewings. Photographs were taken using a Hamamatsu Orca black-and-white camera linked to a Leica DM RXA2 microscope. Imaging software used was Openlab v 3.0.4.

5.2 Results

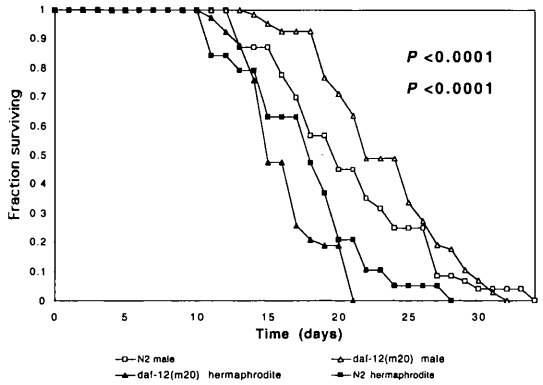
5.2.1 Effect of *daf-12(m20)* on *daf-2*(+) male and hermaphrodite lifespans

daf-12(m20) resulted in a slight (but non-significant overall) decrease in hermaphrodite lifespan relative to N2 (Table 5.1, Figure 5.1a[1]), suggesting that *daf-12* increases wild-type hermaphrodite lifespan, as observed in previous studies on agar plates (Larsen *et al* 1995; Gems *et al* 1998). Conversely, male lifespan was increased by *daf-12(m20)*, although this effect only reached significance in one of two replicates (Table 5.1, Figure 5.1a). This increase in male lifespan due to *daf-12(m20)* is consistent with that due to *daf-12(rh61rh411)* presented elsewhere in this work (Figures 5.1b & c below). This suggests that unlike in hermaphrodites, *daf-12* may act to reduce wild-type male lifespan. However, it should be noted that hermaphrodite lifespan was also increased by *daf-12(rh61rh411)* in Chapter 4 (Figure 5.1b below) and Chapter 2 (Figure 5.1c below), suggesting that the slight lifespan response to *daf-12* mutations may vary according to environmental factors. However, increases in lifespan due to *daf-12(rh61rh411)* were greater in males than hermaphrodites, suggesting that the magnitude of *daf-12* expression and/or function may differ between the sexes.

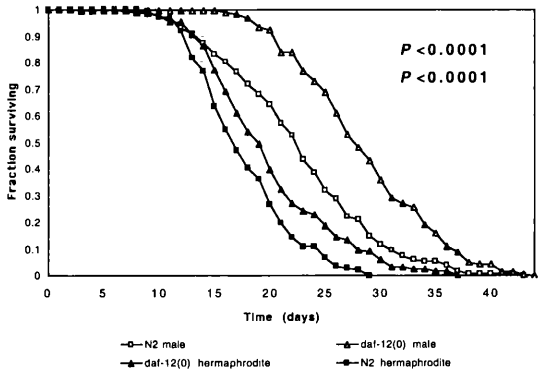
[1] Note that all survival curves in this chapter are based on individual replicates, hence actual values may differ from those presented in Table 5.1

Figure 5.1: Typical survival curves showing effect of (a) *daf-12(m20)* and (b,c) *daf-12(rh61rh411)* on hermaphrodite (closed shapes) and male (open shapes) survival (22.5°C). Upper and lower *P* values apply to the effect of *daf-12(m20)* on male and hermaphrodite survival respectively.

(a) *daf-12(m20)* this chapter



(b) *daf-12(rh61rh411)* reproduced from Chapter 4



(c) *daf-12(rh61rh411)* reproduced from Chapter 2

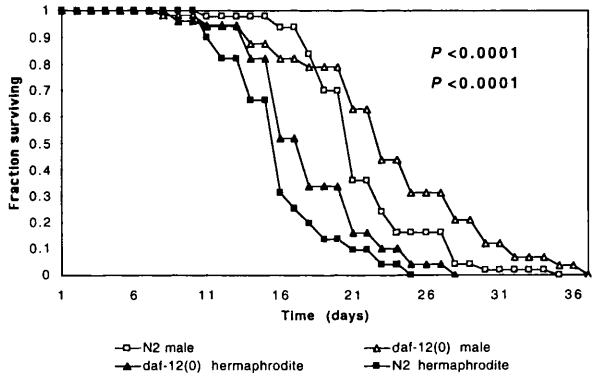


Table 5.1: Effect of *daf-12(m20)* on male and hermaphrodite lifespans in *daf-2*(+), class 1 *daf-2(rf)* and class 2 *daf-2(rf)* genetic backgrounds (22.5°C)

Genotype/ sex	Median lifespan (days) ± 95% C.I.	% effect of *daf-12(m20)* on median	Maximum lifespan (days)	% effect of *daf-12(m20)* on maximum	N*	*P*†
+ H[1]	17.7 (19.0, 16.5)	----	23.0	----	165 (200)	----
+ M[2]	19.8 (21.3, 18.3)	----	29.0	----	135 (211)	----
daf-12(m20) H	16.0 (17.5, 15.0)	-10	20.0	-13	88 (151)	0.56
daf-12(m20) M	23.5 (24.5, 22.1)	+19	29.5	+2	131 (152)	0.07, 0.0001
daf-2(m41) H	31.8 (36.0, 28.8)	----	51.5	----	44 (80)	----
daf-2(m41) M	29.0 (32.0, 26.5)	----	41.5	----	59 (80)	----
daf-2(m41); daf-12(m20) H ‡	18.0 (20.0, 18.0)	-43	22.0	-57	37 (80)	<0.0001
daf-2(m41); daf-12(m20) M	30.0 (33.5, 28.0)	+3	44.0	+6	87 (90)	0.017, 0.74
daf-2(e1370) H	29.3 (32.0, 27.3)	----	45.0	----	124 (332)	----
daf-2(e1370) M	53.0 (56.0, 50.8)	----	105.0	----	209 (262)	----
daf-2(e1370); daf-12(m20) H ‡	54.0 (75.0, 43.5)	+84	113.5	+152	30 (72)	<0.0001
daf-2(e1370); daf-12(m20) M ‡	64.0 (83.0, 55.0)	+21	124.0	+18	40 (72)	0.18

[1]Hermaphrodite; [2]Male; *Senescent deaths (starting population); †Probability that survival curves of a strain with and without *daf-12(m20)* differ by random chance (log rank test). Multiple significance values represent results from trials performed at separate times. ‡Results from only one replicate, due to bacterial contamination in two other replicates.

5.2.2 Effect of *daf-12(m20)* on *daf-2(rf)* lifespan

To determine whether the enhancement of class 2 *daf-2* mutant hermaphrodite lifespan by *daf-12(m20)* was direct or indirect, lifespan was determined for both sexes of class 1 and class 2 *daf-2* mutants with and without *daf-12(m20)* at 22.5°C (Table 5.1).

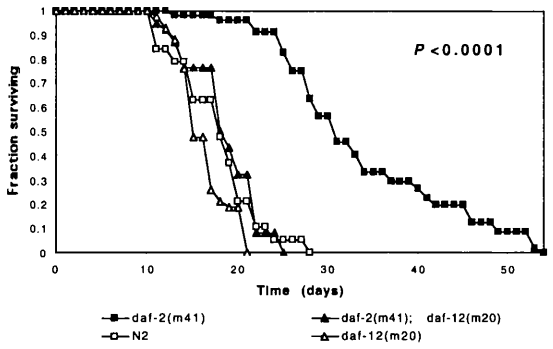
As found previously, *daf-12(m20)* suppressed the Age phenotype of class 1 *daf-2(m41)* hermaphrodites (Larsen *et al* 1995; Gems *et al* 1998) (Table 5.1, Figure 5.2a), with *daf-2(m41)* median lifespan in the present study suppressed by *daf-12(m20)* to a similar extent as in (Gems *et al* 1998). However, maximum lifespan was also suppressed to wild-type levels in the present study, compared with little or no suppression of maximum lifespan in previous studies (Larsen *et al* 1995; Gems *et al* 1998). Thus, *daf-12* was completely required for lifespan enhancement by this class 1 *daf-2* mutation under the conditions of the present study. Unlike in hermaphrodites, *daf-2(m41)* male survival was unaffected by *daf-12(m20)* (Table 5.1, Figure 5.2a), suggesting that *daf-12* does not play a role in the regulation of class 1 *daf-2* male lifespan.

Class 2 *daf-2(e1370)* hermaphrodite lifespan was enhanced by *daf-12(m20)*, in agreement with previous studies on agar plates (Larsen *et al* 1995; Gems *et al* 1998) (Table 5.1, Figure 5.2b). However, this enhancement was much greater in the present study. There may therefore be an interaction between liquid culture and response of class 2 *daf-2(rf)* hermaphrodites to *daf-12(m20)*. Also in contrast to the hermaphrodite result, *daf-12(m20)* did not enhance *daf-2(e1370)* male survival (Table 5.1, Figure 5.2b). This lack of enhancement is consistent with the working hypothesis of this chapter, which is that class 2 *daf-2(rf)* hermaphrodite lifespan is enhanced by *daf-12(m20)* due to suppression of hermaphrodite-specific premature mortality at restrictive temperatures. However, interpretation of this result is confounded by the finding that *daf-2(e1370)* may have resulted in premature mortality of males in the present experiment, since the ratio of median: maximum lifespan of *daf-2(e1370)* males was only 0.50, compared with 0.68 in N2. This finding is considered further in the Discussion. Moreover, the fact that *daf-12(m20)* failed to suppress the *daf-2(m41)* Age phenotype as it did in hermaphrodites (see below) suggests that *daf-12* function may differ fundamentally between the sexes, potentially making comparison of effects in males and hermaphrodites invalid.

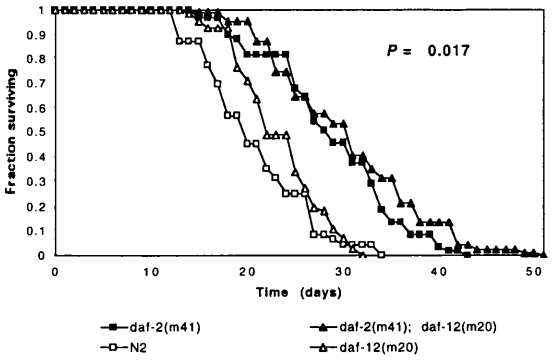
Figure 5.2: Typical survival curves showing effect of *daf-12(m20)* on (a) class 1 *daf-2(m41)* and (b) class 2 *daf-2(e1370)* hermaphrodite and male survival (22.5°C). N2 and *daf-12(m20)* are shown for comparison. *P*= probability that survival of *daf-2(rf)* and *daf-2(rf); daf-12(m20)* differ by random chance (log rank test)

(a) *daf-2(m41)*

Hermaphrodites

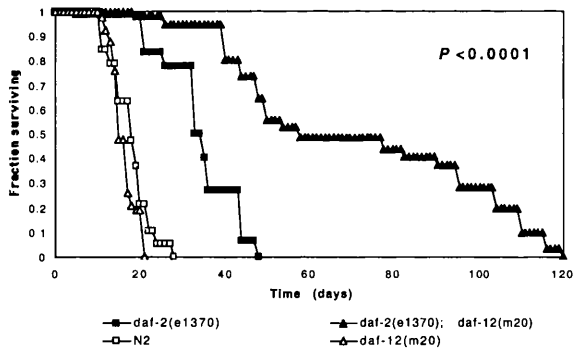


Males

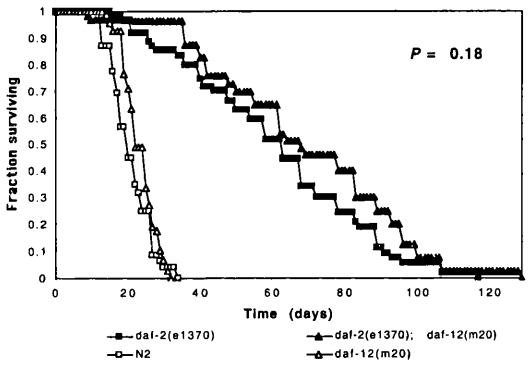


(b) *daf-2(e1370)*

Hermaphrodites



Males



5.2.3 Effect of *daf-12* on hermaphrodite lifespan response to *unc-32(e189)*

Lifespan analyses were also performed to investigate the complex interactions between *unc-32*, *daf-12* and both classes of *daf-2* signalling.

As before (Chapter 3), *unc-32(e189)* extended median and maximum hermaphrodite lifespans to a small extent, an effect unchanged by addition of *daf-12(m20)* (Table 5.2, Figure 5.3a). Also consistent with Chapter 3, *unc-32(e189)* had no effect on class 1 *daf-2* hermaphrodite survival at 22.5°C (Table 5.2; Figure 5.3b). However, in a *daf-12(m20)* genetic background, class 1 *daf-2* hermaphrodite median and

maximum lifespans were markedly extended by *unc-32(e189)* (100% and 105%, respectively) (Table 5.2, Figure 5.3b). Removal of *daf-12* function therefore led to a class 1 *daf-2* hermaphrodite lifespan response to *unc-32(e189)*. Interestingly, the magnitude of *daf-2(m41)* hermaphrodite median lifespan increase due to *unc-32(e189)* in a *daf-12(m20)* genetic background was similar to that of both *daf-2(m41)*; *daf-12(m20)* and *daf-2(m41)*; *daf-12*(+) males, suggesting that the sex difference in response to *unc-32(e189)* had been abolished by *daf-12(m20)* (Figure 5.5).

As in Chapter 3, *unc-32(e189)* markedly extended class 2 *daf-2(e1370)* hermaphrodite survival at 22.5°C, with median and maximum lifespans increased by 188% and 164% respectively (Table 5.2 Figure 5.3c). Conversely, in a *daf-12(m20)* genetic background the increase in class 2 *daf-2* mutant hermaphrodite median and maximum lifespans due to *unc-32(e189)* were only 55% and 33%, respectively (Table 5.2, Figures 5.3c & 5.5), although still notably larger than seen in wild-type or *daf-12(m20)* single mutant genetic backgrounds. This suggests that *unc-32(e189)* and *daf-12(m20)* may enhance class 2 *daf-2* hermaphrodite lifespan via a common mechanism.

5.2.4 Effect of *daf-12(m20)* on male lifespan response to *unc-32(e189)*

As seen in Chapter 3, *unc-32(e189)* extended lifespan to a greater extent in males than hermaphrodites (Table 5.2, Figure 5.5). However, in a *daf-12(m20)* genetic background *unc-32(e189)* increased male and hermaphrodite lifespans to the same small extent (Table 5.2, Figures 5.4a & 5.5). This result implies that *unc-32(e189)* and *daf-12(m20)* act to extend male lifespan via a common mechanism.

Also consistent with Chapter 3, unlike in hermaphrodites, male survival was increased by *unc-32(e189)* in a class 1 *daf-2(m41)* genetic background (Table 5.2, Figure 5.4b), to a similar (but slightly greater) extent than in a *daf-2*(+) genetic background. (Figure 5.5). In a *daf-12(m20)* genetic background, the increases in *daf-2(m41)* male median and maximum lifespans due to *unc-32(e189)* (99% and 100%, respectively) were similar to (although slightly higher than) those in *daf-2*(+) (76% and 100%, respectively) (Table 5.2, Figures 5.4b & 5.5).

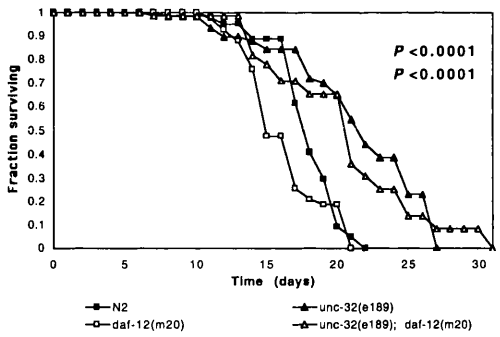
While in Chapter 3 *unc-32(e189)* increased *daf-2(e1370)* male median and maximum lifespans by 5% and 56%, respectively, in the present experiment these

increases were 83% and 24%. The class 2 *daf-2* male lifespan response to *unc-32(e189)* therefore appears to vary in extent. This has implications for the hypothesis in Chapter 3 that the extreme longevity of class 2 *daf-2* males at restrictive temperatures may be the result of the pleiotropic Unc phenotype. This is considered briefly in the Discussion below, and more fully in Chapter 3 Section 3.3.5.

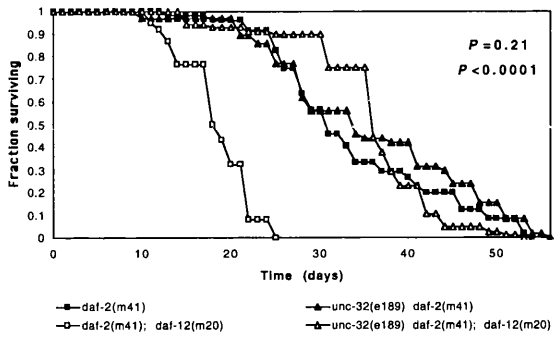
Interestingly, in a *daf-12(m20)* genetic background the increases in *daf-2(e1370)* male median and maximum lifespans due to *unc-32(e189)* were reduced to 42% and 20%, respectively (Table 5.2, Figure 5.4c). Thus, in both males and hermaphrodites, the magnitude of lifespan response to *unc-32(e189)* by *daf-2(e1370)* was reduced by *daf-12(m20)*, the opposite effect to that seen in class 1 *daf-2(m41)*. Although this may simply reflect the variability of the class 2 *daf-2(rf)* male response to *unc-32(e189)*, this finding implies that *daf-12(m20)* and *unc-32(e189)* extend *daf-2(e1370)* lifespan in part via an overlapping mechanism.

Figure 5.3: Typical survival curves showing effect of *unc-32(e189)* on hermaphrodite lifespan both with (open shapes) and without (closed shapes) *daf-12(m20)* in (a) otherwise wild-type (b) *daf-2(m41)* and (c) *daf-2(e1370)* genetic backgrounds (22.5°C). Upper and lower *P* values correspond to effects of *unc-32(e189)* in *daf-12*(+) and *daf-12(m20)* genetic backgrounds respectively.

(a) ***daf-2*(+) hermaphrodites**



(b) ***daf-2(m41)* hermaphrodites**



(c) ***daf-2(e1370)* hermaphrodites**

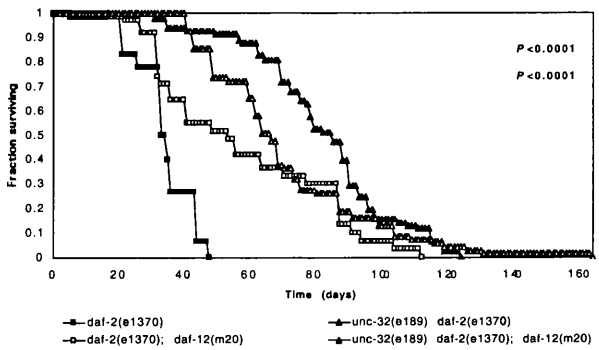
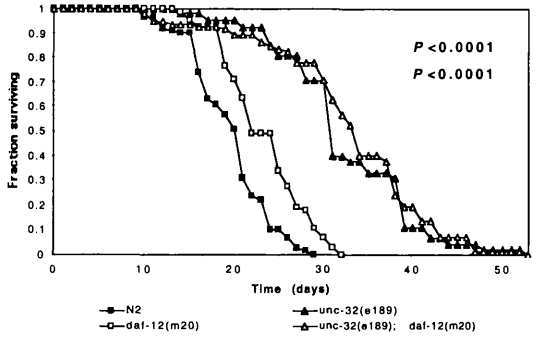
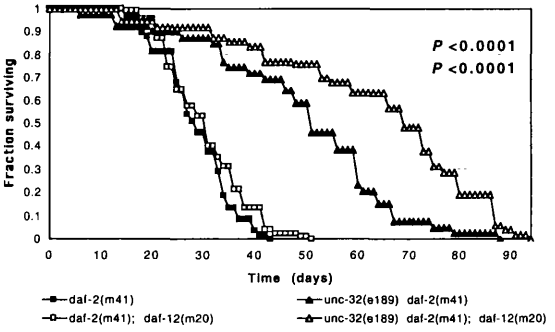


Figure 5.4: Typical survival curves (22.5°C) showing effect of *unc-32(e189)* on male lifespan both with (open shapes) and without (closed shapes) *daf-12(m20)* in (a) otherwise wild-type (b) *daf-2(m41)* and (c) *daf-2(e1370)* genetic backgrounds. Upper and lower *P* values correspond to effects of *unc-32(e189)* in *daf-12*(+) and *daf-12(m20)* genetic backgrounds respectively.

(a) Wild type males



(b) *daf-2(m41)* males



(c) *daf-2(e1370)* males

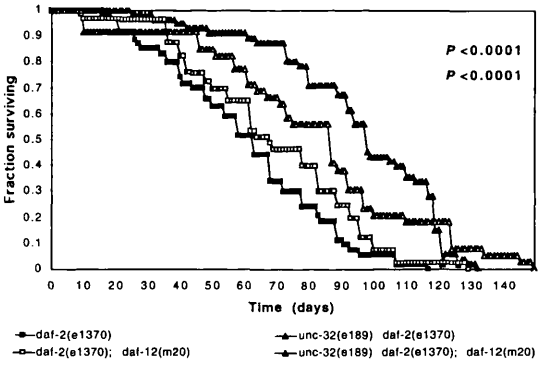


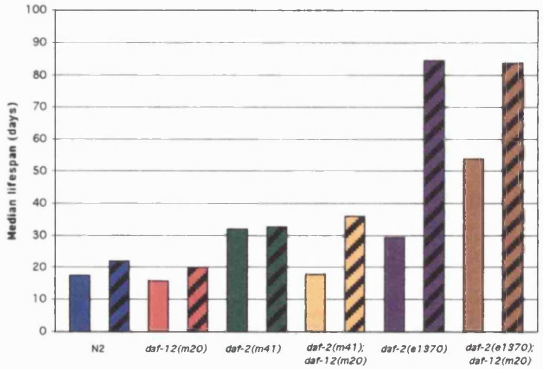
Table 5.2: Effect of *unc-32(e189)* on male and hermaphrodite lifespans in a range of genetic backgrounds (22.5°C)

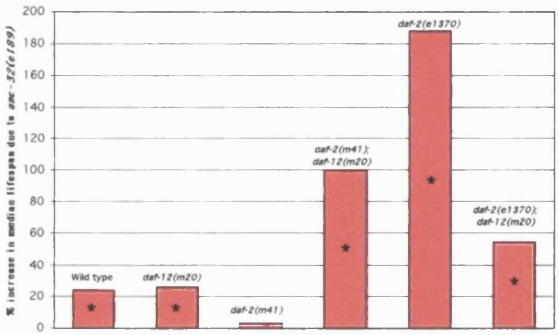
Genotype/ sex	Median lifespan (days) ± 95% C.I.	% effect on median of		Max. lifespan (days)	% effect on max. of		N*	*P*†	*P*#
		m20	*e189*		*m20*	*e189*			
+ H	17.7 (19.0, 16.5)	----	----	23.0	----	----	165 (200)	----	----
+ M	19.8 (21.3, 18.3)	----	----	29.0	----	----	135 (211)	----	----
daf-12(m20) H	16.0 (17.5, 15.0)	-10	----	20.0	-13	----	88 (151)	0.56	----
daf-12(m20) M	23.5 (24.5, 22.1)	+19	----	29.5	+2	----	131 (152)	0.07, <0.0001	----
unc-32(e189) H	22.0 (24.0, 20.3)	----	+24	25.0	----	+9	57 (80)	----	<0.0001
unc-32(e189) M	31.0 (35.5, 27.3)	----	+57	45.0	----	+53	45 (80)	----	<0.0001
unc-32(e189); daf-12(m20) H	20.1 (21.4, 18.9)	-9	+26	26.5	+6	+33	130 (158)	0.39, 0.068	0.009, <0.0001
unc-32(e189); daf-12(m20) M	30.0 (31.6, 28.5)	-3	+28	39.0	-13	+32	129 (192)	0.38, 0.0004	0.001, <0.0001
daf-2(m41) H	31.8 (36.0, 28.8)	----	----	51.5	----	----	44 (80)	----	----
daf-2(m41) M	29.0 (32.0, 26.5)	----	----	41.5	----	----	59 (80)	----	----
daf-2(m41); daf-12(m20) H ‡	18.0 (20.0, 18.0)	-43	----	22.0	-57	----	37 (80)	<0.0001	----
daf-2(m41); daf-12(m20) M	30.0 (33.5, 28.0)	+3	----	44.0	+6	----	87 (90)	0.017, 0.74	----
unc-32(e189) daf-2(m41) H	32.8 (39.5, 28.0)	----	+3	52.5	----	+2	48 (62)	----	0.21
unc-32(e189) daf-2(m41) M	51.0 (60.0, 46.0)	----	+76	83.0	----	+100	39 (43)	----	<0.0001
daf-2(e1370) H	29.3 (32.0, 27.3)	----	----	45.0	----	----	124 (332)	----	----
daf-2(e1370) M	53.0 (56.0, 50.8)	----	----	105.0	----	----	209 (262)	----	----
daf-2(e1370); daf-12(m20) H ‡	54.0 (75.0, 43.5)	+84	----	113.5	+152	----	30 (72)	<0.0001	----
daf-2(e1370); daf-12(m20) M ‡	64.0 (83.0, 55.0)	+21	----	124.0	+18	----	40 (72)	0.18	----
unc-32(e189) daf-2(e1370) H	84.5 (90.0, 76.3)	----	+188	119.0	----	+164	77 (90)	----	<0.0001
unc-32(e189) daf-2(e1370) M	97.0 (107.8, 85.8)	----	+83	130.0	----	+24	51 (100)	----	<0.0001
unc-32(e189) daf-2(m41); daf-12(m20) H	36.0 (37.5, 34.5)	+10	+100	48.5	-8	+105	61 (80)	0.23	<0.0001
unc-32(e189) daf-2(m41); daf-12(m20) M	59.8 (65.0, 55.3)	+17	+99	88.0	+6	+100	119 (170)	0.89	<0.0001
unc-32(e189) daf-2(e1370); daf-12(m20) H	83.8 (89.2, 78.7)	-1	+55	151.5	+27	+33	103 (165)	0.12	0.19, <0.0001
unc-32(e189) daf-2(e1370); daf-12(m20) M	90.8 (100.8, 79.5)	-6	+42	149.0	+20	+20	53 (116)	0.44	<0.0001

* Senescent deaths (starting population); †Probability that survival curves of a strain with and without *daf-12(m20)* differ by random chance (log rank test); #Probability that survival curves of a strain with and without *unc-32(e189)* differ by random chance (log rank test). Multiple significance values represent results from trials performed at separate times, ‡Results from only one replicate, due to bacterial contamination in two other replicates.

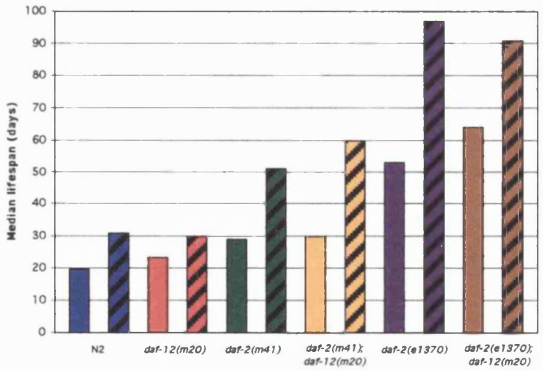
Figure 5.5: Left: actual median lifespans of (a) hermaphrodite and (b) male mutants in *unc-32*(+) (no pattern) and *unc-32(e189)* (striped bars) genetic backgrounds. Right: mean effect of *unc-32(e189)* on median lifespan in class 1 and class 2 *daf-2* mutant (a) hermaphrodites and (b) males with and without *daf-12(m20)* (22.5°C). y axes are on same scale for both sexes, for comparability. Significant effects of *unc-32(e189)* on median lifespan are denoted as a star.

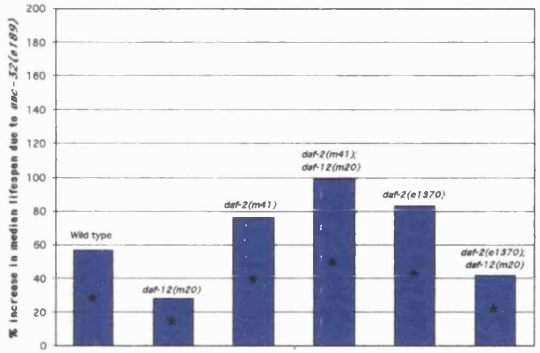
(a) Hermaphrodites





(b) Males





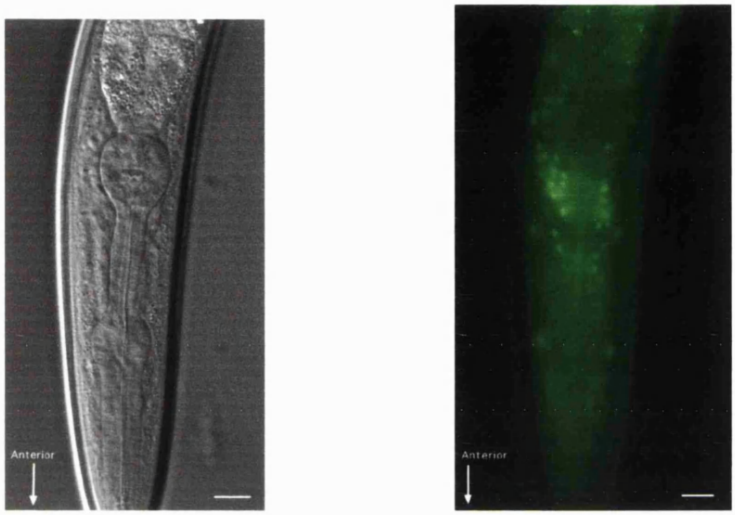
5.2.5 Investigating *daf-12* expression in the two sexes

One possible reason for the sex differences in the role of *daf-12* in ageing is differential *daf-12* gene expression. Preliminary examination of males using the integrated *daf-12*::*gfp* strain AA120 had suggested that *daf-12* expression in the somatic gonad was lower in males than in hermaphrodites (A. Antebi, pers. comm.). Expression of *daf-12* was therefore examined in adult males and hermaphrodites of the AA120 *daf-12*::*gfp* strain.

daf-12 expression occurs in most cell types, especially those which change phenotypically during the dauer transition such as epidermal, intestinal, pharyngeal and somatic gonadal cells (Antebi *et al* 2000). Upon examination of non-gonadal tissues, no difference was apparent between *daf-12*::*gfp* expression patterns or levels of males and hermaphrodites (Figure 5.6). *daf-12*::*gfp* was also expressed in both male and hermaphrodite somatic gonads. However, comparison of the relative expression levels of *daf-12*::*gfp* in the somatic gonads of the two sexes was difficult due to (a) a low general level of GFP expression by this strain, meaning that subtle differences in expression level could pass undetected and (b) considerable variation in levels of *daf-12*::*gfp* expression in the male somatic gonad. A number of males examined displayed virtually no *daf-12*::*gfp* expression in the somatic gonad, while others displayed levels that were very similar to those seen in hermaphrodites (Figure 5.7). In conclusion, therefore, *daf-12*::*gfp* was expressed in non-gonadal tissues and somatic gonad in both males and hermaphrodites. However, it was not possible to draw a general conclusion regarding sex differences in *daf-12*::*gfp* expression levels in the somatic gonad. There may have been a slight reduction in the level of *daf-12*::*gfp* expression in the male somatic gonad compared with hermaphrodites, but such a difference was not readily detectable using the AA120 strain.

Figure 5.6: Expression of *daf-12::gfp* in head neurons of a one-day-old adult (a) hermaphrodite and (b) male (25°C)

(a) Hermaphrodite (scale bars= 20μm)



(b) Male (scale bars= 35μm)

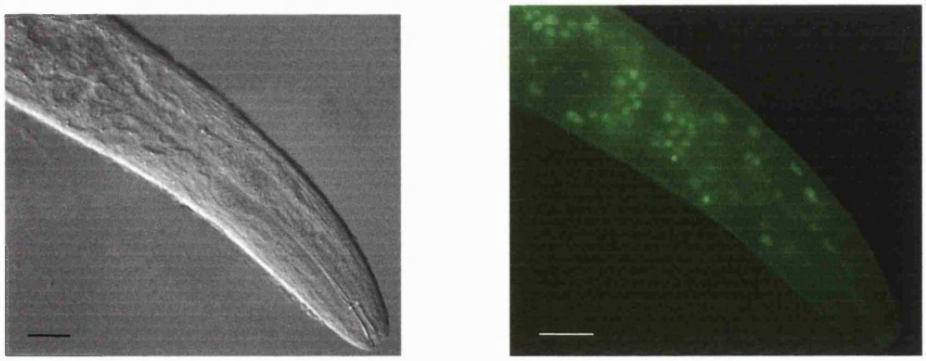
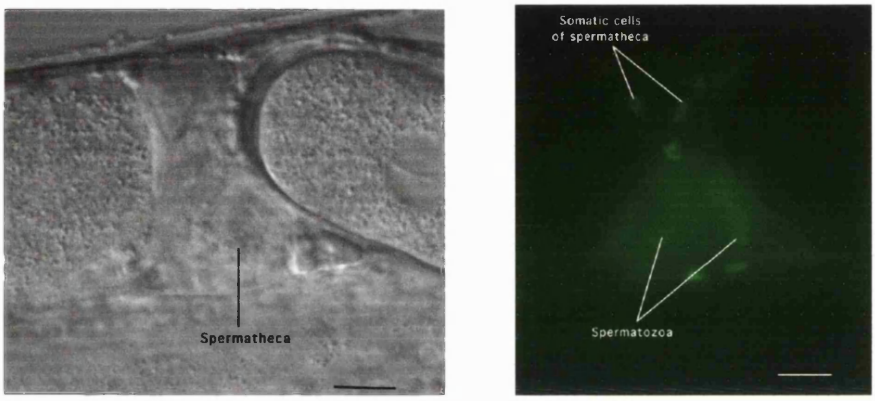
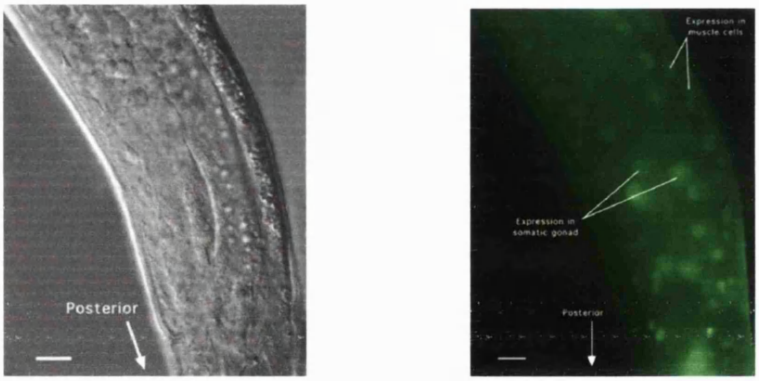


Figure 5.7: DIC and corresponding GFP images of somatic gonadal expression of *daf-12::gfp* in one-day-old adults. (25°C)

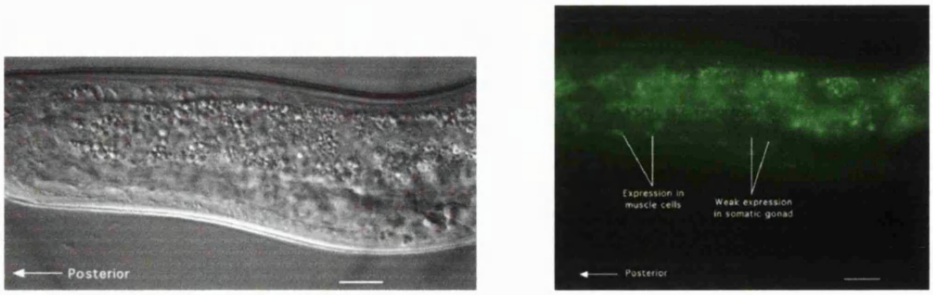
(a) Typical hermaphrodite *daf-12::gfp* expression level. Scale bar= 10μm



(b) Male displaying *daf-12::gfp* expression level comparable to that of hermaphrodites. Scale bar= 20μm



(c) Male displaying very little *daf-12::gfp* expression in the somatic gonad. Scale bar= 20μm



5.3 Discussion

5.3.1 Sex differences in effect of *daf-12(m20)* on wild-type lifespan

daf-12(m20) resulted in a slight decrease in hermaphrodite lifespan (Table 5.1, Figure 5.1a), although this effect was less marked in the present study (10%, 22.5°C) compared with (Larsen *et al* 1995) (28% (mean), 25.5°C) and (Gems *et al* 1998) (35%, 22.5°C). In contrast, male survival was increased by *daf-12(m20)*, although this effect was only significant in one of two replicates (Figure 5.1a). Moreover, in two other experiments (also in liquid culture), *daf-12(rh61rh411)* male median lifespan was 10% and 18% higher than that of wild type (Figures 5.1b & c). These results are consistent with *daf-12* acting to reduce lifespan in wild-type males.

However, while *daf-12(rh61rh411)* had no significant effect on hermaphrodite median lifespan in these other experiments, overall survival was increased in both, and maximum lifespan of *daf-12(rh61rh411)* hermaphrodites was increased by 25% relative to wild type in the Chapter 4 experiment (Figures 5.1b & c). This suggests that *daf-12*(+) slightly increases or decreases hermaphrodite lifespan on agar plates and in liquid culture, respectively. This reversal in mode of action may occur more readily in males than hermaphrodites, explaining why *daf-12* mutations increase male lifespan more readily and to a greater extent than hermaphrodite lifespan. An environmentally regulated role for *daf-12* is proposed in relation to germline signalling in Chapter 4. Specifically, while *daf-12* is required for the lifespan-reducing germline signal under certain conditions (Hsin & Kenyon 1999), under others it is not (Chapter 4). Thus, *daf-12* function could integrate environmental signals into reproductive and longevity responses. Plasticity in the role of *daf-12* signalling in regulation of lifespan is further suggested by the finding that reduction-of-function mutations in *daf-9*, the gene encoding a cytochrome P450 thought to regulate production of the DAF-12 ligand, result in variable degrees of lifespan extension, and even lifespan reduction, in different studies and at different temperatures (Gerisch *et al* 2002; Jia *et al* 2002).

Potentially, sex differences in *daf-12* function could arise through:

(a) Differential *daf-12* expression levels between the sexes (see Section 5.3.7 below).

(b) Differential ligand production between the sexes, as suggested by the DAF-9::GFP study described above, in which no *daf-9* expression was detected in the male somatic gonad compared with strong expression in the hermaphrodite spermatheca (Gerisch *et al* 2002).

(c) Differential expression of co-activators and co-repressors of DAF-12 between the sexes, which could mediate receptor binding to different response elements.

5.3.2 Interaction between *daf-12* and class 2 *daf-2* signalling

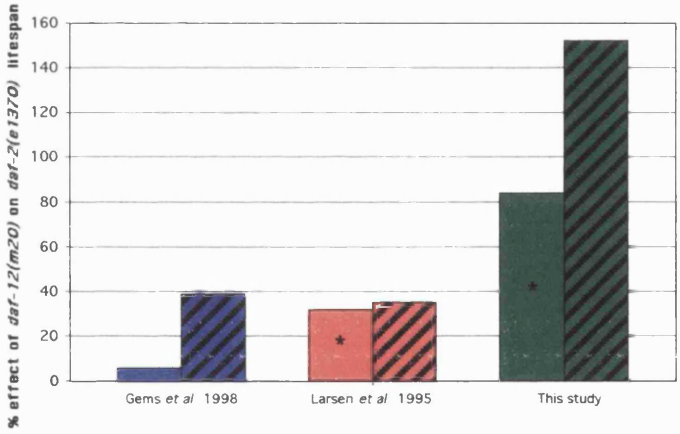
As described in the Introduction, it was of interest to determine whether the enhancement of class 2 *daf-2* mutant hermaphrodite lifespan by *daf-12(m20)* was the result of a direct genetic interaction, or due to suppression of class 2 *daf-2(rf)* hermaphrodite-specific premature mortality (Gems *et al* 1998).

A simple measure of potential premature (non-senescent) mortality is the ratio of median to maximum lifespan of a strain, which decreases as premature mortality increases. While previous ratios of median/ mean to maximum lifespans for *daf-2(e1370)* vs N2 on agar plates were 0.45 vs 0.73 (22.5°C) (Gems *et al* 1998) and 0.38 vs 0.63 (25°C) (Kenyon *et al* 1993), in the present experiment using liquid culture they were 0.64 vs 0.77. *daf-2(e1370)* survival curves were therefore more rectangular in liquid culture than on agar plates. If *daf-12(m20)* does enhance *daf-2(e1370)* hermaphrodite lifespan by suppressing premature mortality, a prediction would therefore be that this enhancement would be smaller in liquid culture. However, this was not the case, since the effect of *daf-12(m20)* on *daf-2(e1370)* hermaphrodite lifespan was much greater in the present study than in previous work (Figure 5.8). Moreover, the shape of the hermaphrodite *daf-2(e1370); daf-12(m20)* survival curve in Figure 5.2b reflects an overall decrease in median: maximum lifespan ratio of this strain (0.48) relative to *daf-2(e1370)* (0.64). Thus, if anything, *daf-12(m20)* enhanced the premature mortality of *daf-2(e1370)* hermaphrodites, suggesting that suppression of premature mortality was not the mechanism of lifespan extension of *daf-2(e1370)* hermaphrodites by *daf-*

12(m20). These findings suggest that *daf-12(m20)* does not extend *daf-2(e1370)* lifespan by suppressing premature mortality, but rather by interacting directly with class 2 *daf-2* signalling or with a common target of class 2 *daf-2* signalling.

In addition to the lifespan data, a direct interaction between *daf-12* and class 2 *daf-2* signalling generally is further implied by the fact that *daf-12(m20)* enhances the class 2 *daf-2* mutant larval arrest phenotype as well as the Age phenotype. For example, *daf-2(e1370)* shows 8.4% early larval arrest (excluding dauers) at 25.5°C, but in combination with *daf-12(m20)* the occurrence of this phenotype increases to 100% (Gems *et al* 1998).

Figure 5.8: Effect of *daf-12(m20)* on *daf-2(e1370)* median/ mean (no pattern) and maximum (striped bars) lifespans in (Gems *et al* 1998) (22.5°C, agar plates, median), (Larsen *et al* 1995) (25°C, agar plates, mean) and the present study (22.5°C, liquid culture, median). Significant effects on median/ mean lifespan are denoted as stars.



As described in the Introduction, because previous work found that class 2 *daf-2* mutant males at restrictive temperatures did not display premature mortality (Gems & Riddle 2000b), enhancement of class 2 *daf-2* male lifespan by *daf-12(m20)* would imply that the effect of *daf-12(m20)* on class 2 *daf-2* mutant hermaphrodite lifespan is a direct, positive effect rather than suppression of premature mortality (assuming a similar mode of action of *daf-12* in both sexes). In disagreement with the findings above, no enhancement of class 2 *daf-2(rf)* male lifespan was seen due to *daf-12(m20)* (Figure 5.4c), suggesting that suppression of hermaphrodite-specific premature mortality was responsible for enhancement of class 2 *daf-2* hermaphrodite lifespan by *daf-12(m20)*.

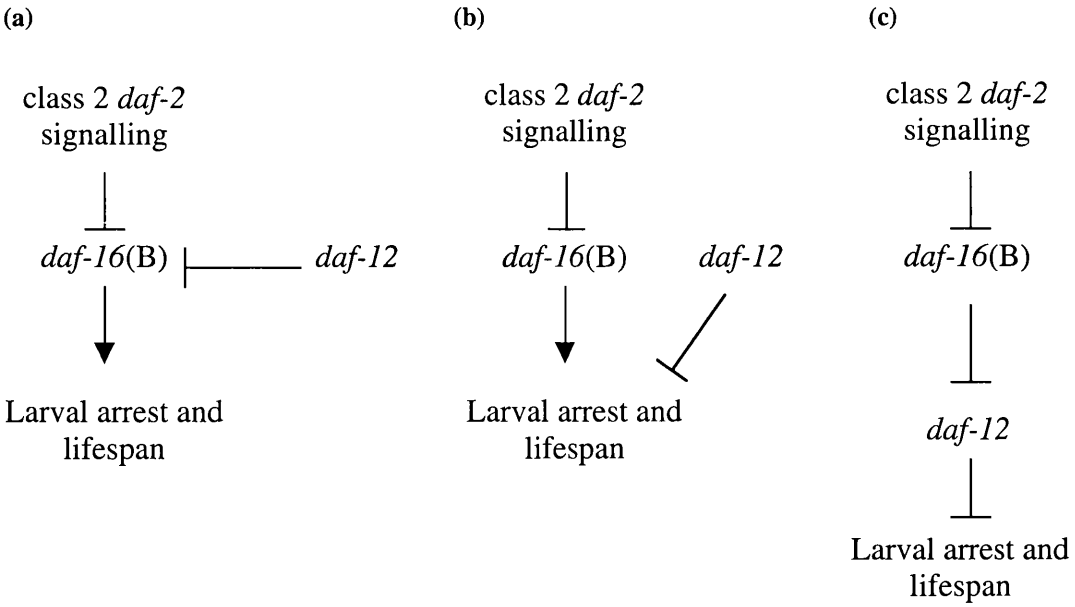
However, it became clear that comparing the effects of *daf-12(m20)* on *daf-2(e1370)* male and hermaphrodite survival in order to test the working hypothesis was invalid, since *daf-2(e1370)* males may have displayed premature mortality (as crudely measured by ratio of median: maximum lifespan) in the present experiment. Moreover, the fact that *daf-12(m20)* also had no effect on class 1 *daf-2(m41)* male lifespan (Section 5.3.3 below) suggests that *daf-12* may function differently in the two sexes, hence inter-sex comparisons may not be valid.

If *daf-12(m20)* does directly enhance the Age phenotype of class 2 *daf-2* mutant hermaphrodites, how might this occur? It has been suggested that *daf-16* acts downstream of class 2 *daf-2* and *daf-12* to regulate lifespan, since the Age phenotype of class 2 *daf-2*; *daf-12* double mutants is suppressed to wild-type levels by *daf-16* null mutations (Larsen *et al* 1995). This could imply that class 2 *daf-2* and *daf-12* are elements of a common pathway, and that mutation of both leads to an additive enhancement of the lifespan and larval arrest phenotypes. However, since (a) *daf-12(rf)* does not cause any marked lifespan increase and (b) *daf-9* (and hence by implication, *daf-12*) is epistatic to *daf-16* (suggesting that *daf-12* acts downstream of or in parallel to *daf-16*) (Gerisch *et al* 2002; Jia *et al* 2002), it seems more likely that the suppression of *daf-2(e1370)*; *daf-12(m20)* lifespan by *daf-16(0)* is solely due to suppression of class 2 *daf-2(rf)* Age by *daf-16(0)*, and that the effect of *daf-12(rf)* on class 2 *daf-2* lifespan is the result of an interaction between two separate pathways which only becomes apparent when class 2 *daf-2* signalling is attenuated. The fact that *daf-12(m20)* enhances *daf-2(e1370)* lifespan suggests that in wild type, *daf-12* either inhibits the element of DAF-16 activity that is also suppressed by class 2 *daf-2* signalling (designated *daf-16*(B)- see (Gems *et al* 1998) Figure 7) (Figure 5.9a) or inhibits a downstream target that is up-regulated by class 2 *daf-2*-regulated DAF-16 activity (Figure 5.9b). Alternatively, class 2 *daf-2* signalling could up-regulate an activity of DAF-16 that suppresses a lifespan-reducing element of DAF-12 activity (Figure 5.9c).

Note that the regulation of separable elements of DAF-16 activity by class 1 and class 2 *daf-2* signalling is speculative, and that the nature of the apparently bi-functional activity of DAF-2 is unclear. Potentially, class 1 and class 2 *daf-2* signalling regulate

different branches of IIS, resulting in different DAF-16 phosphorylation patterns and hence different modes of DAF-16 activity. However, it is also possible that the pleiotropic effects associated with class 2 *daf-2* mutants simply reflect a more severe effect on DAF-2 function, and that the same element of DAF-16 activity is affected by class 1 and class 2 *daf-2* mutations, just to different extents. This latter possibility seems unlikely, however, given that class 1 *daf-2* mutations often have more severe phenotypes than class 2 mutations (Gems *et al* 1998). However, elucidation of the phosphorylation patterns of DAF-16 in different *daf-2* mutants and an understanding of how this regulates lifespan would allow clarification of this point. In this respect, it should be noted that these mutant genetic interactions (as for all such interactions) may not reflect the wild-type situation and may be artefactual.

Figure 5.9: Possible modes of interaction of *daf-12* with class 2 *daf-2* signalling. (a) *daf-12* acts distinctly from *daf-2* and directly affects *daf-16*(B) activity; (b) *daf-12* and *daf-16*(B) share a common output regulating lifespan and larval arrest; (c) *daf-12* acts downsteam of *daf-16*(B). For clarity, only those components of larval arrest and lifespan regulated by class 2 *daf-2* signalling are shown. See also (Gems *et al* 1998) Figure 7.

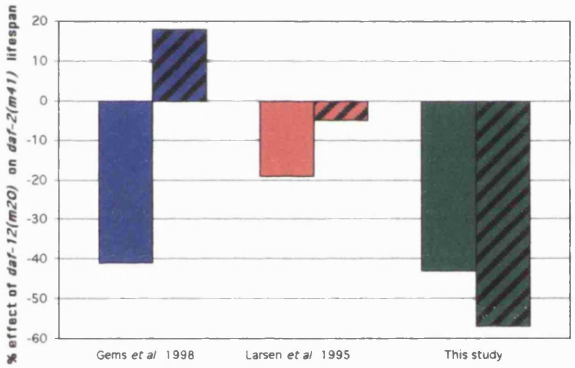


5.3.3 Interaction between *daf-12* and class 1 *daf-2* signalling

While suppression of *daf-2(m41)* hermaphrodite median lifespan by *daf-12(m20)* was identical to that seen in (Gems *et al* 1998), maximum lifespan was also completely

suppressed in the present study, compared with either only partial or no suppression in previous work (Larsen *et al* 1995; Gems *et al* 1998) (Figure 5.10). Thus, the Age phenotype of *daf-2(m41)* hermaphrodites was completely dependent upon *daf-12* in the present study. The fact that the extent of *daf-12*-dependence can vary suggests that *daf-12* is not always required for class 1 *daf-2* signalling. Why the requirement for *daf-12* in lifespan regulation would be increased under the conditions of the present study is unclear, but it is an interesting (but opposite) parallel to the finding in Chapter 4 that germline signalling was no longer dependent upon *daf-12* under similar conditions, and again suggests that *daf-12* may respond to environmental cues to regulate development and lifespan.

Figure 5.10: Effect of *daf-12(m20)* on *daf-2(m41)* hermaphrodite median/ mean (no pattern) and maximum (striped bars) lifespans in this and previous studies. All effects on median/ mean lifespan were significant.



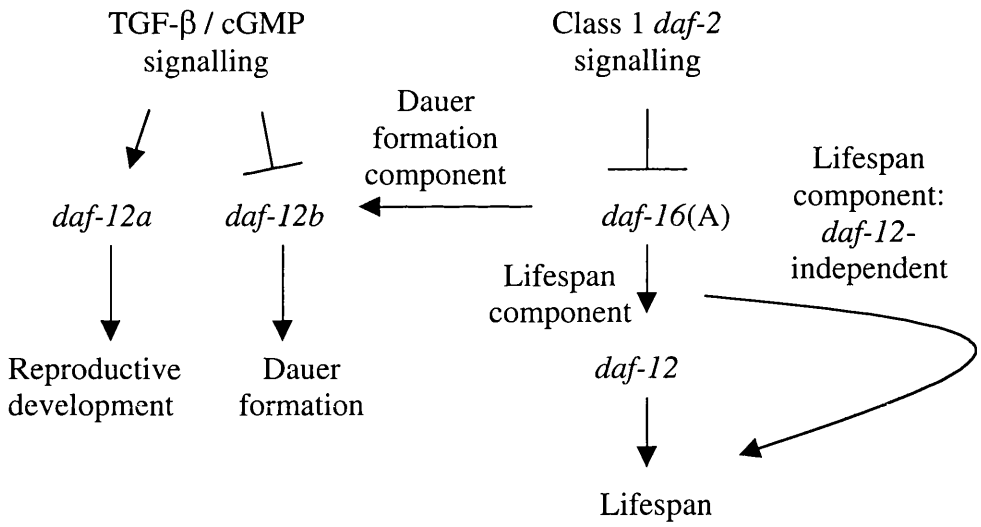
The complete suppression of Daf-c and at least partial suppression of class 1 *daf-2(rf)* Age by mutation of *daf-12* implies that class 1 *daf-2* signalling requires *daf-12* for dauer formation, and at least in part for lifespan regulation. *daf-12* is a candidate gene to act downstream of *daf-16* to regulate dauer formation, since *daf-12* is ubiquitously expressed and could therefore transduce cell-subset-specific IIS dauer formation signals throughout the whole-organism (Apfeld & Kenyon 1998; Antebi *et al* 2000; Gerisch *et al* 2002). It therefore seems likely that the dauer formation component of *daf-12* activity is fully downstream of *daf-16*. Potentially, gene transcription initiated by *daf-16* regulates production of modulators of DAF-12 activity such as *daf-9*. *daf-12b* activity would then transduce the dauer formation regulatory signal from *daf-16* into a whole-

organism response. For simplicity, therefore, dauer formation will always be represented as being mediated by *daf-12* in this chapter.

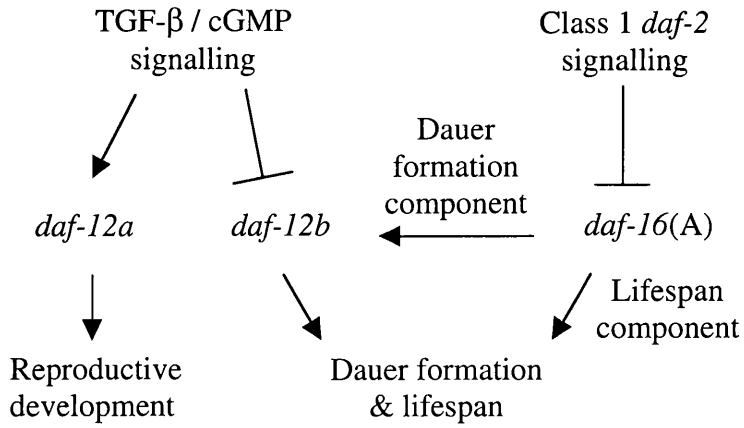
The lifespan-regulatory element of *daf-12* function could be due to either (a) *daf-12* acting downstream of class 1 *daf-2* signalling to transduce part of the lifespan regulatory signal (Figure 5.11a) or (b) DAF-12- and class 1 *daf-2*-mediated DAF-16 activity (designated *daf-16*(A)- see (Gems *et al* 1998) Figure 7) sharing common downstream targets for lifespan regulation, as previously suggested (Gems *et al* 1998) (Figure 5.11b).

Figure 5.11: (a) *daf-12* may act downstream of class 1 *daf-2* signalling to regulate dauer formation and at least in part downstream to regulate lifespan; (b) *daf-12* may act downstream of *daf-16*(A) to regulate dauer formation and in parallel to *daf-16*(A) to regulate lifespan by acting on a common target. See also (Gems *et al* 1998) Figure 7.

(a)



(b)



Although TGF-β and cGMP signalling act via *daf-12* to regulate dauer formation, it seems unlikely that these pathways would be involved with lifespan regulation, since TGF-β signalling mutants are not long-lived (Larsen *et al* 1995). However, in a similar manner to *daf-12(m20)*, *daf-3(rf)* and *daf-5(rf)* enhance class 2 *daf-2* mutant hermaphrodite lifespan, while *daf-3(rf)* suppresses the *daf-2(m41)* hermaphrodite Age phenotype at higher temperatures (King 1998). TGF-β signalling may therefore regulate lifespan, but effects of TGF-β mutations on lifespan may only be apparent once IIS is attenuated.

However, such an interaction between TGF-β signalling and IIS may occur upstream of *daf-12*. For example, mutation of *daf-7*, the gene encoding the TGF-β signalling ligand, results in nuclear accumulation of DAF-16::GFP in a *daf-16(0)* mutant, perhaps due to regulation of *ins* expression by *daf-7* (Lee *et al* 2001). Alternatively, TGF-β signalling may down-regulate PTEN (*daf-18*) expression, and hence increase IIS, as suggested by cell culture work (Li & Sun 1997). It has also been proposed that SMAD proteins involved in TGF-β signalling such as DAF-3 and DAF-8 may form complexes with DAF-16 on the promoters of genes regulating development (Ogg *et al* 1997). That this is possible is demonstrated by the finding that during *Xenopus* development, Smad2 interacts directly with the forkhead FAST1 when binding to an activin response element (Chen *et al* 1996b).

The synergistic interaction between IIS and TGF-β signalling may therefore occur upstream of *daf-12*. If *daf-12* does play a role in regulation of lifespan in parallel to IIS as suggested in Figure 5.11, its activity may therefore be regulated by a pathway other than TGF-β signalling, such as germline signalling. However, although a previous study suggests that *daf-12* may be required for germline signalling (Hsin & Kenyon 1999), results presented in Chapter 4 suggest that *daf-12* may not be required for the germline signal under all conditions. It therefore remains unclear how the role of *daf-12* in lifespan regulation is controlled.

In contrast to the hermaphrodite result, there was no effect of *daf-12(m20)* on *daf-2(m41)* male lifespan. *daf-12* is therefore not required for the class 1 *daf-2(rf)* male Age phenotype. Since the increase in male lifespan due to *daf-2(m41)* (46%) was less than in hermaphrodites (80%), it is possible that *daf-12* is already down-regulated in

males, meaning that mutation of *daf-12* would have no further effect on *daf-2(m41)* male Age. This seems unlikely, however, given that the *daf-2(m41)* hermaphrodite Age phenotype was fully suppressed under conditions of the present study, meaning that a similar effect of *daf-12(m20)* would be expected on *daf-2(m41)* male lifespan. More likely, *daf-12* functions differently in the two sexes.

5.3.4 Effect of *daf-12(m20)* on wild-type male lifespan response to *unc-32(e189)*

As seen previously (Gems & Riddle 2000b; Chapter 3), *unc-32(e189)* extended lifespan more in males than hermaphrodites (Table 5.2, Figure 5.5). In Chapter 3, it was proposed that *unc-32* regulates lifespan by affecting DAF-16 activity, and that sex differences in DAF-16 activity may at least in part underlie the larger effect of neuronal *unc* mutations on male lifespan. Potentially, a higher DAF-16 activity in males (Chapter 2), allows up-regulation of DAF-16 activity by *unc-32(e189)* to reach a threshold level for lifespan extension more readily in males (Chapter 3 Figure 3.11). However, because class 1 *daf-2* mutant hermaphrodite lifespan was not increased by *unc-32(e189)*, despite a presumably high level of DAF-16 activity, it seemed likely that a factor other than or in addition to DAF-16 activity may be responsible for differential responses to neuronal *unc* mutations (Chapter 3 Section 3.3.3).

In this respect, it is interesting to note that in a *daf-12(m20)* genetic background, while the slight increase in hermaphrodite lifespan due to *unc-32(e189)* was unaffected, the increase in male lifespan due to *unc-32(e189)* was reduced (Table 5.2, Figure 5.5). Likewise, *daf-12(m20)* failed to increase male lifespan in an *unc-32(e189)* genetic background as it did in *unc-32*(+). This is consistent with *unc-32(e189)* and *daf-12(m20)* extending male lifespan via a common mechanism, and suggests that sex differences in lifespan response to *unc-32(e189)* may be due at least in part to sex differences in DAF-12 function as well as DAF-16 activity. Since *daf-12(m20)* increases male lifespan (Table 5.1), it is possible that *daf-12* suppresses male lifespan (rather than enhancing lifespan as suggested for hermaphrodites in Figure 5.11), either acting partially downstream of *daf-16* or in parallel to it. Thus, mutation of *unc-32* or *daf-12* would result in up-regulation of transcription of common lifespan-enhancing genes and would increase male lifespan via the same mechanism. Since NHRs such as DAF-12 are able to

promote and suppress transcription of the same genes (Glass & Rosenfeld 2000), the sex difference in DAF-12 function may reflect sex differences in production of DAF-12 ligand(s) or co-regulators.

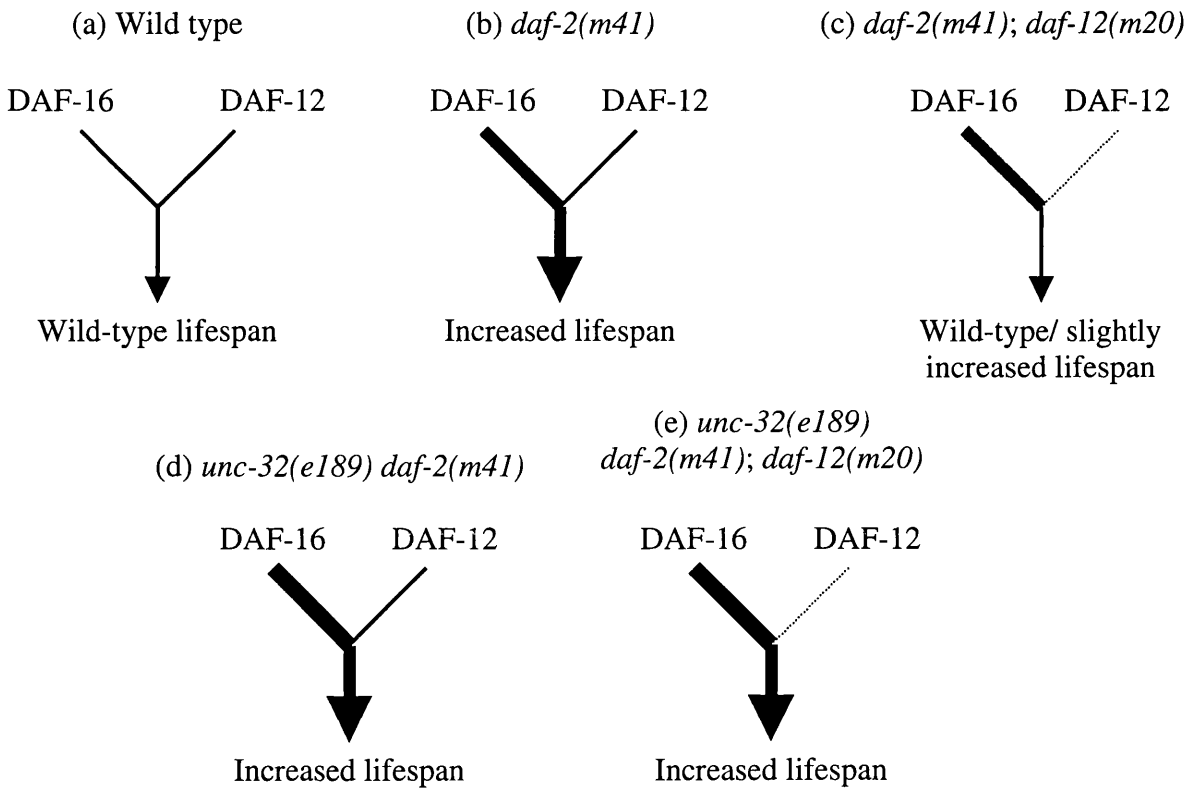
5.3.5 Effect of *daf-12(m20)* on class 1 *daf-2(rf)* lifespan response to *unc-32(e189)*

In agreement with results from Chapter 3 (Table 3.7), *unc-32(e189)* had no effect on class 1 *daf-2(rf)* hermaphrodite lifespan at 22.5°C (Table 5.2, Figure 5.3b). However, in a *daf-12(m20)* genetic background *unc-32(e189)* increased class 1 *daf-2(rf)* hermaphrodite median lifespan by 100% (Table 5.2, Figure 5.3b). Thus, removal of *daf-12* function results in a class 1 *daf-2(rf)* hermaphrodite lifespan response to *unc-32(e189)*. This effect is not seen in *daf-2*(+) hermaphrodites (Table 5.2), hence IIS must be attenuated before *unc-32(e189)* can extend *daf-12(m20)* hermaphrodite lifespan. From another viewpoint, while in an *unc-32*(+) genetic background the Age phenotype of *daf-2(m41)* hermaphrodites was completely suppressed by *daf-12(m20)* (Table 5.2, Figure 5.5), in an *unc-32(e189)* genetic background, *daf-2(m41)* extended *daf-12(m20)* hermaphrodite median lifespan to the same extent (79%) as in a *daf-12*(+) genetic background (80%) (Table 5.2, Figure 5.5). Thus, *unc-32(e189)* prevents suppression of class 1 *daf-2* Age by *daf-12(m20)*, suggesting that under certain conditions, *daf-12* is not required for the Age phenotype of class 1 *daf-2(rf)* hermaphrodites.

How might the above observations be explained? One possibility is that they reflect threshold effects of combined DAF-12 and DAF-16 activity (Figure 5.12). As described above, *daf-12* and *daf-16* may both be required for the Age phenotype of class 1 *daf-2(rf)* hermaphrodites (Gems *et al* 1998) (Figure 5.11), hence without *daf-12*, up-regulation of DAF-16 activity by class 1 *daf-2(rf)* would increase lifespan to a smaller extent (seen as the suppression of class 1 *daf-2(rf)* Age by *daf-12(m20)*) (Figure 5.12c). Potentially, the effect of *unc-32(e189)* on DAF-16 activity is normally masked in hermaphrodites by high levels of DAF-12 activity, which promotes lifespan by regulating transcription of the same genes as DAF-16 (Figure 5.12d). Upon mutation of *daf-12*, the effect of *unc-32(e189)* on DAF-16 activity may be revealed, and lifespan increased, despite the lack of DAF-12 activity (Figure 5.12e). If so, this would suggest that *daf-12* is not absolutely required for the Age phenotype of *daf-2(m41)*

hermaphrodites under conditions where DAF-16 activity is sufficiently high. This scenario is similar to that proposed for the loss of *daf-12*-dependence of the germline signal in Chapter 4: potentially, up-regulation of DAF-16 activity by both germline ablation and (speculatively) culture conditions could have resulted in levels of DAF-16 activity that were sufficiently high to increase lifespan despite the lack of *daf-12* function in the *daf-12(0)* mutant (Chapter 4 Figure 4.18).

Figure 5.12: Hypothetical model of *daf-12*-dependence of *daf-2(m41)* hermaphrodite lifespan response to *unc-32(e189)* at restrictive temperatures. In (d), *unc-32(e189)* does not increase lifespan, since combined effects of DAF-16 and DAF-12 have already maximised the potential for lifespan increase.



While *unc-32(e189)* had no effect on *daf-2(m41)* hermaphrodite lifespan, it enhanced *daf-2(m41)* male median lifespan by 76%. This effect of *unc-32(e189)* on male median lifespan was similar to (but slightly greater than) that seen in a *daf-2*(+) genetic background (+57%). Attenuation of IIS therefore had little effect on the male lifespan response to *unc-32(e189)*, suggesting that if levels of IIS (i.e. DAF-16 activity) regulate the magnitude of the lifespan response to *unc-32(e189)*, this effect is almost maximised

in wild-type males. Based on the model in Figure 5.12, the fact that *unc-32(e189)* is able to markedly extend *daf-2(m41)* male lifespan in a *daf-12*(+) genetic background suggests that *daf-12* activity may be intrinsically down-regulated in males relative to hermaphrodites. Consistent with this, *daf-12(m20)* had little effect on the magnitude of the lifespan increase due to *unc-32(e189)* in *daf-2(m41)* males. Significantly, the increase in *daf-2(m41)*; *daf-12(m20)* hermaphrodite lifespan due to *unc-32(e189)* was comparable with that seen in *daf-2(m41)* males (both *daf-12*(+) and *daf-12(m20)*). This further suggests that the element other than or in addition to sex differences in DAF-16 activity regulating sex differences in response to neuronal *unc* mutations proposed in Chapter 3 Section 3.3.3 could be DAF-12 activity.

5.3.6 Effect of *daf-12(m20)* on class 2 *daf-2* lifespan response to *unc-32(e189)*

In agreement with results in Chapter 3, class 2 *daf-2(rf)* hermaphrodite lifespan was enhanced by *unc-32(e189)* to a much greater extent than was wild-type hermaphrodite lifespan at 22.5°C (Table 5.2). Thus, unlike for *daf-2(m41)* hermaphrodites, *unc-32(e189)* was able to extend *daf-2(e1370)* hermaphrodite lifespan in a *daf-12*(+) genetic background. In this respect, class 2 *daf-2(rf)* hermaphrodites resemble wild-type males, suggesting that *daf-12* may be intrinsically down-regulated in *daf-2(e1370)* relative to wild-type hermaphrodites.

However, since mutation of *daf-12* enhances class 2 *daf-2(rf)* hermaphrodite lifespan (Larsen *et al* 1995; Gems *et al* 1998) (Table 5.2), it was proposed above that *daf-12* and class 2 *daf-2* signalling may act via the same mechanism to regulate hermaphrodite lifespan, with both resulting in suppression of genes downstream of *daf-16*(B) activity (Figure 5.9). Potentially, therefore, *daf-12* may not regulate the magnitude of hermaphrodite lifespan response to *unc-32(e189)* in class 2 *daf-2* mutants as proposed for class 1 *daf-2* mutants (Figure 5.12). *unc-32(e189)* could therefore result in a lifespan increase in class 2 *daf-2(rf)* hermaphrodites (potentially due to further up-regulation of *daf-16*(B) activity) regardless of levels of DAF-12 activity.

daf-2(e1370) male lifespan was also increased by *unc-32(e189)*, to a similar extent as in wild-type and *daf-2(m41)* genetic backgrounds. Thus, as noted above, mutation of *daf-2* has little effect on the magnitude of the male lifespan response to *unc-*

32(e189). The relative magnitudes of this effect in the two sexes will not be considered due to the potentially confounding effects of the class 2 *daf-2(rf)* pleiotropic Unc phenotype, which is discussed in Chapter 3 Section 3.3.5.

In contrast to the enhancement of the class 1 *daf-2(rf)* hermaphrodite lifespan response to *unc-32(e189)* by *daf-12(m20)* described above, the increase in class 2 *daf-2(rf)* hermaphrodite median and maximum lifespans due to *unc-32(e189)* was *smaller* in a *daf-12(m20)* than in a wild-type genetic background (Table 5.2, Figure 5.3c). This effect was also seen in males (Table 5.2, Figure 5.4c). Moreover, the enhancement of *daf-2(e1370)* hermaphrodite lifespan by *daf-12(m20)* was reduced in an *unc-32(e189)* genetic background (Table 5.2). Since *daf-12(m20)* enhances class 2 *daf-2(rf)* hermaphrodite lifespan, it is possible that these effects reflect a shared mechanism of class 2 *daf-2* lifespan increase by *unc-32(e189)* and *daf-12(m20)*, as proposed for wild-type males in Section 5.3.4 above. However, the fact that survival of *unc-32(e189) daf-2(e1370); daf-12(m20)* was reduced relative to that of *unc-32(e189) daf-2(e1370)* in both sexes (Figures 5.3c & 5.4c) suggests that the lifespans of these triple mutant males and hermaphrodites were reduced due to deleterious pleiotropic effects. Potentially, the severity of the class 2 *daf-2* mutation, in combination with loss of both *daf-12* and *unc-32* function, resulted in negative effects on viability that began to override lifespan-enhancing effects.

5.3.7 Expression of *daf-12* in males and hermaphrodites

Since data presented above imply that *daf-12* plays little or no role in the regulation of male lifespan, it seemed possible that *daf-12* expression would be reduced or even absent in male tissues. However, no difference could be detected between expression of *daf-12* by male and hermaphrodite non-gonadal tissues, and although *daf-12* expression was often lower in the male somatic gonad, this effect was variable and the overall level of GFP expression by the strain was low, making detection of sex differences in expression difficult. Moreover, expression data suggest that *daf-12* expression is comparable between the sexes, with, if anything, a slightly higher level of *daf-12* expression in males than hermaphrodites (Stanford Microarray Database- data not shown).

Of relevance, however, is the finding that while *daf-9*, the gene encoding an enzyme probably responsible for the production and/or degradation of the *daf-12* ligand, is expressed strongly in hermaphrodite somatic gonad, it is not expressed at all in the male somatic gonad (Gerisch *et al* 2002). This is consistent with the existence of significant differences between the sexes with respect to *daf-12* activity and/ or function, perhaps due to sex differences in DAF-12 ligand production.

5.4 Conclusions

- *daf-12* mutations can decrease or increase hermaphrodite lifespan according to culture conditions. *daf-12* may therefore integrate environmental signals to regulate reproduction and longevity as it does dauer formation. Male lifespan was increased by *daf-12(rf)* in three separate studies, suggesting that there are sex differences in *daf-12* expression and/ or function.
- There was no effect of *daf-12(rf)* on male lifespan in either class of *daf-2* mutant, indicating that *daf-12* does not play a role in the regulation of *daf-2(rf)* male lifespan Possibly *daf-12* expression/ activity is intrinsically down-regulated in males relative to hermaphrodites. GFP expression studies were inconclusive, although *daf-12* somatic gonad expression may be reduced in males relative to hermaphrodites.
- The effect of *daf-12(m20)* on *daf-2(e1370)* hermaphrodite survival was larger than in previous studies, despite reduced premature mortality in *daf-2(e1370)*, and the fact that premature mortality was higher in *daf-2(e1370)*; *daf-12(m20)* hermaphrodites than in *daf-2(e1370)* alone. This suggests that *daf-12* interacts directly with class 2 *daf-2* signalling to regulate lifespan rather than affecting premature mortality.
- The male-specific element of lifespan increase due to *unc-32(e189)* is reduced by *daf-12* and vice-versa, suggesting that *daf-12(m20)* and *unc-32(e189)* may extend male lifespan via a common mechanism.
- Unlike on agar plates, *daf-12(m20)* fully suppressed the Age phenotype of *daf-2(m41)* hermaphrodites in liquid culture. The extent of the requirement for *daf-12* for the Age phenotype of class 1 *daf-2* mutant hermaphrodites therefore varies according to culture conditions.

- A class 1 *daf-2* hermaphrodite lifespan response to *unc-32(e189)* occurs in a *daf-12(m20)* genetic background. Also, *unc-32(e189)* prevents suppression of class 1 *daf-2* Age by *daf-12(m20)*. Threshold effects of DAF-16 and DAF-12 activity may therefore act together to regulate the magnitude of lifespan response to *unc-32(e189)*.
- *daf-12(m20)* does not affect the magnitude of the *daf-2(m41)* male lifespan response to *unc-32(e189)*, hence *daf-12* does not regulate class 1 *daf-2* male lifespan. The response of *daf-2(m41)*; *daf-12(m20)* hermaphrodites to *unc-32(e189)* is similar to that of males, suggesting that sex differences in *daf-12* activity may regulate the differential responses of males and hermaphrodites to neuronal *unc* mutations.
- Class 2 *daf-2(e1370)* hermaphrodite lifespan is increased by *unc-32(e189)*. These mutant hermaphrodites are therefore similar to wild-type males. The effect of *unc-32(e189)* and *daf-12(m20)* on *daf-2(e1370)* hermaphrodite lifespan is reduced by *daf-12(m20)* and *unc-32(e189)*, respectively, suggesting that *unc-32(e189)* and *daf-12(m20)* enhance *daf-2(e1370)* lifespan via a common mechanism.

5.5 Future directions

Interpretation of results in this chapter was complicated by the fact that the nature of several mutant interactions (such as *daf-16* and *daf-12*) remain unclear. Models presented are therefore based on a number of assumptions, and represent only a few of many potential scenarios. As described in Chapter 3, further investigation into the role of *unc-32* in lifespan regulation would also aid interpretation, since evidence presented earlier suggests that it may interact with IIS and TGF-β signalling to regulate lifespan and dauer formation.

Also fundamental to understanding results in this and other chapters is the molecular basis of the class-specificity of *daf-2* mutations. Class 1 and class 2 components of *daf-2* signalling may represent activities of separate branches of IIS, and potentially differential DAF-16 activation, either spatially, temporally or functionally. Alternatively, they may reflect different degrees of disruption of the same signalling branch. Investigations into the mode of induction of class 2 allele-specific pleiotropic

effects at restrictive temperatures would shed some light onto how characteristics such as reduced motility and fertility may affect germline signalling and response to factors such as *daf-12* and *unc* mutations. It would also be of interest to investigate the generality of the interactions of class 1 and class 2 *daf-2* mutations with genes such as *unc-32* and *daf-12* by repeating some of the above experiments using *daf-2* alleles other than *m41* and *e1370*.

Underlying all chapters in this work has been the effect of liquid culture conditions on responses to a range of mutations. Liquid culture may result in changes in IIS, *daf-12* function or both, and potentially germline signalling. Ideally, comparative experiments would be performed to investigate the effect of liquid culture versus agar plates on lifespan and dauer formation, and attempts made to dissect the underlying gene expression/ function changes under such conditions. The variation in response to different treatments seen between these two culture conditions highlights the importance of standardisation of protocols in comparative studies, and perhaps reflects the phenotypic plasticity evolved by an organism that is likely to encounter frequent and sudden changes in its environment.

Chapter 6
Evolutionary origins of sex differences in lifespan

Contents

6.0 Introduction

Results presented in earlier chapters confirmed the previous finding that *C. elegans* N2 males are longer-lived than hermaphrodites when maintained in isolation (Gems & Riddle 2000b) and suggested the nature of some of the mechanisms that may underlie this difference. In addition to understanding these mechanistic explanations, it is also of interest to understand *why* increased male lifespan would evolve in this species. Is the greater lifespan of *C. elegans* males unusual, or could it be a general feature of nematodes? In this chapter, the evolutionary theory of ageing is employed to test hypotheses as to why *C. elegans* males live longer than hermaphrodites (see also (McCulloch & Gems 2003b).

Evolution of sex differences in lifespan

As described in the Main Introduction, sex differences in lifespan are not uncommon among animals. In addition to *C. elegans*, greater male lifespan has been reported in the housefly *Musca domestica* (Ragland & Sohal 1973), Syrian hamsters and guinea pigs (Committee on Animal Models for Research in Aging 1981), certain strains of laboratory mouse (Oliff 1952; Holzenberger *et al* 2002) and many birds (see (Smith 1989)). Although no exhaustive work has yet been performed for *Drosophila* in this area, data suggest that males are the longer-lived sex in out-bred populations (Clancy *et al* 2001; L. Partridge, pers. comm), although other studies suggest that the opposite may be the case (Lints *et al* 1983). Increased male lifespan is also suggested by measurements of sex ratios in ageing populations of the parasitic nematode *Onchocerca volvulus* (Karam *et al* 1987). Conversely, increased female lifespan has been reported in many fish species (Bidder 1932), flour beetles (Park 1945), spiders (Deevey & Deevey 1945) and gerbils (Committee on Animal Models for Research in Aging 1981). Increased female lifespan is also apparent in humans, with the life expectancy of women born in the UK in the year 2000 exceeding that of men by five years (National Statistics Online).

Unfortunately, such differences are often poorly documented and rely on anecdotal observations, often of wild populations where confounding variables such as

mating effects are not controlled for. However, assuming that at least part of a sex difference in lifespan reflects differences in ageing rates, why might this evolve? According to the evolutionary theory of ageing, senescence is the result of reduced selection pressure at later ages (Medawar 1952). This is because while alleles negatively affecting fitness early in life (especially before or early in reproduction) will rapidly be removed from the population by natural selection, alleles with deleterious effects apparent only later in life (after much reproduction has been completed) will not compromise fitness to such an extent and hence will be selected against less strongly. Thus, an extended period of selection against deleterious mutations in one sex would result in that sex being the longer-lived. Sex differences in the magnitude of selection pressure at different ages could arise for one or both of the reasons described below. The two working hypotheses of this chapter are based on these predictions of the evolutionary theory of ageing.

(a) Sex differences in timing of reproduction

If reproduction occurs later in life in one sex than the other, a prediction of the evolutionary theory of ageing is there will be selection against deleterious mutations for a longer period in the sex with delayed reproduction. Differences in the pattern of reproduction over time (reproductive probability distribution (Williams 1957)) may therefore influence the lifespan of one sex relative to the other. The existence of both dioecious and largely hermaphroditic species within the Nematoda allows this possibility to be investigated.

Dioecy/ gonochorism

With the exception of the Rhabditidae, most nematode species are dioecious (see (Stewart & Phillips 2002)). Dioecious nematodes are amphimictic, and comprise females and (generally smaller) males, with each sex possessing specialised sexual organs. Because both sexes are required for successful reproduction, and at the same time, the reproductive probability distributions of the two sexes are likely to be similar in dioecious species.

Androdioecy (for review see (Pannell 2002))

C. elegans, along with many rhabditids, is an androdioecious species: one composed of hermaphrodites and males (Stewart & Phillips 2002). Androdioecious species (of both plants and animals) are rare, because the benefits to being male in a largely self-fertilising population of hermaphrodites are few (Pannell 2002). In *C. elegans*, one large benefit to males may be that hermaphrodites produce insufficient sperm to fertilise all their own eggs. In addition, because hermaphrodites are unable to mate with one another, males are required for out-crossing if inbreeding depression due to mutation accumulation is a factor. Although *C. elegans* does not appear to suffer from inbreeding depression (as measured by effects of out-crossing on such factors as fertility and lifespan in the laboratory) (Johnson & Wood 1982; Johnson & Hutchinson 1993; Chasnov & Chow 2002), out-crossing would appear to be beneficial to some extent, since fertile males are still generated (albeit at low frequency), and hermaphrodites preferentially use out-cross sperm over their own (Ward & Carrel 1979; Hodgkin & Barnes 1991). Despite this, rates of spontaneous male generation are very low in *C. elegans* (~0.1%) (Hodgkin & Doniach 1997), and males reproduce at a level just below that which would be required to maintain them in a population (Stewart & Phillips 2002). Thus, out-crossing may only benefit hermaphrodites at low frequency.

Apart from nematodes such as *C. elegans* and *C. briggsae*, androdioecy has only been described in a few other animals, including a barnacle (Gomez 1975), a killifish (Turner *et al* 1992) and the freshwater shrimp, *Eulimnadia texana.* Since hermaphrodites of *E. texana* are able to self-fertilise all of their eggs, it is likely that males are maintained due to their role in prevention of inbreeding depression, which is a factor in this species (Weeks *et al* 2001). Out-crossing is therefore more advantageous to hermaphrodite fitness in *E. texana*, perhaps explaining the increased male frequency (up to 42%) (Sassaman 1995) relative to that of *C. elegans.* In contrast to *C. elegans*, in *E. texana* it is hermaphrodites that are the longer-lived sex, by 25-50% (Zucker *et al* 2001).

In *C. elegans*, hermaphrodites begin laying fertilised eggs almost immediately upon moulting into adulthood, and (in the absence of mating with males) deplete their limited supply of ~300 self sperm after approximately one week. Reproduction is therefore rapid in this sex, with each hermaphrodite competing with the others to

produce the earliest progeny in order to give them a competitive advantage during development (Hodgkin & Barnes 1991). However, before males can reproduce, they must locate and successfully mate with a hermaphrodite. In addition, males continue to produce sperm throughout adulthood, and therefore continue to reproduce long after a hermaphrodite has depleted its store of self sperm. Thus, the reproductive probability distribution of males is likely to be skewed to later ages.

It is therefore possible that the increased lifespan of *C. elegans* males is the result of a later period of reproduction, and hence an extended period of selection pressure against deleterious mutations relative to hermaphrodites. If this were the case, it might be expected that in dioecious species, where males and females are likely to have more similar reproductive probability distributions, males and females would have similar lifespans. The first working hypothesis of this chapter is therefore that the increased male lifespan of *C. elegans* is the result of a skew in the male reproductive probability distribution towards later ages due to androdioecy. In order to test this, lifespans were measured for both sexes of a range of androdioecious and dioecious free-living nematode species.

(b) Sex differences in rates of non-senescent death

A second prediction derived from the evolutionary theory of ageing is that the longer-lived sex will be the one subject to a lower rate of non-senescent mortality (Williams 1957). Non-senescent hazards include predation, accidents and disease. Thus, late-acting deleterious mutations will be less exposed to natural selection in the sex that is prematurely removed from the population more frequently by such events. Any rare individuals of that sex not killed by non-senescent factors might therefore be expected to senesce rapidly after the age at which they would normally have been removed from the population, since there would have been little selection against deleterious mutations acting at such advanced ages in that sex.

In light of this theory, it is possible that *C. elegans* hermaphrodites are subject to higher rates of non-senescent mortality than males, since males are the longer-lived sex. The second working hypothesis of this chapter is therefore that increased rates of non-

senescent mortality in hermaphrodites relative to males may have resulted in evolution of increased male lifespan.

One form of non-senescent mortality observed in laboratory populations of *C. elegans* hermaphrodites is internal hatching of eggs (matricide). This occurs as a result of withholding of eggs by hermaphrodites under conditions of low food (Trent *et al* 1983), an effect mediated by serotonin (Horvitz *et al* 1982). Although not an extrinsic hazard in the sense that predation or disease is, matricide does result in hermaphrodite mortality during reproduction and thus is a non-senescent form of death. The natural soil environment of *C. elegans* is likely to be a patchy one, with areas of rich and poor nutrition. Thus, hermaphrodites might be subject to significant levels of matricide in the wild. Although there are likely to be many mechanisms of non-senescent death experienced by both sexes, the existence of matricide in all nematode species tested would provide one candidate mode of non-senescent death that could result in reduced selection for hermaphrodite/ female longevity. Matricide rates were therefore determined for starved hermaphrodites and females of a range of androdioecious and dioecious nematode species.

6.1 Materials and Methods

6.1.1 Isolates and species used

All *C. elegans* wild isolates and other nematode species were phenotyped carefully before use. In the case of wild *C. elegans* isolates, this meant verification of plugging, size and social phenotypes as previously described (Hodgkin & Doniach 1997). For other nematode species, it was verified that each species was dioecious or androdioecious as described in the CGC listing. Certain species were also large (*O. myriophila*) or small (*Oscheius* sp.) relative to *C. elegans*.

Wild isolates of *C. elegans* used and their places of discovery were: AB1 (Adelaide), AB2 (Adelaide), CB4555 (Pasadena), CB4853 (Altadena), CB4854 (Altadena), CB4856 (Hawaii), CB4857 (Claremont), CB4858 (Pasadena), CB4932 (Taunton), RC301 (Freiburg), TR389 (Madison) and TR403 (Madison) (Hodgkin & Doniach 1997). The N2 (Bristol) isolate used was the male stock supplied by the

Caenorhabditis Genetics Center (CGCM). *C. briggsae* (Dougherty & Nigon 1949) isolates studied were: G16 (Gujarat), HK104 (Japan) and VT847 (Hawaii). Other nematode species employed were: CEW1 *Oscheius sp.* (Evans *et al* 1997), DF5018 *O. dolichuroides* (Cunha *et al* 1999), DF5033 *O. dolichura*, EM435 *O. myriophila* (Fitch & Emmons 1995), EM464 *C. remanei ssp. vulgaris* (Baird *et al* 1992) and SB146 *Rhabditis remanei ssp. remanei* (Sudhaus & Kiontke 1996).

Males of androdioecious species were generated by heat shock, and male stocks actively maintained thereafter by sub-culturing L4 males and hermaphrodites in a ratio of 2:1. Stocks of dioecious species were sub-cultured by picking equal numbers of males and hermaphrodites.

6.1.2 Survival analyses

Survival analyses were performed at 22.5°C in standard liquid culture as described in Main Materials and Methods. For survival analysis of the other *C. elegans* wild isolates and of other nematode species, stocks were raised at 20°C and 15°C, respectively.

6.1.3 Dauer formation assays

The ratios of males: hermaphrodites/ females forming dauers for various *C. elegans* isolates and other species were ascertained using the starvation assay described in Chapter 2 Section 2.1.3.

6.1.4 Measurement of matricide frequency

For androdioecious species, hermaphrodites were picked at L4 and allowed to mature into adults overnight on plates at 22.5°C. For dioecious species, L4 males and females were picked in a 1:1 ratio and allowed to mature and mate overnight on plates at 22.5°C. The next day gravid hermaphrodites/ mated females were transferred one-per-well into 96-well microtitre plates containing 50µl S medium (no OP50), at 25°C. Animals were scored daily for matricide for a period of four days. For dioecious species, control mated females were also placed onto streaked plates at 25°C in order to ascertain for how long egg laying continued before the supply of male sperm was depleted. If fertilised eggs were no longer being laid at a point prior to the four-day end point of the experiment,

matricide rates for such species were only scored up until the last day on which fertilised eggs were seen. This prevented the matricide rate from being understated in dioecious species.

6.2 Results

6.2.1 Sex differences in lifespan in other *C. elegans* wild isolates

A large number of *C. elegans* wild isolates have been collected from around the globe. There is variation between these isolates with respect to several traits, including lifespan (Johnson & Hutchinson 1993), dauer formation (M.E. Viney, M.P. Gardner & J.A. Jackson pers. comm), social behaviour (de Bono & Bargmann 1998), body size (Knight *et al* 2001) and production of a copulatory plug during mating (Hodgkin & Doniach 1997). For consistency, the N2 Bristol strain of *C. elegans* has been used as the standard laboratory wild type for many years (Brenner 1973). Although N2 is frequently re-thawed from frozen stocks, it remained possible that generations of laboratory culture had resulted in an atypical relationship between male and hermaphrodite lifespans. That this is possible is suggested by the finding that hermaphrodite lifespan varies considerably between different variants of N2 that arose following isolation and sub-culture of populations of the original N2 isolate in different laboratories (Gems & Riddle 2000a). Differences in response to treatments or mutations may also exist between different laboratory sub-cultures, exemplified by the marked differences in magnitude of hermaphrodite lifespan and body size increase upon germline ablation in a recent study (Patel *et al* 2002). The increased male longevity seen in laboratory sub-cultures of N2 might not therefore be typical of *C. elegans* as a species. Before testing the two working hypotheses of this chapter, therefore, survival analyses were performed for a range of other *C. elegans* wild isolates (Table 6.1). Since these isolates were either obtained directly from the CGC or from frozen laboratory stocks (which had undergone little sub-culture), they would likely display wild-type relationships between male and hermaphrodite lifespans.

Of the other wild isolates tested, nine out of twelve showed significantly increased male relative to hermaphrodite survival, with ratios of male: hermaphrodite

median and maximum lifespans ranging from 1.04 to 1.46 and 1.08 to 1.64, respectively (Table 6.1). Increased male relative to hermaphrodite lifespan would therefore appear to be the general trend in *C. elegans*.

However, note that in AB1 and N2 there was no difference between survival curves of the two sexes, although male maximum lifespans were 12% and 15% longer than that of hermaphrodites, respectively. Since this experiment was performed before the discovery that equal transfer of males and hermaphrodites resulted in greater separation of survival curves, the lack of increased male lifespan in these isolates was probably due to a reduced male transfer frequency, as described in Chapter 1. This possibility is supported by the fact that a repeat of CB4856 lifespan measurement with equal male and hermaphrodite transfer frequencies resulted in a male: hermaphrodite median lifespan ratio of 1.71 compared with 1.11 when males were not transferred daily during hermaphrodite egg-laying (Table 6.1).

In two other isolates (CB4858 and TR389), hermaphrodites were the longer-lived sex, with ratios of male: hermaphrodite median lifespans of 0.79 and 0.65 respectively. Male maximum lifespans were also reduced relative to hermaphrodites in these isolates. While hermaphrodite lifespans were similar to those of other isolates, male lifespans were unusually low, suggesting that the lack of male longevity bias was the result of decreased male lifespan and not increased hermaphrodite lifespan (Figure 6.1).

Figure 6.1: Mean of median lifespans (±s.e.) of all isolates except CB4858 and TR389 (striped bars) compared with median lifespans of CB4858 and TR389 in hermaphrodites (red) and males (blue) (22.5°C).

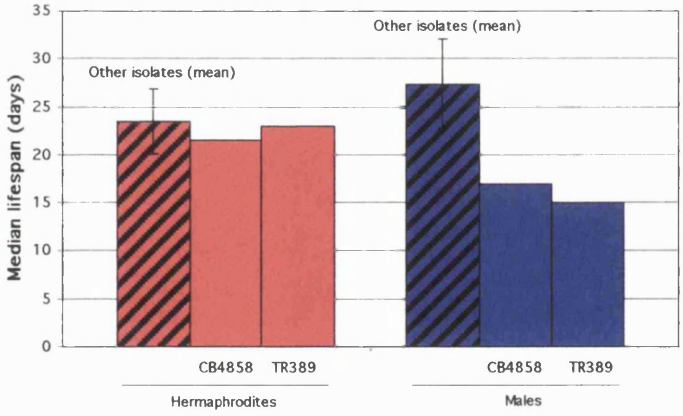


Table 6.1: Median and maximum lifespans for a range of *C. elegans* wild isolates (22.5°C)

Isolate/ origin	Median lifespan (days) ± 95% C.I		Ratio M:H med. lifespan	Maximum lifespan (days)		Ratio M:H max. lifespan	N*		*P*†
	M	H		M	H		M	H	
N2 Bristol	15.7 (17.1, 13.0)	19.0 (22.0, 16.75)	0.83	27.0	23.5	1.15	88 (120)	45 (66)	NS, 0.025
AB1 Adelaide	28.0 (28.0, 26.0)	28.0 (29.0, 26.0)	1.00	37.0	33.0	1.12	57 (60)	21 (39)	0.64
AB2 Adelaide	34.0 (37.0, 32.0)	25.0 (27.0, 24.0)	1.36	46.0	28.0	1.64	60 (61)	26 (36)	<0.0001
CB4555 Pasadena	28.0 (30.0, 26.0)	24.0 (27.0, 22.0)	1.17	43.0	29.0	1.48	53 (60)	31 (36)	<0.0001
CB4853 Altadena	25.5 (26.5, 24.0)	17.5 (21.5, 15.5)	1.46	36.0	31.0	1.16	95 (120)	48 (66)	<0.0001, 0.0002
CB4854 Altadena	29.0 (32.5, 25.0)	28.0 (31.0, 25.0)	1.04	39.0	36.0	1.08	30 (54)	19 (36)	<0.014
CB4856 Hawaii	25.0 (27.0, 24.0)	22.5 (25.0, 19.75)	1.11	36.5	29.0	1.26	105 (120	48 (66)	0.21, 0.0051
CB4856 Hawaii‡	24.0 (24.0, 23.0)	14.0 (16.0, 14.0)	1.71	33.0	28.0	1.18	56 (60)	51 (58)	<0.0001
CB4857 Claremont	28.0 (30.0, 26.0)	25.0 (26.0, 23.0)	1.12	41.0	29.0	1.41	47 (60)	28 (36)	<0.0001
CB4858 Pasadena	17.0 (19.0, 15.5)	21.5 (23.0, 20.0)	0.79	23.5	26.5	0.89	76 (90)	48 (66)	<0.0001, 0.03
CB4932 Taunton	31.0 (31.0, 27.0)	23.0 (26.0, 21.0)	1.35	42.0	28.0	1.50	50 (60)	21 (35)	<0.0001
RC301 Freiburg	26.5 (28.5, 25.0)	21.0 (23.0, 19.0)	1.26	42.0	26.5	1.58	100 (120)	49 (61)	<0.0001, <0.0001
TR389 Madison	15.0 (15.0, 12.0)	23.0 (26.0, 20.0)	0.65	26.0	28.0	0.93	26 (30)	16 (20)	0.0026
TR403 Madison	30.5 (32.5, 29.0)	26.0 (29.5, 21.5)	1.17	40.7	32.5	1.25	95 (131)	42 (66)	0.014, <0.0001

‡ Data from separate trial in which males were transferred daily with hermaphrodites during egg lay; *Number of senescent deaths (starting population); †Probability that male and hermaphrodite survival differ by random chance (log rank test)

6.2.2 Sex differences in lifespan in other nematode species

Having confirmed that there are fundamental lifespan differences between *C. elegans* males and hermaphrodites, it was of interest to address why increased male lifespan has evolved in this species. As described in the Introduction, the first working hypothesis of this chapter is that increased male lifespan in *C. elegans* is the result of extended selection against deleterious mutations in males relative to hermaphrodites, due to a skew in the male reproductive probability distribution towards later ages. To investigate whether this was the case, survival analyses were performed for a range of other nematode species, both androdioecious and dioecious (Table 6.2, Figure 6.2).

Males were the longer-lived sex in three out of four androdioecious species, with male: hermaphrodite median lifespan ratios of 1.12 (*C. elegans*), 1.39 (*O. myriophila*) and 1.50 (*Oscheius sp.*) (Figure 6.3). The exception was *C. briggsae* (Gujarat), in which hermaphrodites were significantly longer-lived, with a male: hermaphrodite median lifespan ratio of 0.56. To verify that this result was characteristic of *C. briggsae* and not due to a peculiarity of the Gujarat strain used, survival analyses were performed for two further *C. briggsae* isolates, Hawaii and Japan. Male: hermaphrodite median lifespan ratios for these isolates were 0.59 and 0.58 respectively, confirming that increased hermaphrodite lifespan is typical of this species under the conditions of the experiment (Figure 6.3).

In all four dioecious species tested males were significantly longer-lived than females (Table 6.2). In three of these species, the effect was more marked than in the androdioecious species, with male: female median lifespan ratios of 1.69 (*C. remanei spp. vulgaris*), 3.48 (*O. dolichura*) and 4.59 (*O. dolichuroides*) (Table 6.2, Figure 6.3). This was not the case in *R. remanei ssp. remanei*, however, where male and female maximum lifespans were almost identical, and the ratio of male: female median lifespan was only 1.21 (Figure 6.3). In addition, survival curves of males and females of this species were only significantly different in one of two trials.

As shown in Figure 6.2i, *O. dolichuroides* females were extremely short-lived relative to males. Females had a sickly appearance, and many extruded part of the gonad through the vulva. Such individuals were censored from the survival analysis. It seemed possible that the gonadal extrusion could be a pleiotropic effect of liquid culture, which

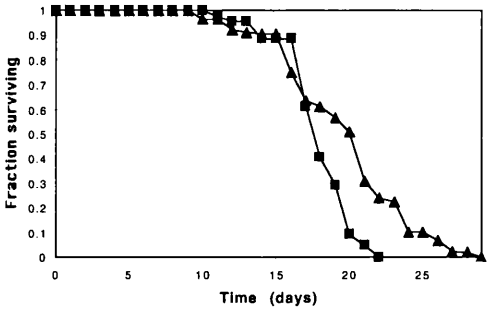
could bias the ratio of male: female lifespan towards males. *O. dolichuroides* female lifespan measurements were therefore repeated on standard NGM plates. Lifespans on agar plates were not significantly different from those obtained in liquid culture (data not shown), and high levels of gonad extrusion were still seen, implying that liquid culture was not responsible for the extremely short lifespan and sickly appearance of *O. dolichuroides* females. It remains possible, however, that both agar plate and liquid culture result in reduced female lifespan relative to males in this species, and that laboratory culture is not representative of sex differences in lifespan.

The results presented above do not support the first working hypothesis of this chapter that the increased lifespan of *C. elegans* males is the result of a skew in the male reproductive probability distribution to later ages, since increased male lifespan was also apparent in dioecious species, where the timing of reproduction in males and hermaphrodites is likely to be similar.

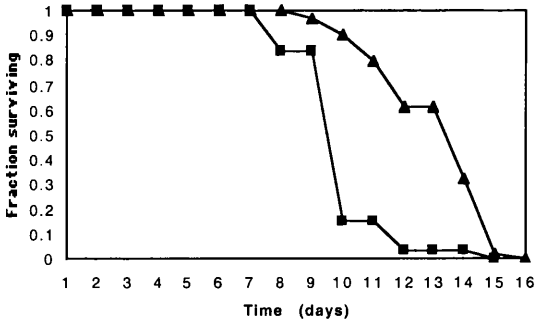
The mean of median lifespans of males from all dioecious species (47.8 days) was significantly greater than that of males from androdioecious species in which males were the longer-lived sex (17.6 days, excluding *C. briggsae*) (Student's *t* test, $P<0.01$). There was no significant difference between the mean of median lifespans of females (22.8 days) and hermaphrodites (13.6 days, excluding *C. briggsae*) (Student's *t* test, $P>0.1$). Thus, although males were the longer-lived sex in these species, androdioecy appears to have resulted in the evolution of shorter male lifespan relative to males of dioecious species, a finding is considered further in the Discussion.

Figure 6.2: Typical survival curves for a range of androdioecious and dioecious nematode species (22.5°C). Triangles= males; squares= hermaphrodites/ females. Significance values comparing male and hermaphrodite/ female survival curves are given in Table 6.2.

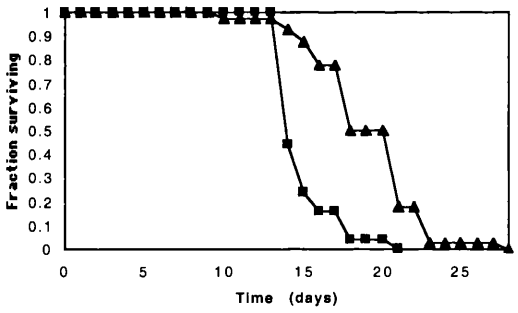
(a) *C. elegans* (Androdioecious)



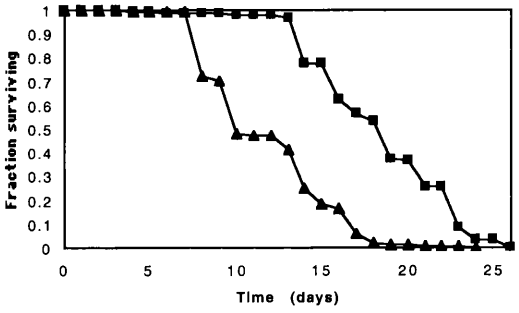
(b) *Oscheius sp.* (Androdioecious)



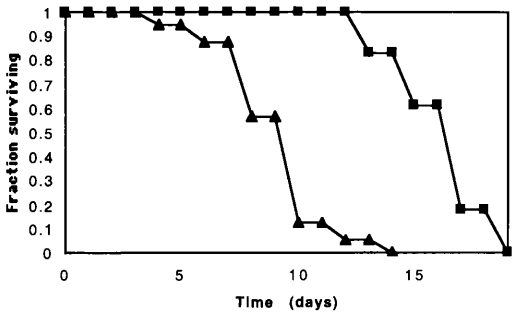
(c) *O. myriophila* (Androdioecious)



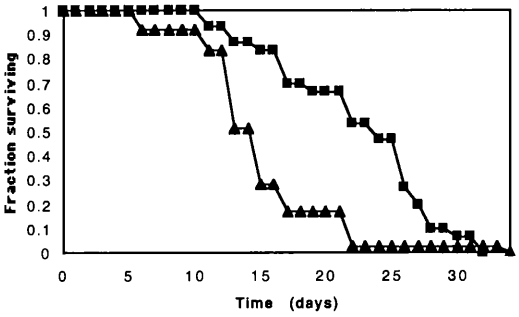
(d) *C. briggsae* Gujarat (Androdioecious)



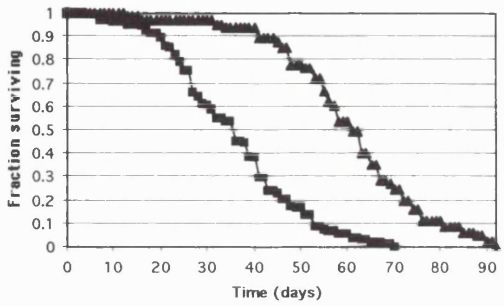
(e) *C. briggsae* Hawaii (Androdioecious)



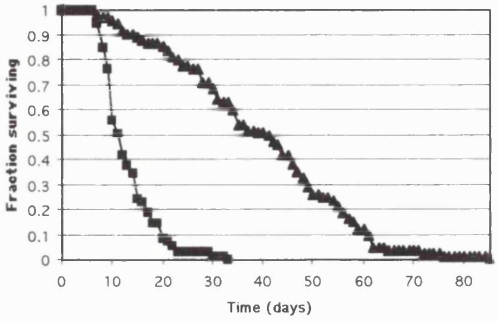
(f) *C. briggsae* Japan (Androdioecious)



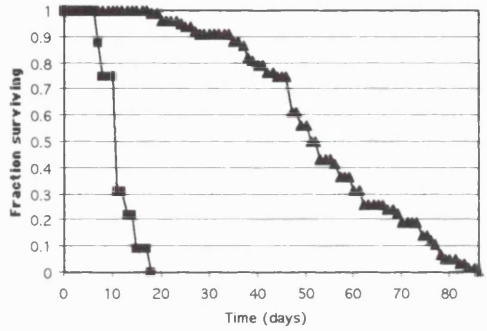
(g) *C. remanei ssp. vulgaris* (Dioecious)



(h) *O. dolichura* (Dioecious)



(i) *O. dolichuroides* (Dioecious)



(j) *R. remanei ssp. remanei* (Dioecious)

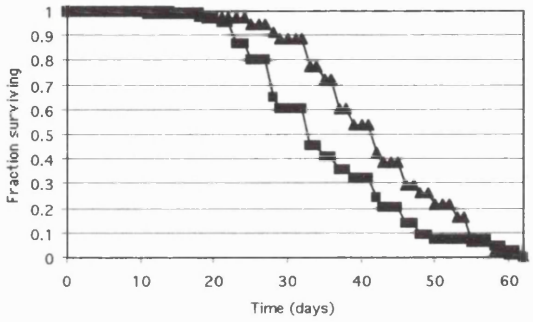


Figure 6.3: Ratios of male: hermaphrodite median (no pattern) and maximum (striped bars) lifespans for a range of androdioecious (blue) and dioecious (red) nematode species (22.5°C).

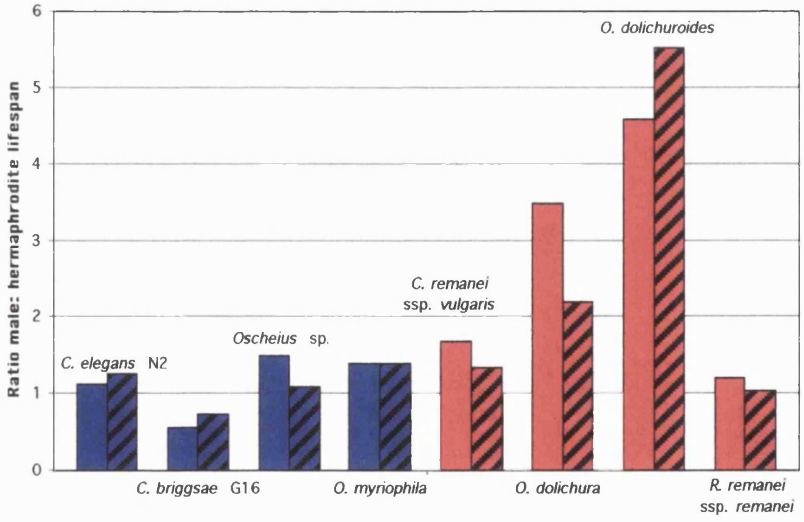


Table 6.2: Median and maximum lifespans for a range of nematode species (22.5°C)

Species (Androdioecious/ Dioecious)	Median lifespan ± 95% C.I. (days)		Ratio M:H/F median lifespan	Maximum lifespan (days)		Ratio M:H/F max. lifespan	N*		*P*†
	H/ F	M		H/ F	M		H/ F	M	
C. elegans (H)	17.7 (19.0, 16.5)	19.8 (21.3, 18.3)	1.12	23.0	29.0	1.26	165 (3)	135 (3)	<0.0001
C. briggsae (Gujarat) (H)	21.0 (22.3, 20.0)	11.7 (13.0, 10.7)	0.56	26.0	19.0	0.73	153 (3)	140 (3)	<0.0001
C. briggsae (Hawaii) (H)	17.0 (17.0, 15.0)	10.0 (10.0, 8.0)	0.59	17.0	12.0	0.71	46 (1)	39 (1)	<0.0001
C. briggsae (Japan) (H)	24.0 (26.0, 20.5)	14.0 (15.0, 13.0)	0.58	30.0	32.0	1.07	30 (1)	36 (1)	<0.0001
Oscheius sp. (H)	9.0 (9.0, 9.0)	13.5 (13.5, 12.0)	1.50	11.0	12.0	1.09	34 (1)	59 (2)	<0.0001
O. myriophila (H)	14.0 (15.0, 14.0)	19.5 (21.0, 18.0)	1.39	18.0	25.0	1.39	27 (1)	40 (1)	<0.0001
C. remanei ssp. vulgaris (D)	35.8 (40.0, 57.0)	60.5 (64.0, 57.0)	1.69	67.0	89.5	1.34	105 (2)	90 (2)	<0.0001
O. dolichura (D)	11.5 (13.5, 10.0)	40.0 (45.0, 33.0)	3.48	35.0	76.5	2.19	70 (2)	95 (2)	<0.0001
O. dolichuroides (D)	11.0 (12.0, 9.5)	50.5 (56.0, 45.5)	4.59	15.0	83.0	5.53	24 (1)	64 (2)	<0.0001
Rhabditis remanei ssp. remanei (D)	33.0 (35.0, 30.0)	40.0 (43.0, 38.0)	1.21	54.5	56.5	1.04	101 (2)	108 (2)	<0.0001, 0.1689

*Number of senescent deaths (number of trials); †Probability that male and hermaphrodite/female survival of each species differ by random chance (log rank test).

6.2.3 Sex differences in dauer formation in other nematode species

In addition to the finding that *C. elegans* males are the longer-lived sex (Gems & Riddle 2000b), previous work has shown that *C. elegans* males form dauers more readily than hermaphrodites (Ailion & Thomas 2000). Since in *C. elegans* both lifespan and dauer formation are regulated by insulin-/IGF-1-like signalling (IIS) to the forkhead transcription factor DAF-16, in Chapter 2 it was suggested that the increased lifespan and dauer formation tendency of males could be the result of constitutively down-regulated IIS (and/or up-regulated DAF-16 activity) in males relative to hermaphrodites. Thus, the increased lifespan of *C. elegans* males could be a correlated effect of selection for increased dauer formation, or vice-versa.

Although this chapter addresses evolutionary rather than mechanistic theories of sex differences in ageing between the sexes, the finding that males were the longer-lived sex in seven of the eight nematode species tested provided the opportunity to investigate whether this might be due to common regulation of both traits by the same underlying mechanism. If the sex that was longer-lived in other nematode species also correlated with the sex that was more likely to form dauers, this could provide evidence for a common element such as IIS and/or DAF-16 activity underlying both traits, as suggested for *C. elegans* in Chapter 2. First, in order to establish that increased male dauer formation was not a peculiarity of N2 following multiple sub-culturing, the ratio of male: hermaphrodite dauer formation was determined for CB4856 (the wild isolate most closely related to N2 (Hodgkin & Doniach 1997)). As well as being the longer-lived sex, males of CB4856 were more likely to form dauers than hermaphrodites (38.2% vs 0% respectively, n=184). This confirmed previous results using N2 that found a male bias to dauer formation (Ailion & Thomas 2000; Chapter 2 Section 2.2.2).

Ratios of male: hermaphrodite/ female lifespan were also determined for several other species (Table 6.3). Although individual replicates showed marked sex biases to dauer formation in most cases, comparison of mean percent dauer formation values across replicates did not always result in a significant *P* value. This was most probably due to the large amount of variation in dauer formation between replicates and because only two replicates were performed per strain.

There was no overall sex-linked correlation of increased dauer formation with increased lifespan, since out of three species in which males were the longer-lived sex, only one showed an increased male tendency to dauer formation (*C. elegans*). In *C. briggsae*, however, the sex that was longer-lived (hermaphrodites) also formed dauers more readily in both isolates tested. Increased male lifespan but decreased male tendency to dauer formation compared with the other sex was the case for dioecious *R. remanei* ssp. *remanei* and androdioecious *O. myriophila*. These findings therefore do not provide support for the hypothesis that differences in IIS may regulate sex differences in lifespan and dauer formation in other nematode species.

Table 6.3: Effects of sex on dauer formation in four nematode species (25°C)

Species (androdioecious/ dioecious)	% dauer formation by males ± s.e.	% dauer formation by hermaphrodites/ females ± s.e.	N	M:H/F[1] dauer formation ratio#	Longer-lived sex	Sex more likely to form dauers	*P*†
C. elegans Bristol (H)	15.1± 9.6	7.5 ± 5.5	1920	2.0	M	M	>0.1
C. briggsae Gujarat (H)	28.9 ± 1.7	36.2 ± 3.6	531	0.8	H	H	>0.1
C. briggsae Hawaii (H)	0.4 ± 0.4	35.4 ± 7.3	439	0.01	H	H	<0.05
O. myriophila (H)	8.1 ± 1.0	49.8 ± 10.6	558	0.2	M	H	>0.05
R. remanei ssp. *remanei* (D)‡	12.5, N/A	41.7 , N/A	608, 53	0.30, 0.6*	M	F	N/A

[1]M; Male, H; hermaphrodite; F, female. #Values larger than one represent an increased tendency by males to form dauers. †Probability that male and hermaphrodite/female dauer formation differ by random chance (Student's *t* test on normalised means); ‡Standard error not applicable as only one replicate performed where number of adults was scored. *Male: hermaphrodite dauer formation ratio based on scoring dauers only and assuming 50:50 sex ratio.

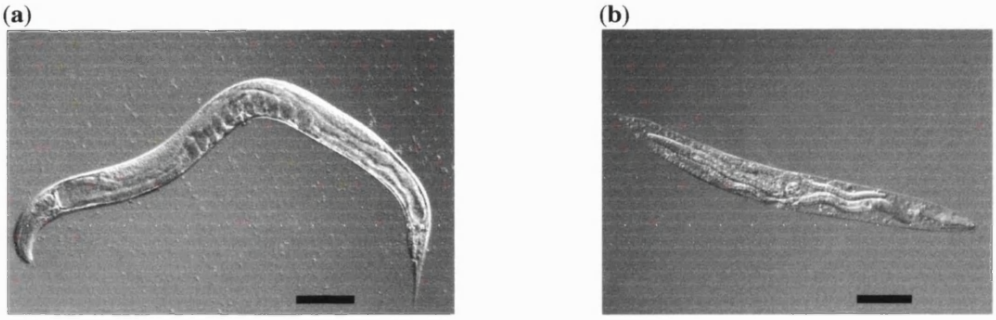
6.2.4 Is increased male lifespan a result of reduced non-senescent mortality?

As described in the Introduction, a prediction of the evolutionary theory of ageing is that a longer lifespan will be seen in the sex subject to lower rates of non-senescent mortality (Williams 1957). Since males are the longer-lived sex in *C. elegans* and all but one of the other nematode species tested, it might be expected that hermaphrodites/females of these species would experience higher levels of non-senescent mortality than males.

One form of non-senescent mortality displayed by *C. elegans* hermaphrodites is internal hatching of larvae (matricide), which occurs in response to low food levels (Figure 6.4). Matricide therefore represents a potential mode of hermaphrodite/ female-

specific non-senescent mortality. As a simplified (and highly artificial) measure of whether matricide is a general feature nematodes, matricide frequencies in the absence of food were determined for each species by placing one-day-old hermaphrodites or mated females in S medium (Table 6.4; Figure 6.5).

Figure 6.4: (a) Gravid young adult *O. myriophila* hermaphrodite raised in the presence of plentiful food; (b) starved *O. myriophila* hermaphrodites corpse containing larvae following internal hatching of eggs. Scale bars= 100μm



Matricide was displayed by all androdioecious species tested, but to varying extents. High levels of matricide (~100% after four days) were seen in three different isolates of *C. elegans*, implying that N2 is not unrepresentative of the species in this respect. Like *C. elegans*, androdioecious *O. myriophila* also reached 100% matricide after four days. In contrast, although hermaphrodites of *Oscheius* sp. did display matricide, overall levels were only 5.8% after four days.

The one nematode species tested above where hermaphrodites are the longer-lived sex was *C. briggsae*. If levels of non-senescent mortality were responsible for sex differences in ageing, a prediction would be that *C. briggsae* hermaphrodites have lower non-senescent mortality rates than males. Rates of matricide might therefore be expected to be reduced in this species relative to others. However, both isolates of *C. briggsae* displayed matricide, with the Gujarat isolate matricide frequency matching that of *C. elegans*.

Matricide also occurred in mated females of all three dioecious species tested. However, overall levels of matricide were generally lower than those seen in hermaphrodites (with the exception of *Oscheius* sp.), ranging from 15.2% to 37.5%.

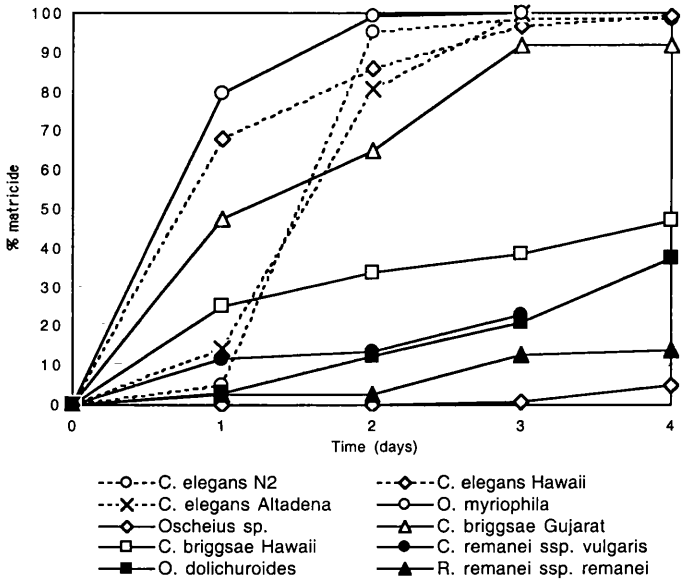
The fact that matricide was seen in all species tested does not exclude the possibility that higher rates of non-senescent mortality may be at least in part responsible for larger male relative to hermaphrodite/ female lifespans. However, it is unlikely to be the sole underlying cause of the sex difference in lifespan because (a) matricide occurs at high levels in *C. briggsae*, in which hermaphrodites are the longer-lived sex, and (b) the degree of matricide measured here does not correlate with the magnitude of lifespan extension.

Table 6.4: Frequency of matricide occurring after four days of starvation for a range of nematode species (25°C).

Species (androdioecious/ dioecious)	% matricide*	N†
C. elegans Bristol (H)	99.1	111
C. elegans Altadena (H)	100.0	118
C. elegans Hawaii (H)	99.2	119
C. briggsae Hawaii (H)	47.1	119
C. briggsae Gujarat (H)	91.7	120
Oscheius sp. (H)	5.8	120
O. myriophila (H)	100.0	116
R. remanei ssp. *remanei* (D)	15.2	79
C. remanei ssp. *vulgaris* (D)‡	22.9	96
O. dolichuroides (D)	37.5	96
O. dolichura (D)	ND	ND

‡Matricide only scored for three days since control mated females on plates ceased laying fertilised eggs after this time; *Weighted mean from two replicates; †Total animals from two replicates; ND- not determined

Figure 6.5: Time-course of cumulative matricide frequency for a range of nematode species (25°C).



6.3 Discussion

6.3.1 Increased male lifespan is a general feature of *C. elegans*

The increased male relative to hermaphrodite lifespan demonstrated for N2 was apparent in nine out of twelve other wild isolates tested from around the globe. Thus, increased male longevity is not just a peculiarity of the much-sub-cultured N2 laboratory isolate, but would seem to be a general feature of the species.

However, it should be noted that in the above experiment, AB1 and N2 male and hermaphrodite survival did not differ. The lack of increased male lifespan in these cases was probably because this experiment was performed before it was discovered that failure to transfer males daily along with hermaphrodites during egg-laying suppressed the lifespan difference between the sexes (see Chapter 1). Male lifespans may therefore have been compromised to some extent in the above experiment. Possibly, there may be variation among isolates in male susceptibility to deleterious effects of liquid culture on lifespan, such as hypoxia or build-up of toxic waste-products. In this respect it is interesting to note that (with the exception of CB4858 and TR389) the male: hermaphrodite median lifespan ratio of N2 was lower than that of any other wild isolate (Table 6.1). This suggests that in the absence of equal transfer frequency for the two sexes, the increased male lifespan is lost most readily in N2. Thus, it is possible that the male longevity bias of the standard laboratory isolate is weaker relative to that seen in other *C. elegans* wild isolates, and that in general the male lifespan bias is stronger in wild populations than seen in N2.

In contrast to the other wild isolates, both median and maximum male lifespans of CB4858 and TR389 were markedly reduced relative to those of hermaphrodites (Table 6.1). Possibly, males of these isolates were particularly susceptible to non-daily transfer in liquid culture, meaning that a repeat of this experiment with daily transfer of males would result in males being the longer-lived sex. However, the fact that male lifespans of CB4858 and TR389 were reduced relative to those of other isolates, while hermaphrodite lifespans were typical of those of other isolates (Figure 6.1), may also indicate the presence of deleterious mutations in these males. That this is possible is suggested by the previous identification of a wild isolate in which males are impotent.

KR314 (Vancouver) produces spontaneous males with abnormal tails that are unable to mate, due to a *mab-23* (male abnormal) mutation (Hodgkin & Doniach 1997). Thus, this wild isolate seems able to survive in nature without any out-crossing with males.

Previous work investigating male and hermaphrodite lifespans of other *C. elegans* isolates found that while males were the longer-lived sex in the isolates PA1 and CL2A, hermaphrodites were longer-lived in N2 (Johnson & Hutchinson 1993). Although these experiments were performed in liquid culture like the present experiment, it is noteworthy that both males and hermaphrodites were maintained in groups. Data presented in Chapter 1 suggest that while interactions between individuals are greatly reduced in liquid culture relative to agar plates, male-male interactions may still reduce grouped male lifespan to some extent. It is therefore possible that interactions between males reduced male lifespan in (Johnson & Hutchinson 1993).

6.3.2 Increased male lifespan is not the result of androdioecy

Having determined that *C. elegans* males are generally longer-lived than hermaphrodites, the question arises as to why this might be. As described in the Introduction, a prediction of the evolutionary theory of ageing is that the sex with a later period of reproduction will be subject to more selection against late-acting deleterious mutations, and hence will be longer-lived. The first working hypothesis of this chapter was therefore that the increased lifespan of *C. elegans* males has evolved as a consequence of delayed male relative to hermaphrodite reproduction. To test this possibility, lifespans were measured for both sexes of a range of androdioecious and dioecious nematode species.

With one exception, males were the longer-lived sex in all species tested, both androdioecious and dioecious (Table 6.2, Figure 6.2). In agreement with this, increased male relative to female lifespan in liquid culture had previously been reported for EM464 *C. remanei* spp. *vulgaris*, and also for CB5161 *Caenorhabditis* sp., which was not tested here (Fletcher & Gems 1998). The exception was *C. briggsae*, in which hermaphrodites were markedly longer-lived than males (Table 6.2, Figure 6.2d-f).

The increased longevity of *C. elegans* males is therefore highly unlikely to be the result of androdioecy and its associated sex differences in reproductive timing, since (a)

in *C. briggsae,* the androdioecious species most closely related to *C. elegans*, hermaphrodites were the longer-lived sex for all three isolates tested and (b) males were the longer-lived sex in all dioecious species tested. Instead, these results imply that increased male longevity may be a typical characteristic of free-living nematodes. It does remain possible that the reasons for increased male lifespan differ between dioecious and androdioecious species, and that in the case of androdioecious species the increased male lifespan arose as a result of later timing of reproduction in males. However, since *C. elegans* and all other Rhabditids probably descended from a dioecious ancestor (Fitch *et al* 1995; Blaxter *et al* 1998), it seems more likely that the increased longevity of males in the species tested is for the same evolutionary reason(s).

It is important to note that the lifespans of the two sexes may respond differently to culture conditions in different species, for example due to changes in levels of neuroendocrine or germline signalling. Examples of potential interactions of liquid culture with IIS, *daf-12* and germline signalling are presented in Chapters 2, 4 & 5. Since there appear to be fundamental sex differences in regulation of *C. elegans* lifespan by neuroendocrine and gonadal signalling (see previous chapters), it is possible that differential responses of the sexes to liquid culture may occur. Thus, culture method may affect which sex is longer-lived, and therefore may affect attempts to explain the evolutionary basis of sex differences in lifespan.

6.3.3 Sex differences in lifespan and dauer formation do not correlate in all species

Because males are both longer-lived and more likely to form dauers than hermaphrodites (Gems & Riddle 2000b; Ailion & Thomas 2000), and because IIS regulates both these traits, it was suggested in Chapter 2 that there may be constitutively reduced IIS/ increased DAF-16 activity in males relative to hermaphrodites. While results implied that sex differences in DAF-16 activity are probably involved, the involvement of IIS was less clear due to the finding that DAF-16::GFP was localised to the nucleus more readily in hermaphrodites, which suggested that IIS may be reduced in hermaphrodites (Chapter 2 Section 2.3.1). Because the significance of the DAF-16::GFP data is unclear, however, the possibility cannot be excluded that sex differences in IIS do underlie increased male lifespan and dauer formation.

If increased male lifespan and dauer formation are both regulated by a common mechanism such as IIS, it is possible that there is selection for only one of these traits, and that the other trait is an unselected result of changes to a common signalling pathway. Thus, there may be an evolutionary advantage to increased male lifespan, with a correlated increased tendency to dauer formation not conferring any benefit to male fitness. Conversely, male fitness may be enhanced by more sensitive regulation of dauer formation, perhaps because males are especially vulnerable to starvation relative to hermaphrodites, and thus would benefit from forming dauers at the first sign of low food levels. Potentially, the increased male lifespan may not therefore be actively selected for, and may not even be a factor in wild populations if males die from non-senescent causes such as predation or mating-associated damage before reaching advanced ages.

It was therefore of interest to see whether males of other nematode species were more likely to form dauers than hermaphrodites/ females in addition to being longer-lived. If so, it would provide further evidence for a common mechanism underlying sex differences in longevity and propensity to dauer formation, although it would not determine whether increased lifespan or increased dauer formation (or both) was the trait being actively selected for. The sex bias to dauer formation was therefore determined for two wild isolates of *C. elegans*, two wild isolates of *C. briggsae* and two other nematode species.

In both *C. elegans* isolates (N2 and CB4856), males were more likely to form dauers than hermaphrodites, in agreement with previous work (Ailion & Thomas 2000; Chapter 2). However, in *C. briggsae*, the species in which hermaphrodites were the longer-lived sex, hermaphrodites formed dauers significantly more than did males. Thus, in these two closely related species, the sex that was longer-lived was also the sex that was more likely to form dauers. It could be that IIS is reduced/ DAF-16 activity up-regulated in *C. briggsae* hermaphrodites relative to males, resulting in the increased hermaphrodite lifespan and tendency to dauer formation.

However, in the two other species tested (*R. remanei ssp. remanei* and *O. myriophila*), the sex that formed dauers more readily (females and hermaphrodites respectively) was not the sex that had previously been found to be longer-lived (males).

In these cases, therefore, there was no suggestion of a common mechanism underlying sex differences in dauer formation and longevity regulation.

Whether IIS plays a role in either of these traits in species other than *C. elegans* has yet to be seen. Very little or no work investigating the genetics of dauer formation and lifespan has been performed using these other nematodes, and their genomes have not been sequenced (except for *C. briggsae*). It is therefore unclear how similar IIS is among different nematodes. However, the high degree of conservation between *C. elegans* and *Drosophila* IIS, and between invertebrate and mammalian insulin and IGF-1 signalling, makes it very likely that IIS among nematodes is also highly conserved. Moreover, in *Drosophila*, IIS regulates both lifespan (Clancy *et al* 2001; Tatar *et al* 2001; Tu *et al* 2002) and a form of larval arrest in response to over-crowding (Britton *et al* 2002), as seen in *C. elegans*. Since the *C. briggsae* genome has recently been sequenced, it should soon be possible to investigate the role of IIS in regulation of sex differences in lifespan and dauer formation in this species at least.

6.3.4 Rates of non-senescent mortality may influence sex differences in lifespan

As described in the Introduction, a prediction of the evolutionary theory of ageing is that increased rates of death due to causes other than senescence (such as predation and disease) will result in evolution of a shorter lifespan. By extending this theory to sex differences in ageing, the prediction is that the sex experiencing higher rates of non-senescent mortality will be the shorter-lived one (Williams 1957). In those nematode species above in which males were the longer-lived sex, therefore, it is possible that an element of being a female/ hermaphrodite increases non-senescent mortality to such an extent that selection against late-acting deleterious mutations is reduced compared with in males. The second working hypothesis of this chapter was therefore that males are the longer-lived sex in *C. elegans* as a consequence of higher rates of non-senescent mortality in hermaphrodites.

As described in the Introduction, a *C. elegans* hermaphrodite trait that is often observed in the laboratory, and hence presumably results in some non-senescent mortality in wild populations, is internal hatching of larvae (matricide). Because matricide does not affect males, it is an example of a non-senescent mode of death

specific to hermaphrodites. It was therefore of interest to determine whether matricide occurred among hermaphrodites and females of the other nematode species employed above. If it did, matricide would be a candidate for a non-senescent mode of death resulting in the evolution of decreased hermaphrodite/ female lifespan relative to males.

Matricide was seen in hermaphrodites of all four androdioecious species exposed to starvation. In *C. elegans*, matricide levels reached ≥ 99% in all three isolates tested. Interestingly, matricide also occurred to a high level in *C. briggsae*, with percent matricide in the Gujarat isolate equalling that seen in *C. elegans*. Thus, hermaphrodites are the longer-lived sex in *C. briggsae* despite the potentially high occurrence of matricide in wild populations. This suggests either that non-senescent hazards do not shape the evolution of sex differences in lifespan in *C. briggsae*, or that in addition to hermaphrodite-specific matricide there are unidentified, more frequent male-specific non-senescent modes of death that result in evolution of a shorter male lifespan. For example, *C. briggsae* males may be particularly susceptible to pathogens encountered in wild populations relative to hermaphrodites, or may be damaged by mating to a much greater extent than are *C. elegans* males.

Matricide occurred extremely rapidly in androdioecious *O. myriophila*, with 100% matricide after just two days. Interestingly, when fully fed hermaphrodites of *O. myriophila* were viewed under a high power microscope, a high proportion of hermaphrodites contained eggs at the point of hatching or larvae that had just hatched. It is therefore possible that matricide occurs more readily in this species even under conditions of high food. Indeed, the percentage of matricide during survival analysis in liquid culture seen in *O. myriophila* (40%) was higher than typically seen in *C. elegans* (~10%). Interestingly, in the nematode *Heterorhabditis bacteriophila*, matricide occurs to such an extent that survival analysis is not possible due to the paucity of senescent deaths (I. Dix, pers. comm.). Matricide may therefore occur constitutively in certain nematode species. Such a trait may be comparable to the mode of reproduction of semelparous species such as Pacific salmon and cuttlefish, in which females die after a single burst of reproduction (see (Finch 1990)). Semelparity is thought to arise in species where the chances of death before a second round of reproduction are extremely high (see (Golding & Yuwono 1994)). Speculatively, different frequencies of matricide in

different androdioecious nematode species may therefore reflect the chances of a hermaphrodite surviving long enough to encounter a rare male and fertilise additional eggs by out-crossing.

Androdioecious *Oscheius* sp. displayed a very low level of matricide (5.8%). Since *Oscheius* sp. has reduced body size relative to the other nematode species, it is possible that only a very small number of eggs are held in the uterus at one time, and are released immediately upon fertilisation. It would therefore be of interest to measure the number of eggs held in the uterus and how long they remain there in this species using high-power microscopy.

Dioecious species in general showed a lower level of matricide than androdioecious ones, but not exclusively, since *Oscheius sp.* displayed the lowest level of matricide overall. However, if *Oscheius* sp. is excluded from Figure 6.5, it is apparent that dioecious species have lower mean matricide rates (25%) than androdioecious species (90%). Although matricide frequency would need to be determined in many more species before it could be concluded that lower matricide rates are typical of dioecious species, it is interesting to speculate that a higher general rate of matricide in androdioecious species may reflect the higher relatedness of self-fertilising hermaphrodites to offspring (100%) than of out-crossing females to offspring (50%). In this respect, it would be of interest to determine whether rates of matricide in hermaphrodites are lower following out-crossing than following self-fertilisation.

The fact that matricide occurred in all species tested suggests that it may have a significant impact on hermaphrodite/ female survival in wild populations. If so, matricide may be a contributing factor to the evolution of sex differences in lifespan. However, the frequency of matricide did not correlate with the magnitude of male lifespan advantage, since the species with the smallest difference between male and hermaphrodite/female lifespan (*C. elegans*) displayed the highest level of matricide, while species showing much greater male lifespan advantages (*C. remanei* and *O. dolichuroides*) had matricide frequencies towards the lower end of the scale. Moreover, *Oscheius* sp. males have a similar lifespan advantage over hermaphrodites as do *C. elegans* N2 males, yet matricide rarely occurred in this species. This suggests that matricide alone cannot account for sex differences in ageing in these nematode species.

However, it is unclear to what extent matricide levels displayed by the above species correspond to those displayed by populations in the wild. The artificial means of inducing matricide in the above experiment may have resulted in matricide occurring more readily in some species than others. In addition, a positive correlation between matricide frequency and the extent of the male lifespan bias might not be expected, since there are likely to be other sex-specific non-senescent modes of death involved with lifespan evolution. Both sexes will experience a range of senescence-independent deaths, such as predation, disease, desiccation and starvation. In some cases, behavioural differences between the sexes may result in one sex experiencing higher levels of a particular form of non-senescent death than the other. Alternatively, there may be forms of sex-specific non-senescent death in addition to matricide. For example, while mating reduces the lifespan of both sexes, only male lifespan is reduced by same-sex interactions (Gems & Riddle 1996; Gems & Riddle 2000b). If differences in frequency of non-senescent death do shape the evolution of sex differences in lifespan, therefore, they are likely to be composed of a number of different components in each sex. According to the second working hypothesis, the sex with the higher rate of non-senescent death overall would be the one subject to reduced selection against deleterious mutations, and hence with a shorter senescent lifespan.

Note, however, that studies of semelparous species suggest that high rates of non-senescent mortality do not necessarily result in reduced selection against deleterious mutations. For example, castration of Pacific salmon, prevention of gonad development in *Octopus* and prevention of male mating in the marsupial mouse *Antechinus* (all of which normally die following a single bout of reproduction) result in increased adult lifespan (see (Golding & Yuwono 1994)). A striking example of this is provided by the polychaete worm *Nereis diversicolor*, which has one reproductive event followed invariably by death due to a loss of capacity to regenerate body parts and a cessation of feeding. This is due to a decline during life in production of a hormone by the cerebral neuroendocrine system, which is required for regenerative capability. However, transplantation of cerebral ganglia from young animals to those nearing reproductive age prevents spawning and allows somatic maintenance and feeding to continue. Such

animals are able to maintain themselves long beyond their usual single season, although disease and physical damage eventually result in death (Golding & Yuwono 1994).

Thus, despite the fact that wild populations of these species die after one season of reproduction, the capacity to survive remains once the mechanism of premature death is removed. This suggests that, in contradiction to the prediction of the evolutionary theory of ageing, reduced selection against late-acting deleterious mutations does not necessarily result in rapid senescence and death, even though wild individuals never survive to advanced ages. However, it has been suggested that those aspects of senescence leading to death need not be synchronised with other, less deleterious senescent symptoms, hence rapid death need not necessarily be expected following removal of the semelparous condition (see (Finch 1994)). It is therefore possible that in semelparous nematodes also, reduced selection against late-acting deleterious mutations due to non-senescent mortality might not immediately result in rapid death in those rare animals that are not removed from the population by non-senescent mechanisms.

6.3.5 Does male rarity influence evolution of male lifespan?

Although males were the longer-lived sex in androdioecious species (except for *C. briggsae*), they were shorter-lived overall than males of dioecious species. By contrast, hermaphrodite and female lifespans were not significantly different from each other.

Why might this be? One obvious difference between androdioecious and dioecious species is that while males comprise approximately 50% of a dioecious population, in androdioecious species they are relatively rare. It is possible that the rarity of males in androdioecious species may have contributed to their reduced lifespan relative to dioecious males. This is because the genomes of males and hermaphrodites are the same (apart from the ratio of A:X chromosomes), hence the genes determining exclusively male traits are all shared with hermaphrodites. If males are very rare, such genes will be carried by hermaphrodites for most of the time, where they will be subject to little or no selection for increased male fitness. Thus, over time, deleterious alleles of these genes may arise, which would not affect hermaphrodite fitness but would reduce male fitness when a rare male is produced. Thus, while in dioecious species genes with effects on males only are subjected to natural selection as often as genes affecting

hermaphrodite traits, in androdioecious species, genes specifying male traits may be subject to weaker selection pressure. In this sense, the increased male lifespan of androdioecious species may be nothing more than an evolutionary vestige from a dioecious common ancestor, which is gradually diminishing as males become rarer.

The finding above that hermaphrodites were the longer-lived sex in *C. briggsae* may relate to the fact males are likely to be very rare in this species, even relative to *C. elegans*. This is because mated *C. briggsae* hermaphrodites preferentially use X chromosome-bearing out-cross sperm before using nullo-X out-cross sperm (LaMunyon & Ward 1997). Thus, whereas in *C. elegans*, 50% of out-cross progeny immediately after mating are male, in *C. briggsae*, production of males is delayed for six hours after mating and only peaks at a level of 40% of out-cross progeny (LaMunyon & Ward 1997). Like that of *C. elegans*, the natural soil environment of *C. briggsae* is likely to be patchy, resulting in "boom and bust" reproductive cycles, hence progeny produced earlier in a period of population growth would be at an advantage. Early male rarity may therefore result in reduced selection against late-acting deleterious mutations in *C. briggsae* males than in *C. elegans* males.

Thus, there may be degrees of male longevity based on their relative rarity in a population. In dioecious species, males are as common as females and are subject to strong selection against late-acting deleterious mutations, leading to long lifespans. Males of androdioecious species such as *C. elegans* are rarer than dioecious males, hence male-specific genes are exposed to natural selection less often and may accumulate some deleterious mutations, resulting in decreased lifespan relative to dioecious males. Finally, males in species such as *C. briggsae* are rarer than males of other androdioecious species such as *C. elegans*, hence are subject to very weak natural selection. Such males may begin to degenerate phenotypically over the generations, and become shorter-lived than hermaphrodites. A prediction might be that male mating efficiency of this species is also reduced due to reduced maintenance of male tail structures.

Potentially, reduced selection pressure in rare males might also explain the short male lifespans of the *C. elegans* wild isolates CB4858 and TR389 described above, if males were unusually rare in these isolates. Rates of spontaneous male generation have

previously been determined for a number of the wild isolates employed above (Hodgkin & Doniach 1997), and are presented in Table 6.5 below. However, spontaneous males are not rarer in CB4858 and TR389 than in other isolates, although it is interesting to note that no spontaneous males were detected in AB1, which is a strain in which male median lifespan was the same as that of hermaphrodites above. Overall, rates of spontaneous male generation do not correlate with the extent of male lifespan advantage among *C. elegans* wild isolates, since certain strains (TR403, CB4856, CB4853 and RC301) produced no or close to no spontaneous males at all, yet still showed increased male relative to hermaphrodite lifespan. Thus, if the rate of spontaneous male generation does play a role in determining sex differences in *C. elegans* lifespan, it is not the only factor.

Table 6.5: Rates of spontaneous male generation for some wild isolates compared with ratios of male: hermaphrodite median lifespan

Strain	% males†	Ratio male: hermaphrodite median lifespan*
N2	0.14	0.83
AB1	0.00	1.00
CB4555	0.88	1.17
CB4853	0.06	1.46
CB4854	0.22	1.04
CB4856	0.06	1.11
CB4857	0.15	1.12
CB4858	0.12	0.79
RC301	0.04	1.26
TR389	0.14	0.65
TR403	0.00	1.17

†Taken from (Hodgkin & Doniach 1997); *Taken from Table 6.1 (22.5°C).

Another possible mechanism that may cause males to be especially rare in certain isolates is if the frequency of hermaphrodite out-crossing with males (which normally generates ~50% male progeny) is reduced relative to that of other isolates. This could be due to evolution of reduced hermaphrodite attractiveness to males in isolates with short-lived males relative to those in which males are the longer-lived sex. It has been suggested that out-crossing is not favoured by *C. elegans* hermaphrodites and that there has been evolution of reduced hermaphrodite attractiveness to males in order to reduce the frequency of out-crossing (Chasnov & Chow 2002). If the extent of this reduction in hermaphrodite attractiveness to males varies among isolates, it is possible that in certain isolates out-crossing occurs so infrequently that males have become particularly rare.

This could then lead to reduced selection against male-specific deleterious mutations as described above. It would therefore be of interest to determine whether rates of out-crossing are reduced in CB4858 and TR389 relative to other wild isolates.

As described above, the isolate KR314 (Vancouver) produces males with abnormal tails which are unable to mate due to the *mab-3(e2518)* mutation (Hodgkin & Doniach 1997). The only source of males is therefore spontaneous non-disjunction of the X chromosome during meiosis, which occurs at a level comparable to N2 in this isolate (Hodgkin & Doniach 1997). Assuming that the *mab-3* mutation is typical of natural populations, the impotence of KR314 males means that all selection for maintenance of male traits has been lost, and that males are a non-viable phenotype. It would therefore be interesting to measure the lifespan of males and hermaphrodites of this isolate (in the absence of the potentially confounding *mab-3* mutation), as a prediction would be that males would be shorter-lived than hermaphrodites due to the complete lack of selection pressure on male-specific genes.

6.3.6 Evolutionary constraints and sex differences in lifespan

A consideration when comparing sex differences in lifespan between different species is that there may be evolutionary constraints affecting sex differences in lifespan (see (Partridge & Sibly 1991)). This is because selection acts upon the same genome in both sexes, with the only difference between the sexes being the ratio of X:A chromosomes. Thus, if some mechanisms of lifespan determination overlap between the sexes, selection for increased/ decreased lifespan in one sex may result in an increase/ decrease in lifespan of the other, and *vice versa*. That such constraints may not necessarily apply to ageing is suggested by the example of the nematode *Strongyloides ratti*, which includes in its life-cycle a free-living adult with a lifespan of one week and a genotypically identical parasitic form with a lifespan of a year or more (Gemmill *et al* 1997). However, such constraints may exist within the different species examined within this chapter, meaning that lifespan may be longer/ shorter in one sex than it otherwise would be due to selection for increased/ decreased lifespan in the other sex. An absolute comparison of sex differences in lifespan may therefore be unrepresentative in some

cases, and sex differences in lifespan reported in this chapter might be understated to some extent as a result of such evolutionary constraints.

6.4 Conclusions

- Increased male relative to hermaphrodite lifespan is a general feature of *C. elegans*, apparent in nine out of twelve other wild isolates tested. Although N2 and one other isolate showed no difference between male and hermaphrodite survival, this may have been the result of unequal transfer of the two sexes in this experiment. However, in two of the isolates males were the shorter-lived sex due to a reduction in male lifespan. Males of these isolates may carry male-specific deleterious mutations.
- Increased male lifespan was seen in all other nematode species examined, both androdioecious and dioecious, bar one. The increased male lifespan of *C. elegans* is therefore unlikely to be a result of androdioecy. In both *C. elegans* and *C. briggsae*, the longer-lived sex (males and hermaphrodites, respectively) was also the sex more likely to form dauers. Possibly, this is due to common regulation of these traits by the same neuroendocrine pathway, such as IIS. This was not the case in other species tested, however, where males were the longer-lived sex but were not more likely to form dauers.
- Increased non-senescent mortality rates among hermaphrodites/ females relative to males in the form of matricide may at least in part be responsible for the increased male lifespan seen in all but one of the species. That matricide rates cannot account for sex difference in lifespan in all cases is implied by the fact that *C. briggsae* displays high levels of matricide, yet shows increased hermaphrodite relative to male lifespan. Moreover, there was no correlation between the degree of matricide and the degree increased male lifespan shown by each species.
- Male rarity may influence sex differences in lifespan. The greater magnitude of the sex difference in lifespan in dioecious compared with androdioecious species may be because increased male lifespan arose in dioecious species and is an evolutionary vestige displayed by androdioecious species. The one species in which males were

the shorter-lived sex was *C. briggsae*. This could be the result of the preferential use of X-bearing out-cross sperm in this species, meaning that males may be exceptionally rare in nature and thus subject to reduced selection against deleterious mutations relative to hermaphrodites.

6.5 Future directions

Since males were shorter-lived than hermaphrodites in two of the *C. elegans* wild isolates tested above, repeat survival analyses for these isolates with equal transfer frequency of the sexes would verify this finding. Male and hermaphrodite lifespans could be measured for more isolates in order to determine whether there are more exceptions to increased *C. elegans* male lifespan. If there are, it would be interesting to attempt to correlate incidence of isolates with increased hermaphrodite lifespan with environment or aspects of population dynamics such as rates of spontaneous male generation or frequency of male production following out-crossing.

Since increased male lifespan appears to be a general feature of free-living terrestrial nematodes, it would be of great interest to perform survival analyses for species from genera other than *Caenorhabditis*, *Rhabditis* and *Oscheius* in order to confirm this. If increased male longevity were a general feature of the Nematoda, it would be of relevance to investigate whether this is true for other invertebrate classes such as Insecta. What are the evolutionary origins of increased male lifespan? Is it a feature of nematodes only, or is it a trait common to the Ecdysozoa?

The mechanism(s) underlying increased male lifespan remain to be defined. It has been proposed in this work that fundamental differences in lifespan regulators such as IIS or gonadal signalling could underlie sex differences in lifespan. As progress is made in sequencing the genomes of other nematode species it should be possible to investigate expression differences between the sexes for potential genes of interest in order to determine whether sex differences in lifespan of different species are determined by common mechanisms.

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Section E: Appendix

Contents

E.1 Reagents employed (in alphabetical order):

M9 buffer (1L in dH_2O):

7.00g $Na_2HPO_4.2H_2O$
3.00g KH_2PO_4
5.00g NaCl
0.25g $MgSO_4.7H_2O$
Autoclave

Minimal medium for E. coli *OP50:*

To 100ml M9 add:

1.00ml 2M NH_4Cl
1.00ml 20% w/v d-glucose
0.20ml 2mg ml^{-1} uracil

Inoculate with ~five single colonies of *E. coli* OP50 and incubate at 37°C in shaker for 24 hours. Store at 4°C.

Nematode growth medium (1L)

3.0g NaCl
2.5g Bactopeptone (Difco)
17g agar (US Biological)
Add 975ml dH_2O and autoclave. Then add (in the following order):

1ml cholesterol (5mg ml^{-1} in ethanol)
1ml 1M $CaCl_2$
1ml 1M $MgSO_4$
25ml 1M potassium phosphate buffer pH6.0

1M potassium citrate pH6 (1L in dH_2O):

20.00g citric acid monohydrate
293.50g tri-potassium citrate monohydrate
Adjust pH to 6.0 using 5M KOH
Autoclave

S Basal solution (1L in dH_2O):

5.85g NaCl
1.00g K_2HPO_4
6.00g KH_2PO_4
1ml cholesterol (5mg ml^{-1} in 100% ethanol)
Autoclave

S Medium (150ml):

To 150ml S Basal solution, add the following using sterile technique:

1.50ml 1M potassium citrate pH6 (autoclaved)
1.50ml trace metals solution (autoclaved)
0.45 ml 1M $CaCl_2$ (autoclaved)
0.45 ml 1M $MgSO_4$ (autoclaved)
Do not autoclave S medium. Store in dark at 4°C.

Standard NGM agar (1.6L in dH_2O):

4.80g NaCl
4.00g Bacto-Peptone
27.20g agar
Autoclave

Place in 55°C water bath immediately following autoclaving and, once agar has cooled to temperature of the water bath, add:

1.60ml cholesterol (5mg ml^{-1})
1.60ml 1M $CaCl_2$
1.60ml 1M $MgSO_4$
40.00ml KH_2PO_4 pH6

Trace metals solution (1L in dH_2O):

1.86g disodium EDTA
0.69g $FeSO_4.7H_2O$
0.20g $MnCl_2.4H_2O$
0.29g $ZnSO_4.7H_2O$
0.025g $CuSO_4.5H_2O$

E.2 Table showing location of primers used during sequencing of *daf-2* ligand-binding and tyrosine kinase domains (Chapter 2 Section 2.2.6)

Primer sequence†	Location in *daf-2* gene sequence	Amplification fragment/ domain*
>>ttgcctcccacccccattgtat>>	Intron 5	Exon 6 LBD
<<tgtcgatttacgcagctgttgtg<<	Intron 6	
>>cccaaaagcggaaaatttacta>>	Intron 6	Exon 7 LBD
<<ttccacatttttcgggaaaa<<	Intron 7	
>>ttcggggcaaaaatcgtat>>	Intron 7	Exon 8 LBD
<<ttacggcggagtagagcaa<<	Intron 8	
>>tctcctgtttcaactgtctcca>>	Intron 8	Exon 9 fragment 1 LBD
<<GCTCTCGAACAAAACAGTGC<<	Exon 9	
>>TGTGATGCTCACCTGTACCTTC>>	Exon 9	Exon 9 fragment 2 LBD
<<ATTGAGGCAAAGTCACTGTTCA<<	Exon 9	
>>GGCTACCTGTTGGTACGTCAAT>>	Exon 9	Exon 9 fragment 3 LBD
<<AAGTCCCACGAATCGATGAG<<	Exon 9	
>>GCGGACTCGGTCTTCTTTAG>>	Exon 9	Exon 9 fragment 4 LBD
<<cgatttgtcggaaattttgatt<<	Intron 9	
>>ctaaaaccctccgaatttgc>>	Intron 13	Exon 14 fragment 1 KD
<<TGTAGATGATCCAGCGTCGA<<	Exon 14	
>>CTGATGGGTGATCGTTTCG>>	Exon 14	Exon 14 fragment 2 KD
<<CGTATGATGCCTGTTCGATG<<	Exon 14	
>>TCGGGATGAGACTGTCAAGA>>	Exon 14	Exon 14 fragment 3 KD
<<TGACGATTCAGAAGCACTGG<<	Exon 14	
>>CCAGCTCGTTCATCTTCTAGC>>	Exon 14	Exon 14 fragment 4 KD
<<ggtttgttccgacttttcga<<	Intron 14	

†Lower case: intronic, upper case: exonic. >> 5' to 3'; << 3' to 5'. * LBD= ligand-binding domain; KD= kinase domain

E.3: Statistical comparisons of percent males vs hermaphrodites and *daf-2*(+) vs *daf-2(m577)* hermaphrodites displaying each level of DAF-16::GFP nuclear localisation following a range of treatments (Chapter 2 Section 2.2.5).

Treatment	Level of NL*	Percentage of animals showing level of NL		N		*P*†
		H	M	H	M	
Control						
	0	83	98	65	63	> 0.05
	1	14	2	65	63	> 0.05
	2	3	0	65	63	> 0.1
Starvation						
15 minutes	0	46	60	67	69	> 0.1
15 minutes	1	37	26	67	69	> 0.1
15 minutes	2	17	13	67	69	> 0.1
15 minutes	3	0	1	67	69	> 0.1
30 minutes	0	24	31	67	74	> 0.1
30 minutes	1	33	49	67	74	> 0.1
30 minutes	2	37	20	67	74	> 0.1
30 minutes	3	6	0	67	74	> 0.1
45 minutes	0	37	54	43	40	> 0.1
45 minutes	1	37	43	43	40	> 0.1
45 minutes	2	26	3	43	40	> 0.1
60 minutes	0	27	44	70	58	**< 0.02**
60 minutes	1	26	40	70	58	> 0.1
60 minutes	2	44	16	70	58	**< 0.01**
60 minutes	3	3	0	70	58	> 0.1
75 minutes	0	28	26	60	73	> 0.1
75 minutes	1	25	42	60	73	> 0.1
75 minutes	2	44	27	60	73	> 0.1
75 minutes	3	3	5	60	73	> 0.1
90 minutes	0	28	37	39	35	> 0.1
90 minutes	1	49	57	39	35	> 0.1
90 minutes	2	18	3	39	35	> 0.05
90 minutes	3	5	3	39	35	> 0.1

*Level of nuclear localisation of DAF-16::GFP as described in Chapter 2 Section 2.25. †Probability that percentage of males vs hermaphrodites or N2 vs *daf-2(m577)* displaying a particular level of DAF-16::GFP nuclear localisation differ by random chance (Student's *t* test on normalised data). Significant differences highlighted in bold.

Treatment	Level of NL*	Percentage of animals showing level of NL		N		*P*†
		H	M	H	M	
Heat stress						
2.5 minutes	0	48	86	40	51	> 0.05
2.5 minutes	1	52	14	40	51	> 0.05
5 minutes	0	7	26	42	39	> 0.1
5 minutes	1	53	69	42	39	> 0.1
5 minutes	2	38	5	42	39	> 0.1
5 minutes	3	2	0	42	39	> 0.1
10 minutes	0	0	8	40	36	**< 0.01**
10 minutes	1	15	59	40	36	**< 0.05**
10 minutes	2	85	33	40	36	**< 0.05**
15 minutes	2	5	0	20	20	**< 0.05**
15 minutes	3	95	100	20	20	**< 0.05**

Treatment	Level of NL*	Percentage of animals showing level of NL		N		*P*†
		H	M	H	M	
Paraquat stress						
0 hours	0	83	93	47	62	> 0.1
0 hours	1	17	5	47	62	> 0.1
0 hours	2	0	2	47	62	> 0.1
1 hour	0	34	92	44	62	> 0.05
1 hour	1	54	8	44	62	**= 0.05**
1 hour	2	7	0	44	62	**< 0.05**
1 hour	3	5	0	44	62	**< 0.01**
2 hours	0	58	80	60	43	> 0.1
2 hours	1	35	16	60	43	> 0.1
2 hours	2	7	2	60	43	> 0.1
2 hours	3	0	2	60	43	> 0.1

Treatment	Level of NL*	Percentage of animals showing level of NL		N		*P*†
		+	*m577*	+	*m577*	
***daf-2(rf)* vs *daf-2*(+) heat shock**						
5 minutes, raised at 15°C	0	51	29	101	105	> 0.1
5 minutes, raised at 15°C	1	43	29	101	105	> 0.1
5 minutes, raised at 15°C	2	6	42	101	105	**< 0.05**
10 minutes, raised at 15°C	1	17	0	42	46	**< 0.02**
10 minutes, raised at 15°C	2	81	76	42	46	> 0.1
10 minutes, raised at 15oC	3	2	24	42	46	> 0.1
5 minutes, raised at 22.5°C	0	73	0	27	28	**< 0.05**
5 minutes, raised at 22.5°C	1	27	25	27	28	> 0.1
5 minutes, raised at 22.5°C	2	0	75	27	28	**< 0.05**

E.4 Table showing nature and location of some *daf-2* mutant lesions characterised to date (Chapter 2 Section 2.2.6).

Allele (reference)	Class	Mutation	Domain
e1365 (1) / *sa193* (2)*	1	A580T	L2
m577 (2)	1	C1045Y	Fibronectin domain
m212 (2)	1	C883Y	Fibronectin domain
sa229 (1)	1	D486N	Cys-rich
m41 (2,3,4)	1	G383E	Cys-rich
e1368 (1)	1	S573L	L2
e979 (2)	2	C146Y	L1
sa187 (1)	2	C469S	Cys-rich
m596 (5)	2	G457S	L2
e1391 (1)	2	P1434L	TK
e1370 (1)	2	P1465S	TK
m579 (5)	2	R437C	Cys-rich
mg43 (1)	Non-conditional	C410Y & P470L	Cys-rich

(1) (Kimura *et al* 1997); (2) D. Patel, pers. comm.; (3) (Yu & Larsen 2001); (4) D. McCulloch, Chapter 2 this thesis; (5) (Scott *et al* 2002) * Subsequently discovered to be allelic.

E.5: Statistical comparisons of percent N2 vs *unc-32(e189)* males and hermaphrodites displaying each level of DAF-16::GFP nuclear localisation following two periods of 35°C heat shock (Chapter 3 Section 3.2.4).

Treatment	Level of NL*	Percentage of animals showing level of NL		N		*P*†
		N2	*unc-32(e189)*	N2	*unc-32(e189)*	
Hermaphrodites						
5 minutes	0	56	72	41	66	> 0.1
5 minutes	1	37	21	41	66	> 0.1
5 minutes	2	7	5	41	66	> 0.1
5 minutes	3	0	2	41	66	> 0.1
10 minutes	0	19	34	74	73	> 0.1
10 minutes	1	37	39	74	73	> 0.1
10 minutes	2	43	23	74	73	> 0.1
10 minutes	3	1	4	74	73	> 0.1
Males						
5 minutes	0	65	32	74	65	> 0.1
5 minutes	1	32	53	74	65	> 0.1
5 minutes	2	3	15	74	65	> 0.1
10 minutes	0	3	1	100	105	> 0.1
10 minutes	1	65	31	100	105	**< 0.02**
10 minutes	2	32	61	100	105	**< 0.02**
10 minutes	3	0	7	100	105	> 0.1

*Level of nuclear localisation of DAF-16::GFP as described in Chapter 2 Section 2.25. †Probability that percentage of N2 vs *unc-32(e189)* animals displaying a particular level of DAF-16::GFP nuclear localisation differ by random chance (Student's *t* test on normalised data). Significant differences highlighted in bold.

E.6 Individual replicates for gonad ablation experiments (Chapter 4 Sections 4.2.1 & 2)

Table E.6.1: Effect of germline ablation on N2 male and hermaphrodite lifespan (22.5°C)

a: Replicate 1

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	16.5 (18.5, 15.0)	----	26	----	32 (51)	----
Z2/Z3(-) H	29.0 (33.0, 26.0)	+76	58	+123	24 (30)	<0.0001
Intact M	20.0 (21.0, 17.0)	----	32	----	57 (58)	----
Z2/Z3(-) M	21.0 (23.0, 18.0)	+5	39	+22	25 (29)	0.052

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

b: Replicate 2

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	18.0 (20.0, 16.0)	----	28	----	49 (60)	----
Z2/Z3(-) H	43.0 (47.0, 36.0)	+139	85	+204	29 (40)	<0.0001
Intact M	23.0 (25.0, 21.0)	----	42	----	79 (81)	----
Z2/Z3(-) M	23.0 (27.0, 21.0)	±0	42	±0	56 (56)	0.34

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

c: Replicate 3

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	15.0 (17.0, 15.0)	----	25	----	32 (58)	----
Z2/Z3(-) H	30.0 (33.0, 28.0)	+100	63	+152	65 (89)	<0.0001
Intact M	24.0 (23.0, 21.0)	----	36	----	58 (61)	----
Z2/Z3(-) M	25.5 (28.0, 21.0)	+6	42	+17	90 (94)	0.60

*N= number senescent deaths (initial sample size) †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

d: Replicate 4

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	P†
Intact H	17.0 (20.0, 15.5)	----	28	----	42 (82)	----
Z2/Z3(-) H	38.0 (40.0, 36.0)	+124	57	+104	38 (44)	<0.0001
Intact M	25.0 (27.0, 23.0)	----	42	----	51 (73)	----
Z2/Z3(-) M	23.0 (27.0, 18.0)	-8	51	+21	37 (46)	0.96

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

Table E.6.2: Effect of whole-gonad ablation on N2 male and hermaphrodite lifespan (22.5°C).

a: Replicate 1

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	16.0 (18.5, 15.0)	----	26	----	32 (51)	----
Z1-Z4(-) H	26.0 (28.0, 22.0)	+63	49	+88	52 (55)	<0.0001
Intact M	20.0 (21.0, 17.0)	----	32	----	57 (58)	----
Z1-Z4(-) M	26.0 (31.0, 23.0)	+30	36	+13	16 (16)	0.0002

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

b: Replicate 2

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	18.0 (20.0, 16.0)	----	28	----	49 (60)	----
Z1-Z4(-) H	32.0 (36.0, 27.0)	+78	65	+132	63 (65)	<0.0001
Intact M	23.0 (25.0, 21.0)	----	42	----	79 (81)	----
Z1-Z4(-) M	22.0 (27.0, 19.0)	-4	46	+10	63 (65)	0.050

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

c: Replicate 3

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	15.0 (17.0, 15.0)	----	25	----	32 (58)	----
Z1-Z4(-) H	21.0 (25.5, 19.0)	+40	57	+128	70 (79)	<0.0001
Intact M	23.0 (24.0, 21.0)	----	36	----	58 (61)	----
Z1-Z4(-) M	23.0 (25.0, 19.0)	±0	51	+42	65 (69)	0.34

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

d: Replicate 4

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	17.0 (20.0, 15.5)	----	36	----	42 (82)	----
Z1-Z4(-) H	31.0 (33.0, 29.0)	+82	62	+72	65 (89)	<0.0001
Intact M	25.0 (27.0, 23.0)	----	42	----	51 (73)	----
Z1-Z4(-) M	24.0 (27.0, 22.0)	-4	44	+5	72 (76)	0.87

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

Table E.6.3: Effect of germline ablation on *daf-12(rh61rh411)* male and hermaphrodite lifespan

a: Replicate 1

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	18.5 (21.0, 16.0)	----	32	----	42 (59)	----
Z2/Z3(-) H	36.0 (43.0, 33.0)	+95	77	+141	61 (66)	<0.0001
Intact M	25.0 (26.0, 23.0)	----	36	----	44 (46)	----
Z2/Z3(-) M	26.0 (28.0, 23.0)	+4	34	-6	36 (38)	0.89

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

b: Replicate 2

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	20.0 (21.0, 17.0)	----	36	----	52 (75)	----
Z2/Z3(-) H	38.5 (47.5, 30.0)	+93	69	+92	40 (70)	<0.0001
Intact M	25.0 (27.0, 21.0)	----	40	----	39 (40)	----
Z2/Z3(-) M	25.0 (27.0, 25.0)	±0	40	±0	30 (49)	0.97

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

c: Replicate 3

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	17.0 (19.0, 16.0)	----	36	----	79 (122)	----
Z2/Z3(-) H	37.0 (40.5, 31.0)	+118	56	+56	32 (65)	<0.0001
Intact M	28.0 (30.0, 26.0)	----	40	----	71 (85)	----
Z2/Z3(-) M	25.0 (28.0, 23.0)	-11	40	±0	42 (68)	0.023

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

d: Replicate 4

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to controls	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	20.0 (20.0, 18.0)	----	30	----	88 (128)	----
Z2/Z3(-) H	47.0 (51.0, 41.0)	+135	77	+157	55 (91)	<0.0001
Intact M	31.0 (34.0, 29.0)	----	43	----	81 (107)	----
Z2/Z3(-) M	27.0 (29.0, 25.0)	-13	42	-2	38 (58)	0.28

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

Table E.6.4: Effect of whole-gonad ablation on *daf-12(rh61rh411)* male and hermaphrodite lifespan

a: Replicate 1

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	18.5 (21.0, 16.0)	----	32	----	42 (59)	----
Z1-Z4(-) H	49.0 (51.0, 44.0)	+165	86	+169	68 (73)	<0.0001
Intact M	25.0 (26.0, 23.0)	----	36	----	44 (46)	----
Z1-Z4(-) M	29.0 (27.0, 32.0)	+16	43	+19	41 (41)	0.003

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

b: Replicate 2

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	20.0 (21.0, 17.0)	----	36	----	52 (75)	----
Z1-Z4(-) H	46.0 (51.0, 37.0)	+130	84	+133	57 (64)	<0.0001
Intact M	25.0 (27.0, 21.0)	----	40	----	39 (40)	----
Z1-Z4(-) M	27.0 (30.0, 21.0)	+8	49	+23	37 (39)	0.69

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

c: Replicate 3

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	17.0 (19.0, 16.0)	----	36	----	79 (122)	----
Z1-Z4(-) H	41.0 (42.0, 36.0)	+141	75	+108	72 (80)	<0.0001
Intact M	28.0 (30.0, 26.0)	----	40	----	71 (85)	----
Z1-Z4(-) M	30.0 (32.0, 28.0)	+7	44	+10	55 (59)	0.52

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)

d: Replicate 4

Treatment & sex	Median lifespan ±95% C.I (days)	% change in median relative to control	Maximum lifespan (days)	% change in max. relative to control	N*	*P*†
Intact H	20.0 (20.0, 18.0)	----	30	----	88 (128)	----
Z1-Z4(-) H	57.0 (62.0, 55.0)	+185	81	+170	48 (57)	<0.0001
Intact M	31.0 (34.0, 29.0)	----	43	----	81 (107)	----
Z1-Z4(-) M	31.0 (34.0, 29.0)	±0	54	+26	46 (48)	0.95

*N= number senescent deaths (initial sample size); †Probability that survival of ablated animals differs from that of intact control of same sex by random chance (log rank test)