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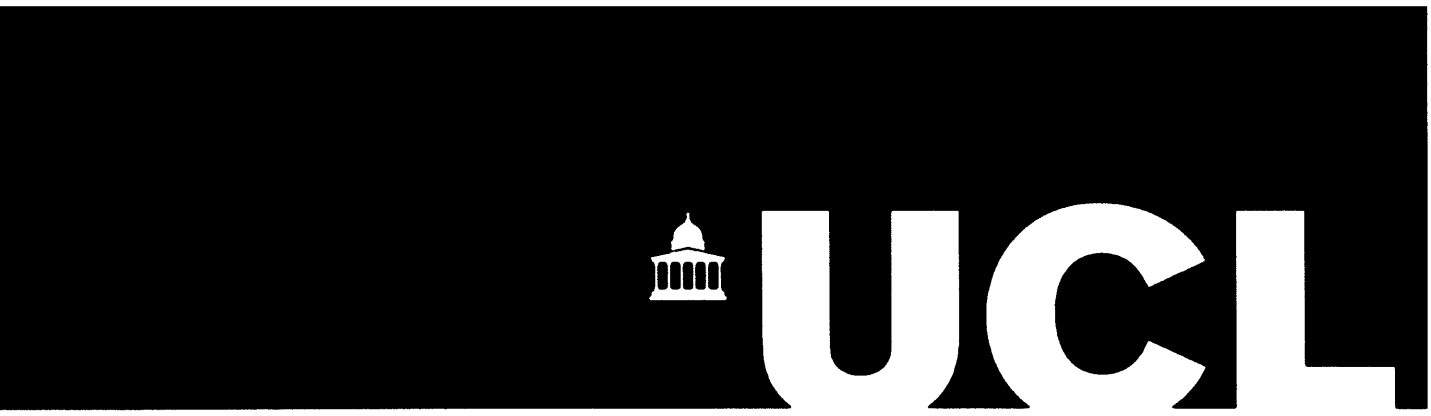

Dietary restriction in *Drosophila melanogaster*

Thesis submitted for PhD

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Dedicated to my father.
14/7/1945 – 10/10/2002

"Oh snail,
Climb Mount Fuji,
But slowly, slowly."

Abstract

Dietary Restriction (DR), the reduction of nutrient intake without malnutrition, was first shown to extend lifespan in rodents in 1935. DR has subsequently proven to be a 'public' method of increasing longevity since its effects are seen in diverse species, ranging from single-celled organisms through to invertebrates and mammals. The appearance of biomarkers of ageing is delayed by DR in non-human primates and indications are that DR may provide health benefits to humans. DR delays the onset of ageing-related pathologies such as cardiovascular disease and cancer, and increases resistance to environmental stresses in rodents. Evolutionary theories of ageing suggest that the effect of DR on longevity represents a trade-off between reproduction and lifespan. In times of famine, an organism's lifetime reproductive success would be increased if reproductive output and the resulting damage were temporarily reduced. Thus, survival to more plentiful times, when reproduction would once again be the most successful strategy, becomes more likely.

In this thesis, I investigate the mechanisms by which DR extends lifespan in the model organism *Drosophila melanogaster*. I use demographic analysis of the effects of applying DR midway though life to show that DR does not slow the rate of ageing but rather removes an acute, transient risk of death that is reversible. I demonstrate that decreased mortality under DR is not the product of lowered mechanical damage resulting from reduced reproductive output. I also show that, unlike the accepted paradigm in mammals, nutrient composition of the food, not calorie intake, is the key determinant of lifespan extension via DR in *Drosophila*. DR flies are shown to be resistant to starvation, which may be indicative of the mechanisms through which DR extends life. However, DR flies are not globally resistant to other environmental stresses including thermal stress, in contrast to DR rodents that have increased thermotolerance. Together, these data give insight into the effects of DR in *Drosophila* and provide a framework for determining the acute risk of death posed by high nutrient intake. They also provide future directions for work in mammalian systems that will enable us to better understand if the mechanisms by which DR extends life really are conserved across species, or whether they are examples of convergent evolution.

Declaration

I declare that the work presented in this thesis is my own except where duly noted.

William Mair.

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Acknowledgements

I would like to thank my mum, brother and sister for always being there when needed over the last four years and my father who, although sadly is not here at the end of the road, was a major factor in encouraging me to start the journey and is sorely missed.

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Publications arising as a result of this thesis

Abbreviations

Dilp – *Drosophila* insulin like peptide

DR – Dietary restriction

ERCs – Extra-chromosomal RNA circles

FF – Full-feeding

HSP – Heat shock protein

IIS –Insulin/ insulin-like signalling

JNK – Jun N-terminal kinase

MAPK – Mitogen activated protein kinase

MRDT – Mortality rate doubling time

PBS – Phosphate buffered solution

RNAi – Ribonucleic acid inhibition

SOD – Superoxide dismutase

SY – Sugar/ Yeast medium

TOR – Target of rapamycin

UV – Ultra violet

Chapter 1. General Introduction

1.1 Introduction to ageing

The inevitability of ageing faces us all and for centuries scientists have sought the key to prolonging human lifespan (Gruman 2003). Oscar Wilde once said 'Those whom the gods love grow younger' and, indeed, one need only look at the rise in cosmetic surgery over recent years (Figure 1.1.1) to appreciate the lengths to which people will go to hold on to their vision of youth. However, despite the many recent improvements in healthcare and medicine, an elixir has so far proved elusive. That life expectancy has increased in the developed world over the last century is the result of reduced mortality rates at all ages, attributable to improved public health, rather than a slowing in the innate ageing process (Wilmoth 2000). Hollywood actors may well look much younger than their years thanks to a decent surgeon, but we can be secure in the knowledge that beyond their Peter Pan exterior they are ageing at the same rate as the rest of us. Ageing could be said to be the worst genetic disease of all. Those of us lucky enough to make it to old age can look forward to a gradual decline in our intelligence, memory, physical fitness, reproductive capacity and sanity, until eventually we fail completely and become the next victim to shuffle off this mortal coil.

'Life's three universal rules – 1) You can't win. 2) You have to lose. 3) There is only one way to get out of the game.' – Anonymous Cynic.[1]

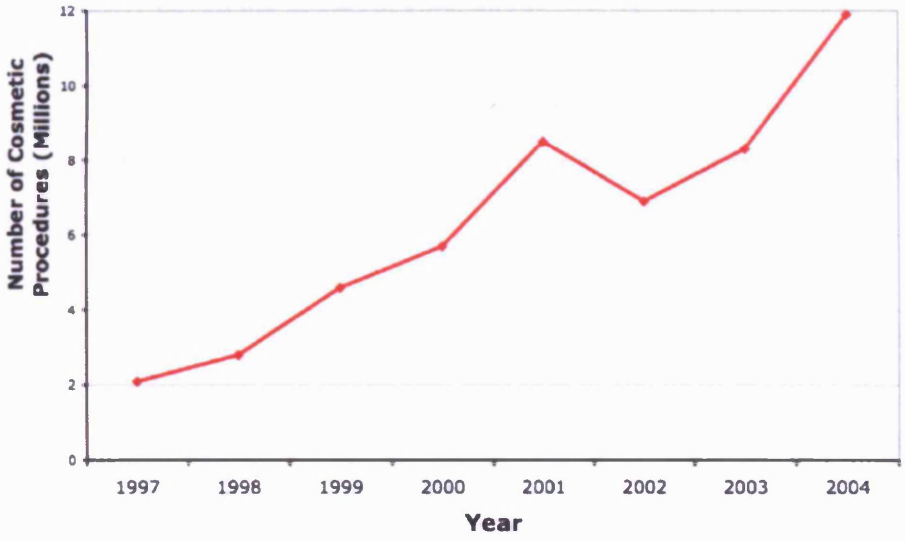
On the face of it then, the ageing process seems to be a universally detrimental trait, but is it really an inevitable one, and why does it exist in the first place?

The common definition of ageing encompasses all things that change with age, both negatively and positively. For example, a wine's flavour may improve with age, and both knowledge and wealth may increase with age for many people, changes few would class as disadvantageous. However, evolutionary biology defines ageing to involve only those factors that deteriorate during the lifetime of an organism and are detrimental to its viability (Ricklefs 1995) and it is this definition that shall be used in this thesis. Ageing in populations can be quantified as the increase in mortality

[1] Taken from Austad (1997), p. 123, chapter 8.

rate and decrease in fecundity with time (Charlesworth 1980; Partridge and Barton 1996) and is seen almost universally amongst different species (Comfort 1979; Finch 1990). Although no detectable senescence was reported in *Hydra* over a four year period (Martinez 1998), metazoans that do not show signs of ageing are rare in the extreme. Even asexually reproducing bacteria, long thought to be effectively immortal, have recently been shown to senesce (Ackermann et al. 2003; Ferber 2005; Stewart et al. 2005).

Figure 1.1.1 The rise in cosmetic procedures in the USA over the last 8 years. These data are taken from the annual statistics of The American Society for Aesthetic Plastic Surgery (ASAPS).



The diversity in the lifespans of different species is startling, with an estimated 1,000,000 fold difference seen across different phyla (Finch 1990; Finch and Ruvkun 2001). More striking still is the existence of related organisms with dramatically different lifespans. For example, the nematode worm *C. elegans* only lives for 2 weeks (Klass 1977), whilst *Loa loa*, also a nematode, can live up to 17 years (Eveland 1975). If *C. elegans* have much the same physiology as *Loa loa*, why is it that their lifespans are so different? Individuals with the same genotype can also show phenotypic plasticity with respect to their rate of ageing; in eusocial insects the lifespan of the queen is invariably greater than that of workers for review see (Finch 1990; Bourke and Franks 1995).

For much of the twentieth century ageing was seen as an evolutionary 'black box', and indeed the existence of any genes that controlled lifespan was questioned (Lints et al. 1979). The discoveries of single gene mutations that extended lifespan such as those in the *C. elegans* gene *age-1* (Friedman and Johnson 1988) were met with much scepticism by the science community (Johnson 2005). Ageing is a highly complex trait and evolutionary theory had suggested it would therefore be polygenetic (Ricklefs 1995). Furthermore, it was thought that genes that affected ageing rates would lie outside of natural selection (see section 1.2.4) and thus show high variation between populations. The identification of mutations that increase lifespan also posed an evolutionary paradox at first glance; if mutating a gene increases lifespan, then the logic follows that the wild type allele functions to decrease longevity. Given the apparent catastrophe that ageing seems to represent for an organism, selection favouring genes that reduce lifespan, rather than extend it, seemed to go against the most fundamental basis of Darwinian evolution, i.e. that natural selection favours those variants that increase fitness and thus their frequency in the next generation.

In the twenty years since lifespan-extending mutations such as *age-1* were reported, the field of ageing research has developed into one of the most exciting and highly funded areas of biological research. The advancement of the genomic era and development of new technologies have allowed the proximate causes of ageing to be examined in much greater detail than previously possible. For a chronological progression of research into ageing since the late 1970's see (Johnson 2002; Warner 2003). To many evolutionary biologists the paradox that ageing represents is enough in itself to warrant its study, but the ultimate aim of much of the field is undoubtedly the extension of human longevity. The ethics of this goal in what is already a 'greying' population is subject to much debate and could be the subject of an entire thesis, with strong opinions both for e.g. (de Grey et al. 2002) and against e.g. (Bruce 2005) the morality of seeking to extend lifespan. There are, however, many pathologies that are strongly associated with age, such as cardiovascular disease, osteoporosis, cancer, stroke, Alzheimer's and type-II diabetes to name just a few. Clearly there is the potential for much medical benefit to be gained by ageing research (Holliday 1998; Holliday 2004). Perhaps the very fact that the population age structure in developed countries is becoming increasingly weighted towards the

elderly makes research into interventions that can reduce the severity of age-related pathologies and promote healthy ageing more necessary than ever.

Theories of ageing generally fall into two categories, loosely classed as the 'why' and the 'how' theories. 'Why' theories are proposed by evolutionary biologists to try to explain the existence and extent of ageing within populations, whilst the 'how' theories describe the underlying mechanisms that cause organisms to senesce. In the next two sections I shall cover each of these in turn.

1.2 Evolutionary theories of ageing

1.2.1 Evidence that ageing is an evolved trait

For ageing to have evolved, it must first have a genetic component. Longevity is a heritable trait, evidence of which is seen in human families; one study reported that children of parents who lived over 80 years themselves live on average 6 years over the population mean, and monozygotic twins die on average within three years of each other compared to six years for dizygotic twins (Ricklefs 1995). Other studies on mono versus dizygotic twins estimate the heritability of human lifespan at between 20-50% (Herskind et al. 1996; Ljungquist et al. 1998; Gudmundsson et al. 2000; Heijmans et al. 2000). Furthermore, laboratory selection experiments in *Drosophila* have yielded long-lived populations from continued selection of old individuals as parents for the next generation (Rose and Charlesworth 1981; Luckinbill et al. 1984; Rose 1984; Partridge and Fowler 1992; Roper et al. 1993; Zwaan et al. 1995; Partridge et al. 1999). There are also now many examples of mutations in single genes that significantly increase the lifespan of model organisms, including *S. cerevisiae, C. elegans, D. melanogaster* and *M. musculus* (Warner 2003). It is of course true that lifespan is not entirely under the control of genetics; clones of genetically identical *C. elegans* reared under laboratory conditions do not all die at the same instant, therefore some factor other than their genes is responsible for the heterogeneity in frailty within those populations (Finch 1990; Rea et al. 2005).

Given that ageing is at least in part genetic, why has it evolved? It is not enough to reason that ageing exists because evolution cannot achieve perfection, and that a

'Darwinian Demon' (Law 1979) that lives long and continues to reproduce is unattainable. Many tumour cell lines (Hayflick 1977) and germ cells (Weismann 1893) are thought to be effectively immortal, thus it is erroneous to assume that the process of natural selection can lead to such complex organisms as those seen in nature, yet continued repair and maintenance is unfeasible. Therefore evolutionary theories of ageing set out to explain why ageing has been selected for during evolution, or perhaps more to the point, why it has not been eliminated by natural selection.

1.2.2 Ageing for the good of the species

That ageing is an evolved trait for the good of the species was an idea first put forward by early evolutionary biologists such as Alfred Russel Wallace and August Weismann in the late nineteenth century. Of ageing Weismann wrote: 'Worn out individuals are not only valueless to the species, but they are even harmful, for they take the place of those which are sound' (Weismann 1891). To many this idea at first sounds plausible. Given that, within its ecological niche, a species is likely to experience limited resources, it makes intuitive sense that by ageing and eventual death, sacrificing the old to free up resources for the benefit of the young will be advantageous for the species. However, this theory is one of group selection and is largely discounted by today's evolutionary biologists because of the inherent assumption that it contains, making the argument circular (Kirkwood 2005) or, as Medawar put it, a 'vicious figure-of-eight'(Medawar 1952).

The idea that the presence of old, fragile individuals is detrimental to their fitter and younger competitors assumes the very thing that it sets out to explain, the existence of ageing (Comfort 1979). Furthermore, group selection theory itself is now largely discounted, with selection thought to work mainly at the level of the gene (Dawkins 1976). A long-lived/ immortal 'cheat' in a population of ageing individuals would rapidly out-compete its peers, thus the genes for immortality would spread as natural selection would favour those with the long-lived genotype (Williams 1957).

1.2.3 Extrinsic hazard and the declining force of natural selection with age

Even in a non-ageing population, the frequency of young individuals will be far greater than that of the old, assuming the population is subject to some level of

extrinsic hazard. This observation was used by Peter Medawar in his inaugural speech at UCL to illustrate that the force of natural selection decreases with age (Medawar 1952), an idea that now underpins much of the evolutionary theory of ageing (Rose 1991).

Medawar used the example of a theoretical population of laboratory test tubes that were not more likely to break with age (non-ageing), but that were subjected to a constant level of extrinsic hazard, i.e. the clumsiness of lab workers and the chance of being dropped. To maintain the number of test tubes at a fixed level, new tubes would have to be added to the 'population' at regular intervals to replace those that had been smashed. The older the test tube, the longer it would have already been exposed to the extrinsic hazard and thus the more likely it would already be broken. Therefore, the age-distribution of the test tube population would quickly become biased towards recent replacements (the young).

Medawar then went on to illustrate the decline in the force of selection with age by adding an ageing component to his model. Suppose a catastrophic trait is added to the test tubes such that, at a specified age, they spontaneously (and this time through no fault of the lab workers) smash. If the age at which this happens is very early in the 'life' of the test tube, the effect on the death rate in the population and the research grant's bank account will be large. However, if this test tube 'death' occurs late in life, the effect on the death rates will be minimal, since most test tubes will not survive extrinsic hazard long enough to be affected by it. Thus, the force of natural selection to remove this catastrophic death event is lower the later the age at which the catastrophe occurs, because the later it occurs the fewer individuals it affects. The persistence of dominant, lethal mutations such as Huntington's disorder in human populations is explained by this principle (Haldane 1941). Huntington's is a late-onset disorder that, although very severe, only exerts its phenotype after the age at which most people have already reproduced. Hence, early onset dominant lethal mutations are rapidly selected against, whilst Huntington's remains within the gene pool.

Comfort argued that, because of the high levels of extrinsic hazard seen in wild populations, ageing is outside of natural selection altogether and hence senescence is

rarely seen in nature (Comfort 1979). However, a decline in survival probability with age has been recorded in nature in mammalian species (Finch 1990; Austad and Fischer 1991; Loison et al. 1999), birds (Gustafsson and Part 1990) and even short-lived invertebrates such as antler flies (Bonduriansky and Brassil 2002). Still, at its most simplistic level, evolutionary theory predicts that as the degree of extrinsic hazard reduces, selection will favour long-lived genotypes (Austad 1997) and indeed some empirical evidence supports this. Island-dwelling Opossums, which suffer low predation rates, show increased lifespan in comparison to mainland populations where predator levels are high (Austad 1993). Bats live 3.5 times longer as a group than rodents, despite the fact that both are mammals and that they are approximately the same size (Austad 2005), and one explanation for this is that the capacity of flight makes bats much better equipped to avoid predation than rodents and therefore they suffer reduced extrinsic hazard (Brunet-Rossinni and Austad 2004). It is interesting to note that one terrestrial rodent, the naked mole rat, lives for over 25 years and experiences very low extrinsic hazard in its natural environment (Sherman and Jarvis 2002).

Different levels of extrinsic hazard may also explain the earlier example of the disparity in lifespan between the nematodes *C. elegans* and *Loa loa*. *C. elegans* are soil dwelling (Riddle et al. 1997) and therefore face higher levels of extrinsic hazard than *Loa loa,* which is an internal parasite (Eveland 1975). The average *C. elegans* lifespan is only 1.5 days in soil conditions within the laboratory, approximately 10 fold less than under standard laboratory culture (Van Voorhies et al. 2005). This short lifespan in wild-like conditions is therefore likely to be due to some increased extrinsic hazard imposed by soil conditions such as desiccation. If worms in the wild also suffer very high levels of extrinsic hazard, selection for lifespan extension beyond two days would be weak, since adults would not live long enough for this extra potential to increase their fitness. In further support of the above hypothesis, altering levels of extrinsic hazard in laboratory populations of *Drosophila* using artificial selection experiments resulted in lifespan being negatively correlated with hazard rates (Stearns et al. 2000). Extrinsic hazard experienced by different castes of ants is also negatively correlated with lifespan when compared under laboratory conditions (Keller and Genoud 1997; Chapuisat and Keller 2002).

However, one recent report did not show the same inverse relationship between extrinsic hazard and the evolution of intrinsic mortality rates in wild guppy populations (Reznick et al. 2004), highlighting the complex relationship between extrinsic mortality, population density and survival probability at different age classes. Fish were caught in the wild from pools with either high or low predation levels. Survival probability in the wild was 20-30 times greater in the low predation pools. However, when maintained in the laboratory for two generations to remove environmental differences, both reproductive and actual lifespan of fish derived from high predation pools was longer than that of the guppies from low predation areas. This is in contrast to the prediction that low extrinsic hazard (predation in this case) would increase selection for long lifespan.

That the study on guppies did not support the original hypothesis may be explained by two different mechanisms. 1) High extrinsic hazard may result in decreased population density that in turn increases *per capita* resource availability. If increased resources benefit older age classes more than the young, greater extrinsic hazard may in fact result in the evolution of delayed senescence (Abrams 1993; Reznick et al. 2004). 2) The effects of environmental hazard may be age-specific. The strictest definition of an extrinsic mortality hazard is something that elevates death rates in an age-independent manner (Partridge 1989; Abrams 1993), but this is clearly not the case for predation, as increased frailty from senescence will increase susceptibility to predators at advanced ages. Increased predation may select for increased escape response and ability to avoid capture. This selection pressure would be greater at ages with the highest reproductive potential and decline with age, a trend that was indeed seen in the guppy populations discussed above (Reznick et al. 2004). Therefore predation could select for reduced intrinsic mortality rate at young ages but accelerated ageing late in life (Williams and Day 2003). Further work is required to test these two hypotheses.

Realisation that the force of selection to remove deleterious traits gets weaker as their age of onset increases led to two separate evolutionary theories as to the existence of ageing in populations. These are the theories of 'mutation accumulation' (1.2.4) and 'antagonistic pleiotropy' (1.2.5), both of which suggest ageing in fact has no biological function (Partridge and Gems 2002).

1.2.4 Mutation accumulation

Mutation accumulation theory states that the selection pressure to remove deleterious alleles from the gene pool becomes progressively less as the age of onset of their phenotype increases. Therefore there will be a build-up of late acting deleterious mutations within the genome that are essentially free from selection and the combined effects of these mutations cause an organism to senesce with age (Haldane 1941; Medawar 1952). Results of empirical studies designed to test this hypothesis have been mixed (Partridge and Gems 2002). If late acting mutations are not under the control of natural selection, their occurrence in the population will be stochastic and show high variation between individuals. By contrast, mutations in early acting genes will be under strong selection because of the severe effect they have on fitness, and will therefore show less variation in the population. Thus, mutation accumulation theory predicts that additive genetic variance (heritability) for ageing-related traits will increase with age (Charlesworth 1990), i.e. the likelihood that an individual shares a mutation with its parents compared to the population as a whole is increased the later its age of onset. However, studies in *Drosophila* failed to support this prediction (Promislow et al. 1996; Shaw et al. 1999).

Furthermore, inbreeding depression, the reduction of fitness in the offspring of closely related parents, should increase with age under this model because late onset deleterious mutations, whose effect will be seen under inbreeding, are more likely to be shared by related individuals than unrelated ones. Although there has been work on *Drosophila* to support this prediction (Hughes et al. 2002), an alternative explanation is that old individuals suffer greater fitness costs from deleterious mutations than young flies because they are inherently more frail (Charlesworth and Hughes 1996).

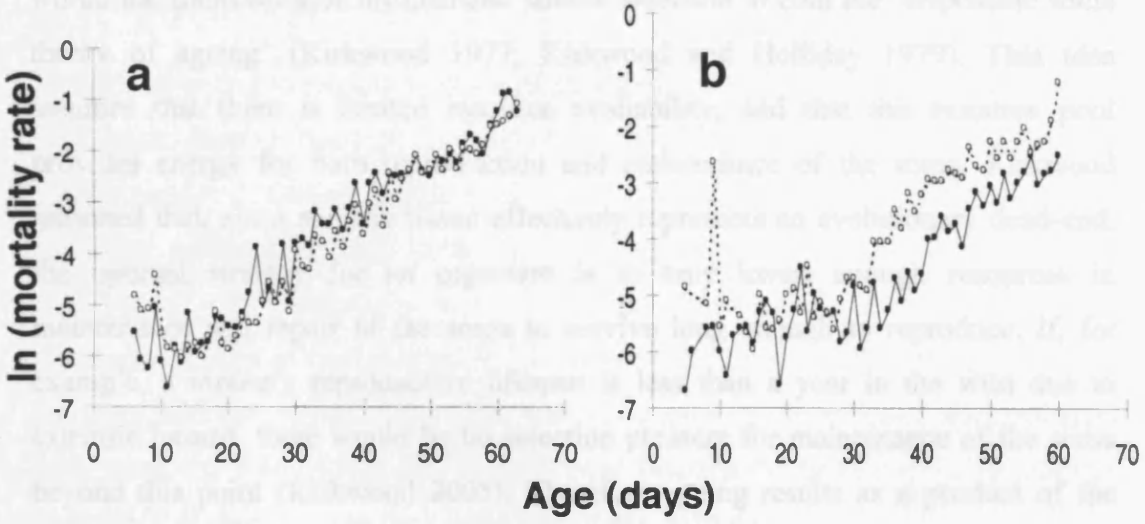
1.2.5 Antagonistic pleiotropy

The concept of antagonistic pleiotropy is that, due to the declining force of natural selection with age, a gene that confers even small fitness benefits early in life will be selected for, even if it has a secondary phenotype that reduces fitness later on (Williams 1957). Therefore genes have not evolved to reduce lifespan, but rather ageing exists as a side effect of otherwise favourable traits (Williams 1957; Partridge and Gems 2002). One of the expectations of this theory, put forward by Williams,

was that 'successful selection for increased longevity should result in decreased vigour in youth' (Williams 1957) and indeed, subsequent experimental data have supported this. In *D. melanogaster*, selection experiments for increased longevity resulted in flies that were long-lived in comparison to controls but had reduced early fecundity (Rose and Charlesworth 1981; Luckinbill et al. 1984; Rose 1984; Partridge and Fowler 1992; Roper et al. 1993; Zwaan et al. 1995; Partridge et al. 1999). These experiments suggest that increased lifespan can be achieved as a trade-off with early reproduction. In support of this hypothesis, the difference in lifespan between control and selection lines is abolished if they are sterilized (Figure 1.2.1) (Sgrò and Partridge 1999).

Figure 1.2.1. Age-specific mortality rates of female *Drosophila* from control and long-lived selection lines.

a. No lifespan difference is seen in control and selection lines when females are sterilised by irradiation. **b**. In fertile mated females, flies from the old selection lines (closed circles) have lower mortality rates than the control line (open circles). Figure taken from (Sgrò and Partridge 1999).



Williams predicted that many aspects of reproduction (e.g. gonadal mass production) would be antagonistically pleiotropic, in that greater current investment would decrease future reproductive potential (Williams 1966), but he found it difficult to provide convincing examples of genes that showed antagonistic pleiotropic effects (Williams 1957). However, recent discoveries of single gene mutations that extend lifespan provide such examples, as they are often associated with some decrease in

early vigour. Mutations in the insulin/ insulin-like growth factor signalling (IIS) pathway have resulted in increased longevity in a variety of species, including *C. elegans* (Johnson 1990; Kenyon et al. 1993; Kimura et al. 1997; Tissenbaum and Ruvkun 1998), *Drosophila* (Clancy et al. 2001; Tatar et al. 2001) and the mouse (Bluher et al. 2003; Holzenberger et al. 2003). That these mutations increase lifespan yet are not the wild type allele suggests they impose some fitness cost. Indeed this is shown as either reduced body size/ fecundity (Clancy et al. 2001; Tatar et al. 2001), reduced ability to compete with the wild type in mixed culture (Jenkins et al. 2004) or constitutive entrance into a dormant larval arrest stage usually seen only under crowded or starved conditions (Kenyon et al. 1993; Gems et al. 1998).

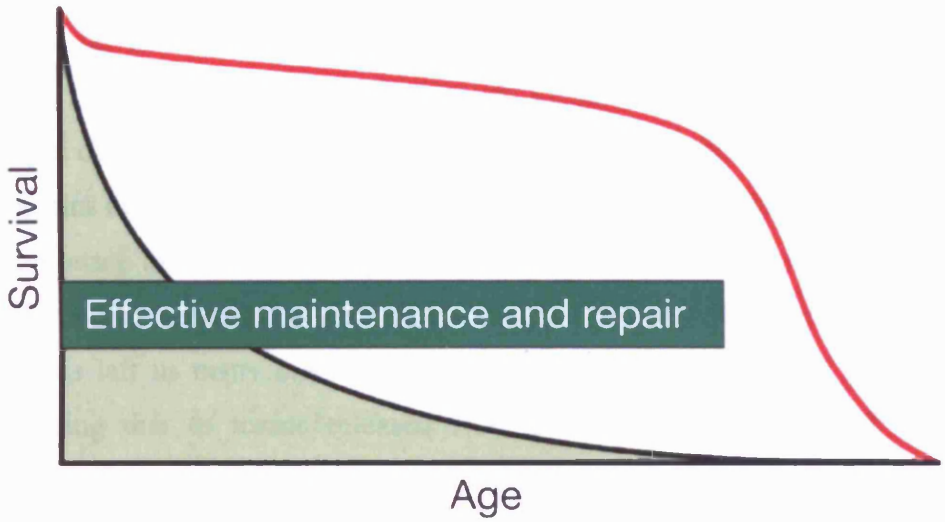
1.2.6 The disposable soma theory

That idea that selection pressure favours early reproduction was first illustrated by Cole (1954) and has since been re-emphasized by many evolutionary biologists and biodemographers (Cole 1954; Lewontin 1965; Williams 1966; MacArthur and Wilson 1967; Charnov and Schaffer 1973; Charlesworth 1980)[2]. Tom Kirkwood then went on to expand the idea of trade-offs between different life history traits within the framework of lifespan and natural selection to coin the 'disposable soma theory of ageing' (Kirkwood 1977; Kirkwood and Holliday 1979). This idea assumes that there is limited resource availability, and that this resource pool provides energy for both reproduction and maintenance of the soma. Kirkwood reasoned that, since somatic tissue effectively represents an evolutionary dead-end, the optimal strategy for an organism is to only invest enough resources in maintenance and repair of the soma to survive long enough to reproduce. If, for example, a mouse's reproductive lifespan is less than a year in the wild due to extrinsic hazard, there would be no selection pressure for maintenance of the soma beyond this point (Kirkwood 2005). Therefore ageing results as a product of the accumulation of cellular and molecular damage after the point at which repairing this damage does not result in increased fitness, i.e. beyond the reproductive life of the organism in the wild (Figure 1.2.2).

[2] Lewontin (1965) and MacArthur and Wilson (1967) cited from Charlesworth (1980)

Figure 1.2.2. Schematic diagram of survival in the wild (black) and under protected conditions (red).

High extrinsic hazard reduces lifespan in the wild, therefore selection pressure for repair mechanisms only exists for as long as an organism is expected survive this hazard and ageing in protected conditions is seen after this point. Figure taken from (Kirkwood and Austad 2000).



1.3 Mechanistic theories of ageing

Rather than trying to explain why the phenomenon of ageing exists, 'mechanistic' or 'proximate' theories describe the deteriorative processes that cause senescence at the organismal level. Gerontologists tend to describe the deterioration with age of the particular process that they themselves are most familiar with, and this leads to proliferation of a multitude of mechanisms that are potentially causal to the ageing process, although in reality much of the work represents correlations of decline in function with age. As summed up in the two quotes below, to date, gerontologists have proposed many (over 300 at last count (Medvedev 1990)) varied proximate theories of ageing, without the emergence of one unifying theory.

'The scientific study of ageing has been an odd mix of the accumulation of mountains of dismal evidence that shows that almost anything you can think of goes wrong with age and proposals of simplistic theories that try to explain ageing in terms of single processes, ranging from defective testicles to shortened telomeres'.

- Professor Brian Charlesworth

'In almost any other important biological field other than that of senescence, it is possible to present the main theories historically and to show a steady progression from a large number of speculative ideas to one or two highly probably, main hypotheses. In the case of senescence this cannot profitably be done'.

- Alex Comfort (1979)[3].

Alex Comfort's somewhat defeatist outlook on the state of the field of ageing research dates to before the recent advances in the genetics of ageing (Finch and Tanzi 1997). Comfort's book, 'The Biology of Senescence', was one of the first collections of data on ageing amongst different species, but the over-riding majority of it contains descriptions of proximate mechanisms, with only five pages given over to evolutionary theory. As Rose points out, detailed description of how something occurs does not necessarily give much insight into why it occurs (Rose 1991). History has left us many colourful examples of proximate theories, ranging from ageing being due to toxins released from gut bacteria (Metchnikoff 1904) to reductions in hormone secretion from the testes. The latter idea lead to the somewhat desperate process of implanting the testes from goats and monkeys into humans in a bid to halt the ageing process (Common 2000). The following section will cover some of the main proximate theories that have been reported during the last century to be the cause of senescence. Although workers in the field have by no means unified upon a central theory, it is perhaps now both possible and profitable to describe a progression towards a small number of main hypothesis when previously it was not (Rose 1991).

1.3.1 Wear and tear

All machines eventually suffer reduced performance due to general damage from use. Any consumer knows that the lifespan of their purchase is finite, and the time will come when a replacement is needed. This timescale will depend upon the initial quality of the product and the ability to repair faults as they occur. Do organisms represent little more than biological machines then, with their eventual decline (ageing) the result of mechanical damage? Despite the fact that organisms have

[3] 1979 refers to the date of the third edition of Alex Comfort's book, however this quote was in the original 1956 version and thus is more a reflection on the state of the ageing field in the mid 20th century.

many repair mechanisms, perhaps these simply cannot function indefinitely and, like any car or washing machine, usage will cause damage and eventual malfunction. The idea is at first compelling. Before the recent advances in dentistry, all humans suffered mechanical damage to teeth, resulting in an aged state 'Sans teeth, sans eyes, sans taste, sans everything'[4]. Without replacements, tooth decay would surely have resulted in eventual starvation and death, i.e. senescence from 'wear and tear'. The argument against 'wear and tear' being the inevitable cause of ageing is that the hypothesis assumes that repair to damage cannot function indefinitely. Yet 'immortal' cell lines such as tumour cells (Hayflick 1977), the non-ageing *Hydra* (Martinez 1998), germ lines (Weismann 1893) and species that exhibit limb regeneration posses very advanced repair capacity and seem to be exceptions to this idea (Rose 1991). Remarkably, recent work suggests that limb regeneration may be possible in mammals (Heber-Katz et al. 2004). Indeed, just because humans cannot replace their adult teeth does not mean that this ability is beyond the reach of evolution, since sharks, as we know, have this capacity.

However, it is certainly true that repair mechanisms become less efficient with age. As previously mentioned, this is predicted by evolutionary theory because of the decline of the force of natural selection with age. Death due to extrinsic hazard renders investment in repair past the natural life expectancy in the wild unbeneficial, with traits that cause early fitness benefits at a cost of reduced repair late in life being selected for (Kirkwood 1981). Organisms undoubtedly suffer wear and tear as they age and this may result in senescence. However, the idea that inevitable wear and tear is the underlying reason why ageing exists in populations is unfounded.

1.3.2 Somatic mutation

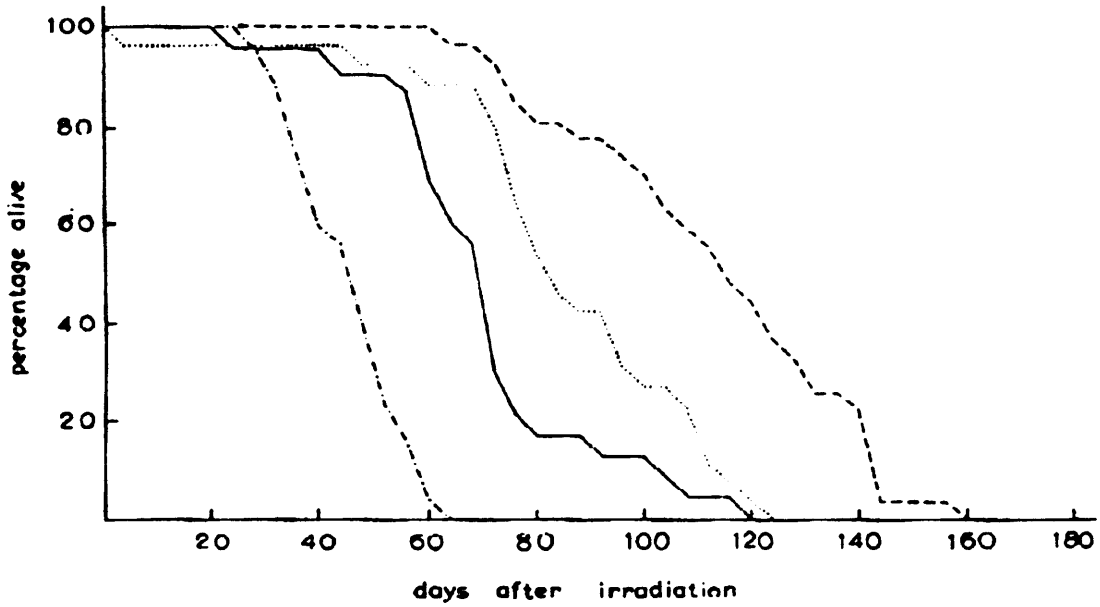
The somatic mutation theory suggests that the proximate cause of ageing is the build-up of mutations to the somatic DNA, leading to incorrect coding sequence and thus reduction/ loss of function of proteins (Szilard 1959; Failla 1960)[5]. Evidence for this idea came from studies in both *Drosophila* (Lamb 1963) and mice (Lindop and

[4] A line from Shakespeare's 'As you like it' and cited in this context from Ricklefs (1995)

[5] Failla 1960 cited from Rose (1991)

Rotblat 1961; Comfort 1979) in which exposure to radiation reduced lifespan (Figure 1.3.1).

Figure 1.3.1. Lifespan of *Drosophila melanogaster* subjected to radiation.
Progressive increases in radiation dose decreased lifespan (dose given to cohorts increases from right to left). Figure taken from (Lamb 1963).



However, there are examples in which low doses of radiation actually *increase* the lifespan of rodents (Carlson et al. 1957; Congdon 1987) and fruit flies (Sacher 1963). Work on the haplodiploid wasp *Habrobracon* has also been used as empirical data against the somatic mutation theory (Rose 1991). In haplodiploid species, the ploidy of the sexes is different; males are haploid and females diploid. The somatic mutation hypothesis predicts that males would age at a faster rate than females in haplodiploid species, since deleterious mutations in the haploid males could not be rescued by the presence of a second non-mutant copy. However, even though male *Habrobracon* wasps show increased sensitivity to radiation compared to females, the lifespan of the un-irradiated sexes are the same (Clark and Rubin 1961). For these data to be true evidence against the somatic mutation hypothesis of ageing there would have to be no difference in intrinsic mutation rate between sexes and this is yet to be tested.

1.3.3 The error catastrophe theory

In 1963 Leslie Orgel proposed his 'error catastrophe' theory of ageing (Orgel 1963; Orgel 1970), the premise being that ageing is caused by errors in the translation efficiency from messenger RNAs into protein. These errors would lead to defective proteins and thus a further increase in translation errors, causing a positive feedback mechanism and the eventual catastrophe. This theory predicts that there will be an increase in the level of abnormal proteins with age, an idea that is not supported by empirical studies (Samis 1978)[6]. Moreover, feeding *Drosophila* amino acid analogues, which get incorporated into proteins and inhibit their function (Richmond 1962), did not reduce lifespan (Dingley and Maynard Smith 1969) as would be expected if ageing is caused by the build-up of damaged, non-functional proteins.

1.3.4 The replicative theory of ageing

Prior to 1960, it was thought that, although whole organisms aged, cells kept in culture could be kept alive indefinitely (Rose 1991). This was based on claims by the Nobel prize winning cell biologist Alexis Carrel, whose laboratory had reportedly kept chick heart cell lines alive and dividing for decades. However, these results could not be repeated by other laboratories (Phillips and Cristofalo 1983), and it has been suggested that either laboratory incompetence was the cause of these immortal cells or that laboratory assistants, too afraid to report the death of cell lines, spiked them with fresh new cells (Austad 1997). Leonard Hayflick discovered that, despite the ability of cancerous cells to continue to divide indefinitely *in vitro,* this is not the case for normal diploid cells (Hayflick 1977). The 'Hayflick limit', the number of cell divisions before cell lines stop dividing and enter the 'phase III' non-growth stage, varies between both cells from different organisms and cells from the same organism taken at different ages. The capacity of human fibroblast cells to divide *in vitro* is inversely related to the age of the donor from which they were taken (Martin et al. 1970). Furthermore, cross-species comparative studies showed that the 'Hayflick limit' of cells is positively correlated with the mean lifespan of the host organism (Hayflick 1977; Rohme 1981) (Figure 1.3.2). This then becomes the basis of the replicative theory of ageing; somatic cells have a finite lifespan in terms of the

[6] Cited from Rose (1991)

number of divisions they can under go *in vivo*, and once this limit is reached the organism will begin to senesce.

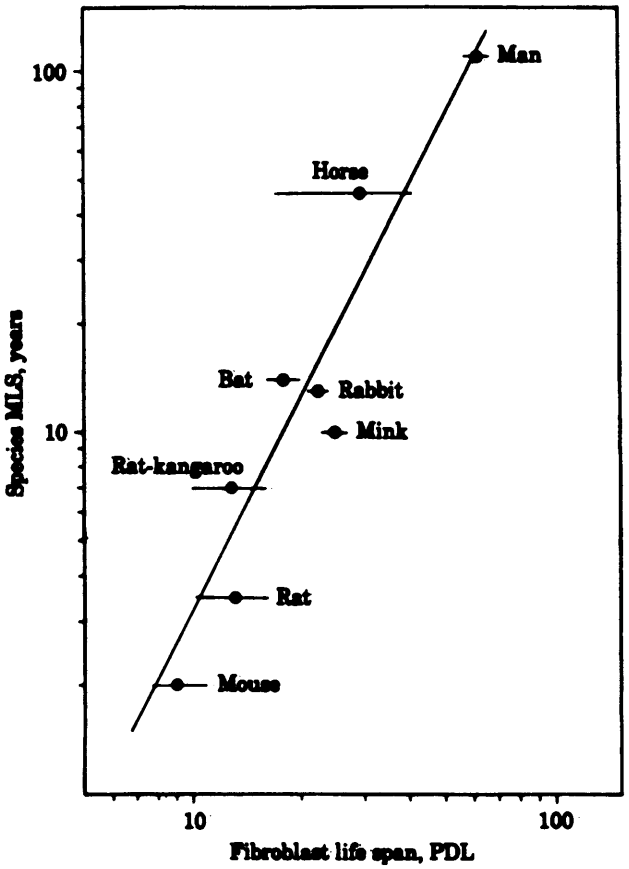


Figure 1.3.2. Mean lifespan (MLS) plotted against fibroblast replicative limit for different species.
A linear positive correlation was seen between the ability of cells to divide in culture and the lifespan of the host organism. Figure taken from (Rohme 1981).

The replicative potential of human cells in culture and *in vivo* is thought to be due to the reduction in telomere length with each cell division (Harley et al. 1990; Allsopp et al. 1992). Telomeres are stretches of heterochromatin found at the tips of the chromosomes and, due to a lack of telomerase activity (Greider 1990; Harley 1991), their length is gradually reduced with every cell cycle. Telomere length is a predictor of human fibroblasts' replicative potential *in vitro* (Allsopp et al. 1992). Furthermore, cells isolated from patients who suffer from the premature ageing syndrome Hutchinson-Gilford progeria have shortened telomeres and a reduced capacity for cell division *in vitro* (Allsopp et al. 1992). There is substantial evidence that telomere length is responsible for the 'Hayflick limit' in human cells (Hornsby 2001); ectopic expression of the reverse transcription subunit of the telomerase complex (TERT) is sufficient to immortalize normal cells (Bodnar et al. 1998; Kiyono et al. 1998). However, the story is not so clear in other mammals. Most rodent cells have long telomeres compared to humans (Blasco et al. 1997; Coviello-McLaughlin and Prowse 1997) and are telomerase-positive (Prowse and Greider 1995), but still have limited replicative capacity in culture (Wright and Shay 2000). Although replicative senescence has been shown to be due to telomere shortening in human cells it cannot be the cause in rodent cells and as such is not a universal

feature. Furthermore, telomere length does not correlate with lifespan in *C. elegans* (Raices et al. 2005).

The 'Hayflick limit' does seem to be real and not an artefact of cell culture (Rose 1991; Hornsby 2001), but its function may be preventing tumorigenesis rather than reducing longevity (Sager 1991; Campisi 1997; Hahn et al. 1999; Campisi 2001; Campisi 2005). Cancer results from uncontrolled cell division, therefore, once an organism has grown to its final size, some inbuilt restriction on further cell division may reduce the likelihood of cells transforming into cancerous growth and thus the 'Hayflick limit' may increase fitness. Further evidence against limited replicative capacity causing ageing is that not all tissues are composed of dividing cells, and structures such as muscle fibres display signs of senescence despite being non-dividing tissue throughout life (Rose 1991).

1.3.5 Rate of living

In the early twentieth century Jacques Loeb and colleagues were the first to show that reducing the ambient temperature at which *D. melanogaster* are housed increased lifespan (Loeb and Northrop 1916; Loeb and Northrop 1917). They reported a 'temperature coefficient', similar to that seen in chemical reactions, in that for every 10 degrees centigrade increase in temperature lifespan was approximately halved. This suggested that housing flies at high temperatures meant that all biological processes (including ageing) proceeded at a faster rate. Rubner showed that there was a correlation between lifespan and resting metabolic rate in mammals and that, of the five species he studied (horses, cows, dogs, cats and guinea pigs), all consumed approximately the same amount of energy in their lifetime (Rubner 1908; Austad 1997)[7]. These studies provided impetus to Raymond Pearl who, with his co-workers, went on to perform a series of studies both on *Drosophila* and canteloup seedlings.

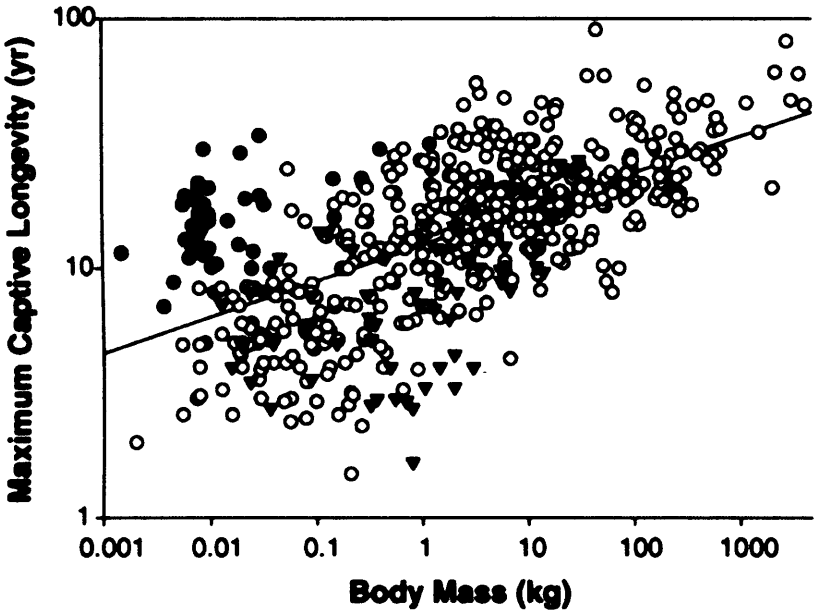
Although in 1919, Pearl intended to work on mice, 'at the end of the first year, when [they] were just ready to start definitive experiments, a devastating calamity wiped out the colony in half an hour', leading to the decision instead to use *Drosophila*

[7] Rubner cited from Pearl (1928)

melanogaster. Pearl's work led him to the conclusion that 'the duration of life varies inversely with the rate of energy expenditure during its continuance. In short, the length of life depends inversely on the rate of living.' (Pearl 1928). Pearl took his idea further and pronounced that lazy people live the longest and that the increased longevity of women compared to men was due to the fact they carried out less physical labour (Austad 1997).

The rate of living theory suggests that there is a fixed lifetime energy expenditure per unit mass and that the lifetime of an organism is dependent on how quickly it uses this energy potential. In other words, live fast and you'll die young. However, although this hypothesis does hold true for some mammals there are exceptions to the rule, which require that the theory is re-evaluated (Sohal 1986).

Figure 1.3.3. Lifespan versus body mass for different mammals.
Bats (closed circles) live longer than predicted by their body size whilst marsupials (closed triangles) live shorter than expected. Figure taken from (Austad 2005).



Most notably, bats and birds live much longer than their metabolic rates would predict if the theory were correct, whilst marsupials are shorter lived than their size predicts (Austad and Fischer 1991; Ricklefs and Scheuerlein 2001; Austad 2005) (Figure 1.3.3). Although birds consume more oxygen than equivalently sized mammals, production of free radicals in avian cells is much less than in mammalian cells and this may explain their longevity (Barja 1998) (section 1.3.6). Therefore,

despite longevity being correlated with the rate of living for poikilotherms at different temperatures and in some groups of mammals, it is not a universally applicable hypothesis.

1.3.6 The free radical/ oxidative stress theory of ageing

In 1956, Harman proposed a mechanism of ageing that was an extension of the rate of living hypothesis and linked metabolic rate to lifespan. Free radicals are highly reactive molecular species containing un-paired electrons. Harman's hypothesis was that ageing was the result of the build-up of damage to DNA with age due to constant bombardment by these radicals (Harman 1956). He based this idea on observations that radiation reduced the longevity of 'all living things' (Hempelmann and Hoffman 1953) in a manner suggestive of accelerated ageing. Thus the 'free radical theory of ageing' was conceived and it remains one of the most widely accepted proximate theories as to why organisms age.

During the course of aerobic respiration, free radicals are produced, commonly in the form of superoxide ($O_2^{\bullet-}$) or the hydroxy radical ($OH^{\bullet}$) (Mathers et al. 2004). Reactive oxidative species (ROS), which include both free radicals and also species that generate them such as H_2O_2, can be highly damaging to other macromolecules in the cell such as proteins, lipids or nucleic acids (Fridovich 1976; Halliwell 1999)[8] since they are powerful oxidising agents. Organisms have in-built defences to neutralise ROS, including the antioxidant enzymes catalase, Cu-Zn superoxide dismutase (SOD), MnSOD, glutathione peroxidases and glutathione S transferases and also free radical scavengers such as glutathione and the flavonoids (Mathers et al. 2004).

Much work has been done to test the free radical theory (also referred to as the oxidative stress theory of ageing) yet, nearly 50 years on, results are still conflicting. One key prediction of the theory is that senescence cannot be retarded without corresponding reductions to oxidative damage/ stress (Sohal et al. 2002). Oxidative damage has been shown to increase with age in different tissues and in different species (Sohal and Weindruch 1996). Furthermore, long-lived flies from artificial

[8] Fridovich 1976 cited from Sohal 2002.

selection experiments have increased levels of SOD activity (Hari et al. 1998). Pigeons and rats are endotherms of similar size, yet pigeons live five times as long as rats and also have decreased ROS generation (Ku and Sohal 1993; Barja 1998). Hypoxia extends the lifespan of *C. elegans* (Honda et al. 1993) whilst hyperoxia increases mortality in worms (Honda et al. 1993) and flies (Landis et al. 2004; Walker and Benzer 2004). Mutant *C. elegans* with increased lifespans have been reported to have reduced metabolic rate, suggested to be causal to their enhanced longevity (Van Voorhies and Ward 1999). However, these findings were not replicated in a recent study (Houthoofd et al. 2005) and neither long-lived *Drosophila* mutants (Tatar et al. 2001; Hulbert et al. 2004), nor long-lived dietary restricted flies (Hulbert et al. 2004) or rodents (Masoro et al. 1982) have decreased metabolic rates.

Stringent testing of any theory of ageing requires more than correlative data and experiments that directly manipulate anti-oxidant defences have sought to provide this for the free radical theory of ageing. *C. elegans* fed synthetic antioxidants have increased lifespan of 44% compared to controls (Melov et al. 2000). Furthermore, both over-expression of the free radical scavenger superoxide dismutase in adult *Drosophila* (Parkes et al. 1998; Sun and Tower 1999; Tower 2000) and over-expression of human catalase in mouse mitochondria extends lifespan (Schriner et al. 2005) (see section 1.3.7). However, the effect of dietary supplementation of antioxidants on lifespan have not been replicated by other laboratories, either in *C. elegans* (Keaney and Gems 2003), *Drosophila* (M. West, unpublished) or houseflies (Bayne and Sohal 2002). Also, ubiquitous over-expression of SOD did not increase lifespan in mice (Huang et al. 2000).

Even staunch supporters of the free radical theory admit 'The strongest evidence supporting the oxidative stress hypothesis consists of correlative relationships involving rates of ROS-production, levels of oxidative damage, and repair capability' (Sohal et al. 2002). Clearly more than correlative evidence is required before the free radical paradigm can be accepted as the fundamental cause of senescence (Lindsay 1999; Wickens 2001). Indeed, rather than being universally detrimental to cells, ROS may act as important cellular signalling molecules, linking changes in the intracellular redox status to multiple signalling pathways, in turn

increasing survival by altering transcription levels or co-ordinating apoptosis in response to stress (Kamata and Hirata 1999; Finkel and Holbrook 2000; Finkel 2003). For a comprehensive review of empirical evidence for and against the oxidative stress theory of ageing see (Beckman and Ames 1998).

1.3.7 The mitochondrial theory of ageing

Mitochondria produce the majority of ROS under normal cellular conditions (Chance et al. 1979), since it is in these organelles that aerobic respiration takes place and this process that is inherently 'leaky' (Cadenas and Davies 2000) in that 1-2% of oxygen molecules consumed are converted into superoxide anions (Kamata and Hirata 1999). It therefore follows that the cellular components most likely to suffer the most damage from free radicals will be the mitochondria themselves. Furthermore, many of the ROS scavenging enzymes, such as MnSOD, are encoded by mitochondrial DNA (mtDNA), as are 13 polypeptides of the electron transport system (Wanagat et al. 2001). The mitochondrial theory of ageing states that free radical damage to mitochondria is the cause of senescence (Wanagat et al. 2001). mtDNA is not protected by histones and as it is damaged by ROS, the production of the defence molecules it encodes will in turn be reduced, resulting in a positive feedback loop. The result would be cellular senescence through reduced ATP production and through protein and lipid damage by the resulting ROS (Miquel et al. 1980) or programmed cell death from apoptosis (Liu *et al.* 1996). Accumulation of ROS damage to mitochondria is thought to be responsible for many age-related pathologies, such as arteriosclerosis, cataracts and neoplasia (Wallace 1992; Finkel and Holbrook 2000).

The rate of oxidative damage of mtDNA is approximately ten times that of nuclear DNA (Ames et al. 1993) and an accumulation of mutations in mtDNA with age has been found in humans (Cortopassi and Arnheim 1990) and in *C. elegans* (Melov et al. 1995). Recent data from rodents lends some support to the mitochondrial theory; transgenic mice that over-express the human form of catalase (a ROS scavenging enzyme) in mitochondria are long-lived (Schriner et al. 2005). However, these results should perhaps be interpreted with caution since the authors report that, nine generations after establishing the transgenic line 'the genetic background was >99% B6 and the mice had been moved to a new facility, and the lifespan extension

phenotype appears to be diminished' (Schriner et al. 2005). Furthermore, ectopic expression of catalase in *Drosophila* mitochondria did not increase longevity (Mockett et al. 2003).

1.3.8 The green theory of ageing (the rubbish theory)

That there were over 300 theories of ageing at the last count in 1990 (Medvedev 1990) does not stop gerontologists adding to the ever-growing list. One such addition is the 'Green Theory', or as it was originally coined by its creators, the 'rubbish theory of ageing' (D. Gems, personal communication). Both hypomorphic mutant alleles of *daf-2*, the insulin/ insulin-like growth factor receptor and *dauer* larvae (a spore-like, diapausal developmental stage) are long-lived in *C. elegans* (Kenyon et al. 1993). By comparative analysis of genome-wide transcription levels between these two long-lived forms of the worm, David Gems and co-workers identified classes of genes that were up-regulated in both (McElwee et al. 2004). One striking finding in both *daf-2* mutants and *dauer* larvae was the up-regulation of genes associated with drug detoxification, such as cytochrome P450's, short-chain dehydrogenase/ reductases and UDP-glutathione S-transferases. The authors then proposed a new proximate theory of ageing; senescence is caused by the build-up of toxic compounds that lead to macromolecular damage. This was dubbed 'The Green Theory' (Gems and McElwee 2005) because the struggle of the organism to get rid of the toxic waste it itself produces is supposedly similar to the problems faced by society with recycling and waste disposable. If this theory is correct, long-lived mutant strains will be resistant to xenobiotic compounds, but this remains to be determined.

1.4 Model organisms used for work on ageing

To investigate the mechanisms underlying human ageing it is necessary to develop model organisms for use in the laboratory, enabling comparative studies of interventions that extend lifespan (Liang et al. 2003). The more related an organism is to humans the more likely it will share common mechanisms but also the more complicated and expensive the system is as a research tool. In ageing research, there are four major model organisms in which the majority of work has been carried out (Austad 2003), ranging from the single-celled budding yeast to rodents, which are multi-cellular and highly complex in comparison. Despite the large difference in

longevity between these organisms, their survival curves share a similar 'rectangular' shape similar to that of human populations (Sinclair et al. 1998a). Each have their own benefits and drawbacks, and in general the procession of science is likely to be advanced more rapidly by a cross-organism, comparative approach, in which consortiums of labs working on different species share information to make best use of each model species[9]. In the next sections I will briefly describe three of the model organisms used before discussing in detail the fruit fly *Drosophila melanogaster,* the species used for all the experimental studies presented in this thesis.

1.4.1 The budding yeast Saccharomyces cerevisiae

Budding yeast are the simplest of the eukaryotes in that they are single-celled and as such do not have complex tissue or organ structures. They are cheap to culture in the laboratory, easy to maintain and the yeast genome was one of the first to be fully sequenced (Goffeau et al. 1996). Furthermore, due to the use of budding yeast for research on biochemistry and metabolism, there are many mutant strains available. *S. cerevisiae* has also lent itself well to work on ageing (Sinclair et al. 1998a; Sinclair et al. 1998b; Sinclair 1999) and there are two distinct methods of determining the lifespan of yeast, measurements of 'chronological lifespan' and those of 'replicative lifespan'.

If *S. cerevisiae* are deprived of nutrients they cease dividing and enter an essentially dormant or stationary phase during which growth slows, they cease to ferment (since sugar is no longer available) and instead begin respiratory metabolism (Werner-Washburne et al. 1993). When transferred back to nutrient-rich medium plates these cells will resume growth once more. However, there is a point at which cells starved of nutrients fail to grow colonies when re-fed, and it is at this point in the 'chronological lifespan' assay that they are scored as dead (Fabrizio and Longo 2003). Aliquots of stationary phase yeast cells are transferred from water to nutrient-rich plates at fixed time points and the percentage of cells that show the capacity for growth (e.g. are alive) is then plotted against time to give a population survival curve. Yeast cells maintained in the stationary phase due to lack of glucose are

[9] One such consortium is 'AgeBase' (http://haldane.biol.ucl.ac.uk)

thermotolerant and are actively maintaining themselves, since if the ROS scavenger SOD is removed they die (Sinclair et al. 1998a; Fabrizio and Longo 2003).

As their name suggests, budding yeast reproduce by segregating 'daughter' cells, and the number of budding events any one cell can go through is limited (Mortimer and Johnston 1959); individual cells have a finite replicative lifespan. The mother cell is easily distinguished from the daughters (Sinclair et al. 1998a) and can thus be removed by micromanipulation. This makes it possible to monitor the exact number of cell divisions a particular yeast cell goes through and this number is termed its 'replicative lifespan'. Cell size increases with successive rounds of cell division and scars build-up on the surface of the mother cell, which can be used as markers of the number of divisions the cell has undergone. As mother cells divide, there is a build-up of extra-chromosomal ribosomal DNA circles (ERCs) within the cytoplasm and it is this that is thought to be the cause of their limited replicative ability (Sinclair et al. 1997).

Although not all strains that show extension of lifespan by one assay also have extension of lifespan using the alternative measure, replicative and chronological lifespan do appear to be at least partly linked (Fabrizio and Longo 2003). Cells that have been through passages of the hypometabolic stationary phase show progressively decreased replicative lifespan (Ashrafi et al. 1999). It may be that replicative ageing in yeast is representative of dividing cells in mammals such as germ cells, whilst chronological lifespan is a better model of ageing in post-mitotic tissues (Kaeberlein et al. 2004). A new approach has recently been suggested that measures both the time taken for a cell to become post-reproductive and also to become metabolically inactive (Minois et al. 2005). This approach may therefore provide a quantitative analysis of life history in yeast that is more representative of those used in other model organisms.

1.4.2 The nematode worm Caenorhabditis elegans

C. elegans are small (approximately 1.2mm), transparent, soil-dwelling nematode worms (Vanfleteren and Braeckman 1999). They are easy to maintain in the lab on agar plates streaked with slow growing *E. coli* bacterial strains on which the worms feed (Brenner 1974). They exist as either self-fertilising hermaphrodites or males,

allowing easy construction of clonal mutant populations that suffer no detrimental effects of inbreeding. Furthermore, strains can be maintained at -80°C and survive on thawing. There are many freely available mutant strains of *C. elegans* and the full genomic sequence has been published (*C. elegans* sequencing consortium 1998). One major strength of the worm as a model organism is the ability to down-regulate gene expression by feeding them bacteria that express double stranded RNA copies of the gene of interest (Carthew 2001). Bacterial libraries of strains that allow mRNA inhibition (RNAi) of all known *C. elegans* coding sequences are available, and can be used for genome-wide screens for phenotypes of interest (Lee et al. 2003; Hansen et al. 2005). *C. elegans* only live for two weeks on average in the laboratory and therefore provide a very convenient model organism for studies on ageing (Vanfleteren and Braeckman 1999; Finch and Ruvkun 2001). Indeed, it was in the worm that the first single gene mutations that extended lifespan were discovered (Tatar et al. 2003), providing impetus for much of the current work in the field of gerontology.

1.4.3 The mouse and rat

Compared to invertebrate models discussed in this section, rodents are expensive to maintain, difficult to handle and require more space to house. Furthermore, since they have lifespans of between 3-5 years (Weindruch and Walford 1988), experiments on rodents take longer to perform than those on invertebrates and, in general, consist of much smaller sample sizes. This makes risky, spontaneous experiments performed on the whim of a researcher unfeasible and therefore rodents are not a good model system for testing new ideas. However, to learn about mammalian ageing it is likely that the ultimate experiments have to be performed in mammals and thus the use of rodents in the study of senescence is fundamental (Miller 2001). The genome sequence of both the mouse (Mouse sequencing consortium 2002) and the rat (Rat sequencing consortium 2004) have now been published, facilitating comparative sequence analysis between both the model organisms discussed in this section and humans.

1.4.4 The fruit fly Drosophila melanogaster

1.4.4.1 The use of *Drosophila* for ageing research

Drosophila melanogaster is a species of fruit fly that is found in both tropical and temperate regions across the globe. The flies are easy to maintain and culture, have high fecundity, and a short life cycle (see section 1.4.4.2) and lifespan (45-60 days) at 25 degrees (Ashburner 1989). Unlike in *C. elegans,* there is no consensus amongst the *Drosophila* research community for one 'wild type' stock and in general, this term is used to describe any strain in which no mutant alleles are described. Wild type stocks are best maintained in large population cages in the laboratory to maintain lifespan and fecundity at levels similar to that of recently caught wild populations (Sgrò and Partridge 2001). *Drosophila* stocks are often contaminated with the internal parasite *Wolbachia* (Clark et al. 2005), and this should be kept in mind when out-crossing stocks because *Wolbachia* are maternally inherited and infection may have an effect on mutant phenotype or lifespan (Fry and Rand 2002).

The use of *Drosophila* as a model organism was pioneered by T. H. Morgan, who chose them in particular because they possess enlarged polytene chromosomes in the salivary glands that can be visualised easily under the light microscope. Many of the major principles of genetics such as the chromosome theory of heredity, gene mapping, multiple allelism and non-disjunction were established using *D. melanogaster* (Ashburner 1989). Even as early as the beginning of the 20th century, *D. melanogaster* were used to study longevity, in particular for examining the effects of temperature on lifespan (Loeb and Northrop 1916; Loeb and Northrop 1917). These studies were followed up by comprehensive work by Pearl in the 1920's, and the use of fruit flies in the laboratory is covered in much detail in his book on the 'rate of living' hypothesis (section 1.3.5) (Pearl 1928). Since these early studies, further work using the fruit fly has done much to progress the field of ageing research (Rose 1999; Stearns and Partridge 2001; Helfand and Rogina 2003b; Helfand and Rogina 2003a). This work is facilitated by the fact that many mutant strains are freely available, there are many powerful genetic tools available to the *Drosophila* researcher and more recently, that the full genomic sequence has been published (*Drosophila melanogaster* sequencing consortium 2000).

1.4.4.2 *Drosophila* life history

There are over 3000 different species of *Drosophila* but the one most commonly used in biological research is *D. melanogaster.* For a comprehensive summary of *Drosophila* life history see (Ashburner 1989). The main details will be described here. At 25 degrees centigrade, the lifecycle from egg to egg is ten days. Fertile eggs are laid immediately after mating. These then hatch after approximately 22 hours at which point the emerging larvae begin feeding. *Drosophila melanogaster* larvae progress through three instars, L1, L2 and L3 that last for 24 h, 24 h and 48 h respectively. L1 larvae feed on the surface of the food medium, whilst L2 and L3 burrow into the food. Feeding lasts for approximately 110 hours and is followed by what is known as the 'wandering stage', where larvae leave the food medium and search for a suitable place for pupation. In laboratory cultures, this is typically towards the top of the vial or bottle in which they are raised. Developing *D. melanogaster* pupate for 4-4.5 days during which they undergo metamorphosis, before eclosing as the imago. Newly emerged flies have un-extended wings and are pale in appearance. Wings expand after approximately one hour and pigmentation occurs in the following two. Female *D. melanogaster* do not mate in the first 6-8 hours following eclosion. Unmated females do lay eggs, but in a pattern markedly different from mated females, who enter a rapid burst of egg laying following mating that peaks at 4-5 days post-copulation.

1.4.4.3 Nomenclature

Historically, gene names in *Drosophila* are often descriptive of the mutant phenotype, such as *Curly* (curly winged), *Tubby* (adults and pupae shorter and thicker than wild type) and *white* (white eyes). Recently, if newly characterised coding sequences are orthologues of genes already described in a different organism, the protocol is to add a 'd' to the original gene name, for example *dFOXO* is the *Drosophila* orthologue of the human *FOXO* transcription factor coding sequence. Gene names are always italicised, and capitalised if the phenotype is dominant but not if it is recessive to the wild type. The wild type allele is characterised by the addition of a '+' to the mutant name, e.g. *w* and w^{+}. Different mutant alleles are distinguished by the addition of superscript letters or numbers, such as $chico^{1}$ and $chico^{2}$. Protein products are described using the same name as the gene that encodes

them, and distinguished by the fact that they are not italicised and are in capital letters.

1.4.5 Potential caveats with using model organisms

As discussed above, the use of model organisms has many advantages when studying ageing and mechanisms that promote longevity (Austad 2003). Not least, their short lifespans allow the direct quantification of the effect of an intervention on population mortality rate and lifespan, something that is not practical when studying humans. However, many organisms used, in particularly the invertebrates, are phylogenetically distant from humans. It is crucial therefore to use comparative studies to determine if a mechanism that affects lifespan is unique to the species in which it was first studied, or if it is evolutionarily conserved across taxa and thus a 'public' mechanism (Martin et al. 1996; Partridge and Gems 2002). When trying to assess which mechanisms may show conserved function in humans the use of phylogenetic trees can be informative (Hedges 2002). There is ongoing controversy about whether insects are genetically closer to humans (Blair et al. 2002) or nematodes (Aguinaldo et al. 1997). However, if a mechanism seen in *Drosophila* is also shared by yeast, it becomes much more likely it will be ancient in origin rather than specific to arthropods and therefore it may also be present in humans. This idea is fallible as it may be that, faced with similar stressors, similar responses could evolve convergently on different branches of the tree. However, independent evolution of the same phenotype on multiple branches of a phylogenetic tree is less likely than one ancient ancestral emergence.

1.5 Dietary restriction - a public mechanism of lifespan extension?

Dietary restriction (DR), sometimes described as 'under-nutrition without malnutrition' (Weindruch and Walford 1988), is one of the few identified public (Martin et al. 1996) mechanisms of lifespan extension and since McCay's pioneering experiments in rats 70 years ago (McCay et al. 1935), DR has been shown to increase lifespan in a range of species. These include commonly used model organisms such as the budding yeast *Saccharomyces cerevisiae* (Jiang et al. 2000; Lin et al. 2002), the nematode worm *C. elegans* (Klass 1977; Houthoofd et al. 2003), the fruit fly *Drosophila melanogaster* (Chippindale et al. 1993; Chapman and Partridge 1996) and rodents (McCay et al. 1935; Weindruch and Walford 1988;

Masoro 2002) along with many species less often used for laboratory research such as spiders (Austad 1989), water striders (Kaitala 1991), water fleas, rotifers, fish, hamsters (Weindruch and Walford 1988; Weindruch 1996; Gerhard 2001; Masoro 2002) and dogs (Kealy et al. 2002).

Three studies on the effects of DR on the lifespan of rhesus monkeys (Ingram et al. 1990; Hansen and Bodkin 1993; Kemnitz et al. 1993) and one on squirrel monkeys (Ingram et al. 1990) are still ongoing. Although too early to detect if DR extends lifespan in these long-term studies, early signs are promising as biomarkers of age seem to be delayed in the DR cohorts (Roth et al. 1999; Lane et al. 2000). One report has described an increase of 7 years in the median lifespan of rhesus monkeys under DR (Bodkin et al. 2003), but this result should be treated as preliminary; out of the 117 monkeys in the study, only 8 were on a DR regime and of those only three had died, making the sample size too small to be conclusive (Lane et al. 2004). It has also been suggested that DR results in health benefits to humans (Walford et al. 1992; Fontana et al. 2004).

However, despite the finding that some form of food restriction increases longevity in such a diversity of species, the mechanisms responsible remain to be fully elucidated in any of them. It is therefore as yet unclear whether these mechanisms are evolutionarily conserved across taxa or if instead life-extension during DR is an example of convergent evolution (Partridge and Brand 2005).

1.6 DR in rodents

Although McCay's early work was on the effect of nutrition on trout (McCay et al. 1929)[10], the study usually regarded as the seminal DR longevity experiment was his 1935 paper on rats (McCay et al. 1935) and historically much of the work on dietary restriction has been carried out on mammals (Masoro 2001). The body of literature is too large to be comprehensively covered here and for two extensive reviews see (Weindruch and Walford 1988; Masoro 2002). In the next few sections I will cover the main findings to date of the effects of DR on the lifespan and physiology of rodents.

[10] Cited from Masoro (2000)

1.6.1 Methods for applying DR in rodents

The protocols used to apply DR vary greatly for different model organisms and, in some cases, for the same species between different laboratories. In mammals, DR is generally applied by the reduction in the quantity of lab chow given to animals. This can be achieved through restricting the DR cohort to a set percentage reduction in food quantity compared to that of the *ad libitum* fed group (Yu et al. 1985), to a quantity that results in a fixed weight reduction relative to controls (Merry and Holehan 1985), or by alternate day feeding (Goodrick et al. 1982). In each of these studies using different methods of DR, both median and maximum lifespan was extended in the restricted animals. The feeding regime for the control animals also varies between labs, with some allowing completely *ad libitum* feeding (Masoro et al. 1982), whilst others restrict the controls to 75% of *ad libitum* levels, and then the DR group to a 40% reduction compared to this control group (Weindruch et al. 1986). This method was used to address concerns that it was in fact the life-shortening pathological effect of over-feeding in *ad lib.* fed groups that was responsible for the apparent extension of life under DR (Cherkin 1979). However, rather than a DR regime returning overfed lab mice to a food intake that is more representative of what they would eat in nature, wild populations actually consume more food than *ad libitum* fed laboratory strains (Austad and Kristan 2003). Laboratory strains have undergone selection for increased food intake however, as they consume more than wild mice brought into the laboratory (Austad and Kristan 2003). Along with reducing the amount of food consumed, DR also alters the feeding behaviour of rats, with DR animals changing to a 'meal eating' pattern from a 'nibbling' pattern (Masoro 2001). This change in eating habit is not responsible for the longevity of DR animals (Masoro et al. 1995; Masoro 2004)

1.6.2 DR slows ageing by retardation of growth/ reducing obesity?

McCay's original experiments were intended to heavily restrict growth, and the animals caloric intake was so limited that eventually they began to fail, at which point food intake was raised. Thus, the rats were maintained for long periods with no growth before calorie intake was increased in bursts, allowing rapid periods of growth before a new plateau in weight (McCay et al. 1935). The resultant animals, though indeed long-lived, were heavily stunted and had very delayed puberty. It has since been shown that the life-prolonging effect of DR in rodents was not due to this

restricted growth however. DR initiated in young adults rather than immediately post weaning extended the lifespan of rats (Yu et al. 1985). Moreover, in mice, applying a DR regime to one year old individuals that were effectively middle-aged also increased both median and maximum lifespan (Weindruch and Walford 1982). In support of this evidence that the effects of DR are not simply the result of reduced growth, invertebrate species such as *Drosophila* that are post-mitotic as adults also show extended lifespan when dietarily restricted only during adulthood (section 1.7.3). Although DR rodents are both smaller and leaner than *ad libitum* fed controls it is not the obesity of *ad libitum* fed animals that causes the difference in lifespan; ob/ob mice that are genetically obese still live longer under a DR regime despite DR individuals weighing more than shorter lived, *ad libitum* fed wild type mice (Harrison et al. 1984).

1.6.3 The effects of DR on metabolic rate

Although initially it was thought that DR extended lifespan by reducing metabolism (Sacher 1977), it is now generally accepted that the metabolic rate of DR rodents per unit mass of *metabolically active tissue* is not decreased compared to controls (Masoro et al. 1982), despite ongoing controversy over the best method to measure it (Greenberg and Boozer 2000). Recent reports using the doubly-labelled water technique (Speakman 1998) suggest that energy expenditure may even be increased by DR (Selman et al. 2005).

1.6.4 Effects of DR on murine physiology

DR maintains rodents in a youthful state for longer than controls, and most physiological processes that decline with age deteriorate at later ages in restricted animals. There is a multitude of reports on aspects of murine physiology that show this pattern, and below is a selection of the key findings:

- DR delays the age-related decline in DNA repair activity (Licastro et al. 1988; Weraarchakul et al. 1989).
- DR increases enzymatic activity relative to controls, for detailed summary see Table 4.2 in (Weindruch and Walford 1988).

- DR attenuates much of the deterioration of immune function that occurs with age (Miller 1995; Pahlavani 2000; Pahlavani 2004).
- DR delays neuronal death in the gut (Cowen et al. 2000) and brain (Hiona and Leeuwenburgh 2004).
- DR attenuates the decline in turnover of damaged proteins with age (Ward 1988).
- DR delays the age-associated decline in the mitogen activated protein kinase (MAPK) pathway (Zhen et al. 1999), whose function promotes stress resistance.
- DR increases apoptosis in liver (Muskhelishvili et al. 1995), small intestine and colon (Holt et al. 1998) cells, a process which is thought to facilitate the removal of damaged cells and thus promote longevity.
- DR delays the age-associated decrease in learning ability and motor function (Ingram et al. 1987; Duffy et al. 1997).

1.6.5 DR delays age-related pathology

The original study of DR in rats by McCay (1935) reported that, along with increasing lifespan, DR reduced the onset and severity of age-associated pathologies. Since those early studies, DR has been shown to have a beneficial effect on a wide range of diseases (for a review see chapter 5 of (Masoro 2002)). These include many forms of cancer (Yu et al. 1982; Hursting et al. 1994; Hursting and Kari 1999; Berrigan et al. 2002), kidney disease (Yu et al. 1982; Maeda et al. 1985), cardiovascular disease (Koletsky and Puterman 1976; Koletsky and Puterman 1977; Maeda et al. 1985), diabetes (Masoro 2001), neurodegenerative diseases (Mattson et al. 2001), auto-immune disease (Fernandes et al. 1976; Kubo et al. 1984), cataracts (Taylor et al. 1989; Taylor et al. 1995), stroke (Stevens et al. 1998) and osteoporosis (Sheldon et al. 1996)[11].

1.6.6 DR and oxidative stress

One much supported paradigm is that DR extends lifespan by reducing oxidative stress, either by decreasing the production of ROS, or increasing repair to damage (Sohal and Weindruch 1996). In keeping with this idea, DR delays the accumulation

[11] Cited from Masoro 2002

of oxidative modification to various macromolecules including protein carbonyls (Youngman et al. 1992; Sohal and Dubey 1994; Dubey et al. 1996; Forster et al. 2000; Goto et al. 2002; Pamplona et al. 2002), peroxidised lipids (Matsuo et al. 1993; Kim et al. 1996; Baek et al. 1999) and DNA damage (Chung et al. 1992; Youngman 1993). Possibly causally associated with the finding of reduced oxidative damage, production of ROS has been reported to be lower in mitochondria isolated from several different tissues from DR rodents than from controls (Sohal and Dubey 1994; Lass et al. 1998; Gredilla et al. 2001; Gredilla et al. 2002; Lambert and Merry 2004). Repair of ROS-damaged molecular species is also increased under DR; protein turnover is higher in DR rodents (Dhahbi et al. 2001; Goto et al. 2002) than controls, and this may function to increase lifespan (Ryazanov and Nefsky 2002; Tavernarakis and Driscoll 2002). Lens epithelial cells from DR individuals were more resistant to hydrogen peroxide *in vitro* than control cells, a result put down to increased anti-oxidant defenses in DR animals (Li et al. 1998). However, increased ROS defense systems in DR tissue was not observed in other studies (Lass et al. 1998; Leon et al. 2001; Judge et al. 2004) and the main cause of reduced oxidative damage in DR individuals is thought to be lowered ROS-production (Gredilla and Barja 2005). Whether this reduced ROS-production and ROS damage is the causal mechanism behind the lifespan extension seen in DR animals remains to be proven.

1.6.7 Genetics of DR in rodents

Down-regulating components of the IIS pathway increases murine lifespan (Bluher et al. 2003; Holzenberger et al. 2003), similar to in worms and fruit flies (Partridge and Gems 2002). However, any role of this pathway in mediating the response of lifespan to DR in mammals is not yet clear. There does appear to be some, but not total, overlap in the effects of DR, mutations in the growth hormone/ IGF-1 axis and insulin signalling (Bartke et al. 2002; Lambert et al. 2004; Tsuchiya et al. 2004; Al-Regaiey et al. 2005).

1.7 DR in invertebrates

In invertebrates, unlike in mammals, DR is generally applied by reducing the quality rather then the quantity of the nutrients given. In this section I will review DR in yeast and worms before concentrating on *Drosophila melanogaster,* which will then be the focus of the rest of this thesis.

1.7.1 DR in the budding yeast

In yeast, dietary restriction is achieved by reducing the glucose concentration of the medium from 2% to 0.5% (or below in some strains), and extends replicative lifespan (Jiang et al. 2000; Lin et al. 2000; Jiang et al. 2002; Lin et al. 2002; Anderson et al. 2003; Kaeberlein et al. 2004; Lin et al. 2004). Osmotic stress from high glucose concentrations also extends the replicative lifespan of *S. cerevisiae* (Kaeberlein et al. 2002). Glucose activates the cAMP-dependent protein kinase pathway (PKA) in yeast and mutations in genes that encode components of this pathway are long-lived and used as genetic mimetics of the DR response (Lin et al. 2000). Reduction of glucose concentration increases lifespan by increasing respiration (Lin et al. 2002). This shift from fermentation to respiration is necessary for the extended longevity under low glucose, since knocking out electron transport by deleting the gene encoding cytochrome c1 blocks the DR response (Lin et al. 2002). Deletion of a gene encoding a histone deacetylase, Rpd3 increases replicative lifespan (Kim et al. 1999; Jiang et al. 2002) and no further extension of lifespan occurs when these mutant yeast are subjected to DR, suggesting that Rpd3 may also be necessary for mediating the response of replicative lifespan to DR (Jiang et al. 2002).

SIR2, which encodes a NAD-dependent histone deacetylase (Imai et al. 2000), has also been implicated in mediating this extension of lifespan by DR. Over-expression of *SIR2* increases yeast replicative lifespan (Kaeberlein et al. 1999), whilst deleting it shortens life (Jiang et al. 2002). The increase in lifespan in response to DR was not observed in a *SIR2* null mutant (Lin et al. 2000). This prompted the suggestion that lifespan extension by dietary restriction functioned via the up-regulation of Sir2 activity (Guarente and Kenyon 2000; Guarente 2005; Sinclair 2005). However, more recent work has cast some doubt on this last finding, since several other strains of yeast, when made null for *SIR2*, show a normal increase in lifespan in response to lowering glucose concentration in the food medium (Jiang et al. 2002; Kaeberlein et al. 2004). Furthermore, the only yeast strain in which life-extension by glucose restriction is shown to require functional *SIR2* does not show increased longevity when *SIR2* is over-expressed (Kaeberlein et al. 2004). Conversely, in other long-lived yeast strains, over-expression of *SIR2* does extend lifespan but Sir2 is not

required for increased replicative lifespan by DR. (Kaeberlein et al. 2004). The *SIR2* independent lifespan extension by DR in some yeast strains may be mediated by other sirtuins such as *HST2* (Lamming et al. 2005), but these results have not been repeated in other laboratories (B. Kennedy, personal communication).

1.7.2 DR in C. elegans

In *C. elegans*, DR is applied by three main methods, which all increase lifespan but that generate some phenotypic differences (Walker et al. 2005). *C. elegans* are routinely supplied with *E. coli* in the laboratory as a food source, and reducing the thickness of the bacterial lawn on culture plates (Hosono et al. 1989) or diluting the concentration of liquid bacterial culture in which the worms are suspended (Klass 1977; Houthoofd et al. 2003) results in increased lifespan and reduced fecundity. Bacterial dilution does not alter metabolic rate (Houthoofd et al. 2002b) and is independent of the IIS pathway (Houthoofd et al. 2003). Another technique that extends the lifespan of nematodes by a mechanism supposedly analogous to DR is feeding worms an axenic (free from living organisms) medium (Vanfleteren et al. 1998). Worms cultured in such a way show delayed development, extreme longevity and low fecundity (Vanfleteren and Braeckman 1999). However, unlike bacterial dilution, culture in axenic medium increases metabolic rate (Houthoofd et al. 2002a) and the medium is actually very nutrient-rich (Walker et al. 2005).

The third protocol for achieving DR in the worm is the use of a class of long-lived mutants named 'Eat' mutants. These worms display reduced pharyngeal pumping rates and are therefore thought to be effectively under DR (Lakowski and Hekimi 1998). Work on Eat mutants in other labs has shown mixed results, some repeating the original life-extension seen (Hsu et al. 2003) while others failed to observe increased lifespan (Walker et al. 2005). Lifespan extension in Eat mutants is *daf-16* independent (Lakowski and Hekimi 1998). *daf-16* is the only *C. elegans* homologue of the FOXO family of forkhead transcription factors. FOXO factors are down-regulated by the IIS pathway, and *daf-16* is required for extension of lifespan by reduced IIS in *C. elegans* (Kenyon et al. 1993).

Pharyngeal pumping rates decrease with age in nematodes (Kenyon et al. 1993; Gardner et al. 2004) and for wild type *C. elegans* the majority of individuals stop

pumping altogether after approximately 9 days (Gems et al. 1998). If pumping rates truly are an indication of food intake, one would expect to see dramatic effects on mortality rates at the age when pumping stops. Potentially these effects may manifest initially by a decrease in mortality as the worms become dietarily restricted, followed by an increase as they enter starvation. However, no such shifts in mortality rates at the time of reduced pharyngeal pumping are seen in *C. elegans* populations (Brooks et al. 1994), hence perhaps pumping rate is not a true indication of food intake.

DR in *C. elegans* is clouded by issues over which protocol best represents actual restriction of food intake (Walker et al. 2005). Despite the fact that the three protocols described above all increase lifespan, it seems likely that they do so by different mechanisms, at least some of which may be specific to the worm. For instance, the tissues of adult worms grown in bacterial culture get increasingly invaded by bacterial cells with age (Garigan et al. 2002), and growing worms on bacteria that have been killed by UV or antibiotics extends lifespan (Gems and Riddle 2000; Garigan et al. 2002). Thus, at least part of the life-extension seen when worms are grown in diluted *E. coli* medium is from reduced toxic effects of bacterial invasion (Walker et al. 2005), which would represent a private rather than public mechanism. All three protocols are independent from the IIS pathway (Lakowski and Hekimi 1998; Houthoofd et al. 2003), but when using the worm to evaluate the role of other genetic pathways implicated in DR, the picture becomes more confused. As discussed previously, in yeast, *SIR2* extends lifespan when over-expressed and is reported to be required for life-extension by DR (Lin et al. 2000). Over-expression of the worm *SIR2* homologue, *Sir-2.1* also extends lifespan (Tissenbaum and Guarente 2001) but, unlike the effect of DR in worms, this is dependent on *daf-16.* Hence, it seems that the DR and *Sir-2.1* pathways are separate in this species. Mutations in the worm homologues of *Indy* (Fei et al. 2004) and *TOR* (Jia et al. 2004) extend lifespan, both of which have been implicated in controlling lifespan extension under DR in fruit flies (see section 1.7.3), but this interaction remains to be studied in *C. elegans.*

1.7.3 Dietary restriction in Drosophila

1.7.3.1 Background

In the laboratory, *Drosophila* are fed a food medium consisting of nutritional components dissolved or suspended in an agar gel (Ashburner 1989). The composition of the food medium varies between different laboratories, but the usual ingredients include sugar, autolysed yeast powder and corn flour/ corn meal. Unlike the protocol used for rodents, DR in *Drosophila* is applied by reducing the quality of the food given to flies in excess, either by altering the availability of the live yeast on the food surface (Partridge et al. 1987; Chippindale et al. 1993) or by the co-ordinate dilution of all the nutrients in food medium (Chapman and Partridge 1996). By either method, the median and maximum lifespan is extended. Since the food dilution method of DR results in flies being given constant access to amounts of food in excess of what they can consume, it has been suggested that they could compensate for altered nutrient content by altering their feeding behaviour to maintain their nutrient intake (Cooper et al. 2004). However, measurements of the time spent feeding on foods of different composition shows this is not the case (Partridge et al. 2005b; Marc Tatar, personal communication). Therefore, *Drosophila* do not compensate when fed a reduced nutrient concentration medium, and food dilution is a reasonable protocol for DR in this species.

1.7.3.2 Sex differences in the response to DR

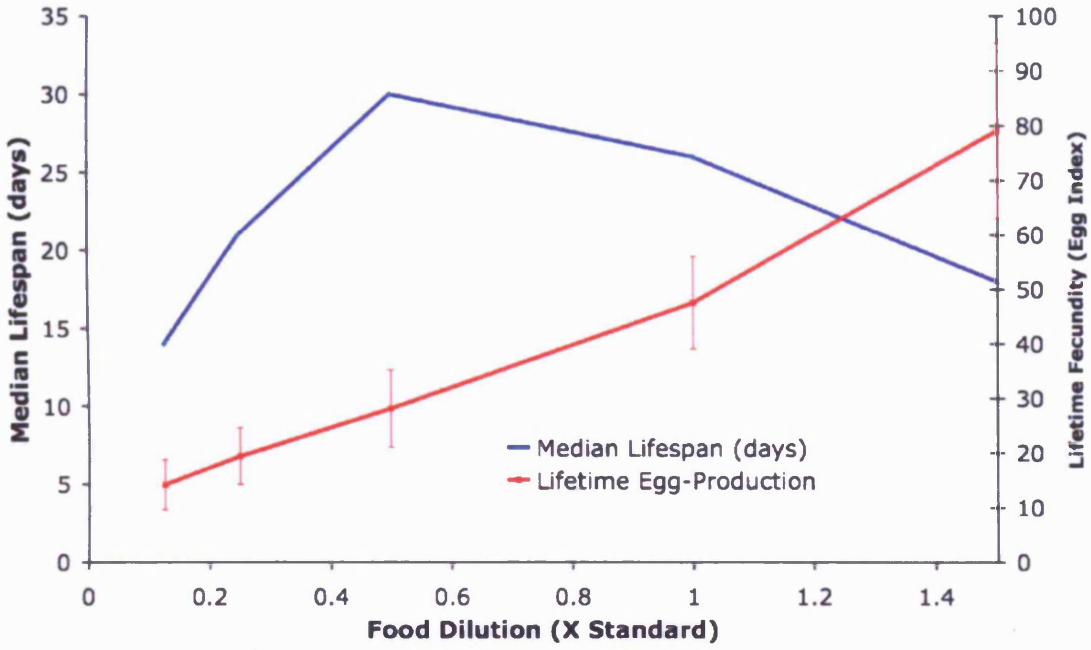
The lifespan of female *Drosophila* increases in response to DR much more than that of males (Magwere et al. 2004). As yet, it is not known why this difference in the response to DR in the two sexes exists. There may be qualitative differences in nutrient use by the two sexes, with females undertaking high levels of anabolism in the production of eggs and males using nutrients mainly for movement and this may have different effects on survival. One possible corollary of these differences is that, since males eat less than females (Richard Wong, unpublished data; Marc Tatar personal communication), the amplitude of the range between full-feeding and the onset of starvation is reduced in males. It is also interesting to note that there are often sex differences in the lifespan extension seen in longevity mutants, with males showing reduced/ no lifespan extension despite a robust response in females (Clancy et al. 2001; Tatar et al. 2001).

1.7.3.3 DR, reproduction and the toxic effects of over-feeding

As food is diluted from a concentrated medium lifespan is extended until it reaches a peak at an intermediate food level, at which point further dilution reduces lifespan, presumably via starvation (Figure 1.7.1). Female fecundity, in contrast, decreases with decreasing food concentration throughout the same nutritional range (Chapman and Partridge 1996) (Figure 1.7.1). The food concentration that maximises lifespan is therefore lower than the concentration that maximises daily and lifetime fecundity.

Figure 1.7.1. Relationship between lifespan (blue), fecundity (red) and food concentration.

Median lifespan of female *Drosophila* peaks at a food dilution less than that required for maximum lifetime egg laying (compare 0.5 SY with 1.5 SY). Figure adapted from (Chapman and Partridge 1996).



It has been suggested that DR increases lifespan by rescuing the toxic and pathological effects of over-feeding (Cherkin 1979; Longo and Finch 2003; Prentice 2005), but the fact that lifespan and reproduction are optimised at separate nutritional levels suggests this is not the case. If increased nutrient intake resulted in progressive poisoning of an organism, one would predict that both lifespan *and* fecundity would decline as food intake increased. For example, in *Drosophila* exposed to increasing doses of X-irradiation, both lifespan and fecundity decline systematically, hence

reproductive capacity is a marker for general frailty in these irradiated flies (Vaiserman et al. 2004). The decrease in female fecundity in response to DR in fruit flies provides further evidence that the intake of nutrients is reduced by this protocol, since reduced egg-production would not be predicted if flies maintained constant nutrient intake levels on different food types by compensatory feeding.

1.7.3.4 The importance of testing a range of food concentrations

The responses of lifespan and fecundity to nutrition in *Drosophila* are affected by long-term laboratory culture on different food concentrations; female flies showed higher adult survival and greater fecundity on the food medium on which they had evolved (Chapman et al. 1994). This must be kept in mind when comparing the effect of DR on flies from different stock centres and laboratories. Different *Drosophila* labs routinely use food media of widely varying composition and mutations in single genes show lifespan peaks at different food concentrations (see section 1.7.3.7). It is therefore crucial to explore a range of nutritional levels when characterising the response of any new strain to DR and to ensure the genetic background is the same when comparing any mutants. Failure to adopt this procedure may explain cases where no response of lifespan to DR has been seen e.g. (Le Bourg and Minois 1996).

1.7.3.5 DR and metabolic rate

In *Drosophila*, measurements of both oxygen-consumption and heat-production in DR and control flies showed no significant effect of DR on mass-specific metabolic rate (Hulbert et al. 2004), as is the case in rodents (section 1.6.3). It is therefore unlikely that a slowed rate of living can account for the increased lifespan associated with DR in this species. This is in contrast to the effect of increased ambient temperature, which decreases the lifespan of *Drosophila* (Miquel et al. 1976) and increases metabolic rate (Berrigan and Partridge 1997), presumably by an increased rate of living.

1.7.3.6 DR and oxidative stress in *Drosophila*

If DR extends lifespan by reducing the levels of oxidative stress, then both the levels of ROS-production and oxidative damage to macromolecules should be reduced by DR. Currently there is little empirical data that tests this theory in fruit flies and

results are still inconclusive. Measurements of ROS-production, measured fluorometrically as hydrogen peroxide in mitochondria isolated from *Drosophila* of different ages (Miwa et al. 2003), did not show any significant effect of DR (Miwa et al. 2004). This finding suggests that the amount of ROS produced by mitochondria may not account for the increased level of protein modifications seen in fully-fed flies compared to DR flies (J. Jacobson, unpublished). Oxidative damage to lipids increases with age in *Drosophila,* and this increase is delayed by dietary restriction (Zheng et al. 2005). As yet, these findings are only correlative and are not sufficient to demonstrate that reduced oxidative damage is causal to life-extension under DR.

1.7.3.7 The genetics of DR in *Drosophila*

To date, five genetic mechanisms have been implicated in mediating the increase in lifespan in response to DR in *Drosophila*: the cotransporter *Indy*, the insulin/ IGF-like signalling (IIS) pathway, the *Rpd3* deacetylase, the *dSir2* (silent information regulator) protein deacetylase and the TOR (target of rapamycin) signalling pathway. I will now briefly discuss each of these in turn.

Both male and female *Drosophila* that are heterozygous for mutations in *Indy* (I'm not dead yet) show a doubling in median lifespan compared to controls (Rogina et al. 2000). cDNA and genomic sequence homology analysis of *Indy* revealed 50% similarity to mammalian renal sodium dicarboxylate cotransporters, which act to take Krebs cycle intermediates into cells. When crossed into long-lived flies from selection experiments (Luckinbill et al. 1984), the life-extension in *Indy* mutants reduced to only 15%. The mutations in *Indy* were the product of insertions of genetically modified, mobile, P elements, and these showed a pattern of expression that included the midgut and the fat body, which is the fly functional equivalent of mammalian liver and white adipose tissue. On standard laboratory medium, long-lived *Indy* flies were also more fecund than controls and their activity levels were normal, thus there seems to be no trade-off between reproduction and lifespan in these flies as evolutionary theory would predict (Marden et al. 2003).

However, this 'cost-free' lifespan extension is conditionally dependent, since *Indy* flies do show lowered fecundity compared to controls on a low nutrient concentration diet (Marden et al. 2003). It is worth noting that in these experiments

the lifespan, activity and metabolic rates were measured in male flies, whilst the fecundity experiments were performed necessarily with females. To make direct comparisons about trade-offs between these life history traits, all physiological tests should be carried out on the same sex. Interestingly, *Indy* mutations extend lifespan by lowering the rate at which mortality increases with age (Marden et al. 2003), while DR results in a reduction in the mortality rate at all ages (Pletcher et al. 2002), therefore perhaps these two interventions are not completely interchangeable. The precise role of *Indy* in mediating the response of lifespan and fecundity to DR awaits further investigation.

In *Drosophila*, the IIS pathway regulates growth and co-ordinates it with nutrient supply during the pre-adult period (Leevers et al. 1996; Bohni et al. 1999; Britton et al. 2002). Reducing flux through the IIS pathway by mutating components within it extends lifespan in *C. elegans* (Klass 1983; Johnson 1987; Friedman and Johnson 1988; Kenyon et al. 1993; Morris et al. 1996; Kimura et al. 1997; Lin et al. 1997; Ogg et al. 1997; Hertweck et al. 2004), *Drosophila* (Clancy et al. 2001; Tatar et al. 2001; Giannakou et al. 2004; Hwangbo et al. 2004) and mice (Bluher et al. 2003; Holzenberger et al. 2003). In *Drosophila* females, null mutations in *chico*, the *Drosophila* orthologue of the mammalian insulin receptor substrate, extends lifespan by 48% (Clancy et al. 2001). When homozygous *chico* females were subjected to DR by the food dilution method, their lifespan peaked at a higher food concentration than did that of control females; at and below the food concentration that maximised the lifespan of control females, *chico* females were short lived (Clancy et al. 2002) (Figure 1.7.2).

Clancy et al. (2002) suggest that *chico* females are therefore partially dietarily restricted by their genotype and that the IIS pathway contributes to mediating the response of lifespan to reduced intake of nutrients. Similar studies in the worm suggest that IIS and DR act in separate pathways (Houthoofd et al. 2003), hence further work on the interaction between IIS and DR in flies that are not dwarf would be illuminating. An alternative explanation of the *chico*/ DR result would be that that *chico* females eat less than controls.

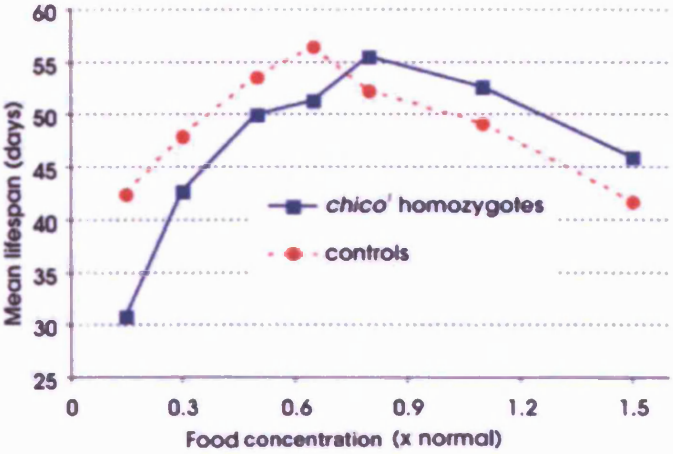


Figure 1.7.2. Mean lifespan of female wild type and *chico* *Drosophila* across a range of food concentrations.
Lifespan of *chico* flies is optimised at a higher food concentration than wild type. Figure taken from Clancy et al. (2002).

Deletion of the histone deacetylase *RPD3* in yeast extends lifespan (Kim et al. 1999) and this has also been reported in *Drosophila*. Heterozygosity for mutations in the fly orthologue of *RPD3* extends lifespan of both females and males (Rogina et al. 2002). When these mutants were put on a DR regime there was no further increase in lifespan. Furthermore, the long-lived mutant flies had lifespans similar to controls subjected to DR, suggesting that *Rpd3* and DR may extend lifespan through similar mechanisms (Rogina et al. 2002). Only two food concentrations were used in these studies of the effects of DR, and it would be informative to measure the response of these flies and controls to a wide range of food concentrations to be sure that the response of lifespan to DR is fully blocked in *Rpd3* mutant flies (section 1.7.3.4). Moreover, if *Rpd3* mutants are already dietarily restricted by their genotype, one prediction would be that feeding them a lower nutrient concentration medium would reduce lifespan due to starvation, rather than have no effect.

Sir2 is a NAD-dependent histone deacetylase required for lifespan extension via reduced glucose intake in some strains of budding yeast (Lin et al. 2000). In *Drosophila*, the closest orthologue of yeast *SIR2*, *dSir2*, has been implicated in controlling the response of lifespan to DR. Increasing *dSir2* expression extends lifespan in both male and female *Drosophila*, while flies that were trans-heterozygous for *dSir2* null mutations did not show extended lifespan under DR (Rogina and Helfand 2004). Characterisation of the lifespans of flies double mutant for both *dSir2* and *Rpd3* suggests that these genes act in the same pathway (Rogina and Helfand 2004). The mammalian orthologue of Sir2 deacetylates and regulates

the activity of FOXO family members (Brunet et al. 2004; Motta et al. 2004), and it may be this activity of Sir2 that is key to its effect upon lifespan (Giannakou and Partridge 2004). *Drosophila* that are null for *dSir2* have normal fecundity and normal or slightly shortened lifespan (Newman et al. 2002; Astrom et al. 2003). RNA expression levels of *dSir2* are increased both by DR (Pletcher et al. 2002; Rogina et al. 2002) and by reduced expression of *Rpd3* (Rogina et al. 2002). However, as for the effects of *Rpd3* mutations, only two food concentrations were used in the experiments. To determine if *dSir2* mutants fully block the response to DR in fruit flies it will be necessary to test a wider range of food concentrations.

The TOR (Target of Rapamycin) signalling pathway has been implicated in the sensing of amino acids (Thomas and Hall 1997; Schmelzle and Hall 2000). In *Drosophila*, the TOR pathway interacts with the IIS pathway in controlling growth and matching it to nutrient supply (Ito and Rubin 1999; Gao and Pan 2001; Potter et al. 2001; Marygold and Leevers 2002; Colombani et al. 2003). In *C. elegans*, reduced activity of the TOR kinase extends lifespan (Vellai et al. 2003). In *Drosophila*, flux of the TOR pathway can be reduced by over-expression of *dTsc1* or *dTsc2* or dominant negative forms of *dTOR* or *dS6K*. In each case, lifespan is increased (Kapahi et al. 2004b). Although it was first reported that down-regulation of the TOR pathway in the fat body was sufficient to extend lifespan, this has since been retracted (Kapahi et al. 2004a).

Over-expressing *dTsc2* increases lifespan to the greatest extent when flies are fed a high yeast concentration food medium, and when no yeast is given reducing TOR signalling decreases lifespan (Kapahi et al. 2004b). As for null mutation of *chico*, this altered pattern of response of lifespan to a range of food concentrations suggests that the TOR pathway may mediate the response of lifespan to DR in *Drosophila* (Kapahi et al. 2004b). However, until there are detailed data on the effect of altered TOR signalling on behaviour it is impossible to determine if this is the case or if TOR changes feeding behaviour. During development, the IIS pathway ligands are responsive to nutrients and act non-cell autonomously to regulate growth and match it to nutrient supply (Brogiolo et al. 2001; Ikeya et al. 2002). The TOR pathway appears to sense nutrients cell autonomously in the fat body of the fly and then act non-cell autonomously to regulate growth in other tissues, possibly by enhancing the

stability of the insulin-like ligands via interaction with the acid-labile subunit (Colombani et al. 2003). A recent report has also suggested that TOR signalling may directly affect the activity of FOXO via interactions with the PH domain protein MELTED (Teleman et al. 2005). Further work is required to determine the exact mode of action of these pathways in regulating the response of lifespan and fecundity to DR in the adult and to determine if other pathways are also important.

One approach to understanding how the response to DR is regulated by global transcription levels is to examine changes in gene expression using micro-array genechip technology. One such study examined RNA transcript levels at different stages throughout life in DR and control female *D. melanogaster* (Pletcher et al. 2002). Approximately one quarter of genes showed changes in expression with age. In lifespan studies, it is possible to describe a population's *chronological* age, the time in days since birth/ eclosion or its *physiological* age, the stage it is at on the Y-axis of a survivorship curve. In the comparisons of control to DR flies, the expression profiles were more similar between flies of the same physiological age rather than chronological age. This finding strongly implies that DR delays the changes in RNA transcript profiles that are characteristic of normal ageing (Pletcher et al. 2002). Analysis of the genes that showed altered expression as a result of DR revealed that the function of those that were down-regulated included DNA repair and replication, cell cycle control, chromosome condensation, chromosome segregation and other cell cycle processes, many developmental processes including nearly all aspects of oogenesis, protein metabolism and ubiquitin-dependent protein degradation. Many of these changes may reflect the reduced reproductive rate of the DR females. Flies are post-mitotic as adults apart from the reproductive system and some cells in the gut, and DNA replication, cell cycle and oogenesis are therefore likely to have occurred mainly in the ovary. Any functional implications of the decrease in protein metabolism and ubiquitin-dependent protein degradation for the increase in survival associated with DR await investigation.

1.8 Chemical DR mimetics

It is of great interest to the pharmaceutical industry to develop drugs whose function mimics that of dietary restriction. In fact, the small print of many academic papers on DR reveals links to biotechnology companies and associated 'competing financial

interests'. The main method of developing these drugs is to identify genes that are thought to be 'master regulators' of the response of lifespan to DR and then find compounds that affect the expression of these genes accordingly. One group of genes thought to be key to the life-prolonging and health improving effects of DR are the sirtuins (Guarente 2005; Sinclair 2005). STACs (sirtuin activating compounds) are chemicals found in red wine that activate Sir2-like proteins and have been reported to extend lifespan of *S. cerevisiae*, *C. elegans* and *Drosophila* (Howitz et al. 2003; Wood et al. 2004). This extension of lifespan by STACs was not seen in flies that were null for *dSir2* (Wood et al. 2004). Dietarily restricted flies fed STACs also showed no further increase in lifespan, suggesting that STACs extend lifespan by the same mechanism as DR (Wood et al. 2004). Similar to the effects of mutations in *Rpd3* and *dSir2*, only two food concentrations were used in these experiments, and characterisation of the response of flies fed STACs to a full range of nutritional levels could provide useful confirmation of the absence of a response to DR. Extension of lifespan by STACs was associated with decreased mortality before day 40 (Wood et al. 2004), unlike the response to DR, where mortality is decreased throughout life (Pletcher et al. 2002), suggesting that the mechanisms may be at least partially different. Recently, it has been reported that STACs do not increase the lifespan of all yeast strains (Kaeberlein et al. 2005) and that the effect of the STACs on Sir2 activity previously reported may have in fact been artefacts of the *in vitro* assay used.

1.9 Thesis outline

The majority of current research into ageing has come from work on rodents (Masoro 2001), a feature which makes the DR field distinct from many other disciplines of biology. Although any potential DR effect(s) in humans may prove to be more similar to those seen in rodents than in invertebrates, there is clearly benefit to be gained from using lower organisms to facilitate research if they are shown to share common 'public' mechanisms. This has already been demonstrated in ageing research by the cross-species conservation of the IIS pathway as a regulator of growth and lifespan (Longo and Finch 2003). Therefore, the broad aim of this thesis is to characterise DR in *Drosophila melanogaster* and utilise the traits that make fruit flies a good model organism to try and elucidate mechanisms by which DR increases

lifespan. Only once the DR response is characterised properly in more than one model organism can prudent decisions be made about the value of comparative analysis between them. DR is often cited as being a truly conserved mechanism of lifespan extension because some form of food restriction extends the lifespan of such a wide range of species, yet it is only in mammals that detailed work has been carried out. The following sections outline the areas of the response of *D. melanogaster* to dietary restriction that are the focus of this thesis.

1.9.1 Does DR slow ageing? (Chapter 3)

Interventions can extend lifespan by either delaying the onset of senescent death or slowing the mortality rate doubling time (MRDT) (Finch 1990). It has been suggested that longevity-extending interventions only represent modifications in the ageing process if they increase the MRDT (Marden et al. 2003). The effects of DR on MRDT in mammals are conflicting (Merry 2005). In rats, DR seems to result in a slowing in the rate of increase of age-specific mortality (Yu et al. 1982; Merry 1987), whilst in mice the effect seems to be mainly a delay in the onset of increased mortality rate (Weindruch et al. 1986). The effect on mortality rate may therefore be strain-specific and dependent on the pathologies that are affected by DR (Merry 2005). However, although it is possible to remodel data to a Gompertzian equation, the rodent studies do not provide sufficient power for answering this question satisfactorily. This is because optimal sample sizes for age-specific mortality trajectories are in the order of tens of thousands (Promislow et al. 1999; Pletcher et al. 2000) and this is beyond the scope of lifespan studies on rodents. Numbers approaching this are achievable in fruit fly studies. Hence, the use of *Drosophila* to investigate whether DR slows ageing rate, and if the effects of *ad libitum* feeding are permanent form the basis of chapter 3.

1.9.2 DR and reduced reproduction (Chapter 4)

One of the hallmarks of DR is that restricted animals, whilst living longer than controls, show reduced reproduction. DR has been shown to reduce both daily and lifetime fecundity in *C. elegans* (Klass 1977) and *D. melanogaster* (Chippindale et al. 1993; Chapman et al. 1995), and to delay reproductive maturity in rats (Osborne et al. 1917; Holehan and Merry 1985). That animals under DR are long-lived yet show reduced fecundity may explain why the plastic response of lifespan to food

levels has evolved across different taxa. Life-extension via DR may be a evolved mechanism to cope with varying levels of nutrition in the wild (Harrison and Archer 1988; Holliday 1989; Masoro and Austad 1996; Shanley and Kirkwood 2000). If food levels are so low that reproducing becomes costly, or the survival of offspring is reduced, shifting the allocation of limited resources from reproduction to somatic maintenance and repair would increase the likelihood of surviving until food is once again plentiful. Therefore, the increased lifespan seen under dietary restriction may be a direct consequence of reduced reproductive rates. This is discussed in greater depth in chapter 4.

1.9.3 Role of calories versus specific nutrient components (Chapter 5)

Unlike in their original study, where they limited growth of rats under DR by reducing all nutrients, in 1939 McCay and colleagues reduced calories alone without restricting the levels of protein, vitamins or minerals and saw life-extension (McCay et al. 1939). This led them to the conclusion that it was the total caloric intake that was the crucial factor in determining lifespan rather than the source of those calories, be they from protein, fat or carbohydrate. Later studies in which either restriction of calorie intake without reduction of protein intake resulted in lifespan extension (Masoro et al. 1989), or where no lifespan extension was seen in rats fed iso-caloric diets in which either the fat or mineral components had been reduced (Iwasaki et al. 1988b) further enhanced this paradigm of calorie restriction.

However, there are exceptions to the rule, and statements such as 'caloric intake is by far the major, if not the sole, dietary factor responsible for [DR's] anti-ageing and life-prolonging actions' (Masoro 2002) may be over-simplistic. Rats fed iso-caloric diets with altered nutritional composition (Dalderup and Visser 1969; Iwasaki et al. 1988a) or reduced protein (Yu et al. 1985) showed lifespan extension. Furthermore, reducing just one amino acid (methionine) increases lifespan in both mice (Miller et al. 2005) and rats (Zimmerman et al. 2003). Hence, it seems that reducing the level of ingested calories may not always be critical for lifespan extension by DR in rodents. Furthermore, there is an important distinction between the actual calorie content of ingested food and that which is available to and used by the organism (Piper et al. 2005). Reducing calorie intake may reduce the ability of an organism to assimilate amino acids and, conversely, reducing the intake of one amino acid such

as methionine may reduce the ability to use ingested calories, despite the fact that methionine-restricted mice do not ingest fewer calories than controls (Miller et al. 2005). The issue of calories versus specific nutrients will be addressed in chapter 5.

1.9.4 DR and hormesis (Chapter 6)

The term 'hormesis' is used to describe the biological phenomenon where by mild exposure to an otherwise detrimental factor is beneficial to survival (Furst 1987). For example, low levels of ionizing radiation extends the lifespan of rodents (Carlson et al. 1957; Congdon 1987) and fruit flies (Sacher 1963), and mild heat stress has also been shown to extend the lifespan of *Drosophila* (Maynard Smith 1958; Khazaeli et al. 1997; Le Bourg et al. 2001; Hercus et al. 2003) and nematodes (Lithgow et al. 1995). Although not a stress likely to be encountered in nature, hypergravity also confers some benefit to the lifespan of fruit flies in mild amounts (Le Bourg et al. 2000). Edward Masoro suggested that lifespan extension under DR may also be an example of hormesis (Masoro 1998). Dietarily restricted rats show elevated afternoon peaks of plasma levels of the hormone corticosterone (Sabatino et al. 1991), which suggests that DR is indeed a mild stress. This may also explain part of the mechanism by which DR functions to extend lifespan, as glucocorticoids are thought to protect against environmental stress (Munck et al. 1984), and in rodents DR does increase resistance to certain stresses (Yu and Chung 2001). The issue of whether this stress resistance is conserved in dietarily restricted *Drosophila* is the topic of chapter 6.

Chapter 2. General materials and methods

2.1 *Drosophila melanogaster* stocks

2.1.1 Dahomey wild type

The wild type (wt) stock used in all experiments was collected in Dahomey (now Benin), West Africa in 1970 and has since been maintained in large population cages (measuring 45x25x25cm) with overlapping generations on a 12:12 light:dark cycle at 25°C and 65% humidity in a controlled temperature (CT) room. Unlike discrete culture, this method has been shown to maintain lifespan and fecundity at levels similar to those seen in freshly collected flies (Sgrò and Partridge 2001). Flies in population cages had constant access to 12 bottles of standard SY food medium (see section 2.2.1), with the three oldest bottles being replaced with fresh medium every week.

2.1.2 ovo^{D1} *stocks.*

The *ovo*^{D1} (1309) mutant was originally obtained from Bloomington stock centre and was twice out-crossed into the Dahomey genetic background. To produce females sterile due to the presence of *ovo*^{D1}, males from the out-crossed *ovo*^{D1} stocks were crossed to Dahomey virgin females (2.3.3). *ovo*^{D1} is located on the X chromosome and maintained in the males, with the genotype of females in the stock being attached XX:Y, thus balancing the mutation and preventing its loss in culture. Stocks were maintained in glass vials containing ASG fly medium (see section 2.2.2).

2.2 *Drosophila* food media

2.2.1 Standard Sugar/ Yeast (SY) medium

SY medium (Ashburner 1989) contained 100g autolysed yeast powder, 100g sucrose granules, 20g agar, 30ml Nipagin (100gL^{-1}), 3ml Propionic Acid, 1 litre distilled water. The distilled water plus agar was brought to the boil at which point the yeast and sugar were added. The medium was then brought back to the boil and allowed to simmer for five minutes before being taken off the heat. When the temperature of the medium cooled to less than 60°C, the Nipagin and Propionic acid (anti-fungal agents) were added.

2.2.2 ASG Medium

Fly stocks were cultured on ASG medium, a softer textured medium designed for maintaining mutant stocks that often show high larval lethality. ASG medium contained 85g sucrose, 20g autolysed yeast powder, 60g maize, 10g agar, 25ml Nipagin ($100gL^{-1}$), 1 litre distilled water. ASG was prepared using the same method as was used for SY (see section 2.2.1).

2.2.3 Variations on standard SY medium for dietary restriction experiments

Nutritional quality of the food medium was varied by adjusting the concentration of the sugar or yeast in comparison to standard SY (Table 2.2.1, Figure 2.2.1).

Table 2.2.1. Food recipes of media used for dietary restriction experiments.
All media were prepared using the same method used for standard SY food (see section 2.2.1).

Food Type	Yeast (g/ litre)	Sucrose (g/ litre)	Agar (g/ litre)
0.65Y 0.65S	65	65	16.5
0.65Y 1.5S	65	150	16.5
1.0Y 1.0 S*	100	100	20
1.5Y 0.65S	150	65	20
1.5Y 1.5S	150	150	20

Y represents yeast, S represents sucrose. * 1.0Y 1.0S is the standard SY medium (section 2.2.1).

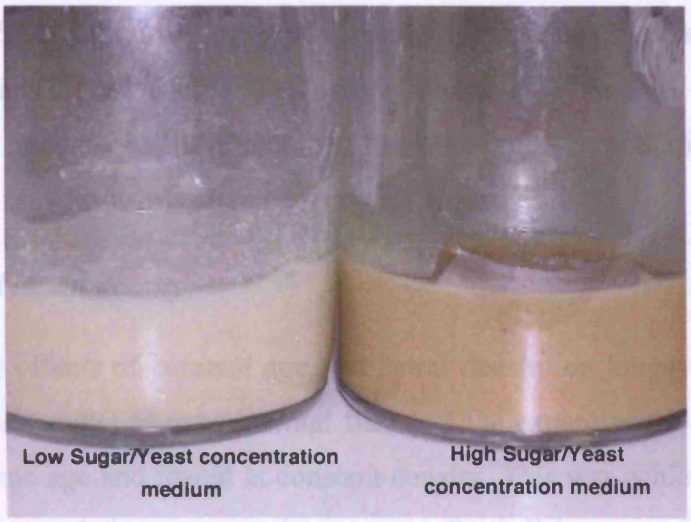
2.2.4 Agar only medium

Agar only medium contained 10g agar, 30 ml Nipagin ($100gL^{-1}$), 3ml Propionic Acid, 1 litre distilled water.

2.2.5 Grape medium

Grape medium contained 1000ml distilled water, 50g agar, 600ml red grape juice, 100ml extra water, 42ml Nipagin ($100gL^{-1}$). The water and agar were brought to the boil before the grape juice was added and the mixture brought back to the boil. The grape medium was then allowed to cool. Any remaining grape juice in the measuring cylinder was rinsed with the extra water and added to the food. When the medium cooled to less than 60°C, the Nipagin was added, after mixing, the solution was poured into plastic Petri dishes where it was allowed to set.

Figure 2.2.1. Photograph of high (Fully-fed) and low (DR) concentration food media.



2.3 General methods and animal husbandry

2.3.1 Stock maintenance

Stocks were maintained in glass shell vials (75mm x 25mm diameter) on 3ml ASG medium. Stocks were kept at 18°C, 65% humidity and on a 12:12 light:dark cycle. Flies were passed onto fresh medium every 4 weeks. This was done either using light carbon dioxide anaesthesia or by rapid transfer without gas. Stocks were checked periodically under a dissecting light microscope to ensure they were of the correct phenotype and free of mites.

2.3.2 Separating males and females

Males and females can be easily identified when anaesthetised with carbon dioxide and viewed under a light microscope. Male *Drosophila* are smaller in size than females, possess 'sex comb' structures on their forelegs used in courtship and a characteristic dark pigmented area on the dorsal posterior section of the abdomen (Ashburner 1989). When under anaesthesia, a fine bristled paintbrush is used to move flies without causing structural damage.

2.3.3 Virgin collection

Female *Drosophila melanogaster* do not mate in the first 8 hours post-eclosion from the pupae at 25°C (Ashburner 1989). Collecting virgin females is therefore achieved by clearing vials of all adults flies before returning to them 6 hours later and collecting any females that have emerged from the pupae over the intervening time period. Virgin females were collected using ice anaesthesia.

2.3.4 Standard larval density

To standardise effects of parental age and larval density on longevity (Priest et al. 2002), both the parents of experimental flies and the experimental flies themselves were of the same age and reared at constant density. This was achieved by allowing flies in plastic population cages to lay eggs on Petri dishes containing grape juice medium (2.2.5) supplemented with live yeast paste. Grape plates were removed after 22 hours and eggs rinsed from the plates into a Falcon tube using phosphate buffered saline (PBS). Eggs were allowed to settle and the remaining supernatant poured off. If necessary, a series of washes was performed to remove any yeast paste that had dissolved in the PBS, resulting in a pellet of eggs in clear solution. Using a Gilson pipette, 20µl of the eggs were aspirated from the solution and squirted into 200ml glass bottles containing 70ml SY medium. This results in a standard density of between 300-350 eggs per bottle (Clancy and Kennington 2001).

2.3.5 Lifespan assays

Unless otherwise stated, all lifespan experiments were performed on once-mated female *Drosophila*. Larvae were reared at standard density in 200ml glass bottles containing 70ml SY medium. Emerging flies were allowed to eclose over a 24-hour period and transferred without anaesthesia to bottles containing fresh SY medium (2.2.1). Flies were then allowed to mate for 48 hours at which point females were separated from males using light carbon dioxide anaesthesia. Female *D. melanogaster* were then placed in groups of 100 into bottles containing 35ml of the appropriate food medium and stored at 25°C, 65% humidity and on a 12:12 hour light:dark cycle. The day at which flies were separated by sex and put into bottles for lifespan assays was designated day zero for experiments. Flies were transferred to fresh bottles every 2 days and deaths scored on 6 out of every 7 days. Initial sample

sizes (N_0) were calculated as the summed death and censor observations over all ages.

2.4 Statistical analysis

2.4.1 Survivorship

Survivorship (lx) is a measure of the probability of an individual surviving from age zero until age x. With no censored observations, l_x is given as the number of individuals alive at age x (N_x) divided by the total number of individuals alive at the start of the experiment (N_0). When an experiment includes censored data, l_x can be calculated from the following formula: $_x$

$$l_x = \prod_0 p_x$$

where p_x is the probability of surviving from age t_{x-1} to age t_x

$$=1 - q_x$$

where q_x = (number of deaths recorded between t_{x-1} and t_x)/N_{x-1}

Statistical analysis of differences between survivorship curves was performed using the Log Rank Test (Peto and Peto 1972) throughout. The test statistic is chi squared (χ^2) and a significant difference between groups is given if the P value is less than 0.05. Statistical analysis was performed using JMP 5.0 statistical software (SAS Institute Inc.).

2.4.2 Age-specific mortality

Age-specific mortality (μ_x) is a measure of the instantaneous risk of death (Lee 1992). μ_x is estimated as $\mu_x = -\ln (p_x)$, where p_x is the probability of an individual alive at age t_{x-1} surviving to age t_x. Age-specific mortality is plotted on the natural log scale as it increases exponentially with age (Lee 1992). To avoid making assumptions about the shapes of mortality trajectories, Cox regression (Cox 1972) analysis was used to analyse statistical differences between age-specific mortality curves, with a P value of less than 0.05 indicating a significant difference. Statistical analysis was performed using JMP 5.0 statistical software (SAS Institute Inc.).

2.4.3 Maximum lifespan

Unless otherwise stated, maximum lifespan is defined as the median lifespan of the longest-lived 10% of individuals. This is because the literal maximum lifespan is based on data from one individual and is sample size dependant. It is therefore more stochastic than median lifespan and is widely agreed to be a bad indicator of lifespan extension (Masoro 2002). Significant difference between the maximum lifespan of two cohorts was tested using the non-parametric Kruskal-Wallis test. Medians and non-parametric tests were used since the last 10% 'tail' of lifespan data is usually not normally distributed, thus the use of means is invalid. Statistical analysis was performed using JMP 5.0 statistical software (SAS Institute Inc.).

2.4.4 Fecundity experiments

Non-parametric Wilcoxon/ Kruskal-Wallis tests were used for analysis of the egg laying data. Statistical analysis was performed using JMP 5.0 statistical software (SAS Institute Inc.).

Chapter 3. Demography of dietary restriction in *Drosophila*

Abstract

Dietary restriction (DR), the reduction of nutrient intake without malnutrition, increases lifespan in organisms ranging from yeast to mammals. DR has been suggested to extend lifespan by slowing the accumulation of ageing-related damage. Here I show that, in *Drosophila*, DR extends lifespan entirely by a reduction in short-term risk of death. Two days after the application of DR for the first time late in life, previously fully-fed flies are no more likely to die than flies of the same age permanently subjected to DR throughout adulthood. Thus full-feeding does not cause any irreversible damage in *Drosophila*. DR of mammals may also reduce short-term risk of death and hence DR/ DR mimetics instigated at any age could generate a full reversal of mortality. These data are published and discussed in (Mair et al. 2003) (See Appendix 1).

3.1 Introduction

DR delays the appearance of age-related pathology in rodents and maintains them in a youthful state for longer than those animals fed *ad libitum* (Weindruch and Walford 1988; Masoro 2002). DR has therefore been suggested to slow the rate of ageing (Finch 1990). Application of a dietary restriction regime to one year old male mice extends average lifespan by 10-20% (Weindruch and Walford 1982). Hence it is not the delay to age at maturity, seen in early experiments (McCay et al. 1935), that is the cause of the extension of lifespan under DR. However, little is known about the mechanism(s) by which DR extends lifespan and whether the decreased mortality seen in DR individuals is due to 1) a reduction in the build-up of irreversible damage caused by over-eating, or 2) the removal of an acute risk factor that increases the likelihood of death only during times of *ad libitum* feeding.

In this chapter, I test which of the above alternatives is the case for life-extension by DR in *Drosophila melanogaster*. The majority of studies present lifespan data as survivorship analysis (2.4.1). Representing the proportion of the original cohort still alive at a given time point, survivorship is a cumulative measure, with each data point being dependent on those previous to it (Lee 1992). Unlike survivorship, age-specific mortality (2.4.2) is a measure of the instantaneous hazard of death for an individual at a given age (Lee 1992) and allows independent comparisons of vulnerability to death at different ages (Vaupel et al. 1998; Carey 2003). To gain accurate resolution of mortality trajectories requires samples sizes much larger than those used in most ageing studies (Promislow et al. 1999), something that can be achieved relatively easily using *Drosophila* as a model organism. Mortality rates during ageing can be described in terms of two important parameters: the initial, baseline mortality rate, which is age-independent, and the rate at which age-specific mortality increases with age (Finch 1990). Interventions, genetic and environmental, that increase lifespan can do so by decreasing the baseline mortality rate, lowering the rate at which mortality increases with age (the slope of the mortality trajectory) or both (Pletcher et al. 2000). It has been argued that a reduction in the slope of a mortality trajectory as a result of an intervention that extends lifespan indicates that longevity has been increased by a reduction in the rate of ageing itself (Finch 1990).

For instance, in *Drosophila*, the mutants *methuselah* (Lin et al. 1998) and *Indy* (Marden et al. 2003), both of which have been shown to increase lifespan, do so by lowering the slope of the mortality trajectory, and have been suggested therefore to reduce the rate of ageing. In contrast, in industrialised human societies worldwide, lifespan has been increasing for over a century, entirely by a reduction in initial mortality rates, with no reduction in the slope of the mortality trajectory (Wilmoth 2000). This lowering of the mortality trajectory has been taken to indicate that overall health at all ages has improved, but that the underlying process of accumulation of ageing-related damage has not been ameliorated.

DR in *Drosophila* increases lifespan entirely by lowering the initial mortality rate in both sexes (Magwere et al. 2004), and a greater effect of DR on initial mortality rate entirely explains the greater extension of lifespan seen in females compared to males. The slope of the mortality trajectory is slightly increased under DR in females compared to controls, but not in males (Magwere et al. 2004). This slope change is in the opposite direction to that required to explain the increase in lifespan, but it does mean that in general median lifespan is extended more than is maximum lifespan in females. The slight increase in slope under DR in females could be accounted for by the age-related decrease in the feeding behaviour (R. Wong, personal communication), which may move them into the starvation range of the response as they age.

The fact that DR in *Drosophila* extends lifespan entirely by a reduction in the initial mortality rate suggests that it might not slow down the rate of accumulation of irreversible, ageing-related damage but it is not in itself proof. Because DR is an environmental intervention, it is relatively straightforward to address this issue (Partridge and Andrews 1985). Interventions can lower adult mortality by slowing the accumulation of the irreversible damage that is characteristic of ageing (ageing-related damage), by reducing short-term vulnerability to death (risk), or by some combination of the two (Partridge and Andrews 1985; Prowse and Partridge 1997). Interventions that lower either acute risk or irreversible damage would both extend median lifespan if applied for the first time at later ages, therefore this observation is not sufficient to distinguish between these two mechanisms. However, these

hypotheses can be distinguished experimentally for DR by examining the effect of past and current nutritional conditions on age-specific mortality (Partridge et al. 2005c).

If full-feeding causes a build-up of irreversible damage, the subsequent mortality rates of individuals 'switched' to DR from full-feeding midway though life would always be higher then that of individuals who had been under a DR regime throughout adult life, since the switched group would suffer the extra deleterious effects caused by their nutritional history. The imprint of the past would never be erased. However, the removal of the damage-inducing conditions (full-feeding) would mean that the subsequent rate of accumulation of damage would be reduced. Thus, mortality rates of the switched and non-switched groups would diverge as the slope of the mortality trajectory is reduced to that of the cohort in which DR has been permanently applied. Still, previous exposure to full-feeding would mean that the switched group would forever show permanently elevated mortality rates compared to the long-lived DR control (Figure 3.1.1a).

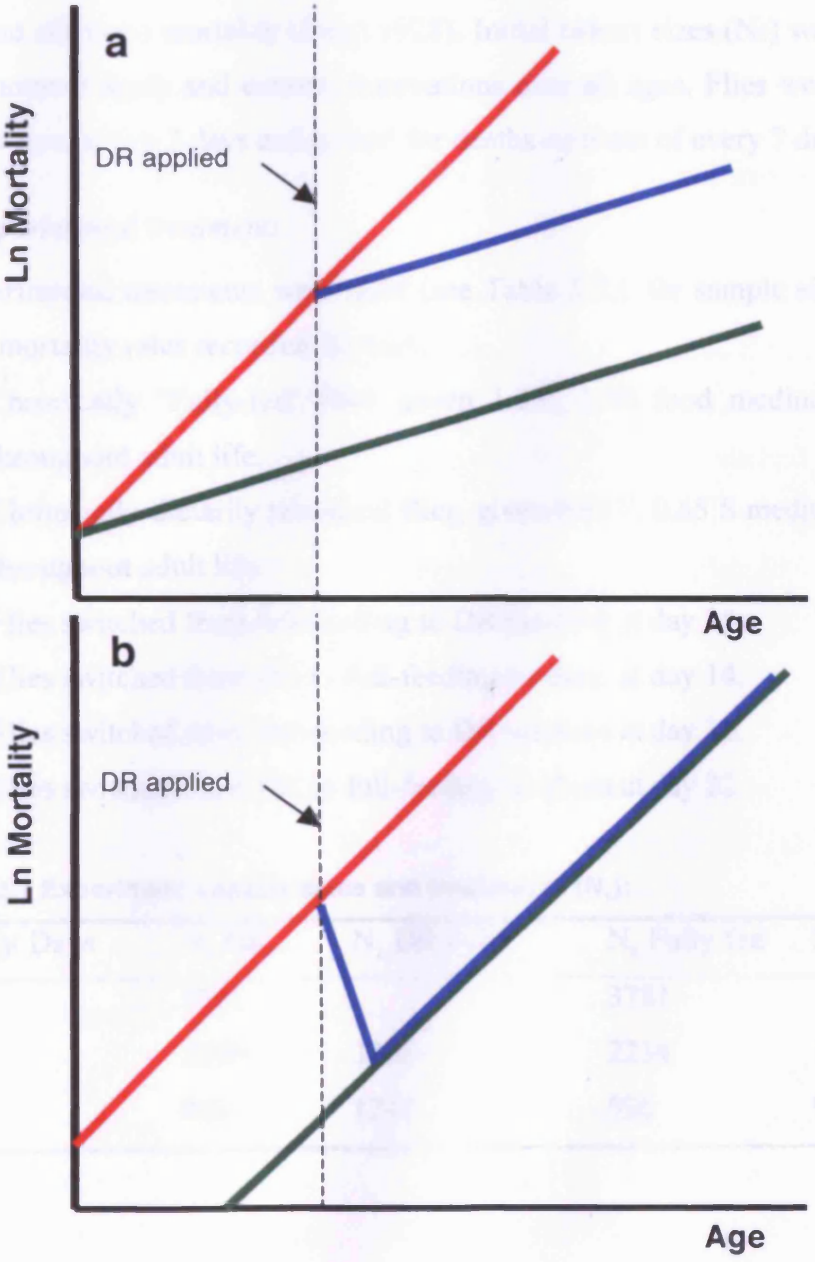
If the alternate hypothesis is true, and full-feeding does not cause irreversible damage but instead increases some acute risk factor, flies switched to DR late in life should not show any effect of nutritional history and have the same subsequent mortality rates as those flies that had been permanently under DR (Figure 3.1.1b). Analogous to the risks associated when crossing roads with different levels of traffic, mechanisms that induce higher levels of acute risk would only increase the chance of death in individuals *currently* exposed to that risk. As long as death is avoided during this exposure, the subsequent chance of dying when the risk is removed is not history dependent; a person's chances of being hit by a car when crossing a country road is not affected by whether they have spent their life crossing quiet lanes or busy motorways. These theories are not mutually exclusive and both acute risk and irreversible damage may be affected. An accurate mortality plot is required to determine precisely what combination of risk and damage is operating.

In this chapter I carried out large sample size lifespan experiments using wild type *Drosophila melanogaster* to investigate the effect on mortality rates of switching nutritional regimes at different stages in adulthood and thereby determine by which

method DR extends lifespan in fruit flies, decreasing the build-up of irreversible damage or reducing the transient risk of death.

Figure 3.1.1 Schematic diagram of predicted changes to mortality rates if DR is applied midway through life.

Red line represents permanently fully-fed, green represents permanently DR and blue represents group switched to DR late in life. **a.** Predicted response of the mortality trajectory if DR reduces the accumulation of irreversible damage. **b.** Predicted response of the mortality trajectory if DR reduces the acute risk of death.



3.2 Methods

3.2.1 Experimental procedure

Eggs were collected from stock cages over an 8 hour period and reared on standard sugar/ yeast medium (see 2.2.1) at a density of 400-450 eggs per 200ml bottle at 25°C (see 2.3.4). Flies eclosing over an 8-hour period were collected, transferred to fresh bottles without CO_2 and left to mate for 24 hours. Females were then sorted on CO_2 diffusers and randomly allocated to one of the treatments and its corresponding food nutrient concentration medium. Experimental flies were kept in 200ml bottles on 35ml food at a standard density of 100 (±10) throughout, since density has a significant effect on mortality (Pearl 1928). Initial cohort sizes (N_0) were calculated as the summed death and censor observations over all ages. Flies were transferred onto new food every 2 days and scored for deaths on 6 out of every 7 days.

3.2.2 Experimental treatments

Six experimental treatments were used (see Table 3.2.1 for sample sizes) and age-specific mortality rates recorded for each.

1) Chronically 'Fully-fed' flies, given 1.5Y, 1.5S food medium (see 2.2.3) throughout adult life.
2) Chronically dietarily restricted flies, given 0.65Y, 0.65 S medium (see 2.2.3) throughout adult life.
3) Flies switched from full-feeding to DR medium at day 14.
4) Flies switched from DR to full-feeding medium at day 14.
5) Flies switched from full-feeding to DR medium at day 22.
6) Flies switched from DR to full-feeding medium at day 22.

Table 3.2.1 Experiment sample sizes and treatments (N_x):

Age (x)/ Days	N_x DR	N_x DR to FF	N_x Fully-fed	N_x FF to DR
0	3711		3781	
14	2199	1332	2234	1261
22	809	1249	596	937

3.3 Results

3.3.1 Lifespan of females subjected to dietary restriction.

Maintaining flies on a DR medium throughout adult life significantly extended lifespan (P<0.0001, Log Rank Test). Median lifespan increased by 48% in the DR group compared to the fully-fed control (figure 3.3.1a). Maximal lifespan (median lifespan of the longest lived 10%) was also significantly extended by DR (maximal fully-fed = 34 days, maximal DR = 51, χ^2 = 149.37, df = 1, P<0.0001, non-parametric Kruskal-Wallis Test, 50% extension). Age-specific mortality trajectories for female flies subjected to DR from the onset of adulthood showed the characteristic (Pletcher et al. 2002) delay in the onset of detectable ageing-related mortality compared to those maintained on full-feeding (Figure 3.3.1b).

3.3.2 Age-specific mortality of Drosophila *switched between different nutritional regimes during adulthood.*

Previously fully-fed flies showed a rapid and complete reduction in mortality when switched to DR at 14 or 22 days of adulthood (Figure 3.3.2a). Cox regression was used to avoid making assumptions about the shape of the trajectories post-switch. Within 48 hours of the switch, mortality trajectories of switched cohorts were indistinguishable from those of same-age flies maintained on DR throughout adulthood, as shown by a risk ratio between the groups of 1 (day 14 switch, p=0.79, DR control n = 2137, switch n = 1245, Risk Ratio = 0.994 [95% Confidence Intervals: 0.952, 1.039]; day 22 switch, p = 0.94, DR control n = 912, switch n = 798, Risk Ratio = 0.998 [95% Confidence Intervals: 0.952, 1.047]). After both switch points, fully-fed and DR flies differed significantly in mortality (day 14, p<0.0001, DR control n = 2137, fully-fed control n = 2198, Risk Ratio = 0.419 [95% Confidence Intervals: 0.397, 0.442]; day 22, p<0.0001, DR control n = 798, fully-fed control n = 501, Risk Ratio = 0.491 [95% Confidence Intervals: 0.460, 0.525], Cox regression/ proportional hazard test).

Reciprocal switches from restricted conditions to full-feeding resulted in rapid and substantial increases in log mortality rates within 48 hours (Figure 3.3.2b). However, at both switch points, mortality of the DR to full-feeding switch group remained

significantly lower than that of fully-fed controls ($p<0.0001$, Cox regression). Protection offered by DR increased with time spent on the regime: 48 hours after the day 22 switch, risk ratios of switched cohorts compared to fully-fed controls were: 14 days of DR, ratio = 0.831 [95% Confidence Intervals: 0.787, 0.878], 22 days of DR, ratio = 0.763 [95% Confidence Intervals: 0.723, 0.806]. There was a significant difference in mortality between the switched cohorts during this time ($p<0.0001$).

Figure 3.3.1. Survivorship/ age-specific mortality of female *Drosophila melanogaster* on full-feeding and dietary restriction.

a. Dietary Restriction had a significant effect on survival, extending median lifespan by 48% and maximal lifespan by 50%. **b.** DR resulted in a characteristic delay in the onset of increases to mortality rates.

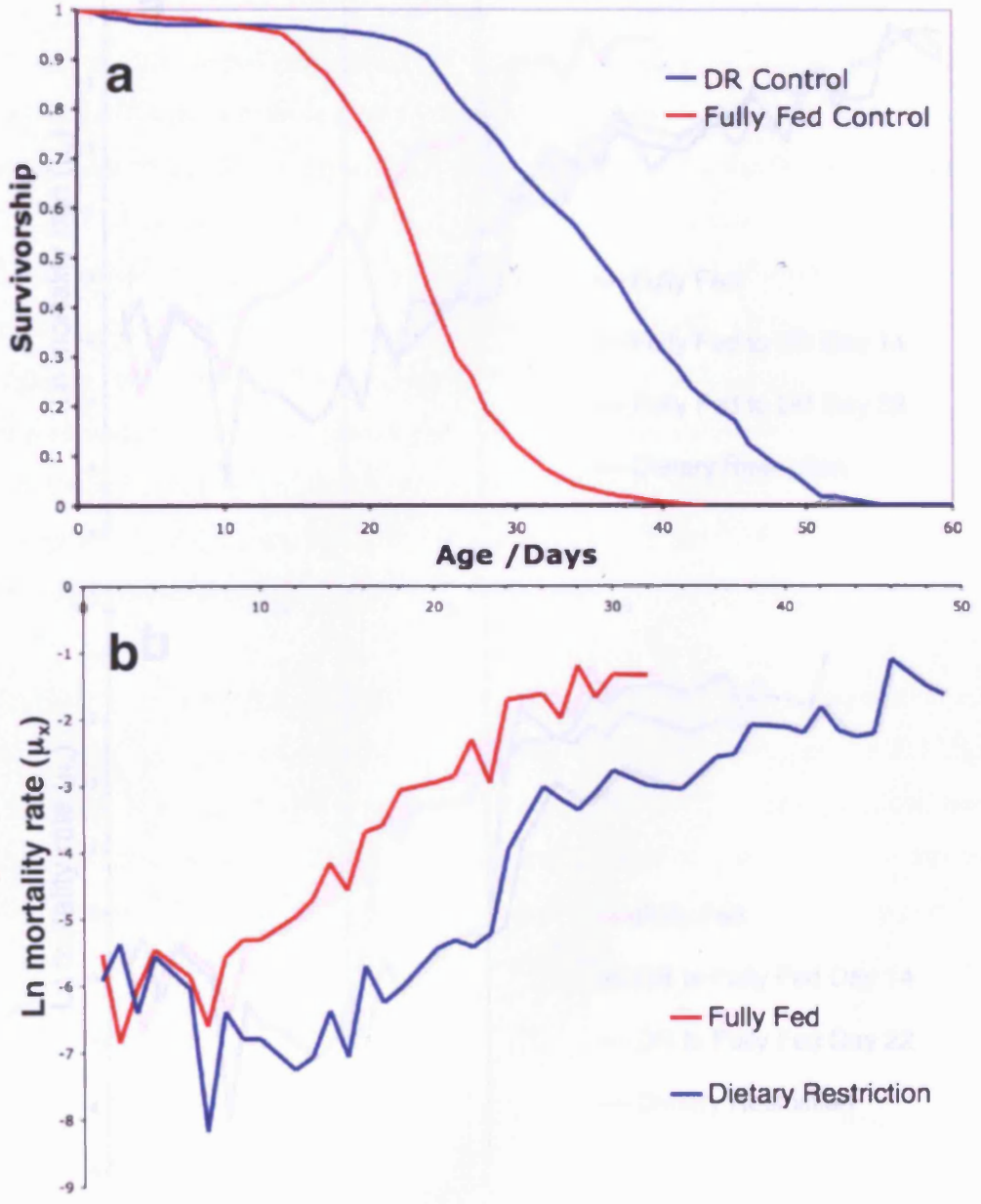
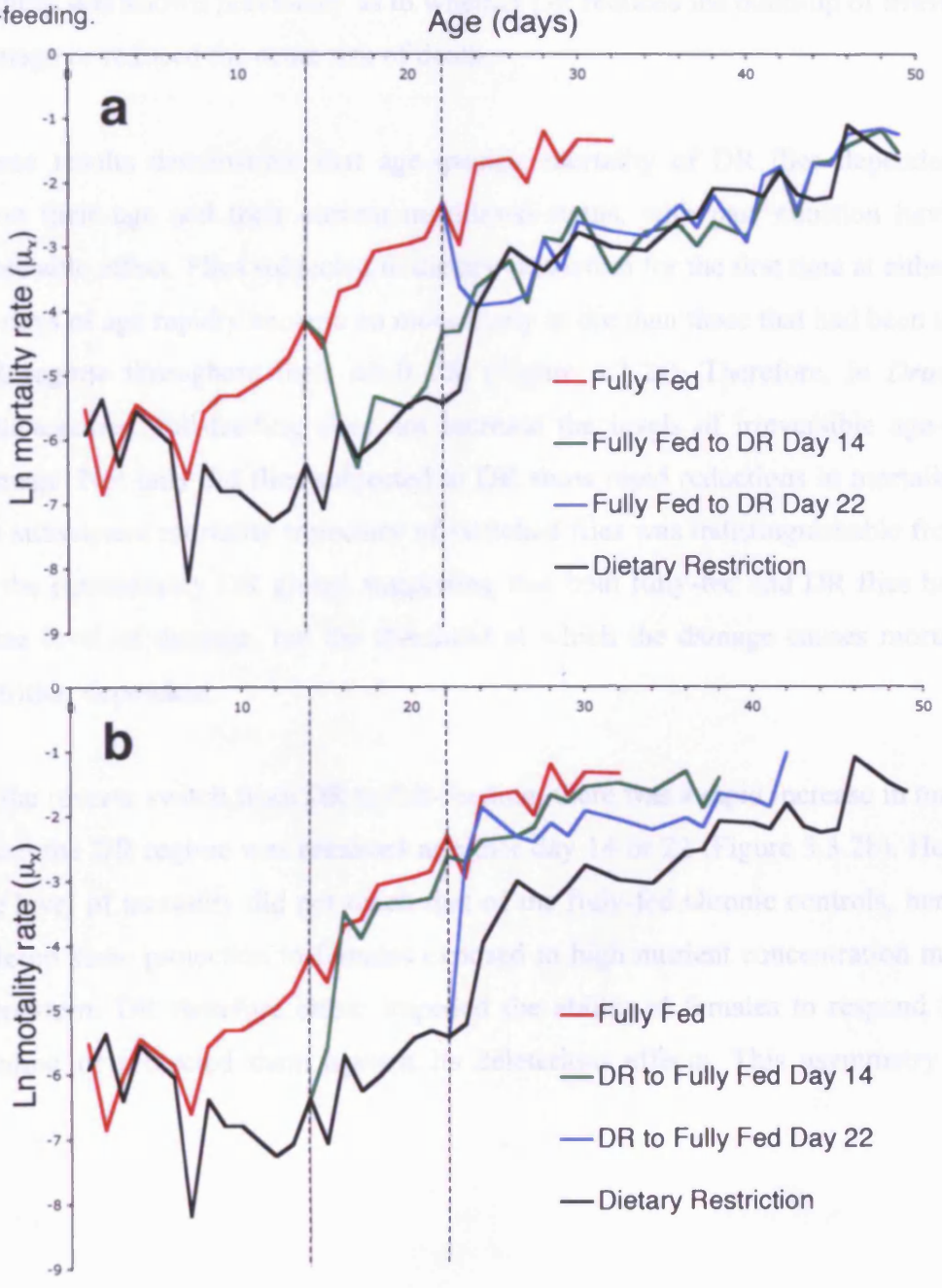


Figure 3.3.2. Age-specific mortality rates of female *Drosophila* in response to the instigation of a DR regime.

Mortality rate (μx) is plotted on the natural log scale since it increases exponentially with age (Lee 1992). Dotted vertical lines represent switch days. Mortality curves were truncated when n<50. **a.** Within 48 hours of being switched from full-feeding to DR, flies became no more likely to die than those that had been under DR throughout adulthood, and subsequent mortality trajectories became indistinguishable. This effect was the same if flies were switched to DR after either 14 or 22 days of full-feeding. Full-feeding did not cause any permanent damage. **b.** Switching flies from DR to full-feeding at either 14 or 22 days resulted in rapid and dramatic increases in mortality rates. However, these did not reach the level of the fully-fed control group, thus DR gives females some protection to subsequent full-feeding.



3.4 Discussion

3.4.1 Demography of DR in Drosophila

Dietary restriction in female wild type *Drosophila* extended median and maximal lifespan (Figure 3.2.1a), as has been seen previously when food nutrient concentration is diluted (Chippindale et al. 1993; Chapman and Partridge 1996; Pletcher et al. 2002). This extension was seen by an apparent delay in the onset of exponential increase in age-specific mortality (Figure 3.3.1b), characteristic of the response to DR in this species (Pletcher et al. 2002; Magwere et al. 2004). However, nothing was known previously as to whether DR reduced the build-up of irreversible damage or reduced the acute risk of death.

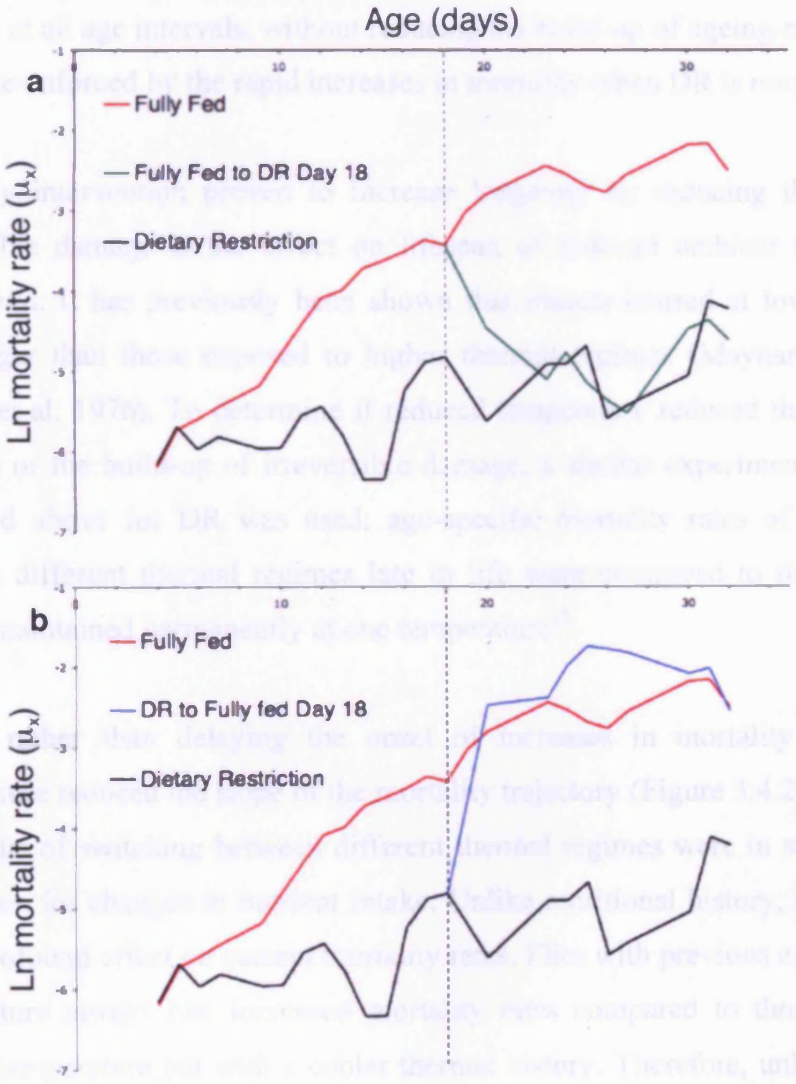
These results demonstrate that age-specific mortality of DR flies depended only upon their age and their current nutritional status, with past nutrition having no detectable effect. Flies subjected to dietary restriction for the first time at either 14 or 22 days of age rapidly became no more likely to die than those that had been under a DR regime throughout their adult life (Figure 3.3.2a). Therefore, in *Drosophila melanogaster,* full-feeding does not increase the levels of irreversible age-related damage. Not only did flies subjected to DR show rapid reductions in mortality rate, the subsequent mortality trajectory of switched flies was indistinguishable from that of the permanently DR group, suggesting that both fully-fed and DR flies have the same level of damage, but the threshold at which the damage causes mortality is nutrition dependent.

In the reverse switch from DR to full-feeding, there was a rapid increase in mortality when the DR regime was removed at either day 14 or 22 (Figure 3.3.2b). However, the level of mortality did not reach that of the fully-fed chronic controls, hence DR offered some protection to females exposed to high nutrient concentration medium. Long-term DR therefore either impeded the ability of females to respond to full-feeding or protected them against its deleterious effects. This asymmetry in the

response of mortality to switches in nutrient concentration was not seen in male *Drosophila* (Figure 3.4.1)[12].

Figure 3.4.1. Age-specific mortality rates of male *Drosophila* in response to the instigation/ removal of a DR regime.

Dotted vertical lines represent switch day. **a.** Flies switched from full-feeding to dietary restriction at day 18 showed rapid and complete reduction in mortality. **b.** In the reciprocal switch from DR to fully-fed conditions at day 18, mortality rates rapidly increased. Subsequent mortality was higher in the switched flies. This experiment was terminated on day 32. N.B - This figure is from (Mair et al. 2003), figure produced by W. Mair, experimental work carried out by S. D. Pletcher.



[12] The experimental work described on DR of male *Drosophila* was published in (Mair et al. 2003) but carried out by S.Pletcher. All analyses of the data and production of figures were done by W. Mair.

The data in this chapter data show that in *Drosophila*, DR extends lifespan by reducing the acute risk of death associated with full-feeding and that this effect is rapidly reversible. This is concordant with the hypothesis set out in Figure 3.1.1b. The implications of these data and how they fit with results in the literature on DR in mammals will be discussed later in this chapter.

3.4.2 Interventions that affect ageing-related damage

The data presented here for DR in *Drosophila* show that DR does not reduce the rate of accumulation of irreversible damage and it can therefore be deduced that DR does not slow the rate of ageing in this species. Moreover, analogous to improved public health in humans (Wilmoth 2000), DR increases lifespan by reducing the likelihood of dying at all age intervals, without reducing the build-up of ageing-related damage, a point re-enforced by the rapid increases in mortality when DR is removed.

The only intervention proven to increase longevity by reducing the build-up of irreversible damage is the effect on lifespan of reduced ambient temperature in *Drosophila*. It has previously been shown that insects housed at low temperatures live longer than those exposed to higher thermal regimes (Maynard-Smith 1958; Miquel et al. 1976). To determine if reduced temperature reduced the transient risk of death or the build-up of irreversible damage, a similar experimental protocol as presented above for DR was used; age-specific mortality rates of flies switched between different thermal regimes late in life were compared to those of control groups maintained permanently at one temperature[13].

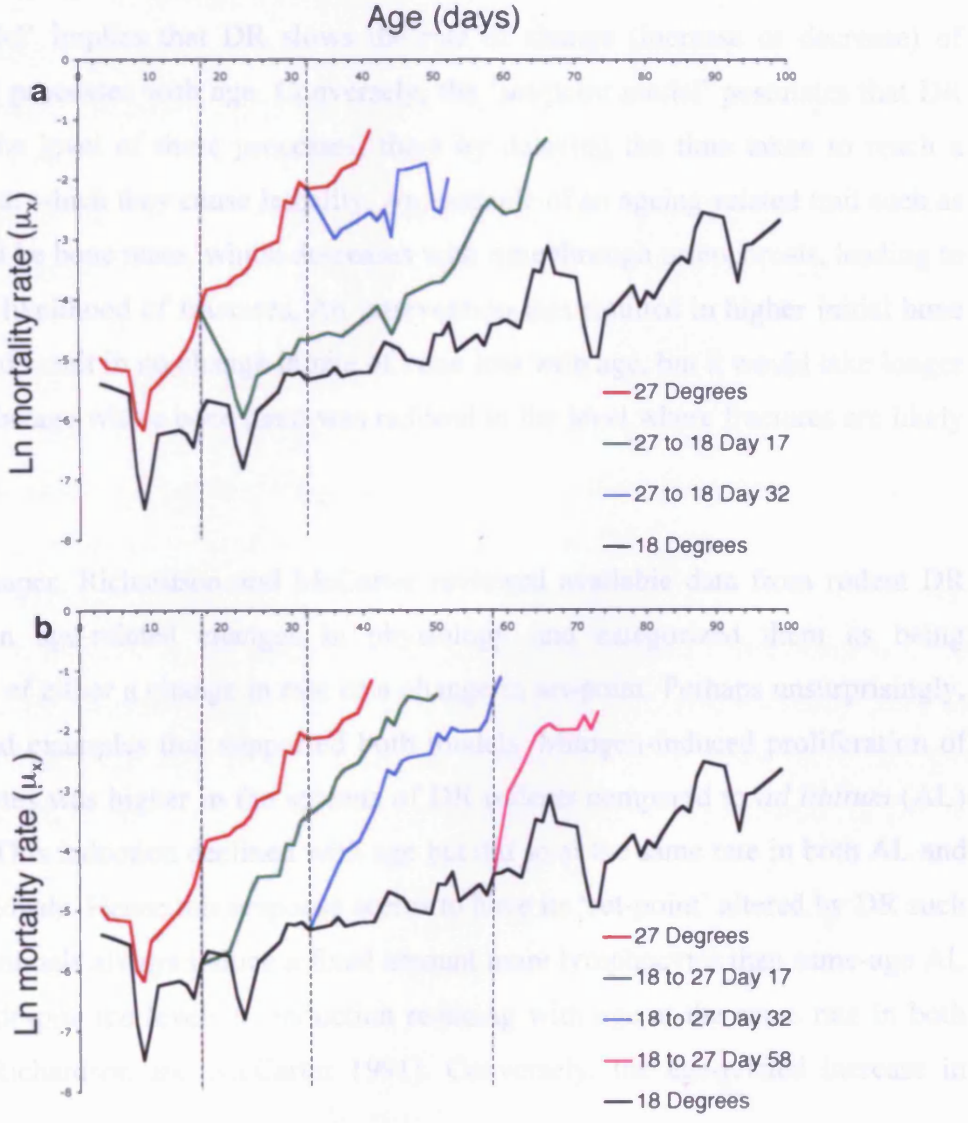
Firstly, rather than delaying the onset of increases in mortality rate, lowered temperature reduced the slope of the mortality trajectory (Figure 3.4.2). Furthermore, the results of switching between different thermal regimes were in stark contrast to those seen for changes in nutrient intake. Unlike nutritional history, thermal history had a profound effect on current mortality rates. Flies with previous exposure to high temperature always had increased mortality rates compared to those at the same current temperature but with a cooler thermal history. Therefore, unlike DR, colder

[13] The experimental work described in this section on temperature switches was published in (Mair et al. 2003) but carried out by P. Goymer. All analyses of the data and production of figures were done by W. Mair.

ambient temperature increases the lifespan of the ectothermic species *Drosophila* by reducing the build-up of irreversible damage, most likely by a reduction in the 'rate of living' (Section 1.3.5) and a slowing of all life history traits.

Figure 3.4.2. Age-specific mortality rates of male *Drosophila* in response to switches in ambient temperature.

Dotted vertical lines represent switch days. **a.** Lower experimental temperature reduced the slope of the mortality trajectory. Mortality of flies switched from 27°C to 18°C remained higher than that of flies maintained at 18°C throughout adulthood. **b.** In the reciprocal switch from 18°C to 27°C mortality trajectories of flies with a history of low temperature remained lower than 27°C controls. N.B - This Figure is from (Mair et al. 2003), figure produced by W. Mair, experimental work carried out by P. Goymer.



The contrast in responses of mortality rates to switch experiments for the DR and temperature experiments suggest that the mechanisms by which DR extends lifespan, at least in *Drosophila*, are fundamentally different from those occurring at low temperatures. Therefore the theory that DR extends lifespan by reducing the rate of living (Sacher 1977) is not supported by the work presented here on *Drosophila*. Whether this is the case in mammals is as yet unknown and will be discussed later in this chapter (Section 3.4.5).

3.4.3 The set-point model of lifespan extension by DR

In their 1991 paper entitled 'Mechanism of food restriction: change of rate or change of set-point?', Arlan Richardson and Roger McCarter put forward two potential theories as to how DR could extend lifespan (Richardson and McCarter 1991). The 'rate model' implies that DR slows the rate of change (increase or decrease) of biological processes with age. Conversely, the 'set-point model' postulates that DR changes the level of these processes, there by delaying the time taken to reach a threshold at which they cause lethality. An example of an ageing-related trait such as this would be bone mass, which decreases with time through osteoporosis, leading to increased likelihood of fractures. An intervention that resulted in higher initial bone mass could result in no change in rate of bone loss with age, but it would take longer to reach the age where bone mass was reduced to the level where fractures are likely to occur.

In their paper, Richardson and McCarter reviewed available data from rodent DR studies on age-related changes in physiology and categorized them as being examples of either a change in rate or a change in set-point. Perhaps unsurprisingly, they found examples that supported both models. Mitogen-induced proliferation of lymphocytes was higher in the spleens of DR rodents compared to *ad libitum* (AL) animals. This induction declined with age but did so at the same rate in both AL and DR individuals. Hence this response seems to have its 'set-point' altered by DR such that DR animals always induce a fixed amount more lymphocytes than same-age AL animals, despite the levels of induction reducing with age at the same rate in both groups (Richardson and McCarter 1991). Conversely, the age-related increase in

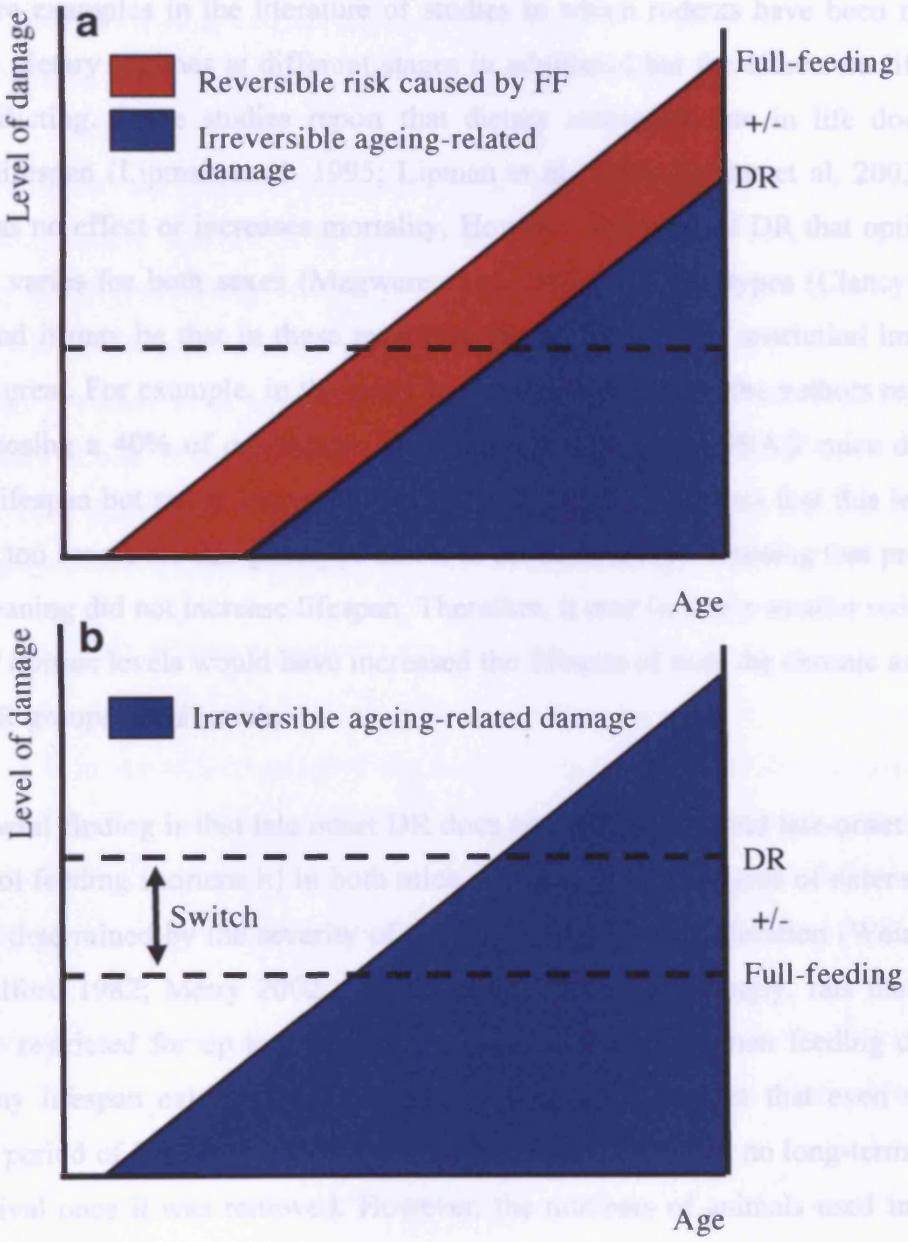
severity of chronic nephropathy legions is slowed by DR in rats, implying that DR alters the 'rate' of change for this pathology.

The data shown above from DR in *Drosophila* suggest that the underlying processes (i.e. ageing-related damage) that change with age in fully-fed and DR flies proceed at the same rate *and* at the same level in both groups. The mechanisms that generate the acute effect of DR and the irreversible increase in death rate with age are different from one another. This leads to two possible models. 1) Two types of damage occur, irreversible ageing-related damage that is present in both DR and fully-fed groups *and* a reversible, age-independent, full-feeding related damage that is produced at a constant level only when flies are fully-fed and that ceases when DR is applied (Figure 3.4.3a). 2) Both DR and fully-fed flies have the same amount of total damage but the threshold at which this damage causes lethality is higher under DR (Figure 3.4.3b), in which case, unlike the osteoporosis example, the 'set-point' is not the level of the process (damage) but the level at which the process causes death. This could be due to increased levels of damage repair under DR, for example, proteasome activity declines with age in rodents and this decline is not only delayed in DR animals but short-term DR can restore activity levels (Goto et al. 2002).

DR in *Drosophila* therefore increases lifespan by a method similar to the 'set-point' model and late-onset DR is as effective as chronic DR in this species. However, this model differs from the example given above for bone-mass and osteoporosis, where the initial level of the process was altered, because for DR in fruit flies, the ageing-related irreversible damage that increases with age in fully-fed and DR flies proceeds at the same rate *and* at the same level in both groups. For this phenomenon to be conserved in mammals, late-onset DR must similarly decrease mortality and, as Richardson and McCarter postulated in the summary to their paper, those changes that are causal to this change in set-point must occur rapidly after induction of DR.

Figure 3.4.3. Models of the roles of ageing-related damage and acute risk of death in *Drosophila* subjected to different dietary regimes.

In both cases, horizontal dashed lines represent the threshold at which death occurs. **a.** Irreversible ageing-related damage builds up at the same rate in both fully-fed and DR animals, but full-feeding induces an additional level of damage (risk factor) that is acute and reversible and increases the risk of death at all ages. **b.** Irreversible ageing-related damage builds up at the same rate in both fully-fed and DR animals but the threshold at which this begins to cause lethality is increased under DR, and this threshold can be rapidly raised or lowered in response to changes in diet.



3.4.4 The effect of late onset DR on lifespan in rodents

Accurate resolution of age-specific mortality plots requires vary large sample sizes (Promislow et al. 1999), hence *Drosophila* as a model organism lends itself very well to experiments of this kind. For this reason, similar experiments in rodents have not been performed because running costs and animal handling have been prohibitive. The total sample size used in the experiment reported here was approximately 7500 (Table 3.2.1), much greater than those used in rodent studies.

There are examples in the literature of studies in which rodents have been moved between dietary regimes at different stages in adulthood but the effects on lifespan are conflicting. Some studies report that dietary restriction late in life does not extend lifespan (Lipman et al. 1995; Lipman et al. 1998; Forster et al. 2003) and either has no effect or increases mortality. However, the level of DR that optimizes lifespan varies for both sexes (Magwere et al. 2004) and genotypes (Clancy et al. 2002) and it may be that in these examples the severity of the restriction imposed was too great. For example, in the study by Forster et al. (2003) the authors reported that imposing a 40% of *ad libitum* DR regime late in life in DBA/2 mice did not extend lifespan but rather increased mortality. However, it appears that this level of DR was too severe for this genotype since, in the same study, imposing that protocol from weaning did not increase lifespan. Therefore, it may be that a smaller reduction from *ad libitum* levels would have increased the lifespan of both the chronic and late onset DR groups of this strain.

The general finding is that late onset DR does extend lifespan (and late-onset return to control feeding shortens it) in both mice and rats, with the degree of extension of lifespan determined by the severity of the DR imposed and its duration (Weindruch and Walford 1982; Merry 2002; Dhahbi et al. 2004). Interestingly, rats that were dietarily restricted for up to a year and then moved to *ad libitum* feeding did not show any lifespan extension (Merry 1987). These data suggest that even such a lengthy period of DR, up to 34% of the rat median lifespan, had no long-term effect on survival once it was removed. However, the numbers of animals used in these studies have been insufficient to determine the relative contributions of risk and damage to the reduction in mortality by DR.

In *Drosophila*, the fully reversible effects of DR are associated with extension of lifespan by a reduction in the initial mortality rate, rather than a reduction in the slope of the mortality trajectory (Pletcher et al. 2002). As stated earlier, this is not enough to show that an intervention extends lifespan by reducing risk of death rather than irreversible damage, but it may be indicative. The data from rodents on whether DR affects initial mortality rate or the slope of the trajectory are somewhat mixed (Merry 2005). Data from rats suggest that DR may both lower initial mortality rate and decrease the slope of the mortality trajectory (Merry and Holehan 1981; Yu et al. 1982; Holehan and Merry 1986; Merry 1987; Pletcher et al. 2000; Merry 2005), while in mice lifespan is extended almost entirely by a decrease in initial mortality rate (Weindruch et al. 1986; Hursting et al. 1994) (Figure 3.4.4).

However, there are potential complexities in the interpretation of the data. As mentioned, sample sizes have in general been too low for an accurate mortality trajectory to be constructed. In addition, DR can change the shape of the mortality trajectory, with the result that a simple comparison of elevation and slope becomes impossible (Pletcher et al. 2000). DR in rodents has also been shown to reduce core temperature (Duffy et al. 1997) and this may lead to some reduction in irreversible damage. Data from the mortality trajectories of un-switched animals should therefore be interpreted with some caution.

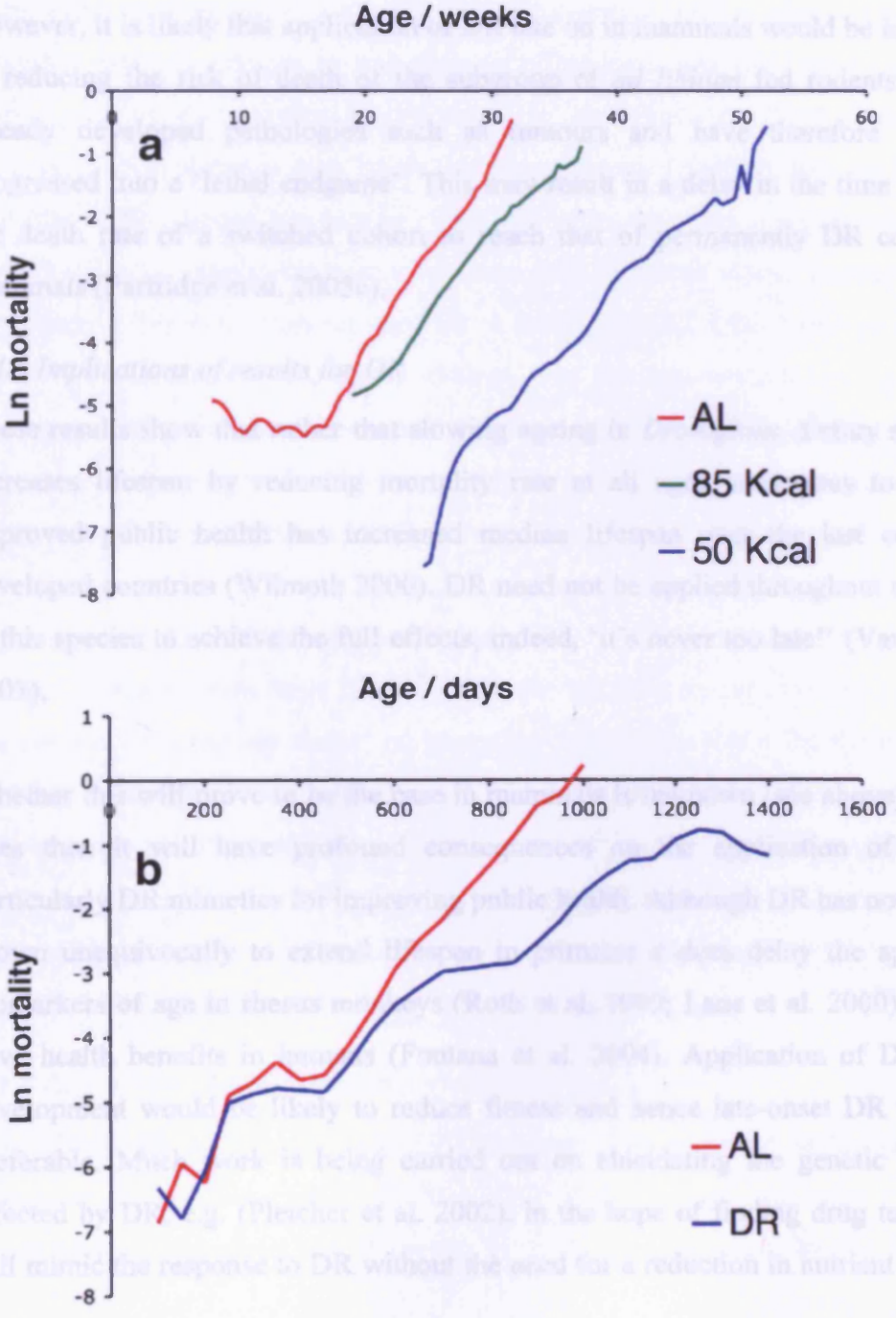
3.4.5 Acute effects of dietary restriction on mammals

Without large-scale experiments on rodents involving switching between dietary regimes it is impossible to gauge if the same effects on age-specific mortality seen here in *Drosophila* would be conserved in mammals. However, it is possible to look at the physiological changes that occur in mammals exposed to short-term DR in comparison with those maintained under dietary restriction through their adult life.

Some of the molecular traits associated with DR in rodents show rapid switching with diet. For instance, the RNA expression profiles from liver of mice subjected to short-term DR within 4 weeks came to resemble the profiles seen under long-term DR (Cao et al. 2001). Over a period of 8 weeks of late-onset DR both liver RNA profiles and incidence of tumours came to resemble those seen in chronic DR mice (Cao et al. 2001; Dhahbi et al. 2004; Tsuchiya et al. 2004; Spindler 2005). Dietary

Figure 3.4.4. Age-specific mortality trajectories (-ln μx) of DR rodents in comparison to *ad libitum* fed controls.

a. As female C3B10RF1 mice are subjected to increasing levels of DR, the mortality trajectories shift to the right whilst remaining parallel to the control group. Data kindly supplied by R. Weindruch and taken from (Weindruch et al. 1986). Data was analysed by S. D. Pletcher and smoothed using the Muhaz package in R. **b.** In contrast, DR of male rats results in an apparent reduction in the slope of the mortality trajectory. Data kindly supplied by B. Merry and taken from (Merry 1987). This figure was made by W. Mair and taken from (Partridge et al. 2005c).



restriction in rats is associated with lowered plasma insulin and lowered peroxidisability of mitochondrial membranes. The mitochondrial phenotype is reversed by two weeks administration of exogenous insulin (Lambert et al. 2004). Oxidative damage to proteins in mice is lowered under DR, and is reversible within 3-6 weeks of a change in nutritional regime (Dubey et al. 1996; Forster et al. 2000). None of these phenotypes have been directly demonstrated to be causal in the extension of lifespan by DR. None the less, the rapid switching in physiology with a change in diet could provide a mechanism for a reversible effect upon mortality rate, and motivates a study of the effects of DR reversals on mortality itself in rodents. However, it is likely that application of DR late on in mammals would be ineffective in reducing the risk of death of the subgroup of *ad libitum* fed rodents that had already developed pathologies such as tumours and have therefore in effect progressed into a 'lethal endgame'. This may result in a delay in the time taken for the death rate of a switched cohort to reach that of permanently DR controls in mammals (Partridge et al. 2005c).

3.4.6 Implications of results for DR

These results show that rather that slowing ageing in *Drosophila*, dietary restriction increases lifespan by reducing mortality rate at all ages, analogous to the way improved public health has increased median lifespan over the last century in developed countries (Wilmoth 2000). DR need not be applied throughout adulthood in this species to achieve the full effects, indeed, 'it's never too late!' (Vaupel et al. 2003).

Whether this will prove to be the case in mammals is unknown (see above), but if it does then it will have profound consequences on the application of DR and particularly DR mimetics for improving public health. Although DR has not yet been shown unequivocally to extend lifespan in primates it does delay the appearance of biomarkers of age in rhesus monkeys (Roth et al. 1999; Lane et al. 2000) and may have health benefits in humans (Fontana et al. 2004). Application of DR during development would be likely to reduce fitness and hence late-onset DR would be preferable. Much work is being carried out on elucidating the genetic pathways affected by DR, e.g. (Pletcher et al. 2002), in the hope of finding drug targets that will mimic the response to DR without the need for a reduction in nutrient intake. If

DR extends lifespan by reducing a transient risk of death in mammals then, consequently, the length of time a DR mimetic would be needed to generate health benefits is reduced. Woody Allen once said of ageing 'You can live to be a hundred if you give up all things that make you want to live to be a hundred.' The alternate view was taken by Mae West, who said 'You're never too old to become younger.' For *Drosophila* at least, it looks as though Mae West was right.

3.4.7 DR as an adaptive strategy – late onset DR and the plasticity in response

The work presented here put focus on the ability of DR to rapidly reduce mortality rates when applied midway though life. *Drosophila* show a plastic response of mortality rates to changes in nutrition, in effect switching between two different ageing profiles. Evolutionary theory states that the ability to extend lifespan under periods of food shortage may be an adaptive strategy (Harrison and Archer 1988; Holliday 1989; Masoro and Austad 1996). A shift in the allocation of resources from reproduction to somatic maintenance when food is scarce would increase lifetime reproductive success, allowing survival to more plentiful times when reproduction would again be the most successful strategy. The link between DR and reproduction in *Drosophila* is discussed in chapter 4. However, it is worth noting the conditions necessary for the response of lifespan to DR to be an evolutionary adaptive strategy. First, reproduction should be costly during times of low resource availability and second, the survival of offspring must be impaired when food is limited (Shanley and Kirkwood 2000; Kirkwood and Shanley 2005). If this is indeed true, it suggests that the effects of adult onset DR are more relevant from an evolutionary perspective than chronic DR studies started on immature individuals. Since the theory predicts that few offspring will be produced in times of famine and that their survival rates will be poor, the effect of DR on lifespan can only be adaptive if beneficial effects are seen when adults are subjected to DR midway through life. This may therefore make it likely that the rapid reductions in risk of death seen in *Drosophila* when DR is applied late in adulthood are conserved across species.

3.4.8 Loss of heterogeneity and late life mortality plateaus

Population-wide age-specific mortality rates are often presented as fitting a simple Gompertz model (Lee 1992), in which, once they begin to accelerate they do so exponentially throughout life. However, in many population studies and large scale

laboratory experiments it has been shown that, rather than increasing throughout life, mortality rates can decrease at very advanced ages (Pletcher and Curtsinger 1998; Johnson et al. 2001; Gendron et al. 2003; Vaupel et al. 2004). Although the cause of this 'negative senescence' remains unknown, one theory is that it is due to a loss of heterogeneity within cohorts at advanced ages (Brooks et al. 1994; Khazaeli et al. 1998; Service 2000; Service 2004). If a population is heterogeneous with respect to the fragility of individuals within it then, as the population ages, the most fragile individuals are likely to die first. This will have the effect of gradually reducing the heterogeneity over the course of time, leading to populations comprised of very old individuals that represent the most hardy of the initial cohort. It is this loss of heterogeneity that had been suggested to be the underlying cause of late life mortality plateaus.

However, the data presented in this chapter on late life DR in *Drosophila* do not support this hypothesis. The loss of heterogeneity theory would predict that the cohort of flies with a history of full-feeding subsequently switched to DR late in life would be *less* heterogeneous that the chronically dietarily restricted control group, since the more fragile individuals would have died whilst exposed to the mortality inducing full-feeding. These weaker individuals would still be present in the DR control group since they have no history of full-feeding and have therefore not been filtered out. Loss of heterogeneity theory therefore predicts that the age-specific mortality trajectory of the cohort switched from full-feeding to DR would plateau earlier than the DR control group because the switch group is less heterogeneous. However, as shown, the mortality rates of the switched and non-switched groups are indistinguishable 48 hours after the switch (Figure 3.3.2a). That there is an apparent plateau in these mortality trajectories late on, and that two cohorts display indistinguishable trajectories despite the disparity in heterogeneity between them is an interesting observation and experiments of this kind could be used to test the underlying causes of negative senescence further. Other theories on why mortality rates plateau late on are based on evolutionary theory of ageing and the decline in the force of natural selection with age (see Section 1.2.3), and it may be that these, rather than loss of heterogeneity, will ultimately prove to be responsible for the reduction in mortality rate at very late ages (Partridge and Mangel 1999; Rose et al. 2002).

3.4.9 Application of mortality switch experiments to investigate the mechanisms behind other modifiers of lifespan

The finding that the effect of DR can be acute raises the issue of whether the same is true of the effects of mutations that increase lifespan. Directly addressing this issue is much more difficult than for an environmental intervention such as DR, because the effect of the mutation would have to be switched part way though life. However, methods for inducible induction or suppression of gene expression are now widely available in model organisms, and should be used to address the issue.

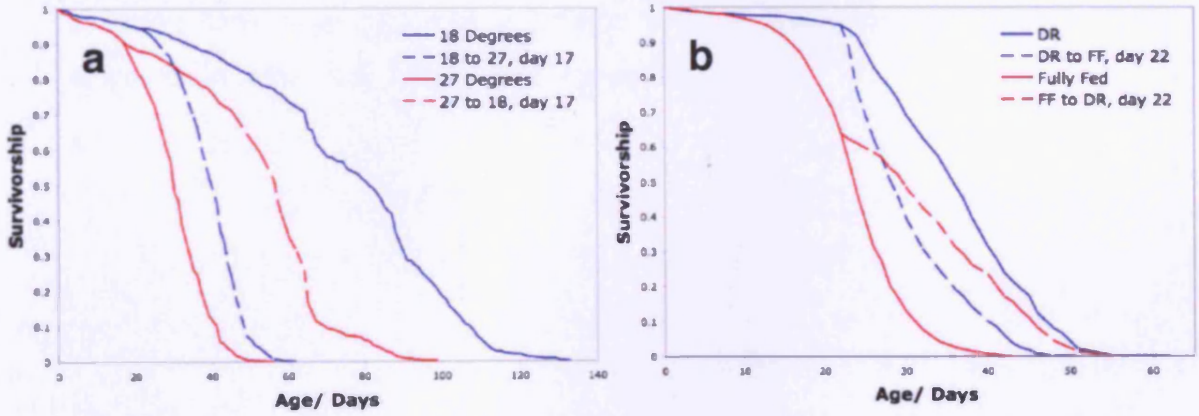
There are technical difficulties associated with this experimental design that must be considered however. For example, mRNAi inhibition of gene expression (via degradation of the transcript) can be achieved in the model organism *C. elegans* by growing the worms on bacterial lawns of *E. coli* that express double-stranded RNA sequence from the gene of interest (Carthew 2001). This elegant system therefore seems to lend itself well to studying the effects of late onset inhibition of genes that extend lifespan. Indeed it has been used in the past to determine when in life reducing insulin signalling (by reducing transcription of the insulin-like receptor *daf-2*) results in an increase in lifespan (Dillin et al. 2002). However, the response to mRNAi in *C. elegans* is highly heterogeneous within a population (J McElwee, personal communication), leading to problems with using this method of mRNAi in switch experiments of the type described for DR.

In an experiment comparing two cohorts of worms, one with lifetime exposure to mRNAi of a gene that increases lifespan and one in which this mRNAi had recently been applied, the cohorts would differ not only with respect to their history of expression levels of this gene, but also in their heterogeneity in the ability to respond to mRNAi. Any deaths in the control group that was subjected to mRNAi throughout life would more likely be in individuals that did not respond well to the mRNAi, since these worms would not have the reduction in gene expression that extends lifespan and reduces frailty. Therefore, this chronic group would become progressively less heterogeneous in the ability to respond to mRNAi and become biased towards individuals that responded well to the treatment. The switched cohort would still contain the original mix of responders and non-responders however.

Despite the need for experimental caution when designing switch experiments to test other modifiers of ageing, only by doing switches between treatments midway though life and looking at age-specific mortality can we distinguish between interventions that slow the rate of irreversible damage accumulation and those that remove a transient risk factor. Both types of intervention increase median lifespan when applied midway through life, as can be seen by the effects of DR or reduced temperature in *Drosophila* (figure 3.4.5). Therefore this observation is not sufficient to distinguish between these two mechanisms. At present, it is not proven that any genetic intervention that extends lifespan does so by reducing the rate of accrual of irreversible, ageing-related damage.

Figure 3.4.5. Survivorship of *Drosophila* switched between either thermal or dietary regimes midway though life.

a. Temperature slows the rate of ageing (Mair et al. 2003) and switching from 27 to 18 degrees at day 17 extended median lifespan, whilst the reciprocal switch from 18 to 27 degrees reduced it. These data were taken from (Mair et al. 2003) and the experiment carried out by P. Goymer. **b.** Similar patterns were seen in the DR switch, despite DR reducing an acute risk of death and not slowing the rate of ageing. Switching from full-feeding to DR at day 22 extended median lifespan, whilst the reciprocal switch from DR to FF reduced it.



3.4.10 Conclusions and future directions – identifying the nutrient related acute risk of death

The data presented here show that, not only does DR extend lifespan in *Drosophila* when applied midway through life, but that within 48 hours of switching to a DR

regime flies are no more likely to die than those that have been on DR throughout their adult life. Full-feeding does not cause any irreversible damage in this species but rather induces an increased risk of death at all ages that is acute and reversible.

These data also give direction as to how to identify the possible mechanism(s) by which DR extends lifespan in fruit flies. As is the case for mammals (Richardson and McCarter 1991), many physiological factors may both change with age and have this change delayed by DR in *Drosophila*, either by altering the set-point or rate. However, only those that reverse rapidly when dietary regime is switched, mapping to the mortality rate changes, can be the nutrient dependent risk factor that is removed to cause life-extension by DR in *Drosophila melanogaster*. An obvious candidate for this risk factor is reproductive output, which rapidly changes in response to nutrient intake in fruit flies (Partridge et al. 1987; Chippindale et al. 1993; Chapman et al. 1994) and will be discussed in the next chapter.

Chapter 4. Lifespan extension via dietary restriction in *Drosophila* is not due to reduced egg-production, movement or courting behaviour.

Abstract

Dietary restriction (DR) extends lifespan in a wide range of organisms. DR also reduces daily and lifetime fecundity. The latter may be an evolutionary adaptation to survive periods of food shortage. Reproductive rate is often negatively correlated with lifespan, and a reduced cost of reproduction could be the mechanism by which DR extends lifespan. I show here that the rapid (48 hour) changes in age-specific mortality, previously seen in fertile female *Drosophila* switched between full-feeding and DR, were not accompanied by concurrent changes in egg-production. Furthermore, these rapid changes in age-specific mortality in cohorts of fertile, wild type females were also seen in females in which vitellogenesis was blocked by the mutant ovo^{D1}. DR did not alter activity or same-sex courtship rates in male *Drosophila*. These results demonstrate that: 1) Reduced reproduction is not necessary for lifespan extension by DR in *Drosophila* females, or that the relevant aspects of reproduction act upstream of the ovo^{D1} mutation and were therefore not blocked in these experiments, 2) DR does not extend lifespan of male *Drosophila* by reducing costs associated with courtship/ activity. These data are published and discussed in (Mair et al. 2004b) (See Appendix 3).

4.1 Introduction

The physiological mechanisms by which DR reduces the risk of death remain unclear. Although organisms are longer-lived under DR, they are often less fecund. DR has been shown to reduce daily and lifetime fecundity in *C. elegans* (Klass 1977) and *Drosophila* (Chippindale et al. 1993; Chapman and Partridge 1996), and to delay reproductive maturity in rats (Osborne et al. 1917; Holehan and Merry 1985). Experimentally increased reproductive activity is associated with decreased lifespan in a diversity of species (Williams 1966; Rose 1984; Gustafsson and Part 1990; Stearns 1992; Leroi 2001; Lessells and Colgrave 2001; Nager et al. 2001; Barnes and Partridge 2003; Partridge et al. 2005a).

That nutrition, reproduction and lifespan are so clearly interlinked prompted the suggestion that lifespan extension by DR is an evolutionary adaptation to withstand periods of food deprivation. When food is scarce, re-allocation of resources away from current reproduction to somatic maintenance would increase survival and future fecundity, and could therefore increase lifetime reproductive success (Harrison and Archer 1988; Holliday 1989; Masoro and Austad 1996; Shanley and Kirkwood 2000; Kirkwood and Shanley 2005). If, in comparison to situations in which resources are high, the numbers of offspring that can be produced in times of famine are lowered, their survival rates reduced or the threat of reproduction to parental survival is increased, it would be more profitable for an individual to lower or cease reproduction and divert resources to maintaining the soma (Shanley and Kirkwood 2000). This would allow survival to more plentiful times when increased reproduction would once again be the most successful strategy.

In this chapter I tested the idea that increased survival under DR is a consequence of the removal of the risk of death caused by high reproduction activity. In *Drosophila* females, egg-production (Maynard-Smith 1958; Lamb 1963; Partridge et al. 1987; Sgrò and Partridge 1999), exposure to males (Partridge and Fowler 1990) and mating (Fowler and Partridge 1989; Chapman et al. 1995) increase mortality and are therefore costly aspects of reproduction. Both egg-production and mating rate increase with increasing nutrition (Partridge et al. 1987; Harshman et al. 1988; Trevitt et al. 1988; Chippindale et al. 1993; Chapman and Partridge 1996).

Furthermore, egg-production has been shown to be rapidly inducible/ reversible within two days of a change in nutritional yeast intake in female *Drosophila* (Chippindale et al. 1993; Chapman et al. 1994; Drummond-Barbosa and Spradling 2001; Good and Tatar 2001). This is similar to the time required for mortality rates to dramatically reduce after the application of a DR regime in adult *Drosophila* (chapter 3). Hence increased reproductive output is an obvious candidate for the nutrient-dependent reversible risk factor that causes high mortality under fully-fed conditions (Vaupel et al. 2003; Mair et al. 2004a; Rauser et al. 2004).

I tested this hypothesis in *Drosophila* by performing two experiments. First, if lifespan extension by DR is a consequence of a reduced cost of reproduction, then changes seen previously (chapter 3) in mortality rates when dietary regime was switched during adulthood should be mirrored by concurrent changes in egg-production. I therefore measured changes in age-specific mortality *and* fecundity in response to switches in dietary regime of middle-aged, once-mated, wild type, female, Dahomey flies. Second, if the hypothesis is correct, removing egg-production, a costly aspect of reproduction in female *Drosophila* (Maynard-Smith 1958; Lamb 1963; Partridge et al. 1987; Sgrò and Partridge 1999), should partially or completely block the longevity-extension seen under DR. I tested whether halting egg-production in female flies using the female-sterile mutation ovo^{D1} 1309 blocked the acute responses of age-specific mortality to changes in dietary regime seen previously in middle-aged, fully-reproductive flies (chapter 3). ovo^{D1} encodes a zinc finger protein required for female germ line development (Oliver et al. 1987; Mevel-Ninio et al. 1991). Oogenesis is arrested prior to or at stage 4 (King 1970) in ovo^{D1} females and vitellogenesis is completely blocked. The mutant has no effect on somatic ovary tissue (Oliver et al. 1987). If the rapid reduction in mortality seen in fertile females switched to DR in chapter 3 was due to the cessation of vitellogenesis and egg-production, I would not expect to see such a rapid response in the ovo^{D1} mutant in which these processes are blocked.

Male *Drosophila* elicit mating with females by a series of behavioural displays including wing vibrations (courtship 'songs'), wing extensions, chasing, licking and attempted copulations (Hall 1994). This courting activity is costly to males (Cordts and Partridge 1996) and can reduce their ability to respond to infection (McKean and

Nunney 2001). Male courting activity may increase as food nutrient concentration is increased and it has therefore been suggested that males kept on a DR diet show reduced mortality rate due to reduced reproductive activity (Vaupel et al. 2003). Males in single-sex groups show lifespan extension under DR (Mair et al. 2003; Magwere et al. 2004) and only juvenile males elicit courtship from other males, with mature males producing a pheromone that inhibits courtship (Tompkins 1984; Curcillo and Tompkins 1987). This makes male courting activity an unlikely candidate for the increased risk of death of males on control food compared to those under DR. However, male:male interactions can reduce longevity in insects (Gaskin et al. 2002) and these interactions may be effected by nutrition. Therefore, to test if the response of lifespan to DR in males was due to reduced male:male interactions I measured courtship/ aggression activity and movement in male *D. melanogaster* maintained on different dietary regimes.

4.2 Methods

4.2.1 Fly stocks

The ovo^{D1} 1309 mutant was obtained from Bloomington stock centre. The mutant was twice backcrossed into the Dahomey genetic background. To produce females that were sterile due to the presence of ovo^{D1}, males from the out-crossed ovo^{D1} stock were crossed to Dahomey virgin females (see section 2.3.2).

4.2.2 Effects of the implementation/ removal of a DR regime during adulthood on fecundity and age-specific mortality of fertile females

To collect sufficient numbers of experimental flies for age-specific mortality analysis, larvae were raised on bottles of standard SY food medium (section 2.2.1) at a density of 400-450 per 200ml bottle at standard larval density (section 2.3.4). After eclosion, flies were mated for 24 hours on standard SY food. Females were then separated using light CO_2 anaesthesia and allocated to either a fully-fed (1.5Y 1.5S) or DR (0.65Y 0.65S) regime (see Table 4.2.1 for sample sizes). Experimental females were kept in 200ml bottles on 35ml food at a standard density of 100 (±10) throughout at 25°C on a 12:12 light:dark cycle. Initial cohort sizes (N_0) were calculated as the summed death and censor observations over all ages. Flies were transferred onto new food every 2 days and scored for deaths on 6 out of every 7

days. At days 14 & 22, subgroups were switched to the opposite food regime. Age-specific mortality was measured throughout in all treatments.

Female fecundity was measured at time points throughout the experiment: before switches in food regime and over the course of the switch to assess if changes to age-specific mortality were mirrored by changes in reproductive output. Fecundity was measured by counting the number of eggs produced by female *D. melanogaster* in 24 hours. Females were taken from the lifespan experiment and housed individually in vials containing the appropriate food medium and left at 25°C for 24 hours. Females were then removed and the number of eggs per vial was counted using a dissecting scope at low magnification. Hand counters were used to improve accuracy. Any vials not processed on the day that the females were removed were stored at -80°C and processed later. Any vials in which females died during the 24 hours used for the fecundity assays were discarded from the experiment. All females removed from the lifespan assays for fecundity measurements were censored from the lifespan data on the day of removal and were not returned to the experiment.

4.2.3 Effects of the implementation/ removal of a DR regime during adulthood on age-specific mortality of sterile females

Larvae were raised on bottles of standard SY food medium (section 2.2.1) at a density of 400-450 per 200ml bottle at standard larval density (Section 2.3.4). Experimental flies were collected over an 8-hour period at eclosion and transferred without anaesthesia to standard SY food for 24 hours. ovo^{D1} 1309 females were then collected using light CO_2 anaesthesia and assigned to either a fully-fed (1.5Y 1.5S) or DR (0.65Y 0.65S) regime. Experimental females were kept in 200ml bottles on 35ml food at a standard density of 100 (±10) throughout at 25°C on a 12:12 light:dark cycle. Initial cohort sizes (N_0) were calculated as the summed death and censor observations over all ages (see Table 4.2.2 for sample sizes). Flies were transferred onto new food every 2 days and scored for deaths on 6 out of every 7 days. Subgroups of the fully-fed and DR cohorts were reciprocally switched between food treatments at days 27 & 35 post-eclosion. Age-specific mortality (section 2.4.2) was measured throughout in all treatments.

Table 4.2.1. Wild type female experiment sample sizes (Nx):

Age (x)/ Days	Nx DR	Nx DR to FF	Nx Fully-fed (FF)	Nx FF to DR
0	2967		2971	
14	1809	889	1708	791
22	678	690	435	412

Table 4.2.2. *ovo*^{D1} female experiment sample sizes (Nx):

Age (x)/ Days	Nx DR	Nx DR to FF	Nx Fully-fed (FF)	Nx FF to DR
0	3506		3569	
27	2074	1176	1964	849
35	794	873	503	439

4.2.4 Male:male courtship and activity on different nutritional regimes

Male Dahomey *Drosophila* were raised at standard density (2.3.4) on standard SY medium (2.2.1) at 25 degrees centigrade. Flies were left to eclose over a 24-hour period and then transferred without anaesthesia to fresh bottles of SY medium where they were left for a further 24 hours to mate. At this point flies were separated by sex (2.3.2) and male flies were put into vials containing either 1.5Y 1.5S medium or 0.65Y 0.65S medium at a density of 10 flies per vial. 50 vials of each food type were set up, a total of 500 flies per treatment. Flies were tipped onto new food vials three times a week and 10 days after being put onto the different food media, courting, movement and flight assays were performed.

Activity and courtship of male flies fed different food concentrations was measured by direct observation. Prior to mating, male *Drosophila* undergo a characteristic courtship display that includes wing vibrations (courtship 'songs'), wing extensions, chasing, licking and attempted copulations. The wing extension behaviour is easily identifiable to the naked eye. Ten flies were placed in shell vials and a total of 40 vials per food concentration were used. Vials were set up on shelves within the controlled temperature and humidity room at 10am and left undisturbed for one hour. At this point vials were observed in turn and the number of flights made in a 10 second time-period was recorded. In addition to this, the fly nearest to a marked spot on the vial wall was observed and categorized as either moving (walking) or

stationary. This fly was also scored as having its wings in the normal retracted position, the courting-related one wing extension (Hall 1994) or the aggressive 'scissor' position (Hoffmann 1987; Chen et al. 2002). Vials were progressively scanned one at a time and once all vials had been observed the process was repeated. Observations were carried out once in the morning and once in the afternoon on three consecutive days such that for each treatment there was a total of 480 observations. For these assays, 40 vials from each food treatment group were selected and any dead flies in these vials were replaced with same age live flies from the remaining 10 'spare' vials from that treatment, such that throughout the assays all vials contained 10 live flies.

4.3 Results

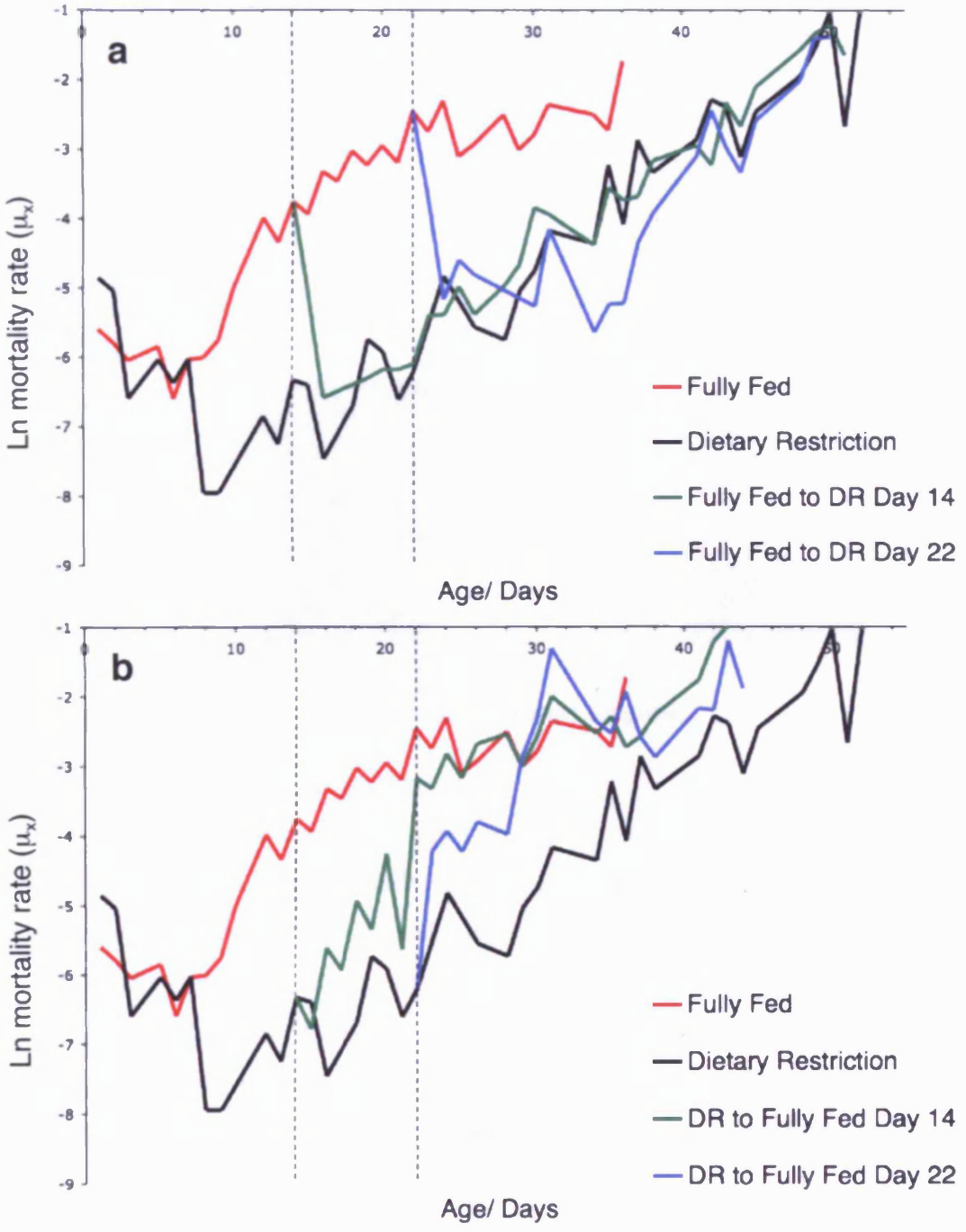
4.3.1 Effects of the implementation/ removal of a DR regime during adulthood on fecundity and age-specific mortality of fertile females

Switching once-mated females from full-feeding to DR at day 14 or 22 of adulthood resulted in rapid (within 24 hours) reductions in age-specific mortality to those of females maintained permanently under DR throughout their adult life (Figure 4.3.1a, Cox regression. Day 14 switch: P = 0.22, DR n = 1809, fully-fed to DR switch n = 791, risk ratio = 1.043 [95% Confidence Intervals: 0.975, 1.116]; Day 22 switch: P = 0.052, DR n = 678, fully-fed to DR switch n = 435, risk ratio = 0.918 [95% CI: 0.840, 1.001]) in a repeat of the result seen in previous work (chapter 3).

When formerly DR flies were switched to full-feeding, mortality increased rapidly in comparison to that of permanently DR flies (Figure 4.3.1b). However, mortality remained lower than that of flies that had been fully-fed throughout adult life (day 14 switch, P < 0.0001, fully-fed n = 1708, DR to fully-fed switch n = 889, risk ratio = 0.769 [95% CI: 0.723, 0.819]; day 22 switch, P = 0.002, fully-fed n = 435, DR to fully-fed switch n = 690, risk ratio = 0.849 [95% CI: 0.782, 0.924]) also seen previously (chapter 3). After both switch points, permanently fully-fed flies had significantly higher mortality rates than those flies permanently under DR (from day 14 switch onwards: P < 0.0001, DR n = 1809, fully-fed n = 1708, risk ratio = 2.553 [95% CI: 2.376, 2.744]; from day 22 switch onwards: P < 0.0001, DR n = 678, fully-fed n = 435, risk ratio = 2.056 [95% CI: 1.875, 2.252]).

Figure 4.3.1. Age-specific mortality rates of female *Drosophila melanogaster* in response to the instigation or removal of a DR regime.

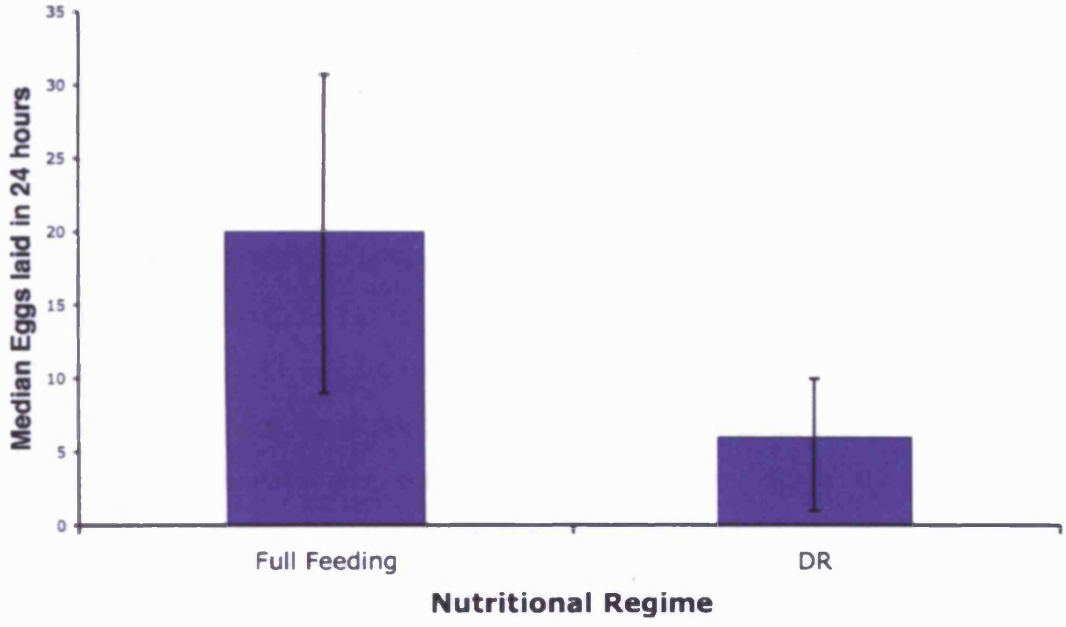
Mortality rate (μx) is plotted on the natural log scale, because it increases exponentially with age. Dotted vertical lines represent days on which food regimes were switched. Mortality curves were truncated when n<40. **a.** Previously fully-fed reproductive females showed a rapid reduction in mortality rate when switched to DR at 14 or 22 days of adulthood. 48 hours after the switch, mortality trajectories of switched flies were indistinguishable from those of flies permanently under DR. **b.** Reciprocal switches of reproductive females from DR to full-feeding resulted in rapid, marked increases in mortality rate.



Fully-fed, young, once-mated, wild type females laid significantly more eggs than same-age females on DR (Wilcoxon/ Kruskal-Wallis test: chi squared = 32.93, 1.d.f, $P<0.0001$). At day 7 post-eclosion, the median number of eggs laid per female per day was 20 in the fully-fed food group (n = 60) and 6 for females under DR throughout adulthood (n = 60) (Figure 4.3.2). For both treatments, the percentage of 7-day old females laying eggs was 97.1%.

Figure 4.3.2. Median number of eggs laid in 24 hours by 7 day old females on different dietary regimes.

Error bars represent inter-quartile range. Flies on full-feeding laid significantly more eggs than DR flies.



However, the percentage of females laying eggs decreased with age in all groups and, after day 14, many females had ceased egg-production entirely. This makes tables of median eggs laid uninformative and therefore the data is presented as the percentage of females laying eggs at each time point measured (Table 4.3.1). There was no significant difference in the percentage of females laying eggs on different food regimes on any of the four days following the food switches (Chi squared test, 3 degrees of freedom, $P>0.05$ in all cases, see Table 4.3.1 for details).

Table 4.3.1. Percentage of females that laid eggs in 24-hour periods on different food regimes at different ages.

a. There was no significant difference in the percentage of females laying eggs on different food regimes during the four days after the 1st food switch. **b.** There was no significant difference in the percentage of females laying eggs on different food regimes during the four days after the 2nd food switch.

a.

Age/ Days	Fully-Fed (FF)	FF to DR on day 14	Dietary Restriction	DR to FF on day 14	Chi sq	P Value
14-15	65	60	55	46.7	4.489	0.213
15-16	31.7	55	50	46.7	7.457	0.059
16-17	34.5	40	40.7	50	3.030	0.387
17-18	37.3	41.7	51.6	46.6	2.790	0.426

b.

Age/ Days	Fully-Fed (FF)	FF to DR on day 22	Dietary Restriction	DR to FF on day 22	Chi sq	P Value
22-23	44.1	35.6	41.4	44.8	1.284	0.733
23-24	38.3	32	31.3	30	0.876	0.831
24-25	40.4	46.9	38	34	1.763	0.623
25-26	20	38.8	32	36	4.951	0.175

No significant difference was seen between the numbers of eggs laid by females switched on day 14 from DR to full-feeding and the permanently DR females on each of the four days post-switch (Wilcoxon/ Kruskal-Wallis test, modified Bonferroni adjustment for multiple comparisons, $P>0.05$ in all cases, $n=60$ for each treatment on each day). For the reciprocal switch, no significant difference in numbers of eggs laid was seen between females switched from full-feeding to DR on day 14 and the permanently fully-fed flies in the four days following the switch except for day 15 (Wilcoxon/ Kruskal-Wallis test, modified Bonferroni adjustment for multiple comparisons, $P>0.05$ in all cases, $n=60$ for each treatment on each day). On day 15, a significantly higher number of eggs were laid by females that had been switched from full-feeding to DR on day 14 compared to those permanently fully-fed throughout adulthood (median number of eggs laid in 24 hours: switched group= 1, fully-fed= 0, Wilcoxon/ Kruskal-Wallis test $P = 0.017$, $n = 60$ for each treatment). On day 14, the fully-fed females laid a significantly greater number of eggs than those permanently under DR ($P= <0.001$), but this difference was not apparent on days 15-17 ($P>0.05$ in all cases). In the late switch at day 22, there was no significant difference in the numbers of eggs laid between any of the treatments on

any of the four days following the switch (Wilcoxon/ Kruskal-Wallis test, modified Bonferroni adjustment for multiple comparisons, $P > 0.05$ in all cases, $49 < n < 59$ for each sample). In summation, reciprocal switches in food nutrient concentration caused mortality changes that were rapid, dramatic and consistent at both day 14 and day 22 but these were not mirrored by consistent patterns in egg laying.

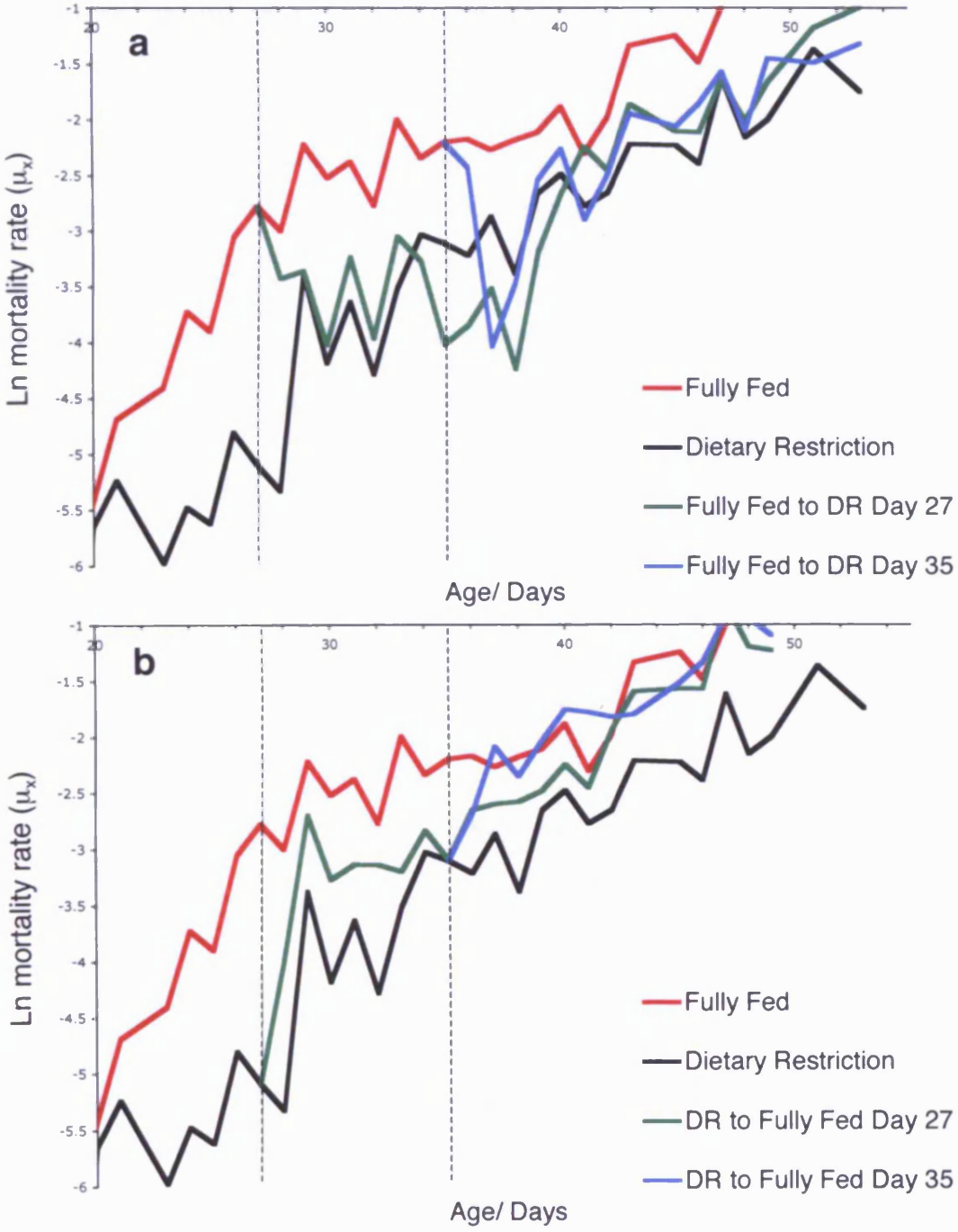
4.3.2 Effects of the implementation/ removal of a DR regime during adulthood on age-specific mortality of sterile females

Females heterozygous for the dominant ovo^{D1} 1309 mutation did not lay any eggs, yet DR extended the lifespan of sterile females and switches between full-feeding and DR at days 27 and 35 of adulthood generated the same rapid switch in age-specific mortality (Figure 4.3.3) previously seen in fertile wild type females (chapter 3). Within 48 hours of being switched to DR, previously fully-fed ovo^{D1} 1309 females were no more likely to die than flies under DR throughout adulthood as shown by a risk ratio between the groups of 1 (Figure 4.3.3a, Cox regression. Day 27 switch: $P = 0.15$, DR $n = 2074$, full-feeding to DR switch $n = 849$, risk ratio = 1.034 [95% Confidence Intervals: 0.988, 1.082]; Day 35 switch: $P = 0.153$, DR $n = 794$, full-feeding to DR switch $n = 439$, risk ratio = 1.045 [95% CI: 0.984, 1.110]).

Reciprocal switches from DR to full-feeding resulted in rapid increases in age-specific mortality (Figure 4.3.3b). Flies switched from DR to full-feeding at day 27 maintained a lower mortality than that of flies that were fully-fed throughout adult life (as indicated by a lowered risk ratio), whilst for the day 35 switch, the mortality of flies switched from DR to full-feeding became indistinguishable from that of the permanently fully-fed flies within 48 hours (day 27 switch, $P < 0.001$, Fully-fed $n = 1964$, DR to fully-fed switch $n = 1176$, risk ratio = 0.807 [95% CI: 0.775, 0.840]; day 35 switch, $P = 0.943$, fully-fed $n = 503$, DR to fully-fed switch $n = 873$, risk ratio = 1.001 [95% CI: 0.945, 1.060])

Figure 4.3.3. Age-specific mortality rates of female *Drosophila melanogaster* in response to the instigation or removal of a DR regime.

Mortality rate (μ_x) is plotted on the natural log scale, because it increases exponentially with age. Dotted vertical lines represent days on which food regimes were switched. Mortality curves were truncated when n<40. **a**. Previously fully-fed sterile ovo^{D1} 1309 females showed a rapid reduction in mortality rate when switched to DR at 27 or 35 days of adulthood. 48 hours after the switch, mortality trajectories of switched flies were indistinguishable from those of flies permanently under DR. **b**. Reciprocal switches of sterile ovo^{D1} 1309 females from DR to full-feeding resulted in rapid, marked increases in mortality rate.



4.3.3 Male: male courtship and activity on different nutritional regimes

A total of 480 observations were made for each behaviour on each food treatment. Extension of one wing is part of the courtship process in *Drosophila* (Hall 1994) and there was no significant difference in the number of observations of wing extensions between male flies kept on high or low nutrient concentration food (χ^2 = 0.504, 1 df, P= 0.478) (Table 4.3.2). The frequency of wing extensions was less than 1.1% for both treatments however, and the majority of any wing movements seen were 'scissor' wing movements that are a sign of aggression in fruit flies (Hoffmann 1987; Chen et al. 2002). Since this movement is likely to be as costly as the courting-related single-wing extension, the numbers of wing extensions and scissor movement observations were pooled for each treatment. Again, there was no significant difference in the number of wing movements seen in male flies kept on the two different food treatments (χ^2 = 0.081, 1 df, P= 0.776) (Table 4.3.2).

Table 4.3.2. Courtship activity, wing extension behaviour and movement of male *Drosophila* kept in single-sex groups on different food regimes.

No significant difference was seen between flies on full-feeding or DR for any of the behaviours assayed.

	Courting	Not Courting
Full-feeding	5	475
DR	3	477
	Wing Movement	No Wing Movement
Full-feeding	25	455
DR	27	453
	Moving	Stationary
Full-feeding	179	301
DR	157	323

No significant difference was seen in the movement of flies on the two food treatments (χ^2 = 2.216, 1 df, P= 0.137) (Table 4.3.2). The number of flights observed was not normally distributed, because the majority of observations were zero (Table

4.3.3). However, non-parametric analysis revealed that there was no significant difference in the flight activity between male flies on full-feeding and DR (Wilcoxon/ Kruskal-Wallis test P =0.665, $\chi^2 = 0.187$).

Table 4.3.3. Interquantile range of the observed number of flights made by male *Drosophila* on different food regimes.

	10%	25%	Median	75%	90%	Maximum
Full-feeding	0	0	0	1	2	6
DR	0	0	0	1	2	6

4.4 Discussion

I showed in chapter 3 that, in *Drosophila*, DR extends lifespan by removing some increased risk of death that is seen on high nutrient concentration food. Previous work has shown that egg-production can be induced/ reversed rapidly in female *Drosophila melanogaster* by changing nutritional regime (Chippindale et al. 1993; Chapman et al. 1994; Drummond-Barbosa and Spradling 2001; Good and Tatar 2001). Hence increased egg-production has been suggested to be the nutrient-associated risk factor that is removed under DR (Vaupel et al. 2003). If this were the case, 1) whenever there is a switch in mortality rate in flies moved between nutritional regimes there would be concurrent changes in egg-production and 2) female flies that do not lay eggs would not show the same rapid response of mortality rate to changes in nutrition as fertile flies. Neither of these predictions were supported by the data in this chapter.

4.4.1 Effects of the implementation/ removal of a DR regime during adulthood on fecundity and age-specific mortality of fertile females.

I saw typical patterns of age-specific mortality when fertile flies were switched to DR from full-feeding (Figure 4.3.1), but these were not accompanied by rapid switches in egg-production. At the first switch point, the chronic fully-fed and DR groups differed in their rates of egg-production, with the fully-fed females laying more eggs, but this difference was not maintained in the four days following the

switch. There was no evidence of a rapid change in the rate of egg-production in the switched females. At the time of the first switch, many females were still fertile, but their rates of egg-production did not switch over with the changes in mortality.

At the second switch point, most females were sterile and there were no significant differences between any of the groups in their rates of egg-production, yet their mortality rates showed the usual rapid response to switches in nutritional regime. Although fully-fed flies did show higher levels of egg-production early in life (Figure 4.3.2), in middle-age, when reciprocal switches in food regime were performed, flies were no longer laying eggs in any great number and there was no consistent pattern in egg-production at either switch. The lack of correlation between mortality rates and fecundity is in contrast to what would be predicted if egg-production was responsible for the elevated death rates of fully-fed female *Drosophila* compared to those on DR.

4.4.2 Effects of the implementation/ removal of a DR regime during adulthood on age-specific mortality of sterile females

Despite the fact that oogenesis is blocked at stage four (King 1970) in ovo^{D1} females, I observed the same rapid response of mortality to switches in nutritional regime (Figure 4.3.3) as seen previously in fertile, wild type females (Chapter 3 & section 4.4.1). 48 hours after the application of DR for the first time at day 27 or 35 of adulthood, previously fully-fed ovo^{D1} females were no more likely to die than those that had been subjected to DR throughout adult life. Since the response of age-specific mortality seen in ovo^{D1} females subjected to changes in nutritional regime was the same as that seen previously in fertile, wild type females in chapter 3, the data provide further evidence that the increased risk of death in fully-fed females could not have been due to increased vitellogenesis or egg-production.

4.3.3 Male:male courtship and activity on different nutritional regimes

Previous work has shown mortality also decreases in dietarily restricted male *Drosophila* kept without females (Mair et al. 2003; Magwere et al. 2004). The main costs of reproduction in male *Drosophila* are courting and movement (Cordts and Partridge 1996). Only juvenile males elicit courtship from other males, with mature males producing a pheromone that inhibits courtship (Tompkins 1984; Curcillo and

Tompkins 1987). In accordance with this, these results demonstrated that males kept in single-sex groups showed very low levels of courtship activity and that there was no difference in male:male courtship between males kept on different food regimes. Physical activity of male *Drosophila* was not greater on high nutrient concentration food than under DR. It is therefore unlikely that reduced reproductive activity is responsible for lifespan extension by DR in male flies kept without females as previously suggested (Vaupel et al. 2003), unless the relevant aspect is physiological rather than behavioural, for example if different diets result in different rates of sperm/ accessory protein production.

4.4.4 The effect of removing different costs of reproduction on the extension of lifespan by dietary restriction in Drosophila

The data presented in this chapter demonstrate that experimentally reducing egg-production in female flies did not prevent lifespan extension by DR. However, they do not show whether blocking costly aspects of reproduction can partially block this lifespan extension. To address this issue, the effect of DR on lifespan was measured in four cohorts of female *Drosophila melanogaster* in which different aspects of reproductive activity were independently suppressed (Mair et al. 2004b)[14]. 1) Fully reproductive wild type females in mixed-sex groups. 2) Once-mated fertile females thereafter kept without males, removing both the cost of mating and exposure to males. Once-mating establishes a rate of egg-production that is significantly greater than that of virgin flies (Ashburner 1989). 3) Two separate stocks of the dominant sterile mutant ovo^{D1} (Oliver et al. 1987; Mevel-Ninio et al. 1991) in which egg-production is abolished. 4) Females whose germ line had been ablated via X-irradiation as late pupae (Ashburner 1989). At eclosion, stage 7 (King 1970) is the most advanced stage of oocyte development observed in females irradiated using this technique (Ashburner 1989), hence all vitellogenesis is also blocked.

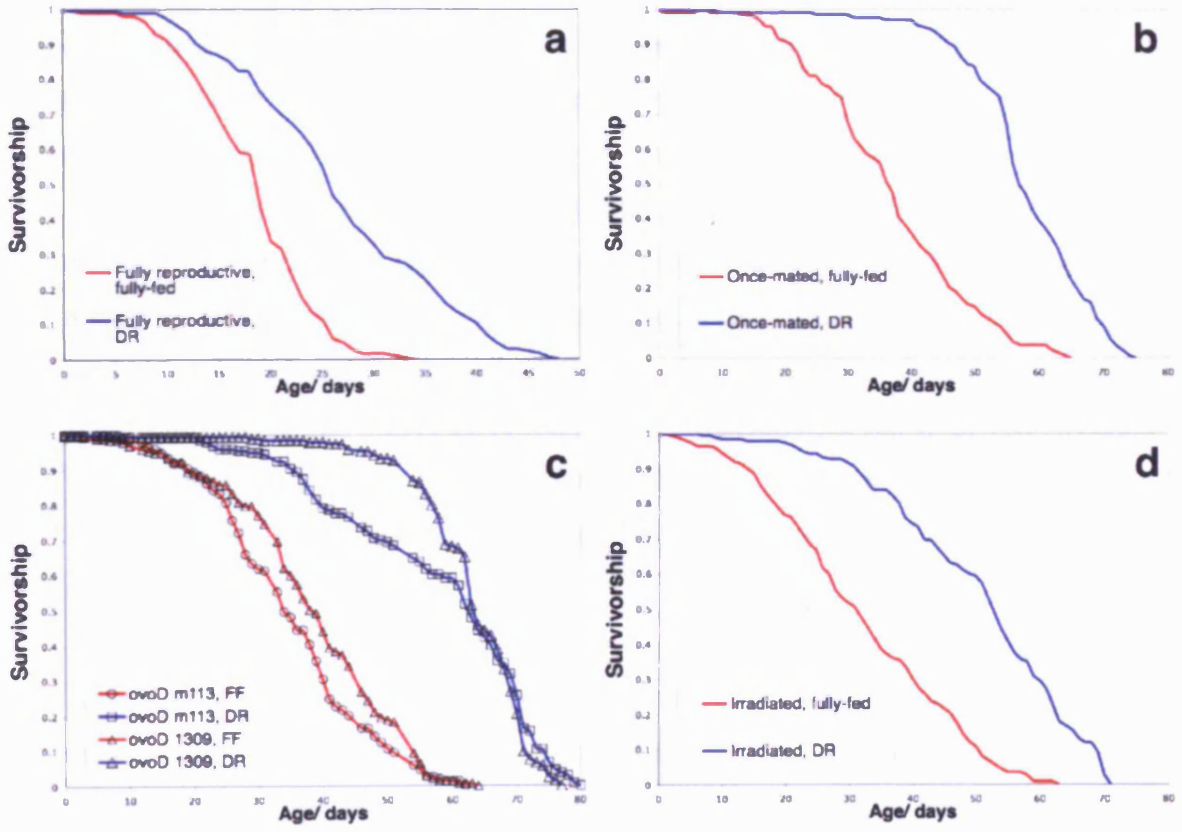
Experimentally reducing costly aspects of reproduction in female flies did not block or reduce lifespan extension by DR. Dietary restriction extended the lifespan in all female flies, regardless of the experimental manipulations of reproduction (Figure

[14] The experimental work described in this section on blocking costly aspects of reproduction was published in (Mair et al. 2004b) but carried out by T. Chapman. All analyses of the data and production of figures were done by W. Mair.

4.4.1). Percentage lifespan extension under DR was greatest in the ovo^{D1} females, despite oogenesis having been arrested and vitellogenesis completely blocked in these flies. The weakest extension of lifespan by DR was seen in the fully reproductive females kept with males throughout life. However, the lifespan of these females was greatly reduced in comparison to all the other groups of females, whose exposure to males had been removed. The main cost of exposure to males in *Drosophila* females results from mating (Fowler and Partridge 1989), attributable to a deleterious effect of seminal fluid components on survival (Chapman et al. 1995). The increased death of the females kept with males in this study may have limited extension of their lifespan by DR.

Figure 4.4.1. Response of lifespan to chronic DR of female *Drosophila melanogaster* that had increasing levels of suppression of reproductive activity.

Survivorship (l_x) is the fraction of the original cohort still alive at age x. **a**. Fully reproductive females kept with males. **b**. Once-mated, reproductive females kept without males. **c**. Sterile females due to the presence of either ovo^{D1} M113 or ovo^{D1} 1309 kept without males. **d**. Sterile females due to ablation of the ovaries by X-irradiation kept without males. N.B - This Figure is from (Mair et al. 2004b), figure produced by W. Mair, experimental work carried out by T. Chapman.



These results also showed that egg-production in once-mated females was associated with reduced lifespan, but only on a DR regime. Only ovo^{D1} females were used for this comparison, because X-irradiation had a deleterious effect on lifespan. The once-mated females lived significantly less long than either group of ovo^{D1} females when under DR, with no significant difference on full-feeding. This suggests that the levels of oogenesis or vitellogenesis undertaken by normal, fertile females are more hazardous under low nutritional conditions than they are in fully-fed females that are producing eggs at a maximum rate. Similar results have been found in female Mediterranean fruit flies (Carey et al. 2001). Indeed in general, the cost of reproduction has been suggested to be greater under conditions of low nutrition (Van Noordwijk and de Jong 1986; de Jong and Van Noordwijk 1992; Reznick et al. 2000).

4.4.5 Implications of results on evolutionary theory of DR; trade-offs, resource allocation and non-cell autonomous signalling

The 'Darwinian Demon' is a hypothetical organism that both reproduces at high frequencies from birth and is long-lived (Law 1979). Selection would favour such an organism and it would soon out-compete all else. One explanation as to why no such organism exists is that there is a 'Loi de Balancement' (Leroi 2001); organisms are constrained by the level of resource available to them, with reproduction and maintenance of self competing for limited resources from the same pool in a Y allocation model (Van Noordwijk and de Jong 1986; de Jong and Van Noordwijk 1992). The disposable soma theory of ageing suggests that, when faced with limited resources, an organism's reproductive success is increased by investing only enough resources in maintenance to facilitate reproduction, since somatic tissue is an evolutionary dead-end (Kirkwood 1977). This theory was given weight by empirical studies in *Drosophila,* where selection for early reproduction led to shorter lifespan, whilst selection for late reproduction resulted in long-lived flies (Rose 1984; Partridge et al. 1999; Sgrò and Partridge 1999), demonstrating a trade-off between these life history traits.

Extension of these ideas further led to two models as to why life-extension is seen under dietary restriction (Figure 4.4.2). First, life-extension via DR evolved as a mechanism to cope with varying levels of nutrition (Harrison and Archer 1988;

Holliday 1989; Masoro and Austad 1996; Shanley and Kirkwood 2000) and represents a shift in the allocation of resources from reproduction to somatic maintenance during food shortage (Figure 4.4.2a). Second, reduced resource availability leads to an increase in lifespan that is mediated by reduced reproduction but that is not a direct result of a trade-off with somatic maintenance (Barnes and Partridge 2003; Partridge et al. 2005a). In this model, reproduction causes damage that increases mortality (Figure 4.4.2b). When food supply is restricted there are not enough available resources for reproduction to take place, thus reproductive rate (and the levels of damage it induces) decreases, resulting in lifespan extension. The results described in this chapter, demonstrate that young, fully-fed female flies laid more eggs than those on DR, but still nutritional history had no effect on their mortality when switched to DR later in life. Although these data do not rule out either of the above hypotheses, they do suggest that the any damage caused by reproduction and responsible for the high death rates under full-feeding cannot be permanent.

Uncoupling egg-laying and nutritional uptake using sterile females did not prevent age-specific mortality changing rapidly in accordance with nutrient intake or block the extension of lifespan under DR. Thus, the plasticity of lifespan with respect to food intake is not directly linked to reproductive activity and any damage caused by high levels of reproduction cannot be the result of increased egg-production or vitellogenesis. However, not all aspects of females' reproduction were abolished in the experimental females in this study and those relevant to extension of lifespan by DR may act upstream of the interventions. In the ovo^{D1} females, proliferation of the germ line and somatic stem cells in the ovary could continue (Drummond-Barbosa and Spradling 2001), and stem cell proliferation has been shown to be critical for extension of lifespan by ablation of the germ line in *C. elegans* (Hsin and Kenyon 1999), although this is not true for *Drosophila* (A. Barnes, unpublished).

Life-extension under DR could still be mediated by reproduction in a manner unrelated to mechanical damage. In *C. elegans,* ablation of all four precursor cells that give rise to the gonad had no effect on lifespan, yet ablating the germ line precursors cells while leaving an intact somatic gonad resulted in a 60% extension of lifespan (Hsin and Kenyon 1999). Hence these authors suggest it is not the reduction

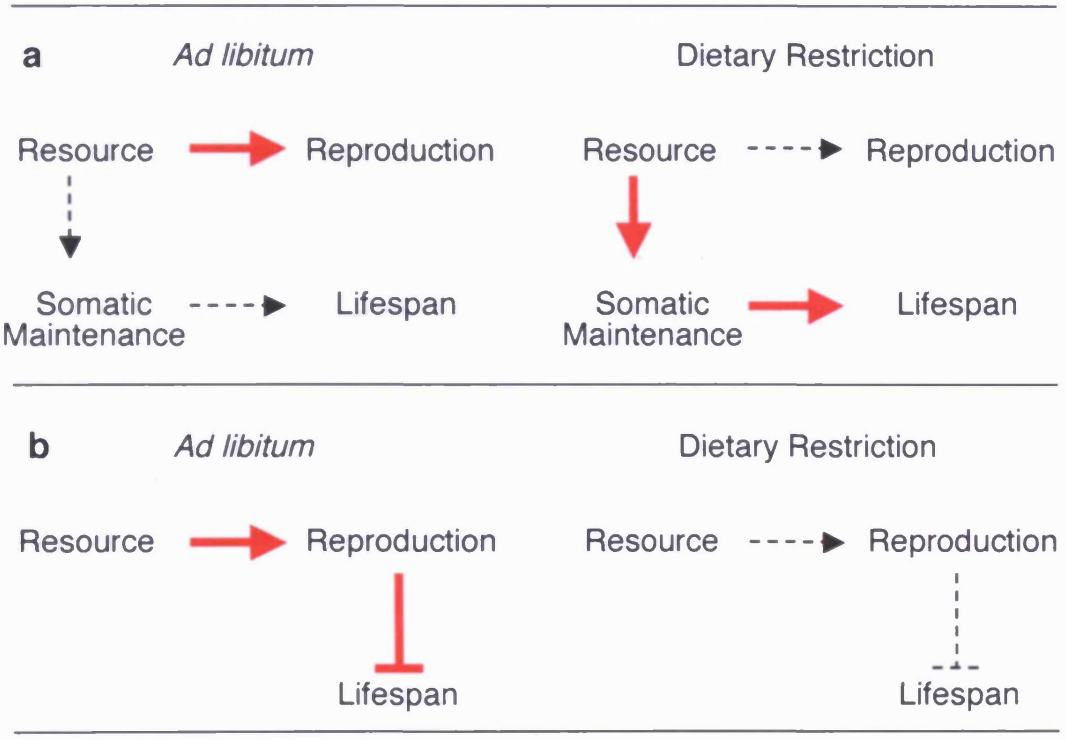
in reproduction-induced mechanical damage that extends lifespan in these worms, but rather the removal of a life-shortening signal secreted from the germ line. Extension of lifespan in these ablated worms is daf-16 (a forkhead transcription factor) dependent and, since lifespan extension from reduced insulin/ insulin like signalling (IIS) functions through activating daf-16 (Kenyon et al. 1993), it is suggested that this germ line signalling is upstream of the IIS pathway (Hsin and Kenyon 1999). It could be that in ovo^{D1} mutants there is a nutrient-dependent signal that acts upstream to determine lifespan, and that this signal functions normally despite the flies being sterile. To paraphrase a previously used analogy, if a container is being filled by a tap, removing that container will not have any effect on the flow of water from the tap (Lessells and Colgrave 2001; Barnes and Partridge 2003). Blocking reproduction may therefore not necessarily generate more resources for somatic maintenance and removing the ability to produce eggs may not block the increased cost of reproduction on full-feeding.

4.4.6 Conclusions and future directions

In summary, these results provide no support for the idea that lifespan extension via dietary restriction in female *Drosophila* is a consequence of reduced reproductive output. Increased lifespan under DR is still seen in non-laying females and there was no concurrent switch in egg-production rates when mortality rates changed in response to switches in dietary regime. Furthermore, male *Drosophila* did not show reduced levels of activity or courtship when on a DR regime. Therefore DR does not extend lifespan by changing reproductive behaviour in this species as has been suggested (Vaupel et al. 2003). Work in the Mediterranean fruit fly demonstrates that organisms can switch between different modes of ageing in response to changes in diet and reproduction (Carey et al. 1998). These data show that in *Drosophila*, even sterile females can switch between different ageing modes and that the underlying mechanism behind this is therefore more than one of simply an increased cost of reproduction when nutrients are plentiful.

Figure 4.4.2. Models of the extension of lifespan under dietary restriction mediated by reduced reproduction.

a. Extension of lifespan under DR represents a trade-off between reproduction and somatic repair. Under DR, resources are reallocated away from reproduction towards maintenance, thus increasing lifespan. **b.** Reproduction causes lifespan-reducing damage. During periods of food shortage (DR), the resource pool is insufficient for reproduction to take place, thus the damage caused is also reduced and lifespan extended. Diagram is adapted from (Partridge et al. 2005a).



Chapter 5. Lifespan extension by dietary restriction without reduced caloric intake in *Drosophila*

Abstract

Dietary restriction is often also known as calorie restriction, because it has been suggested that reduction of calories, rather than of particular nutrients in the diet, mediates extension of lifespan in rodents. I test this theory in fruit flies and show that extension of lifespan by DR in *Drosophila* is not attributable to the reduction in calorie intake. Reduction of either dietary yeast or sugar can reduce mortality and extend lifespan, but by an amount that is unrelated to the calorie content of the food, and with yeast having a much greater effect per calorie than does sugar. Calorie intake is therefore not the key factor in the reduction of mortality rate by DR in this species. Together with recent data from rodents highlighting the importance of specific amino acids on both longevity and stress resistance, these data suggest that the calorie content of food may not explain the widespread response to DR. The full extension of lifespan by DR may therefore be achievable by reducing the intake of key nutrients without reducing overall calorie intake. These data are published and discussed in (Mair et al. 2005) (See Appendix 7).

5.1 Introduction

As discussed in chapter 1, the diversity of species in which some form of food restriction has been shown to increase lifespan is staggering. Lifespan extension by DR has been reported in yeast (Jiang et al. 2000; Lin et al. 2002), nematodes (Klass 1977), fruit flies (Chippindale et al. 1993; Chapman and Partridge 1996) and mice (Weindruch and Walford 1982) along with many species less often used for laboratory research such as water fleas, spiders, fish (see Weindruch and Walford 1988 for review) and dogs (Kealy et al. 2002). However, the protocols by which DR is applied vary greatly depending on the organism studied, and still the mechanisms responsible for the resulting lifespan extension remain to be fully elucidated in any of them. This makes it unclear whether these mechanisms are evolutionarily conserved across taxa or if instead life-extension during DR is an example of convergent evolution.

Dietary restriction is often termed 'calorie restriction' because, in rodents, daily calorie intake *per se* has been implicated as the key determinant of lifespan, with the source of these calories (i.e. carbohydrate, protein or fat) being considered irrelevant (Masoro 2002). Evidence for this point of view came from two types of experiment on rats: 1) restriction of calorie intake without reduction of protein intake resulted in lifespan extension (Masoro et al. 1989), 2) no lifespan extension was seen in rats fed iso-caloric diets in which either the fat or mineral components had been reduced (Iwasaki et al. 1988b). This work has prompted many bold statements from researchers, for example:

'It should be clear by now that the sole, critical factor responsible for CR's anti-ageing action is reduced calories, not dietary nutrients' - (Yu and Chung 2001)

If the ingested calorie intake is indeed the critical factor, non-malnourished rodents of the same strain fed iso-caloric diets should invariably have the same lifespan, irrespective of the nutritional composition of their diet. However, in some experiments, rats fed iso-caloric diets with altered nutritional composition (Dalderup and Visser 1969; Iwasaki et al. 1988a) or reduced protein (Yu et al. 1985) showed lifespan extension. Furthermore, recent studies have shown that reducing just one

amino acid (methionine) increases lifespan in both mice (Miller et al. 2005) and rats (Zimmerman et al. 2003), despite methionine-restricted animals consuming more calories than controls (Miller et al. 2005). There is an important distinction between the actual calorie content of ingested food and that which is available to and used by the organism (Weindruch and Walford 1988; Piper et al. 2005). It may be that animals that are limited for methionine have reduced capacity to utilise ingested calories and are therefore effectively calorie restricted, or, conversely, that reducing calorie intake restricts an organisms' ability to assimilate amino acids. It is worth noting that the methionine-restricted rats described above showed severe growth retardation that could not be reversed by supplementation with extra dietary calories. However, it is clear that reducing the level of *ingested* calories is not always critical for lifespan extension by DR in rodents and that the effect of different nutritional components on lifespan requires further work. The intriguing possibility still remains that food components can affect physiology in many ways to promote longevity.

In this chapter, I investigated whether calories or specific nutrients were the key mediator of lifespan extension by DR in *Drosophila melanogaster*. I varied the sugar or the yeast component in the food independently to generate media that were iso-caloric, yet had different nutritional compositions, and measured the effect these media had on mortality and survival, both for chronic feeding of one medium throughout life and for flies subject to switches in food regime during adulthood.

5.2 Methods

5.2.1 Lifespan experiments

Two experiments were conducted, the first testing the lifespan of flies fed different diets chronically throughout life, whilst the second repeated the first and added four cohorts of flies that were subjected to changes in food medium during adulthood (see Table 5.2.1 for details). In both experiments, experimental flies were raised at a standard density of 400-450 eggs per 200ml bottle (section 2.3.4) on standard SY medium (section 2.2.1). Adults were collected over a 24-hour period and transferred without anaesthesia to fresh SY food for 48 hours and allowed to mate. Females were then collected using light CO_2 anaesthesia and assigned randomly to the food regimes (Table 5.2.1). All experiments were done with mated females. Flies were

kept on 35ml of food at an initial density of 100 individuals per 200ml bottle and transferred without anaesthesia to fresh food every 2-3 days. Deaths were scored 5-6 days a week and initial sample sizes (N_0) were calculated as the summed death and censor observations over all ages. To minimise any density effects on mortality, two bottles within cohorts were merged when the density of flies reached 50±10. To standardise the effects of parental age on offspring fitness (Priest et al. 2002), parents of experimental flies were of the same age and reared at a constant density.

Table 5.2.1. Sample sizes and treatments

Food Type	Experiment 1	Experiment 2
DR SY	1000	2037
DR Y, Control S	962	674
Control Y, DR S	967	688
Control SY	957	2101
Control SY until day 25 then Control Y, DR S *		460
Control SY until day 25 then DR Y, Control S *		489
DR SY until day 25 then Control Y, DR S *		675
DR SY until day 25 then DR Y, Control S *		678

* These represent the number of flies switched between treatments (i.e. N_{25}) and were sampled from the original chronic controls (Control SY or DR SY) and censored from the lifespan data of these treatments at day 25.

5.2.2 Iso-caloric food types

The nutritional composition of yeast was estimated using values from Lange and Heijnen(2001). This allowed the calculation of the caloric content of one gram of dried yeast, with estimations of the caloric value of protein, lipid and carbohydrate taken from (Southgate and Durnin 1970)[15]. This gave values of 4Kcal per gram sucrose and 4.02 Kcal per gram yeast, hence iso-caloric food types were generated (see Table 5.2.2).

Table 5.2.2. Nutritional composition and caloric content of experimental food types.

Food Type	Nutritional Content (grams of components per litre water)	Est. Protein (g/l)	Est. Carbohydrate (g/l)	Est. Lipid (g/l)	Est. Caloric Content (Kcal/litre)
DR SY	65g Y, 65g S	27.755	89.96	5.59	521.17
DR Yeast/ Control Sugar	65g Y, 150g S	27.755	174.96	5.59	861.17
Control Yeast/ DR Sugar	150g Y, 65g S	64.05	122.6	12.9	862.7
Control SY	150g Y, 150g S	64.05	207.6	12.9	1202.7

[15] These calculations were performed with the assistance of M. Piper.

Food media were based on standard sucrose/ yeast (SY) medium as described in Chapman and Partridge(1996). 'Y' refers to autolysed yeast powder and 'S' refers to sucrose

5.3 Results

5.3.1 Lifespan of female Drosophila *given foods of different caloric value*

Lifespan of female *Drosophila* was extended much more by reduction of yeast from control to DR concentration than by the equivalent reduction in sugar (Figure 5.3.1, Table 5.3.1) and median lifespan therefore did not correlate with caloric content of the food medium to which the flies were exposed (Figure 5.3.2). In two independent experiments, reducing yeast concentration from control to DR levels whilst keeping sugar levels constant significantly increased lifespan ($P<0.0001$ in both cases, Log Rank Test). Lowering caloric content to the same extent by reducing sugar from control to DR levels increased lifespan at DR yeast levels in both experiments ($P<0.0001$ in both cases, Log Rank Test) but the effect on median lifespan was much less than that of changing yeast levels (Figure 5.3.2, Table 5.3.1). Reducing sugar from control to DR concentrations whilst keeping yeast at control levels significantly increased lifespan in experiment 1 ($P<0.0001$, Log Rank Test) but again the effect on median lifespan was much less that that of changing yeast levels (Figure 5.3.2, Table 5.3.1). Reducing sugar from control to DR concentrations whilst maintaining yeast at control levels increased median lifespan in experiment 2 (Figure 5.3.2), but the effect on lifespan was not significant ($P>0.05$, Log Rank).

Table 5.3.1. Median and maximum lifespan of flies on fed different food media as adults

Food Type	Median Lifespan (Days)	Maximum Lifespan* (Days)	Median lifespan extension relative to Control SY
DR SY	42, 48	54, 58	82.6%, 60.0%
DR Y, Control S	38, 43	52, 56	65.2%, 43.3%
Control Y, DR S	25, 35	38, 48	8.7%, 16.7%
Control SY	23, 30	37, 48	-

'Y' refers to autolysed yeast powder and 'S' refers to sucrose. * Maximum Lifespan is the median lifespan of the longest-lived 10% of individuals. In each case, the pairs of values represent results of two independent repeats (experiment 1 & 2 respectively).

Figure 5.3.1. Survivorship (l_x) analysis of lifespan of female *Drosophila* on different food regimes.

Colour/ symbol of the curves shows yeast levels whilst the line type represents sugar levels in the respective foods. Panels A & B are independent repeats. In both cases, changing caloric content of the food by altering yeast levels had a much greater effect on lifespan than that seen when caloric content was changed by the same amount by manipulating sugar levels.

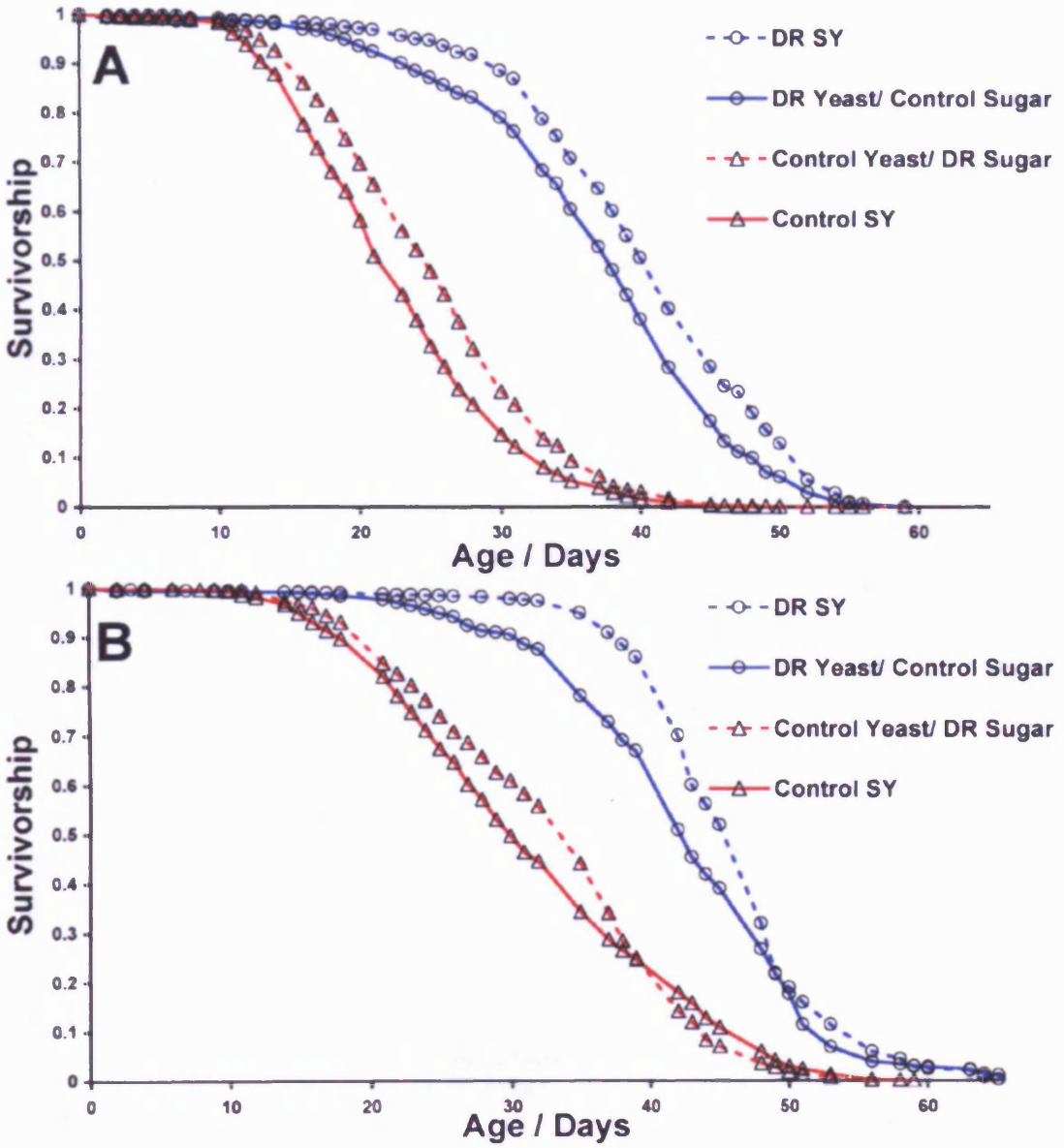
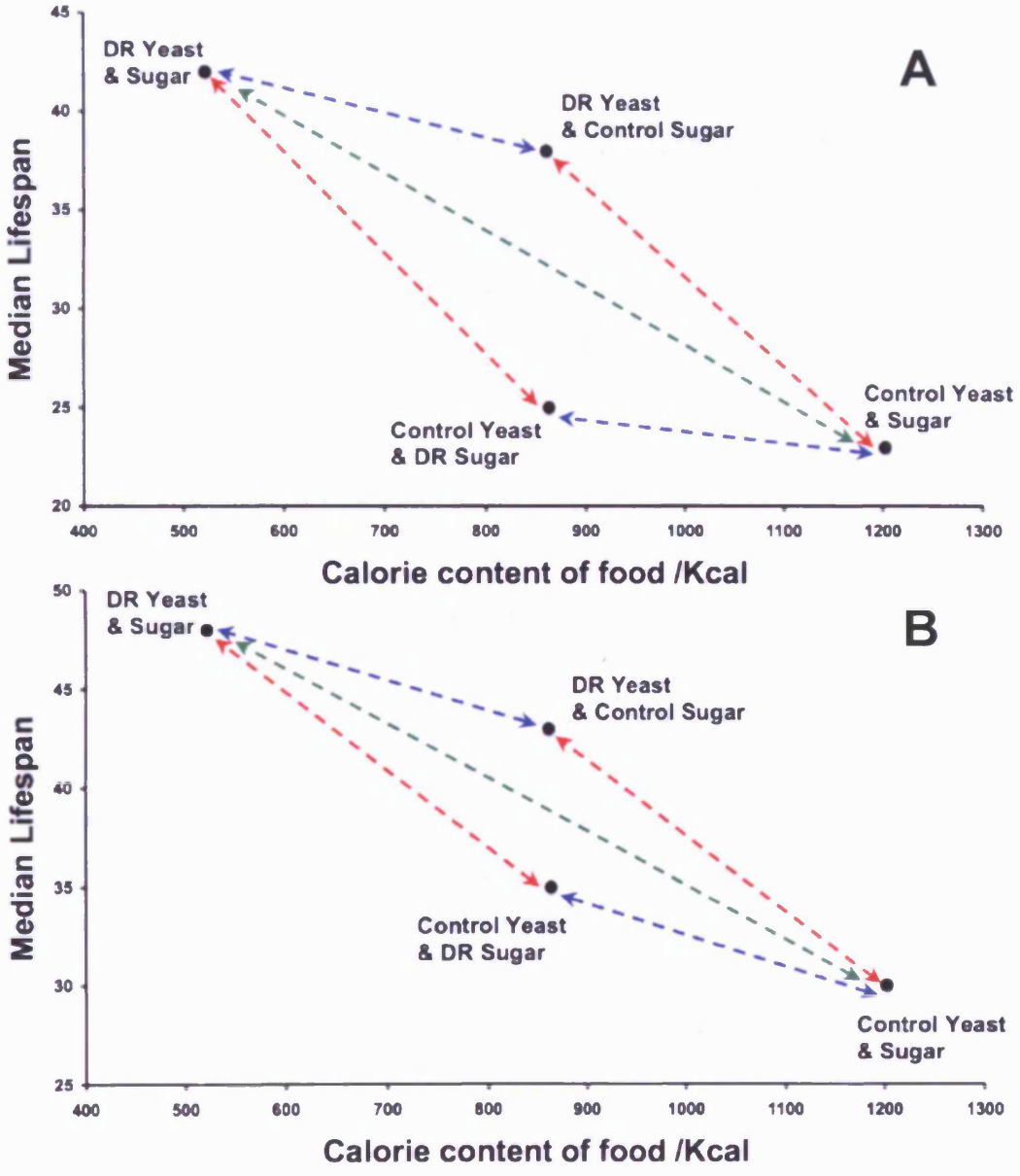


Figure 5.3.2. Plot of median lifespan (days) of female *Drosophila* against the estimated caloric content of the food medium.

Panels A & B represent independent repeats. Red arrows link pairs of food types where differences in caloric content are due to different yeast concentrations. Blue arrows link pairs of food types where differences in caloric content are due to different sugar concentrations. Green arrow links food types where differences in caloric content are due to both different sugar and yeast concentrations. Lifespan is extended to a greater extent per calorie by reducing yeast concentration from control to DR levels than by reducing sugar. This is in contrast to what would be predicted if calorie intake were the key mediator of lifespan extension by DR in fruit flies.



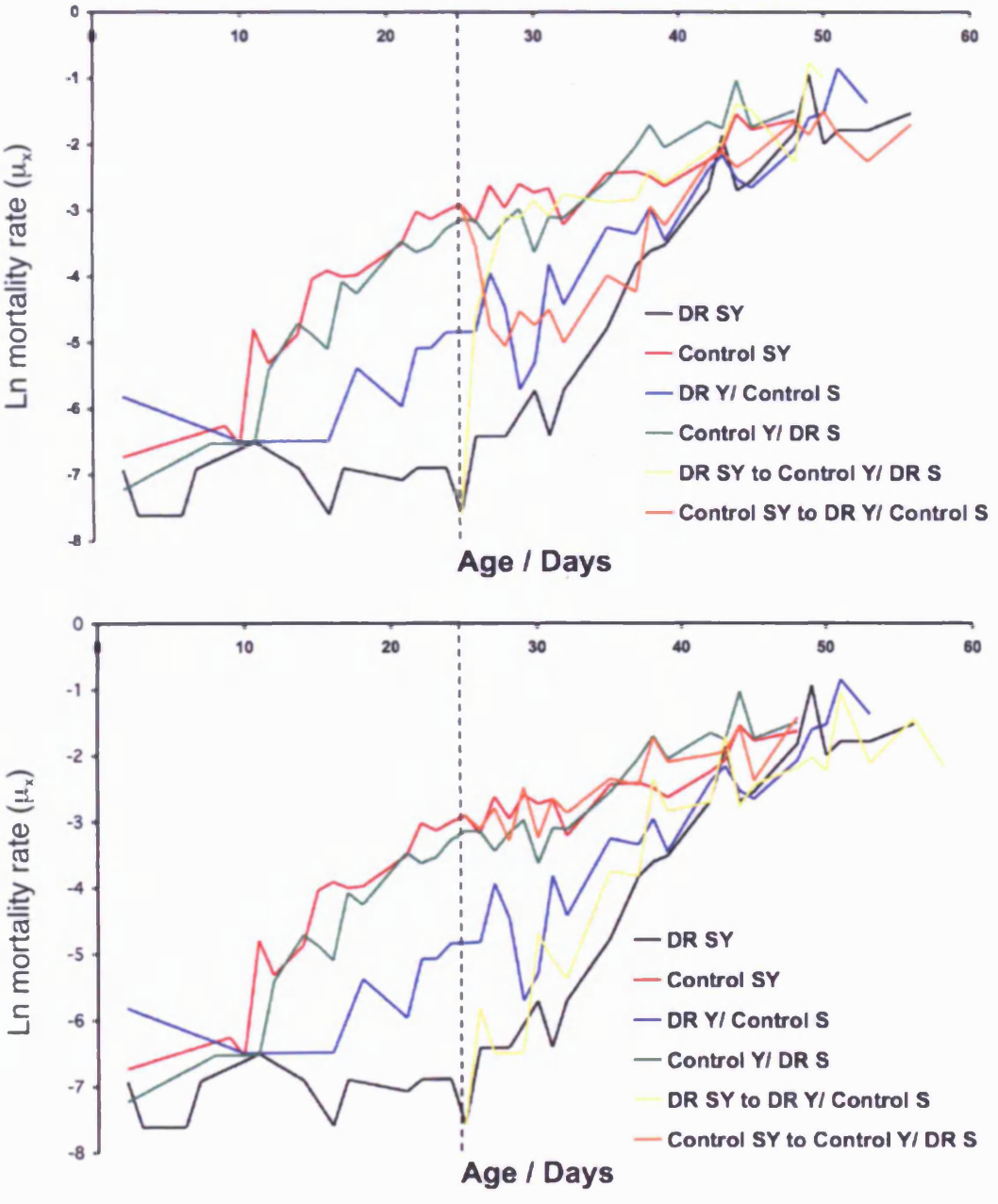
5.3.2 Effects on mortality of switching yeast and sugar

The effect of DR on mortality in *Drosophila* is acute; within 48h flies switched between Control and DR diets adopt the mortality rates characteristic of flies chronically exposed to the nutritional regime that the switched flies have joined (Chapter 3). I therefore measured the acute effects on mortality of switching the yeast and sugar components of the diet separately. When yeast was switched, mortality rates responded similarly to the responses to switches between control and DR SY food medium. 48 hours after being switched from control SY medium to DR yeast/ control sugar medium at day 25, flies were no more likely to die than those maintained on DR yeast/ control sugar medium throughout adulthood (Cox regression. P = 0.22, n DR yeast/ control sugar chronic group = 626, n switch group = 475, risk ratio = 0.96 [95% CI: 0.91, 1.02]) (Figure 5.3.3a). In the reciprocal switch, flies moved from DR SY medium to control yeast/ DR sugar medium showed a rapid increase in mortality rate, although this did not quite reach the level seen in flies that had been on control yeast/ DR sugar medium throughout adult life (Cox regression. P <0.05, n Control yeast/ DR sugar chronic group = 480, n switch group = 668, risk ratio = 0.88 [95% CI: 0.83, 0.93]) (Figure 5.3.3a).

In contrast, switching of sugar had no significant effect on mortality. From 48h after being switched from control SY medium to control yeast/ DR sugar at day 25, no significant difference was seen between the mortality of switched flies and the unswitched group maintained on control SY medium (Cox regression. P = 0.34, n Control SY = 427, n switch group = 440, risk ratio = 0.97 [95% CI: 0.91, 1.04]) (Figure 5.3.3b). Similarly, flies switched to DR yeast/ control sugar from DR SY medium at day 25 did not show increased mortality in comparison to unswitched controls (Cox regression. P = 0.41, n DR SY = 615, n switch group = 676, risk ratio = 0.98 [95% CI: 0.93, 1.03]) (Figure 5.3.3b).

Figure 5.3.3. The acute effects on age-specific mortality in *Drosophila* of changes in nutritional content of the food midway through life.

Vertical line represents switch day. Mortality trajectories were truncated when n<40. **A.** Control yeast (Y) intake caused no irreversible damage since flies switched from control yeast to DR yeast at day 25 rapidly became no more likely to die than those flies given DR yeast levels throughout adulthood. Flies with a history of DR yeast levels showed rapid increases in mortality rate when moved to control yeast levels at day 25, but mortality rates did not become as high as those of flies that had been maintained on control yeast levels permanently. **B.** Changing caloric intake to the same extent via changes to sugar (S) levels rather than yeast did not cause rapid changes in mortality rate. Despite flies chronically fed control sugar and DR yeast having increased mortality rate compared to the DR control, switching from DR to control sugar late in life did not increase mortality rate.



5.4 Discussion

5.4.1 Lifespan is not related to calorie intake

Flies fed food media with very similar caloric content showed marked differences in their lifespans (Figure 5.3.2). This finding is in direct contrast to what would be predicted if ingested calories were the key mediator of lifespan in *Drosophila melanogaster* and demonstrates that the nutritional composition of the diet affects lifespan extension by DR in this species. Reduction in the concentration of either sugar or yeast levels increased lifespan (Figures 5.3.1 & 5.3.2). However, the magnitude of the effects on lifespan when the caloric content of the food was changed via altering yeast concentration far exceeded that seen when calories were changed to the same extent via manipulation of sugar levels, suggesting that protein/ lipid levels have a greater effect on *Drosophila* survival than does carbohydrate.

The differing effect of sugar and yeast on mortality in *Drosophila* could occur if different pathways sense nutrients during DR, possibly with different outputs affecting lifespan. Sir2 (Rogina and Helfand 2004; Wood et al. 2004), Rpd3 (Rogina et al. 2002), the insulin/IGF-like signalling (Clancy et al. 2002) and TOR pathways (Kapahi et al. 2004b) have all been implicated in mediating the response of lifespan to DR in *Drosophila*, with the latter two suggested to interact in the fly to control growth in response to nutrient levels (Colombani et al. 2003). The role of these and other candidate pathways in mediating the response of lifespan to specific nutrients should be investigated further. Sugar and yeast could affect mortality rates differently if they differentially modulate metabolic or other processes that increase risk of death.

Experimentally increased reproduction has been shown to decrease lifespan in a variety of species (Williams 1966; Rose 1984; Gustafsson and Part 1990; Nager et al. 2001; Barnes and Partridge 2003) and the level of dietary yeast and egg-production are positively correlated in *Drosophila* (Chippindale et al. 1993; Chapman and Partridge 1996). Therefore an obvious hypothesis as to why there is a greater response of lifespan in *Drosophila* to changes in yeast than sugar is that the increased mortality on control yeast levels represents the cost of reproduction, which

correlates with yeast intake and not sugar. However, since lifespan extension via DR in *Drosophila* occurs normally when egg-production or vitellogenesis are blocked genetically (Chapter 4), the greater response of lifespan to changes in yeast is not directly attributable to the reduction of reproductive output. Furthermore, although the magnitude of the response to DR in male *Drosophila* is less than that of females (Magwere et al. 2004), males do live longer if nutrient levels are reduced and they show the same rapid changes in mortality as females when dietary regime is changed (Mair et al. 2003), yet they do not suffer the high costs of producing eggs on high yeast.

5.4.2 Rapid changes in mortality in response to DR are attributable solely to yeast content

DR acts acutely to extend lifespan in *Drosophila*; it does not slow the accumulation of irreversible damage with age (Chapter 3). Flies subjected to DR for the first time in mid-life rapidly become no more likely to die than those than have been under DR throughout adulthood (Chapter 3). I investigated the roles of the sugar and yeast components of the diet in producing this rapid change in mortality rate in flies switched between control and DR conditions. When flies previously subjected to control SY food were switched to DR yeast levels, there was a rapid (within 48h) drop in mortality rates to those seen in the flies chronically exposed to DR yeast/ control sugar food (Figure 5.3.3a). A similar rapid increase in mortality rates was seen when flies exposed to DR food were switched to control yeast levels (Figure 5.3.3a) although, as seen previously using whole food dilutions (Chapter 3), a history of low yeast gave slight protection to female *Drosophila* moved to control yeast late in life.

However, when caloric content of the food given to flies was changed to the same extent midway though life by changing sugar rather than yeast levels, no change in mortality rate was seen (Figure 5.3.3b). Therefore the acute mortality 'switch' phenotype in response to dietary restriction is attributable to changes in the level of the dietary yeast alone. That chronically reducing sugar intake of flies can extend lifespan, yet reducing sugar intake late in life does not cause rapid changes to mortality rates suggests the deleterious effects of sugar may occur mainly early in adult life. The mortality trajectories in Figure 5.3.3 support this conclusion, by

showing that the lowering of mortality rate in response to reduced sugar is most obvious early in the trajectory, when mortality rates in all groups of flies are low. More work is needed using accurately defined media to investigate this effect. Rapid reductions in mortality rate have been seen previously in *Drosophila* by altering the intake of yeast only (Good and Tatar 2001). However, the results of the previous study differ from those here in that reduced mortality was achieved by *increasing* the nutrient intake of flies that had previously been deprived of yeast, rather than by reducing the nutrient intake of control fed flies.

5.4.3 Feeding rates of flies on different food types

Unlike in rodents, where dietary restriction can be achieved by directly reducing the quantity of food eaten in comparison to animals given *ad libitum* access (Masoro 2002), DR is achieved in *Drosophila* by reducing the quality (nutrient concentration) of the food given to the flies (Chapman and Partridge 1996) with the quantity maintained in excess of that which they can consume. Despite the fact that fecundity correlates with food medium concentration (Chapman and Partridge 1996), it has been suggested that flies may be able to compensate when faced with reduced nutrients by increasing feeding rates, and therefore they may not be dietarily restricted (Cooper et al. 2004). This issue becomes critical when altering the nutritional composition of the food fed to *Drosophila* but maintaining calorie content constant, since if feeding rates are determined by the sensing of a particular nutrient the calorie intake of flies fed these iso-caloric diets may well differ. However, the results of behavioural assays of flies fed the different media used in this chapter suggest that feeding activity was unaltered by the nutritional composition of the food, as measured by time spent on the food with the proboscis extended[16] (Figure 5.4.1).

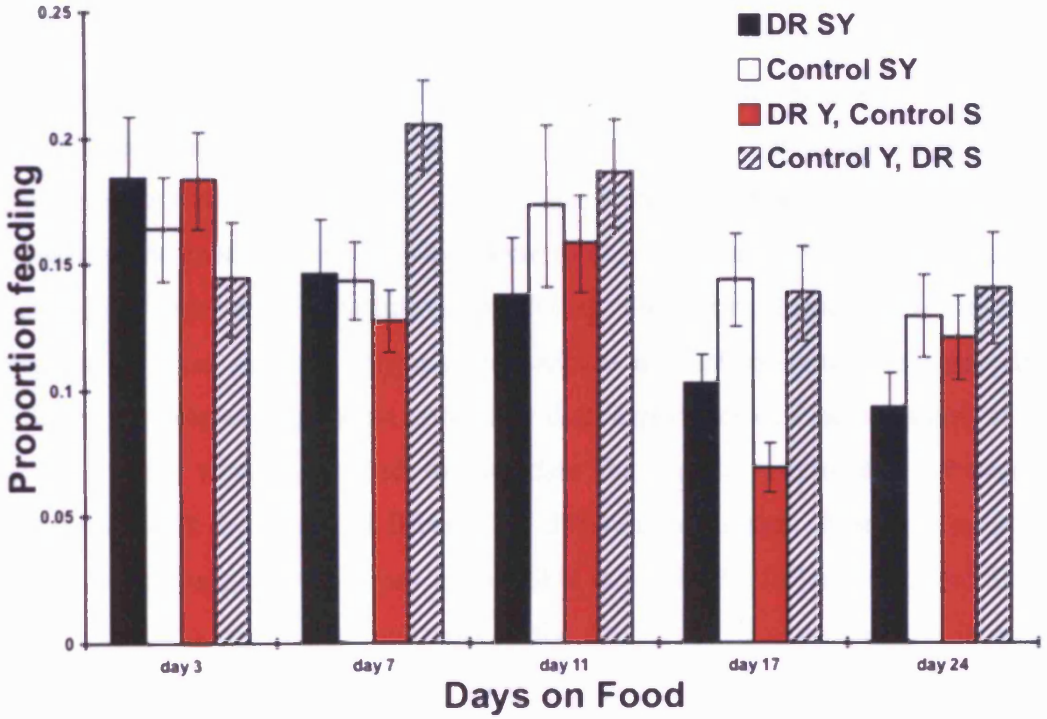
It is possible *Drosophila* can alter the rate of food uptake per unit time that the proboscis is extended, in which case the behavioural assays would not detect these changes. More direct approaches to quantify feeding rates require radio-labelling the food (Brummel et al. 2004) or the addition of coloured food dye (Edgecomb et al.

[16] All results discussed in this section on feeding rates are published in Mair (2005) but are from experiments conducted by M. Piper.

1994), with uptake rates assessed upon short-term exposure to labelled food. However, our own unpublished observations show that flies moved to fresh food medium display elevated feeding behaviour that is unrepresentative of the steady-state situation and that leads to a highly non-linear relationship between time and uptake of the food label. Hence, the behavioural measure described here better represents the normal feeding of the flies. The feeding assay results suggest that altering the nutrient composition of the food medium whilst keeping caloric content constant does indeed cause flies to eat the same levels of calories but different proportions of yeast and sugar.

Figure 5.4.1. Feeding rates of female *Drosophila* on food media with different nutrient concentrations.

Feeding rates were recorded by direct observation as the proportion of time flies spent on the surface of the medium with their proboscis extended and touching the food (Y Axis). Replicate measurements of the proportion of females feeding versus those not feeding were recorded during a two-hour period on the days shown. No significant different was seen between flies fed different diets on days 3, 7, 11 & 24. There was a significant difference in feeding rates on day 17 ($P = 0.0068$) with flies on the DR yeast/ Control sugar medium eating less. These data show that *Drosophila* do not exhibit compensatory feeding behaviour for the DR regime imposed. This figure is from (Mair et al. 2005), figure produced by W. Mair, experimental work carried out by M. Piper.



5.4.4 Conclusions

The response of *Drosophila* lifespan to nutrition is not governed by calories, but rather by specific nutritional components of the food. This finding represents a departure from the generally accepted model in rodents, where it has been suggested that the level of calorie intake *per se*, not the source of calories, is critical for lifespan extension (Masoro 2002). The apparent disparity between the factors in the diet that effect lifespan in fruit flies and rodents leads to two possible conclusions. First, the mechanisms by which these organisms respond to food shortage could be different. Second, the long held view that calorie intake is the critical variable in the response of mammalian lifespan to DR may require further evaluation.

Despite some reports in the literature where DR did not extend lifespan (Lipman et al. 1995; Le Bourg and Minois 1996; Lipman et al. 1998; Forster et al. 2003; Cooper et al. 2004), the overwhelming majority of data support the idea that dietary restriction in some form extends lifespan across diverse taxa. However, it is still unknown if lifespan extension under DR is achieved through common mechanisms in different species. A case for conservation of the mechanisms by which DR extends lifespan can be made from evolutionary considerations. Evolutionary theory suggests that, during times of famine, diversion of resources away from reproduction towards somatic maintenance will increase the chances of an organism surviving to more plentiful times and thus increase long-term reproductive success (Harrison and Archer 1988; Holliday 1989; Masoro 1996; Shanley and Kirkwood 2000). The selective advantage of shifting resources from reproduction to maintenance when food is restricted could be the "public" factor shared between diverse organisms. However, the mechanisms by which extension of lifespan is achieved could be an example of convergent evolution, producing the same plasticity of lifespan in response to food shortage through mechanisms at least to some extent specific to different organisms, dependent upon their diet, experience of food shortages and life history. More work is needed to elucidate the precise relationship between the composition of the diet and lifespan in different organisms, including mammals. These results suggest that it may be possible to obtain the full extension of lifespan by DR by reducing critical nutrients in the food without any reduction in overall calorie intake.

Chapter 6. Stress resistance of dietary restricted *Drosophila*

Abstract

Increased lifespan is often associated with increased resistance to environmental stress. Oxidative stress of mammalian cells is positively correlated with the lifespan of the host organism and selection for increased lifespan in fruit flies results in long-lived flies that are also stress resistant. Furthermore, decreased insulin signalling extends lifespan and confers increased stress resistance in *C. elegans*, *Drosophila* and mice. Dietary restriction increases lifespan in a range of taxa and is known to improve the stress resistance of rodents. This increase in stress resistance may be causal to the life-prolonging effect of a restricted diet. I show here that DR does not increase the global stress resistance of *Drosophila*. DR flies do not have increased ability to withstand desiccation, heat, cold, or oxidative stress compared to controls. DR does increase the ability of flies to withstand starvation and the mechanisms by which this is achieved may also be relevant to the mechanisms via which DR extends lifespan. I also show that feeding flies an antibiotic does not increase lifespan or have any effect on lifespan extension by DR. Hence, increased bacterial infection is not responsible for increased death rates of flies on control food medium compared to those on a DR regime. Together these results imply that DR does not enhance global stress resistance in fruit flies. The same stresses tested in DR rodents and *Drosophila* do not result in conserved effects. Either increased stress resistance is not responsible for lifespan extension under DR, or the mechanism by which DR extends lifespan is not conserved between rodents and fruit flies.

6.1 Introduction

If dietary restriction is a public mechanism of lifespan extension, the mechanisms via which it extends lifespan should be conserved across different taxa. Therefore, it is of interest to investigate if changes seen in long-lived DR rodents are also seen in other model organisms subjected to dietary restriction. If these mechanisms are shared between different species, they may be good candidates for the underlying cause of reduced mortality that is induced by DR. If mechanisms are not shared between different dietarily restricted organisms then, either they are not causal to lifespan extension under DR, or the method by which this extension is seen is not the same in different species.

6.1.1 Stress resistance correlates with lifespan

Resistance to environmental stress is often positively correlated with lifespan in a range of organisms. Selection experiments in *Drosophila melanogaster* for increased longevity generated flies that were not only long-lived compared to control lines, but that were also starvation and desiccation resistant (Rose 1984; Service et al. 1985) and, *vice versa*, selection for increased starvation and desiccation resistance resulted in flies that were both stress resistant and long-lived (Rose et al. 1992; Chippindale et al. 1993). Reduced IIS increases lifespan in a variety of model organisms (Partridge and Gems 2002), and this reduced mortality is often coupled with an increase in stress resistance. *C. elegans* with reductions in IIS were also resistant to heat shock, ultraviolet (UV) light, hydrogen peroxide (H_2O_2) and paraquat (oxidative stress) (Larsen 1993; Lithgow et al. 1995; Murakami and Johnson 1996; Guarente and Kenyon 2000; Houthoofd et al. 2005), whilst reducing IIS signalling in fruit flies also increased resistance to starvation (Clancy et al. 2001) and increased levels of superoxide dismutase (SOD), a free radical defence mechanism (Tatar et al. 2001). Flies in which the *Drosophila* insulin-like peptide (dilp) producing cells were ablated were long-lived and also resistant to starvation and oxidative stress (Broughton et al. 2005). Similarly, insulin receptor knock-out mice had extended lifespan and increased resistance to oxidative stress (Holzenberger et al. 2003), suggesting that this correlation is conserved in mammals.

Organisms also show progressive deterioration in stress resistance with age (Yu and Chung 2001) and the immune system, crucial for resistance to stress from infection, becomes less effective as an individual gets older (Miller 1991). Resistance to oxidative stress of skin fibroblasts from a variety of mammals is positively correlated with the lifespan of the host organism (Kapahi et al. 1999). Over-expressing mitochondrial heat shock protein in *Drosophila* extends lifespan and increases stress resistance (Morrow et al. 2004) and heat shock proteins may represent a global defence mechanism, where by exposure to one stress increases defence levels not only to that particular stressor but to environmental stress in general (Ray 1999).

6.1.2 DR and Hormesis

Increased stress resistance has been suggested to be partly responsible for the reduced mortality of dietarily restricted rodents (Yu and Chung 2001), leading to the hypothesis that the effect of DR on lifespan is an example of hormesis (Masoro 1998; Turturro et al. 2000). The term 'hormesis' is used when mild exposure to an otherwise detrimental factor is beneficial to survival (Furst 1987; Calabrese 2004). For example, low levels of ionizing radiation extend the lifespan of rodents (Carlson et al. 1957; Congdon 1987) and fruit flies (Sacher 1963), and mild heat stress has been shown to increase the lifespan of *Drosophila* (Maynard Smith 1958; Khazaeli et al. 1997; Le Bourg et al. 2001; Hercus et al. 2003) and nematodes (Lithgow et al. 1995). Furthermore, mild exposure to hypergravity also confers some benefit to the lifespan of fruit flies (Le Bourg et al. 2000). Dietarily restricted rats show elevated afternoon peaks of plasma levels of the hormone corticosterone (Sabatino et al. 1991), which implies that DR is indeed a mild stress and hence may induce a hormetic response. If DR does induce a hormetic response and increases lifespan by up-regulating general stress resistance, it follows that DR individuals should show an increased ability to cope with environmental stress compared to controls.

6.1.3. Increased stress resistance of DR animals

Studies in which the ability of DR rodents to withstand environmental stress has been directly tested are limited in number, due to the ethical considerations associated with subjecting live animals to such a challenge. However, that DR increases general stress resistance in rodents in comparison to controls is widely

accepted (Yu and Chung 2001) and has been reported in the literature. Glucocorticoid levels were higher in DR rats than in controls and these are thought to protect against environmental stress (Munck et al. 1984; Frame et al. 1998). Chemically induced tumorigenesis was lower in DR mice than in controls (Tannenbaum 1942; Pariza and Boutwell 1987)[17] and DR significantly reduced the level of radiation-induced tumours in mice challenged with gamma rays (Gross and Dreyfuss 1990). Thermotolerance in old rats was increased by dietary restriction; experimentally increased core temperature killed 50% of the control cohort whilst all DR individuals survived heat treatment (Hall et al. 2000). Power failure in one laboratory also led to an unplanned thermotolerance test in rats; the facility's ambient temperature was raised to above 33°C for several hours and 75% of DR rats survived, as opposed to only 16% of the *ad libitum* fed group (Heydari et al. 1993).

DR also increases the resistance of rodents to the administration of toxic drugs (Duffy et al. 1995) such as ganciclovir sodium (Berg et al. 1994). Furthermore, moderate DR (65% *ad lib.*) in rats also increased tolerance to toxic compounds compared to controls (Keenan et al. 1997). Dietary restriction attenuates the age-related decline in immune function (Pahlavani 2000; Pahlavani 2004) in rodents and makes rats less prone to stress-induced apoptosis (Ando et al. 2002). Although not calorie-restricted, methionine-restricted mice are long-lived and show increased resistance to oxidative-stress-induced liver cell damage *in vivo* (Miller et al. 2005).

The effect of dietary restriction on rodent wound repair capacity and ability to withstand cold are mixed. Some studies suggest that DR rodents show decreased cold tolerance (Muralidhara and Shetty 1987; Muralidhara and Shetty 1990), yet others reported that intermittent feeding in mice increased both lifespan and ability to cope with cold (Talan and Ingram 1985). DR has been shown both to decrease (Harrison and Archer 1987; Reiser et al. 1995) and have no effect on (Harrison and Archer 1987; Emery and Sanderson 1995) the wound healing capacity of rats, measured as collagen deposition after injury or tensile strength of collagen at the site of the wound 7 days after surgery.

[17] Tannenbaum (1942) cited from Yu (2001).

Limited data suggest that DR may increase stress resistance in yeast (Lin et al. 2000) and worms (Houthoofd et al. 2002a), but no comprehensive study has been published. In this chapter, I investigated the effects of dietary restriction on stress resistance in *Drosophila melanogaster* to determine if the resistance phenotype seen in DR rodents was evolutionarily conserved. The ability of control and DR flies to resist desiccation, starvation, heat, cold, and oxidative stress was compared. It has also been suggested that higher nutrient concentrations in fly food may lead to higher proliferation of bacteria on the medium, and that therefore increased infection is responsible for the elevated death rates in control *Drosophila melanogaster* compared to those under DR (Cooper et al. 2004). This hypothesis predicts that 1) flies fed antibiotics will live longer, and 2) the lifespan extension seen when nutrient concentration is reduced will be blocked when antibiotics are present. In this chapter, I tested this hypothesis by feeding flies control and DR medium either with or without antibiotics. Even if the levels of bacterial found on control and DR fly medium are the same, DR may increase immunity to infection and this may be causal to life-extension. Again, if this is the case, adding antibiotics to the food should increase the lifespan of *Drosophila* and this was also tested here.

Resistance to environmental stress may change with age, and the inherent frailty of old control flies relative to DR individuals may thus result in apparent increased stress resistance of the DR cohort. This may be unrelated to the mechanism by which DR reduces inherent frailty; old individuals may be less able to cope with being hit over the head with a stick than the young, but that does not make being hit with a stick the cause of ageing. Thus, some caution should be applied when looking at stress resistance assays performed on one age class. If DR does increase global stress resistance then this phenotype should be apparent at all ages, therefore in this chapter, where possible, stress tests were carried out both early in life and at a point where mortality rates in the control and DR cohorts have diverged.

6.2 Methods

All stress tests were carried out on Dahomey wild type stock (section 2.1.1) grown as larvae on standard SY medium (section 2.2.1). On emergence, flies were allocated to either control (1.5Y 1.5S) or DR (0.65Y 0.65S) medium in single-sex groups as described in section 2.3.5. Stress tests were then carried out on either males or females at set ages as described below.

6.2.1 Heat resistance assay

A water bath was held at 38.5°C with a thermo-regulator. Flies were transferred via aspiration to 5ml empty glass vials that were sealed with cotton wool pushed half way down the vial. Density of the flies was ten per vial, n = 50 flies per treatment. Heat knockdown experiments were performed 'blind', in that flies were put into coded vials by someone other than WM[18]. These vials were then arranged randomly on a weighted plastic frame that held the bottom half of the vials under water. The time taken for each fly to be knocked down was scored by progressively scanning along the vials, removing one at a time and recording how many flies were still moving (Hoffmann et al. 2005). Heat resistance was assayed for female flies after 7 and 15 days of control or DR feeding.

6.2.2 Cold resistance assay

When chilled to 4 degrees centigrade, *Drosophila* stop moving and enter what is known as a chill coma (Hoffmann et al. 2005) from which they recover when returned to room temperature. The ability to recover from the coma varies between *Drosophila* from different geographic locations and can be readily assayed (Hoffmann et al. 2005). To achieve this, flies were transferred using ice anaesthesia to dry, empty glass vials and placed on ice for 4 hours at a density of 6 flies per vial, 56-60 flies per treatment. After cold stress, vials were removed from the ice and placed on viewing racks in a 25 degree centigrade controlled temperature room. Vials were progressively scanned and the time taken for flies to stand was recorded (Hoffmann et al. 2005). Chill coma recovery was assayed for female flies after 7 or 8 days of either control or DR feeding.

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[18] Yasmine Driege coded & labelled vials before heat assay was carried out by William Mair.

6.2.3 Desiccation resistance assay

Desiccation/ dry-starvation resistance was assessed using 10 vials of 10 flies for each treatment. Flies were stressed in empty vials covered with gauze placed at 25°C in a sealed tank containing silica gel, generating a relative humidity of less than 10%. Desiccation was assayed for females maintained on either control or DR food for 8 or 19 days and for males fed control or DR food for 19 days. Mortality was scored every 30 minutes until the flies had died in the 19-day trials. For the 8-day trial, deaths were scored first at 12 hours, then again at 14, 15.5, 17 and 18 hours when remaining flies were censored. Desiccation experiments were performed 'blind' in that flies were put into coded vials by someone other than WM[19].

6.2.4 Starvation resistance assay

The starvation (without desiccation) assay was achieved by exposing flies to a 1% agar food medium (2.2.4). Flies were kept at a density of 10 per vial and each treatment had 10 vials. Deaths were scored daily or twice daily and live flies were tipped to fresh agar vials three times weekly for the duration of the assay. Starvation resistance was assayed at both 5 days and 14 days of DR or control feeding regime for females and either 6 or 14 days of DR for males.

6.2.5 Resistance to paraquat feeding

To measure resistance to oxidative stress, female flies were put on DR or control (1.5Y 1.5S) food for 7 days and then moved to standard SY medium containing 30 mM methyl viologen (paraquat) (Broughton et al. 2005). Subsequent lifespan was then recorded. Density per vial was 10 and sample size per treatment was 100. Flies were moved to vials containing fresh paraquat medium every 2 days.

6.2.6 Effect of tetracycline on lifespan

Tetracycline is a general antibiotic that inhibits ribosomal translocation and acts on both gram positive and negative bacteria (Davies and Smith 1978). A tetracycline solution was made up in 70% Ethanol and added to the food medium after it had been boiled and cooled to 60°C[20]. The final concentration of tetracycline in the

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[19] Yasmine Driege coded & labelled vials before desiccation assay was carried out by William Mair.

[20] Method received as personal communication from G. Hurst.

medium was 0.025% weight/volume (Hurst 2000), 5 times more than that used when tetracycline resistance is utilised as a selectable marker for bacterial transformation (Sambrook et al. 1989) and thus sufficient to kill all non-tetracycline-resistant bacteria. The wild type stock Dahomey is infected by the cytoplasmic bacteria *Wolbachia* (unpublished). A 0.025% tetracycline solution is sufficient to remove bacteria such as *Wolbachia* from *Drosophila* stocks if fed to larvae (Hurst 2000) and can suppress *Wolbachia* in other insects when fed to adults only (Hurst et al. 1992). Therefore flies fed tetracycline media as adults may not only have reduced exposure to external microorganisms on the food surface compared to controls, but also reduced *Wolbachia* infection. 7ml of food was poured into 30ml glass vials and the lifespan of flies measured with 92-101 flies per treatment and 10 flies per vial. Fresh food was prepared once a week and flies moved onto new media three times per week.

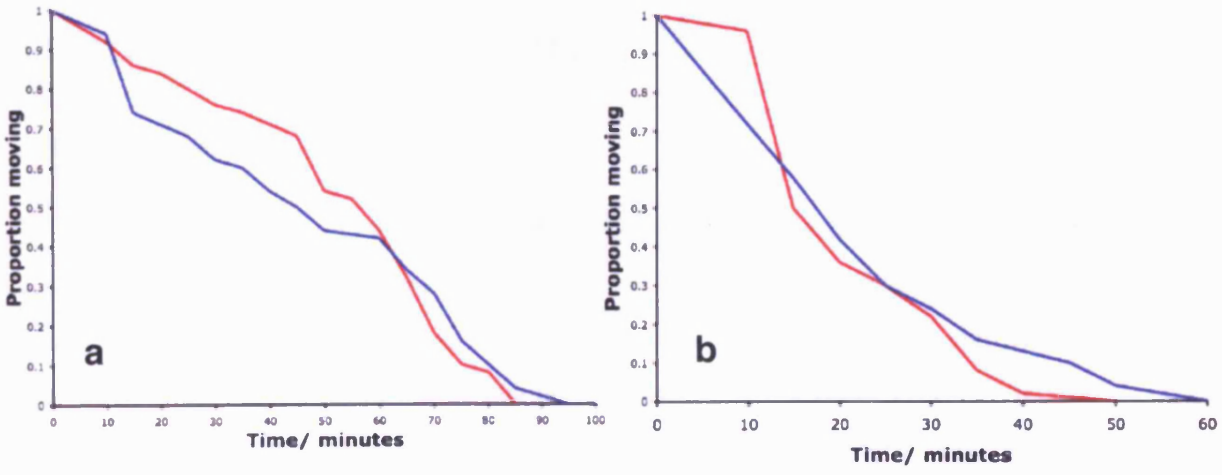
6.3 Results

6.3.1 Heat resistance

No significant difference was seen in the time taken to heat knockdown for females that had been on control or DR food for either 7 days (Log Rank, P= 0.15, n = 50 per treatment) or 15 days (Log Rank, P = 0.824, N = 50 per treatment) (Figure 6.3.1).

Figure 6.3.1. Heat resistance of wild type female *Drosophila* on either a control (red) or DR (blue) diet.

DR feeding for either 7 (a) or 15 (b) days did not increase the time taken to heat knock down compared to control flies.

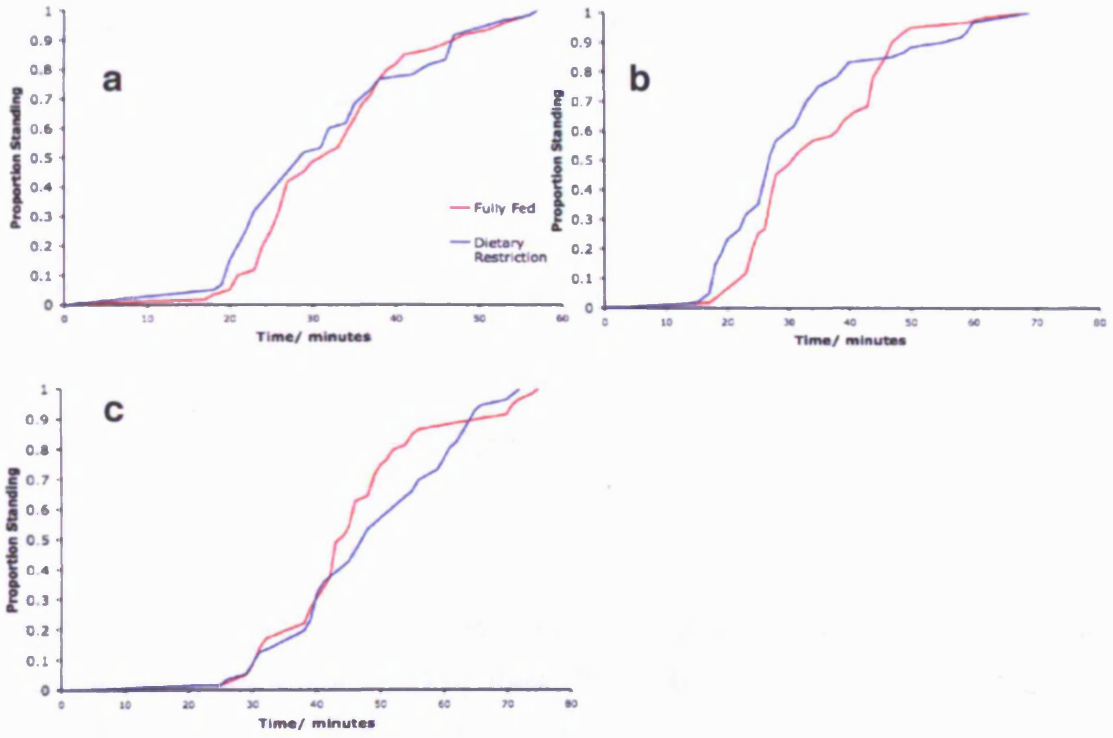


6.3.2 Cold recovery

The time taken for females to recover from cold coma did not significantly differ between those that had been fed a DR or control diet (Figure 6.3.2). This was the case for females that had been on a DR regime for 7 days (repeat 1; Log rank, chi squared = 0.1341, DF = 1, P = 0.7142, n = 60 per treatment, repeat 2; Log rank, chi squared = 0.5753, DF = 1, P = 0.4482, n = 60 per treatment) or 8 days (Log rank, chi squared = 0.210, DF = 1, P = 0.4704, DR n = 56, FF n = 59).

Figure 6.3.2. Time taken to recover from chill coma of DR and control fed wild type female *Drosophila* of different ages.

No significant different was seen in the ability to recover from chill coma between DR (blue) and control (red) fed flies that had been on the respective food regime for 7 (a & b), 8 (c) days.

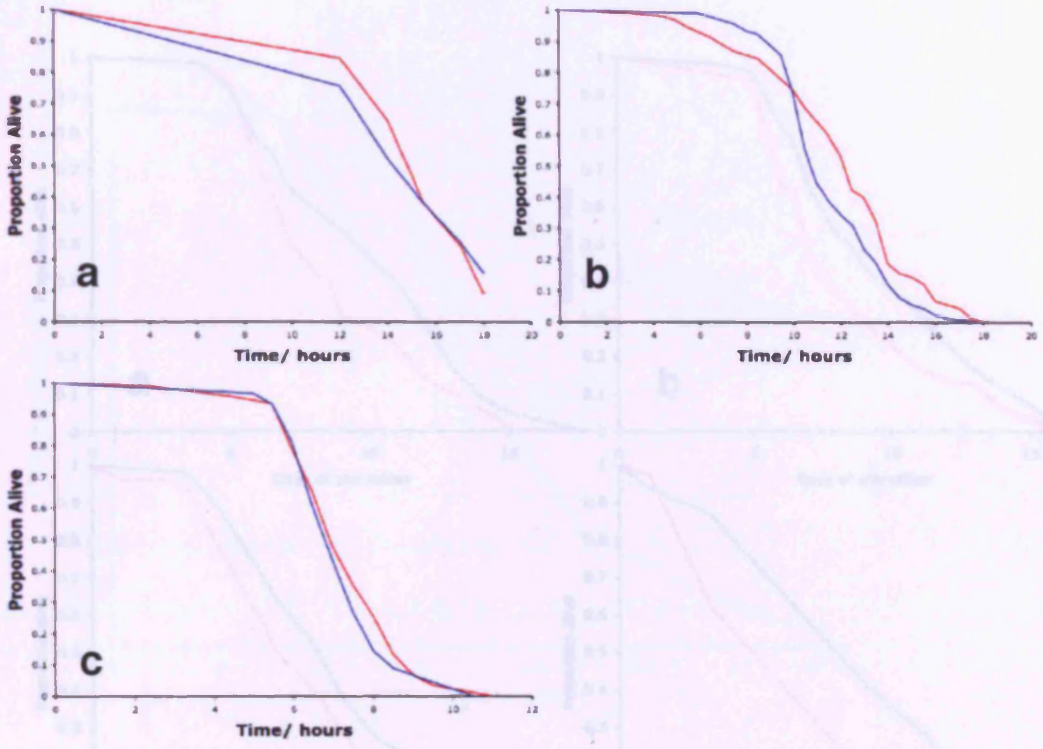


6.3.3 Desiccation resistance

Desiccation resistance was not significantly different between females that had been on a DR or control food regime for 8 days (Log Rank Test, P = 0.964, N = 100 per treatment) (Figure 6.3.3a). Females on a DR regime for 19 days were significantly less desiccation resistant than same age control females (Log Rank Test, P = 0.0306, n = 100 per treatment) (Figure 6.3.3b). However, this difference was not seen

between males that had been on a DR or control regime for 19 days (Log Rank Test, P = 0.320, N = 100 per treatment) (Figure 6.3.3c).

Figure 6.3.3. Desiccation resistance of DR and control *Drosophila melanogaster*.
a. No significant difference in desiccation resistance was seen between DR (blue) and control (red) females that had been on their respective food regimes for 8 days. **b**. Females that had been on a DR regime for 19 days were less resistant to desiccation than controls. **c**. Males on control and DR food for 19 days showed no difference in their ability to withstand desiccation.

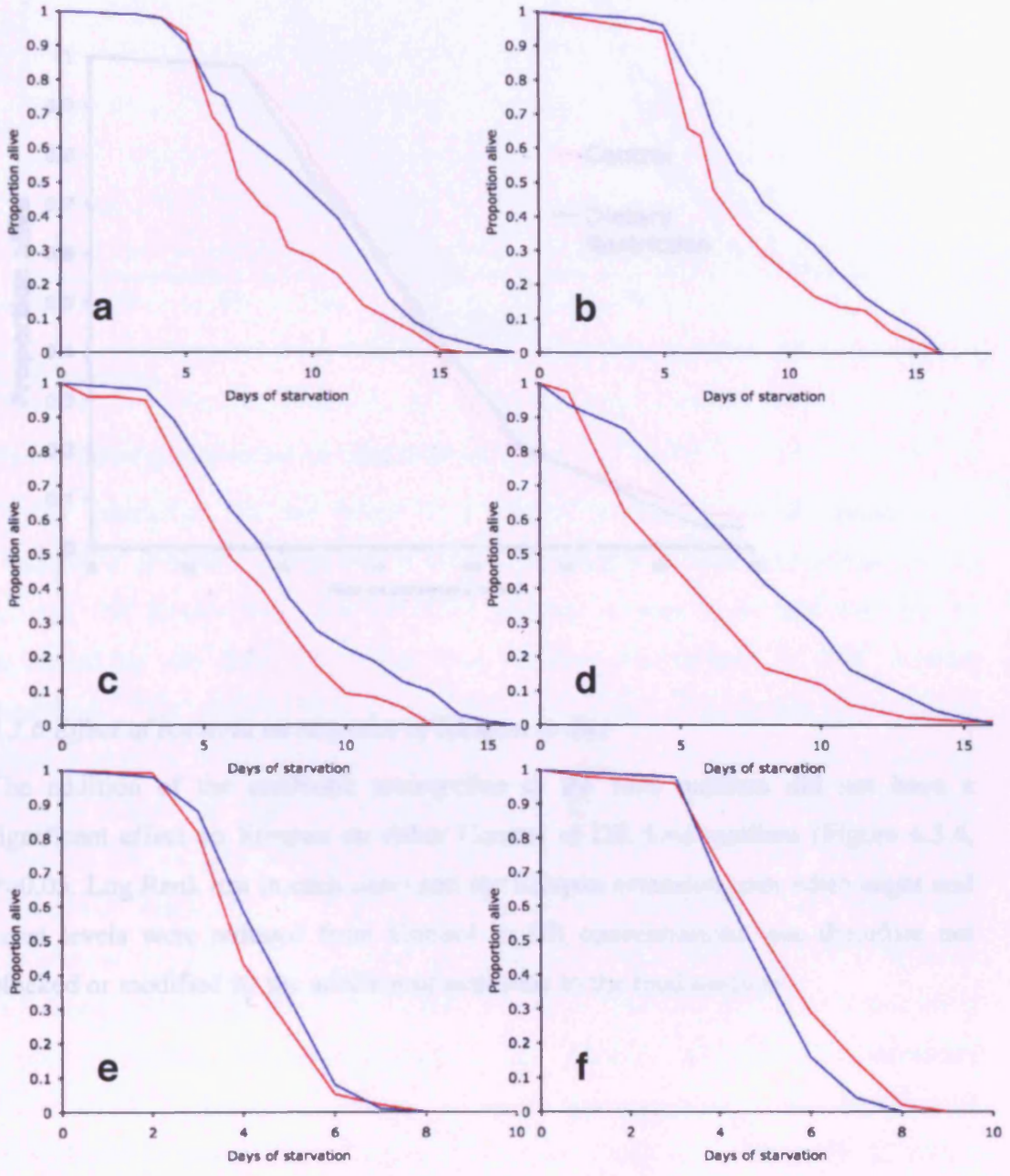


6.3.4 Starvation resistance

Dietary restriction for 5 days significantly increased the starvation resistance of female *Drosophila* (Repeat 1, Log Rank, P = 0.007, n DR = 99, n control = 102; Repeat 2, Log Rank, P = 0.025, n DR = 101, n control = 97) (Figure 6.3.4 a & b). Dietary restriction for 14 days also significantly increased the starvation resistance of female *Drosophila* (Repeat 1, Log Rank, P = 0.0029, n DR = 102, n control = 100; Repeat 2, Log Rank, P < 0.0001, n DR = 195, n control = 194) (Figure 6.3.4 c & d). Dietary restriction for 6 days did not significantly increase the starvation resistance of male *Drosophila* (Log Rank, P > 0.05, n DR = 99, n control = 97) (Figure 6.3.4e). Dietary restriction for 14 days did not significantly increase the starvation resistance

of male *Drosophila* (Log Rank, P > 0.05, n DR = 100, n control = 103) (Figure 6.3.4f).

Figure 6.3.4. Starvation resistance of DR (blue) versus control (red) fed *Drosophila*. **a** & **b.** 5 days of DR increases starvation resistance of females relative to controls. c & d. 14 days of DR increases starvation resistance of females relative to controls. **e**. Males on a DR regime for 6 days do not show significantly increased starvation resistance relative to controls. **f.** Males on a DR regime for 14 days do not show significantly increased starvation resistance relative to controls.

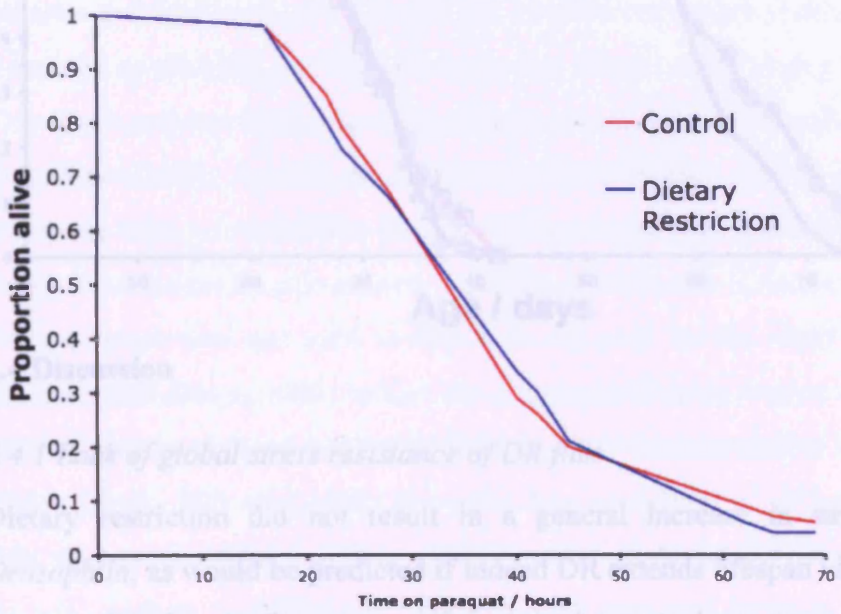


6.3.5 Resistance to paraquat feeding

There was no significant difference between survivorship of *Drosophila* females moved to standard SY medium containing 30mM paraquat after 7 days of a control or DR food regime (Log Rank, P< 0.0001, n = 100 per treatment) (figure 6.3.5).

Figure 6.3.5. Resistance of DR and control females to oxidative stress.

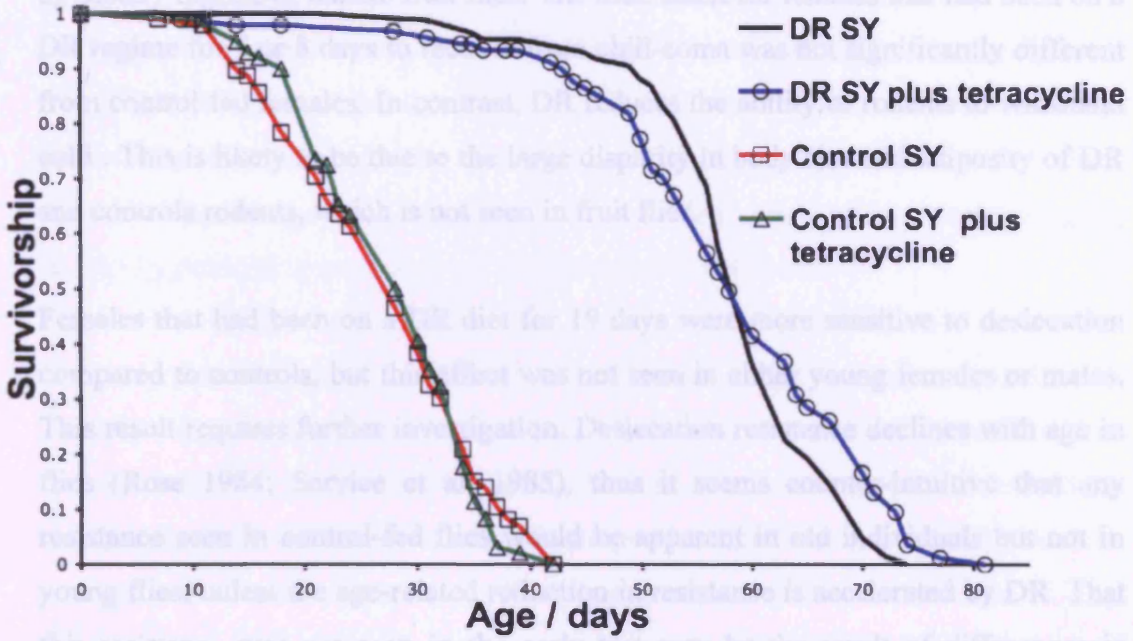
After 7 days of DR or control feeding, no significant difference was seen between the survival of females moved to standard SY media containing 30mM paraquat.



6.3.6 Effect of bacteria on response of lifespan to diet

The addition of the antibiotic tetracycline to the food medium did not have a significant effect on lifespan on either Control or DR food medium (Figure 6.3.6, P>0.05. Log Rank test in each case) and the lifespan extension seen when sugar and yeast levels were reduced from Control to DR concentrations was therefore not blocked or modified by the addition of antibiotic to the food medium.

Figure 6.3.6. Effect of tetracycline on lifespan of female *Drosophila melanogaster*. The addition of the antibiotic tetracycline to the food media did not have a significant effect on lifespan at either Control or DR concentration food media.



6.4 Discussion

6.4.1 Lack of global stress resistance of DR flies

Dietary restriction did not result in a general increase in stress resistance in *Drosophila*, as would be predicted if indeed DR extends lifespan via hormesis in this species. DR female flies were no more resistant to heat stress than controls, as measured by the time taken until they became immobilized at 38.5 degrees centigrade. This was true both for young (7 days) flies and those that had been fed either control or DR food for 15 days, sufficient time for mortality rates to diverge between cohorts on these two food regimes (Chapter 3). This is in contrast to the effect of DR on heat resistance in rodents (Hall et al. 2000). One caveat of the *Drosophila* assay is that, since it is a score of immobility rather than death, performing the test with ten flies per vial may mean that interaction between individuals affects the result; previously immobile flies may temporarily recover if jostled by others. An improvement to the protocol used here, subsequently implemented in our laboratory, is to assay heat knock-down for flies housed individually in sealed vials submerged in a glass water tank held at 39 °C with a

thermo-regulator. The time taken for each fly to be knocked down and stop moving can then be scored by watching the flies through the glass of the tank and progressively scanning along the vials, thus reducing interference during the assay (Hoffmann et al. 2005). The ability to recover from chill-coma was also not affected by dietary regime in female fruit flies. The time taken for females that had been on a DR regime for 7 or 8 days to recover from chill-coma was not significantly different from control fed females. In contrast, DR reduces the ability of rodents to withstand cold . This is likely to be due to the large disparity in body size and adiposity of DR and controls rodents, which is not seen in fruit flies.

Females that had been on a DR diet for 19 days were more sensitive to desiccation compared to controls, but this effect was not seen in either young females or males. This result requires further investigation. Desiccation resistance declines with age in flies (Rose 1984; Service et al. 1985), thus it seems counter-intuitive that any resistance seen in control-fed flies would be apparent in old individuals but not in young flies, unless the age-related reduction in resistance is accelerated by DR. That this resistance was not seen in the early test may be the result of differences in experimental design, since unlike the later test, this run was not scored every 30 minutes and was set up in the evening and left overnight until it was first scored at twelve hours. This may make the assay less sensitive and subsequent preliminary data suggests that this sensitivity of DR females to desiccation is also seen in young flies (C. Milton, unpublished). DR flies have increased levels of stored lipids and carbohydrates compared to controls (Simmons and Bradley 1997), but reduced protein levels and lower dried weight, which may be the cause of their sensitivity to desiccation. It may also be the case that fully-fed females can re-absorb eggs that are not fully-developed, and this may confer some increased resistance to desiccation not available to DR flies. It would be interesting to test the desiccation resistance of ovo^{D1} sterile females that have oogenesis blocked at early stages.

There was no difference in oxidative stress resistance between DR and control females as measured by survival rates when fed paraquat. However, any stress resistance test that involves feeding the stressor can be potentially confounding when assaying control and DR flies using the food dilution DR protocol, for example, paraquat may affect feeding rates. Furthermore, in the assay protocol used here,

paraquat was fed to flies in standard SY medium. *Drosophila* rapidly switch mortality rates when moved between dietary regimes and there is no effect of former feeding (Chapter 3). Hence, 48 hours after moving control and DR flies to standard SY containing paraquat, any difference between their ability to cope with oxidative stress may be diminished. It is possible to add paraquat to either a control or DR medium, but feeding activity when transferred to new vials differs for control and DR flies (M. Piper, unpublished) and this would confound results of the assay. Alternative assays of the ability of control and DR flies to cope with oxidative stress are microinjecting paraquat solution directly into the thoracic cavity or subjecting the flies to hyperoxygen, and these are potential future directions.

The only positive effect of DR on stress resistance was the increased ability of dietarily restricted females to survive starvation. This has been seen previously, with increased dietary yeast in *Drosophila* causing increased egg-production whilst reducing both starvation resistance and storage of metabolic reserves (Chippindale et al. 1993; Simmons and Bradley 1997). Flies fed foods with reduced levels of yeast extract were also starvation resistant relative to controls (P. Kapahi, personal communication). Indeed, it has been suggested that the diversion of metabolite use from reproduction to storage, and the resultant starvation resistance, is the causal factor in lifespan extension via DR for *Drosophila* (Rauser et al. 2004). However, the positive correlation between starvation resistance and lifespan is not linear, and long-term selection for starvation resistance can lead to an uncoupling of the two phenotypes (Phelan et al. 2003). Furthermore, there is not a direct quantitative relationship between reproduction and stored metabolites in *Drosophila*, with the energy required for increased egg-production in flies given a high level of dietary yeast greater than the deficit of stored metabolites between these individuals and those on restricted yeast (Simmons and Bradley 1997). Increased storage may be the mechanism by which starvation resistance is increased under DR. However, although this mechanism may be indicative of preparation for times of famine, it is unclear why it should decrease mortality when flies are under DR but not malnutrition.

Tetracycline did not extend the lifespan of flies, nor did it block the DR response, meaning either that reduced bacterial challenge is not the mechanism by which

diluting food medium extends lifespan in *Drosophila*, or that the relevant microorganisms are tetracycline-resistant. Previous work has suggested that exogenous bacteria *increase* the lifespan of *Drosophila*, with flies grown axenically having reduced longevity (Brummel et al. 2004). The difference in the results by Brummel et al. and the data presented here may be the result of different methodologies. Although tetracycline was added in high doses in the present work, the flies were not grown axenically and the levels of bacteria present on the flies themselves was not measured. The study mentioned above used tetracycline along with rifamycin and ampicillin in food medium that had been autoclaved, and irradiated with a radioactive cesium source. Furthermore, eggs were washed with bleach, ethanol and water to remove any bacteria on the resulting experimental flies. It could be that this treatment reduced lifespan or that the exogenous bacteria that increase lifespan of flies were not killed in our study. Similar experiments to those presented here, using different antibiotics, had no effect on lifespan in another fly lab (S. Pletcher, personal communication).

6.4.2 Effect of age on stress resistance

Starvation resistance was the only stress response that was enhanced by dietary restriction, and this increased ability to survive without food of DR flies compared to controls was seen in young and old DR individuals. Thus DR flies were better equipped to deal with starvation both at old ages when mortality rates of control flies are higher than those on DR feeding (Chapter 3) *and* at young ages when there is no difference in the death rate of DR and control groups (Chapter 3). This is important, as it means that the difference in resistance between control and DR cohorts is not simply due to the inherent frailty of the control individuals. In rodents studies, thermotolerance of dietary restricted cohorts was seen in old rats, potentially mediated by increased expression of heat shock proteins (Hall et al. 2000), but at advanced ages *ad lib.* fed rats are much less healthy than DR individuals. It would be of interest to see if these findings are also seen at young ages, when then the mortality rates of control and DR groups are the same.

Casual inspection of the data presented in this chapter reveals that for each stress test that was performed on more than one age class, the resistance of both control and DR flies to the stressor decreased with age. However, quantitative analysis of the

data is unjustified, since these tests were carried out at different times of year and with flies from different parents. It is obviously not possible to compare the stress resistance of flies at different age classes without either using flies from different parents or doing the tests at different times. However, one potentially interesting future direction would be to repeat some of these tests over a wider range of ages to test the relationship between age and stress resistance in a more controlled manner.

6.4.4 Conclusions

DR does not increase the global stress resistance of fruit flies in a manner predicted by the hormesis theory. However, DR may increase resistance to stressors not tested here and it would be of interest to extend this study to include different stresses that would perhaps better represent what *Drosophila* experience in the wild. These could include the ability of flies to clear bacterial infection or deal with either low pH or high alcohol levels. The green theory of ageing predicts that the ability to deal with xenobiotics is up-regulated in long-lived animals (Gems and McElwee 2005) and as such, testing the ability of DR and control flies to withstand a stress such as tannic acid may be of interest. As mentioned above, it would also be of value to try different protocols for testing the ability of flies to cope with oxidative stress, such as injecting paraquat directly into the thorax or exposing the flies to hyperoxygen, which drastically reduces lifespan in *Drosophila* (Walker and Benzer 2004). Increased exercise also reduces lifespan of house-flies (Yan and Sohal 2000) and *Drosophila* (Magwere et al, in press) and therefore it would be of interest to see if DR affects the ability of flies to cope with experimentally induced continual flight.

In the supplementary data to their report on TOR signalling and lifespan in *Drosophila*, Kapahi *et al.* (2004b) refer to decreased stress resistance of flies fed high levels of yeast extract. However, there is no published data on stress resistance of DR flies and during personal communication, Kapahi wrote, "I have done heat, paraquat, oxygen and starvation tests. We have done starvation tests most thoroughly and I can say with confidence that's where we see a big difference'. Hence, as with the data presented here, increased starvation resistance seems to be the main benefit of DR in flies.

The results presented here suggest that the increased ability of DR rodents to cope with environmental stress is not seen in fruit flies under DR. This finding can lead to two interpretations. 1) If the increased lifespan seen in DR flies and rodents is indeed the result of similar mechanisms, then the increased stress resistance seen in rodents is not the causal factor in life-extension by DR. 2) Dietary restriction extends lifespan by different mechanisms in these species, and the mechanism in fruit flies is unrelated to the ability to cope with the stressors tested here. Further work testing different stressors on *Drosophila* along with the interaction between mutants in the JNK signalling pathway, which functions to up-regulate stress resistance (Wang et al. 2003), and DR may shed light on this issue.

Chapter 7. General discussion and conclusions

7.1. Summary of findings

In this thesis, I demonstrate that DR extends lifespan in *Drosophila* by a transient reduction in the risk of death at all ages. There is no memory effect of past full-feeding in this species; in contrast to what might have been expected, full-feeding does not cause any permanent damage. In females however, a history of DR conferred some protection to the damaging effects of full-feeding and this protection was greater the longer flies were on a DR regime. The nutrient related risk factor is not increased mechanical damage resulting from the increased egg-production seen in control compared to DR females. Females that are sterile due to the presence of the mutation ovo^{D1} showed extension of lifespan by DR and exhibit the same rapid changes in mortality rates when subjected to changes in feeding regime seen in wild type, fertile females. Caloric intake is not the key mediator of lifespan extension via DR in this species, since reducing calorie intake to the same extent through reduced dietary yeast had a much greater effect on lifespan than reducing carbohydrate intake. DR flies were not globally resistant to environmental stress as might have been predicted by the hormesis theory of lifespan extension via DR.

7.2 Discussion and future directions

Work presented here has further advanced our knowledge of DR in *Drosophila,* but the mechanisms by which life-extension is achieved when nutrient intake is reduced still remain to be determined. However, the results in this thesis do provide a useful framework on which future experiments designed to identify these mechanisms can be based.

The data in chapter 3 showed that the effect of food regime on mortality rate in flies is transient and reversible. The use of 'switching' experiments provides a powerful method to separate factors that merely correlate with diet from those that are causally involved in the DR effect. To be causally associated with lifespan extension by DR in fruit flies, a factor must be shown not only to differ between chronically control and DR fed flies, but also to reverse in at least the same time frame as mortality rates in flies moved between diets. For example, if levels of damage markers such as protein carbonyls are elevated in control flies compared to those on DR, but this elevated level does not reduce to that of the DR group within 48 hours when flies are

switched from control feeding to DR, then protein carbonyls cannot be responsible for the increased mortality rates on control food. Experiments such as these that test the effect of switches in diet on various physiological markers are yet to be published and may prove to be illuminating.

Genome wide profiling of the transcript levels of flies fed a control or DR medium have already been published (Pletcher et al. 2002). This work allowed genes to be classed into different groups based both on the changes in their expression with age and on the difference in their expression levels between flies fed different food regimes. Genes were grouped into those whose transcription changes mapped to chronological age in both treatments, those with patterns of gene expression that mapped to physiological age (i.e. mapped to mortality rates) and, importantly, those that showed rapid changes in expression levels when food regime was changed but that did not alter with age. Genes that are involved in the nutrient dependent risk factor, and could therefore provide a starting point for future investigation, are likely to show rapid changes in expression levels when food regime is altered, and may therefore fall into the last category (Partridge et al. 2005c). This same experimental procedure could also be used with proteomic and metabolomic profiling techniques.

'Switch' experiments may also provide a method for rapid screening of genes associated with the effect of DR on lifespan. Since age-specific mortality analysis is more sensitive than survivorship analysis, it is possible to carry out switch experiments early on in the lifespan of flies and be able to detect the wild type response to the application of DR in a control group in which over 90% of the original cohort is still alive. It may therefore be possible to run batch experiments on mutant flies and test if particular mutations blocks the switch phenotype without the need to run full lifespan experiments. To gain sufficient resolution on the mortality trajectories these experiments would require large sample sizes but their speed may make this a worthwhile trade-off.

The use of age-specific mortality analysis in combination with switching experiments may provide insight into the mechanisms by which other modifiers of lifespan increase longevity. Reduced insulin/ insulin-like signalling increases lifespan in invertebrates (Johnson 1990; Kenyon et al. 1993; Clancy et al. 2001;

Tatar et al. 2001) and mammals (Bluher et al. 2003; Holzenberger et al. 2003). However, it is not known if this lifespan extension is the result of decreased accumulation of irreversible damage (similar to reduced ambient temperature in flies) or a reduction in the transient risk of death (similar to the effect of DR in flies). Tissue-specific, inducible induction or suppression of single genes is now becoming increasingly feasible in biological research. Work in *C. elegans* on the stages in life in which reduced IIS functions to extend lifespan demonstrated that the later the reduction of IIS flux occurred in the life of the worms, the smaller the life-extension seen (Dillin et al. 2002). However, this was determined by analysing the effect of progressively delayed reduction of IIS flux on median lifespan, and both interventions that reduce risk and those that reduce permanent damage would have this effect on median lifespan. Therefore applying the methodology used in chapter 3 may be productive in determining if reducing signalling through the IIS pathway decreases the levels of irreversible damage or, like DR in fruit flies, reduces the transient risk of death.

The fact that both males and sterile females have increased longevity under dietary restriction suggests that this lifespan extension is not the result of decreased egg-production when nutrients are reduced. Indeed, many of the dietary restriction studies performs on rodents are done on male animals, and lifespan extension is usually seen (Weindruch and Walford 1988; Masoro 2002). Evolutionary theory suggests that the plasticity of lifespan in response to reduced food intake may represent the diversion of resources from reproduction to maintenance (Holliday 1989). In chapter 4, blocking egg-production did not block lifespan extension via DR. However, removing an aspect of a fly's physiology such as egg-production that uses up much of its resources does not necessarily free up more resources for other functions, such as repair and maintenance (Lessells and Colgrave 2001; Barnes and Partridge 2003). Indeed, it may be that although sterile flies do not lay eggs, upstream signals associated with reproduction are fully functional and respond to differences in diet. Hence, the increased longevity of flies on DR may still be associated with a decrease in their reproductive state, even if it is not due to decreased mechanical damage from reproductive output. Work on a mutant that could not sense changes in food medium may yield informative results, i.e. a mutant that does not 'know' if it is on full-feeding or DR. It would be of interest to see if

flies with reduced olfactory ability change their reproductive output when fed different media and if not, if they still show increased lifespan under DR. *Pox neuro* mutants, which have reduced chemosensory ability, respond normally to dietary restriction (A. Barnes, unpublished), but their fecundity also changes in a wild type manner with altered dietary regime, suggesting that they can detect changes in food nutrient concentration.

The fact that the level of dietary yeast, rather than caloric intake *per se*, is the key determinant of lifespan in flies fed different food media asks the obvious question of what it is in the yeast that is responsible for this effect. Since autolysed yeast powder contains some carbohydrate as well as all the protein and lipid in fly food medium, the most productive way to answer this question will be to use a defined or semi-defined medium in which levels of individual dietary components can be altered in a quantitative way. This will allow the effect on lifespan of reducing a particular nutrient, such as one amino acid, to be determined in a manner similar to the recent reports of methionine-restriction in rodents (Zimmerman et al. 2003; Miller et al. 2005).

7.3 Food for thought, does dietary restriction really extend lifespan in *Drosophila*?

The aim of this thesis was to add to the known data in the field and further characterise the response of *Drosophila melanogaster* to DR, with a view to better understanding the mechanisms by which it extends lifespan in this species. DR has been reported to increase the lifespan of fruit flies in a multitude of studies by different laboratories (Chippindale et al. 1993; Chapman and Partridge 1996; Clancy et al. 2002; Pletcher et al. 2002; Rogina et al. 2002; Mair et al. 2003; Bauer et al. 2004; Kapahi et al. 2004b; Magwere et al. 2004; Mair et al. 2004b; Rogina and Helfand 2004; Wood et al. 2004; Mair et al. 2005; Piper et al. 2005). However, these results have recently been called into question (Le Bourg and Minois 2005), and in particular the protocols and methods used in this thesis have been criticised. In this section, I will respond to the arguments raised by these authors and their statement that 'published papers have not shown without ambiguity that DR increases longevity in *D. melanogaster*'.

7.3.1 Failure to confirm DR effect in Drosophila

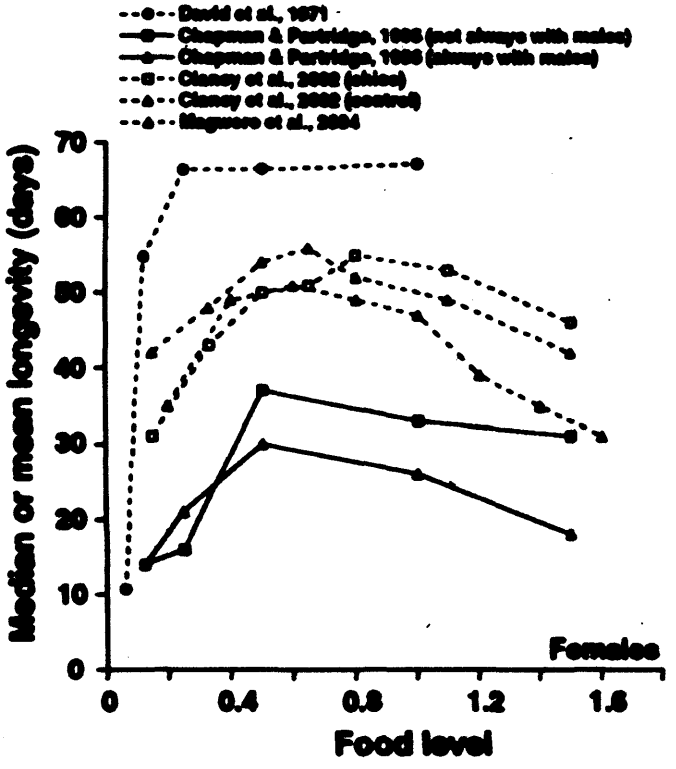
One key argument of Le Bourg and Minois is that DR does not extend the lifespan of either male or virgin female *D. melanogaster,* and that any extension of lifespan seen in fertile females is the result of reduced egg-production. This statement is incorrect and based on one study published by these authors (Le Bourg and Minois 1996), which was aimed at repeating earlier work by Chippendale at al. on the effects of varying the levels of live yeast fed to fruit flies (Chippindale et al. 1993). In their study, Le Bourg and Minois (1996) increased the numbers of flies per vial used by Chippendale et al. from two to ten. Yeast concentrations given to flies were then determined by calculating the concentration of yeast *per fly* in the Chippindale et al. experiment and multiplying it accordingly. Therefore, for yeast levels to be comparable between the two studies, flies would need be eating the same amount and consuming *all* the given yeast, neither of which were demonstrated.

These two studies are not direct repeats, and any failure to see a response to DR may have been due to the range of food dilutions being insufficient. The fly strain used in by Le Bourg and Minois (1996) differed from that used by Chippendale et al. (1993) and sample sizes were low (n = 40, two orders of magnitude less than the experiments in this thesis), reduced further by the fact 'some flies escaped' and had to be censored (Le Bourg and Minois 1996). Lifespan does not peak at the same nutrient levels for all genotypes (Clancy et al. 2002; Magwere et al. 2004), and it is therefore imperative that for all studies on the effects of diet on lifespan, a full range of nutrient concentrations is first tested and the nutrient level that maximises lifespan is first determined empirically (Clancy et al. 2002; Partridge et al. 2005b; Piper et al. 2005). Furthermore, in the work by Chippendale et al. , yeast paste was added to the surface of a charcoal medium that already contained some nutrients in the form of sugar and dead yeast, (Rose and Charlesworth 1981; Rose 1984; Chippindale et al. 1993), whereas Le Bourg and Minois supplemented a synthetic medium originally described in (Pearl et al. 1926) with yeast, and this medium alone was not nutritious enough for larvae to develop (Le Bourg and Minois 2005). The source of 'live yeast' also varies between labs and there are no data comparing the two fly strains used in these conflicting studies. The results from this work are therefore far from conclusive proof that DR does not extend lifespan in *Drosophila.*

The only other example of a published report of food dilution failing to increase the lifespan of wild type *Drosophila* is by (David et al. 1971). Dilution of the standard fly medium in this experiment resulted in progressive reduction of both fecundity and lifespan. However, as demonstrated in the review by Le Bourg and Minois (2005) (Figure 7.3.1), this may have been due to a failure to increase the food nutrient concentration past the point at which lifespan is maximised.

Figure 7.3.1. Comparison of the effects of varying food concentration on the median lifespan of female *Drosophila* in different experiments.

Figure taken from (Le Bourg and Minois 2005). The study by David et al. only looked at food concentrations that represent the left side of a 'tent shaped' response of lifespan to diet.



The experimental flies used by David et al. (1971) were the F1 generation of a cross between a wild type stock and a *vestigial* strain. The long lifespan of these flies may therefore may have been due to hybrid vigour (heterosis), which can result in lifespan extension of over 100% (Clarke and Maynard Smith 1955; Comfort 1979). Thus, heterosis may result in flies that can withstand levels of yeast that would be detrimental to the lifespan of the parental stocks. Finding the food concentration that optimises lifespan for each different strain examined and then testing a wide range of food concentrations either side of this optimum must become a prerequisite in DR

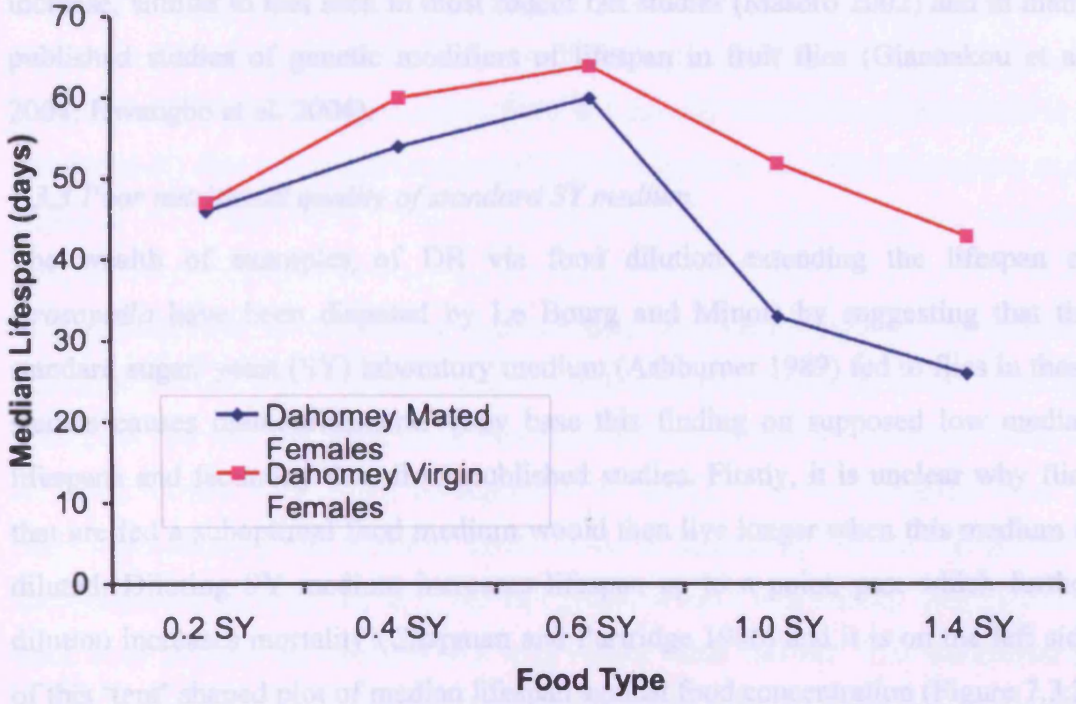
studies on *Drosophila* (Piper et al. 2005). Failure to do so may result in false negative responses to diet being reported.

7.3.2 DR does extend the lifespan of both male and virgin female Drosophila

Virgin female *Drosophila* do show a response to DR, with lifespan extended as food concentration is diluted (Clancy 2002; A. Barnes, unpublished; Figure 7.3.2), contrary to the findings of Le Bourg and Minois (1996).

Figure 7.3.2. Median lifespan of mated and virgin female wild type *Drosophila* on different food regimes.

The DR response of mated females is greater than that of virgins. However, contrary to Le Bourg et al. (1996), virgin females do live longer under DR. These data are from unpublished work by A. Barnes and printed with his kind permission.



Le Bourg and Minois state, without any experimental evidence, that reduction in egg-laying must be the cause of the response of lifespan to DR in *Drosophila*. DR produces a full extension of lifespan of female flies that are sterile due to either the presence of the dominant mutation ovo^{D1} or X-irradiation as late pupae (Mair et al. 2004). Not only do ovo^{D1} flies not lay any eggs, they do not undergo vitellogenesis, ruling out mechanical damage from egg-production as the risk factor associated with full-feeding. DR has been shown to increase survival in male flies in several studies

(Chippindale et al. 1993; Rogina et al. 2002; Mair et al. 2003; Magwere et al. 2004; Rogina and Helfand 2004), although the magnitude of this lifespan extension is less than in females (Magwere et al. 2004). Interestingly, the magnitude of lifespan extension in virgin females is also less than that of mated flies (A. Barnes, unpublished, Figure 7.3.2) and it seems likely that reproduction does play some part in this difference.

A complete comparative study of once-mated females, virgin females and males is as yet unpublished using the DR protocol presented in this thesis and may well prove informative. In a dismissal of the work by Magwere et al. (2004), Le Bourg and Minois state that 'the longevity increase observed in wild type males is not very important' because it is only an increase of ten days. However, this represents a 32% increase, similar to that seen in most rodent DR studies (Masoro 2002) and in many published studies of genetic modifiers of lifespan in fruit flies (Giannakou et al. 2004; Hwangbo et al. 2004).

7.3.3 Poor nutritional quality of standard SY medium.

The wealth of examples of DR via food dilution extending the lifespan of *Drosophila* have been disputed by Le Bourg and Minois by suggesting that the standard sugar/ yeast (SY) laboratory medium (Ashburner 1989) fed to flies in these studies causes malnourishment. They base this finding on supposed low median lifespans and fecundity data from published studies. Firstly, it is unclear why flies that are fed a suboptimal food medium would then live longer when this medium is diluted. Diluting SY medium increases lifespan up to a point, past which further dilution increases mortality (Chapman and Partridge 1996) and it is on the left side of this 'tent' shaped plot of median lifespan against food concentration (Figure 7.3.2) that the medium is likely to be malnourishing flies. Indeed, in comparison to other *Drosophila* food media, the nutrient content of SY medium is markedly concentrated[21]. Secondly, the statements regarding the low lifespan and fecundity of flies fed SY medium in Le Bourg and Minois (2005) show a lack of attention to the differing culturing and husbandry techniques used in these studies, and an

[21] Food comparison taken from Bloomington Stock Centre website (http://fly.bio.indiana.edu/media-recipes.htm)

inaccuracy with regard to reporting fecundity data published by Chapman and Partridge (1996).

As Le Bourg and Minois point out, 'the mean lifespan of flies can strongly vary, depending on rearing conditions and strain' (Le Bourg and Minois 2005). For this reason, wide scale comparisons of lifespan data from different experiments, as was done by Le Bourg and Minois (2005), are inadvisable without taking experimental differences into account. The density of flies in bottles/ vials is inversely proportional to their lifespan (Pearl 1928) and fecundity (Pearl et al. 1926). In addition, flight activity also reduces the lifespan of house flies (Yan and Sohal 2000) and *Drosophila* (T. Magwere, in press). In the experiments in this thesis, high sample sizes were needed for demographic analysis and hence the flies were cultured in 200ml bottles containing 100 individuals. Increased flight activity and interaction with other flies may explain the lower median lifespans of flies in these experiments compared to those fed the same medium but housed in smaller (30ml) vials at a density of 10 flies per vial (Clancy et al. 2001; Clancy et al. 2002; Broughton et al. 2005).

The concentration of the control medium used also varied between the different published studies. The 'fully-fed' controls in the experiments in this thesis were fed a medium that was 1.5 times more concentrated than control SY used in (Clancy et al. 2001; Clancy et al. 2002; Broughton et al. 2005). This was purposely done in order to enhance the magnitude of the extension of lifespan by DR and was therefore suboptimal for fly lifespan, as it was this phenomenon I was testing. The Dahomey stock used in these experiments is a wild caught strain that has been maintained in large population cages with overlapping generations since its collection in order to maintain lifespan and fecundity at levels similar to freshly caught stocks (Sgrò and Partridge 2001). It is therefore representative of wild *Drosophila* populations and is as long-lived, if not more so, than other wild type strains such as Canton-S used in different laboratories (Mair, unpublished). The longevity of Dahomey flies when maintained on normal SY medium and in vials at low density is in the order of 45-60 days (Clancy et al. 2001; Clancy et al. 2002; Broughton et al. 2005), the lifespan regarded as 'normal' by Le Bourg and Minois.

Le Bourg and Minois (2005) suggest that further evidence of the poor nutritional quality of the SY medium is provided by the low fecundity of females fed this food. As part of their evidence for this, they cite lifetime fecundity data from Chapman and Partridge (1996) despite the fact that no such data existed in that paper or were recorded in that experiment. Chapman and Partridge (1996), only counted eggs every three days, and the lifetime fecundity index published in their study represents approximately a third of the potential lifetime egg-production of the experimental females. When this is taken into account, fecundity of these females is approximately 420 eggs, which is standard for flies that do not have access to live yeast (T. Chapman, personal communication). Le Bourg and Minois also state that the value of 20 as the median egg-production of 7-day old female flies on control food for 24 hours, shown in chapter 4, is very low, and again that the food source is therefore suboptimal. Females in this experiment were housed with males for 48 hours before being kept in single-sex groups on the respective diets for 7 days, whereupon fecundity was measured. Once-mated females store sperm after mating but this runs out after 10-11 days as they become 're-virginised' (Kaufman and Demerec 1942; Ashburner 1989), and it may be that subsequent egg-production is less than continuously mated females.

7.3.4 The effects of live yeast on lifespan and fecundity

Le Bourg and Minois state that live yeast promotes longevity and fecundity in *Drosophila* and that without it lifespan is less than 30 days. "Clearly speaking, live yeast is an essential nutrient and studies totally removing live yeast give information on malnutrition rather than on DR' (Le Bourg and Minois 2005). However, this is not the case, and a medium containing dead, autolysed yeast as a source of protein is sufficient to support healthy lifespans (Ashburner 1989). Flies fed a sugar only medium have no access to protein, fat, vitamins or minerals in their diet and as expected have elevated mortality rates that can be rapidly reduced when fed live yeast (Good and Tatar 2001). However, in this case live yeast is promoting longevity by rescuing the effect of nutrient deprivation. SY medium is indeed limiting flies with respect to egg-production, but importantly not with respect to lifespan, since adding live yeast to such a medium will increase fecundity but *decrease* longevity (S. Pletcher, unpublished), in contrast to adding live yeast to a starvation medium which increases both parameters (Good and Tatar 2001).

7.3.5 Conclusions

The review by Le Bourg and Minois highlights the need to keep in mind the ecology of the model organism when designing experiments and comparing results between laboratories. For example, throughout this thesis the experiments were done on flies in single-sex groups. This is because, when comparing flies with different nutritional histories, is important that they have identical mating histories. Many previous studies have shown that nutritional intake has an effect on mating rates in fruit flies (Harshman et al. 1988; Chapman and Partridge 1996) and that mating reduces the longevity of both male (Partridge and Andrews 1985; Prowse and Partridge 1997) and female *Drosophila.* Studies using mixed sexes are also confounded by differences in the sex-ratio of treatments that emerge as the experiments progress, since those treatments with high mating rates will become male biased earlier in the study than those with low mating rates, as females suffer the deleterious effects of being mated at high rates (Fowler and Partridge 1989). Thus performing DR experiments on flies in mixed-sex groups results in comparing flies subjected to different nutritional *and* mating histories.

Intermittent feeding extends lifespan in rodents (Goodrick et al. 1982) but not in *Drosophila* (Le Bourg and Medioni 1991) or the Mediterranean fruit fly (Carey et al. 1999; Carey et al. 2005). A possible explanation of these findings is that these insects need to maintain a steady food intake if they are to avoid starvation. Periods of food consumption, during which all the food is eaten, interspersed with periods when no food is available may not have the same effect on lifespan as the consumption of the same amount of food at a continuous low rate. Indeed, in the work on intermittent feeding in medflies, there were pronounced mortality oscillations linked to food availability, with food removal resulting in instantaneous spikes in death rates (Carey et al. 1999). This may also explain one report on houseflies where reducing the quantity of food available per day did not extend lifespan (Cooper et al. 2004).

Le Bourg and Minois suggest that the ability to increase survival rates when nutritional availability is limited may only be seen in species that do not have the ability to 'flee' areas with poor resources to find new food patches. With little

empirical basis, they suggest that insects have this capacity to flee but that rodents do not. *Drosophila* from temperate regions do not migrate during winter but instead enter a diapause-like state that enables them to survive with limited nutrient availability and at low temperatures (Bouletreau-Merle and Fouillet 2002). Thus, it seems likely that the ability to increase survival at a cost of reproduction when faced with restricted food levels would be strongly selected for in this species. Historically, times of famine may have been primarily influenced by seasonal fluctuations in the climate and thus this may be a key factor in the strength of selection for the ability to live-long under DR (de Grey 2005). Little is known about the relative abilities of insects and rodents to flee their current habitat when it is depleted of resources, but it seems unlikely that flies could migrate the distance that would be needed to find food rich patches during periods of drought etc.

Le Bourg and Minois add to their theory that species that can flee areas of low nutrition will not show increased lifespan under DR to suggest that DR will not extend human lifespan because of the ability of humans to emigrate when faced with starvation. They give the example of the fact that, during the 19th century, people encountering famine in Ireland had the ability to emigrate, and those that did increased their chance of surviving, whilst those who remained in starvation 'did not establish the longevity record of that time' (Le Bourg and Minois 2005). Firstly, this anecdote fails to appreciate that during the 3 million years or so that humans have been in existence, it is only in the last few hundred that mass migration has been a realistic option to avoid famine, and as people in the third world sadly still demonstrate, this is by no means a ubiquitous ability. Thus, any effect of emigration ability on the evolution of plasticity of lifespan in response to nutritional intake in humans is likely to be negligible and indeed famine is likely to have exerted strong evolutionary pressures on humans (Prentice 2005). Secondly, and perhaps more importantly, it is an example of the general lack of distinction that the authors show in their paper between DR, which is reduced intake without malnutrition and starvation. In the same way that feeding starving flies live yeast makes them live longer, of course, moving away from a country where food is low in supply to one where it is more plentiful will be beneficial. What this observation has to do with the phenomenon of dietary restriction is unclear.

The weight of studies in support of the life-prolonging effect of diluting the food medium fed to *Drosophila* seem to far out weigh the single negative result published by Le Bourg and Minois in 1996. Indeed, the lack of an effect in that study may be explained by the experimental design used and a wider range of dilutions may have yielded a positive result. The logic that the food medium is malnourishing fruit flies in all the published studies in which DR has increased their lifespan is flawed, because of the very fact that dilution of such a medium extends lifespan. It is indeed crucial when interpreting data on any intervention that extends lifespan, that the lifespan extension is seen in long-lived wild type animals and not just in a sick, short-lived strain, since for the latter the intervention may just be rescuing the deleterious traits associated with that particular strain. However, the 'short' lifespans of control flies in the experiments in this thesis are not due to poorly nutritious food but rather the effects on fruit flies of eating high levels of dietary yeast. Elucidating mechanisms by which this nutrient-related increase risk of death occurs was the basis for the study. The correspondence from Le Bourg and Minois (2005) highlights the ever present need in the field of ageing research for a clearer thinking about the causal relationships between genotypes, nutrition and lifespan.

7.4 DR, *truly* a public mechanism?

The aim of this thesis was to further characterise DR in *Drosophila* to gain some insight into the mechanisms via which it extends lifespan, with the ultimate goal of comparing these mechanisms to those seen in rodents. If the mechanisms are shared between two distantly related organisms such as flies and rats, perhaps the likelihood of work on DR resulting in health benefits to humans becomes real and rapid progress in characterising the mechanisms can be made. The results here on specific nutrients and stress resistance seem to be examples of differences between DR rodents and DR flies, but this does not mean that the mechanisms through which DR extends lifespan are necessarily different in insects and mammals. I have shown that a remarkable phenotype of DR in flies is the rapid and reversible effect of diet on mortality rate in this species. Therefore, a true test of conserved mechanism between rodents and flies would be to examine the effect on mortality rates of moving rodents to a DR regime in middle-age. However, the large sample sizes and labour costs required for such an experiment may prove prohibitive. Furthermore, the reduced

core temperature of DR rodents compared to controls (Duffy et al. 1997) may mean that nutritional history will have an effect on mortality rates.

Although it has been suggested that DR gives some health benefit to humans (Fontana et al. 2004), the lifespan studies on the effects of DR on non-human primates remain inconclusive (Lane et al. 2004). In fact, it has been argued that selection pressure for a substantial lifespan extension by DR in humans is weak (de Grey 2005). If the plasticity of lifespan in response to diet is a mechanism that has evolved to survive periods of food shortage (Holliday 1989), it follows that the strength of selection for this trait is influenced by the length of time historically that animals have been subjected to famine during evolution.

Seasonal fluctuation in the weather may be one major cause of reduced resource availability, but would be unlikely to result in poor food yields for many years in succession. Therefore, natural selection may favour those individuals that have the capacity to survive these seasonally induced food shortages, but not those with the potential to survive long periods of starvation of tens of years, as such long periods of food shortage would be too seldom to make this capacity beneficial. Under this hypothesis, the extension of lifespan we can expect in different species under DR will be the same in absolute, rather than proportional time and will only be of the magnitude of the length of time that food deprivation occurred historically. This may be a marked increase in the lifespan of a mouse, but much less of an extension in percentage terms for a human (de Grey 2005). The idea that the ability to increase survival under DR is an adaptive response, and that the length of time that this reduced mortality can be maintained is determined by how long it was needed during the course of evolution is only true if this ability is costly; entering an effectively dormant stage with lowered reproduction and increased survival during a two year famine may increase fitness, but having the *capacity* to maintain this for longer may not reduce fitness.

Much work is still needed on comparing the effects of DR between different species before any claim can be made about the likelihood of these effects also being applicable to humans. Despite the fact that it is still unclear if the same mechanisms that facilitated lifespan extension under DR in McCay's original work in rats are the

same as those that function in *Drosophila*, it is to be hoped that the results presented here may form some part of the chain of experiments that will eventually resolve this question.

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Appendix 1

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Appendix 2

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Appendix 3

Mair, W., C. M. Sgro, A. P. Johnson, T. Chapman and L. Partridge (2004). Lifespan extension by dietary restriction in female *Drosophila melanogaster* is not caused by a reduction in vitellogenesis or ovarian activity. Exp Gerontol 39(7): 1011-1019.

Appendix 4

Hulbert, A. J., D. J. Clancy, W. Mair, B. P. Braeckman, D. Gems and L. Partridge (2004). Metabolic rate is not reduced by dietary restriction or by lowered insulin/IGF-1 signalling and is not correlated with individual lifespan in *Drosophila melanogaster*. Exp Gerontol 39(8): 1137-43.

Appendix 5

Partridge, L., S. D. Pletcher and W. Mair (2005). Dietary restriction, mortality trajectories, risk and damage. Mech Ageing Dev 126(1): 35-41.

Appendix 6

Partridge, L., M. D. Piper and W. Mair (2005). Dietary restriction in *Drosophila*. Mech Ageing Dev 126(9): 938-950.

Appendix 7

Mair, W., M. D. Piper and L. Partridge (2005). Calories do not explain extension of life span by dietary restriction in *Drosophila*. PLoS Biol 3(7): e223.

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Calories Do Not Explain Extension of Life Span by Dietary Restriction in *Drosophila*

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Dietary restriction (DR) extends life span in diverse organisms, including mammals, and common mechanisms may be at work. DR is often known as calorie restriction, because it has been suggested that reduction of calories, rather than of particular nutrients in the diet, mediates extension of life span in rodents. We here demonstrate that extension of life span by DR in *Drosophila* is not attributable to the reduction in calorie intake. Reduction of either dietary yeast or sugar can reduce mortality and extend life span, but by an amount that is unrelated to the calorie content of the food, and with yeast having a much greater effect per calorie than does sugar. Calorie intake is therefore not the key factor in the reduction of mortality rate by DR in this species.

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Introduction

Dietary restriction (DR), the extension of life span by reduction of nutrient intake without malnutrition, is often used as a benchmark comparison for interventions that extend life span [1–3]. Since McCay's pioneering experiments in rats 70 years ago [4], some form of food restriction has been shown to increase life span in commonly used model organisms such as yeast [5,6], nematodes [7], fruit flies [8,9], and mice [10], along with many species less often used for laboratory research such as water fleas, spiders, fish (see [3] for review), and dogs [11]. Preliminary data also suggest that DR may extend life span in nonhuman primates [12,13] and potentially give health benefits in humans [14]. Despite the finding that restricting diet increases longevity in such a diversity of species, the mechanisms responsible remain to be fully elucidated in any of them. It is therefore as yet unclear whether these mechanisms are evolutionarily conserved across taxa or if instead life extension during DR is an example of convergent evolution.

DR is often termed 'calorie restriction' because, in rodents, daily calorie intake per se has been implicated as the key determinant of life span, with the source of these calories (i.e., carbohydrate, protein, or fat) being considered irrelevant [1]. Evidence for this point of view came from two types of experiment on rats: (1) restriction of calorie intake without reduction of protein intake resulted in life-span extension [15]; (2) no life-span extension was seen in rats fed isocaloric diets in which either the fat or mineral components had been reduced [16]. However, in other experiments, rats fed isocaloric diets with altered nutritional composition [17,18] or reduced protein [19] showed life-span extension. Furthermore, reducing just one amino acid (methionine) increases life span in both mice (R. Miller, personal communication) and rats [20]. Hence, it seems that reducing the level of ingested calories may not always be critical for life-span extension by DR in rodents. Here we address this issue in the fruit fly *Drosophila melanogaster*.

Results

DR can be applied in *Drosophila* by the simultaneous dilution of the nutrients in a standard sugar yeast (SY) food medium [9] in which the yeast is the only source of protein and lipid. As food concentration declines from maximum, life span first increases in response to DR, becoming greatest at an intermediate food concentration, before declining due to starvation at lower concentrations [9,21]. We tested the separate effects of sugar and yeast on life span at the concentrations that maximise life span (DR) and under full feeding (control).

Feeding Rates of Flies on Different Food Types

Because flies may respond to changes in dietary composition by altering their feeding behaviour, thereby potentially compensating, we determined the effect of food composition on the amount of time that the flies spent feeding on different diets. Varying the proportions of sugar or dead yeast fed to adult *Drosophila* females did not have a significant effect on feeding behaviours (Figure 1; $p > 0.01$ in all cases, chi-squared test, Bonferroni adjustment for multiple comparisons). A significant difference was seen on day 17 (chi-squared, $p = 0.0068$) with flies on DR yeast/control sugar food eating less. However, this difference was in the opposite direction to that expected if flies on low-nutrient diets compensated by increasing feeding rates. Hence, the flies did not compensate for decreased nutrient content of the food medium by increasing the time that they spent feeding.

Caloric Content of Dead Yeast/Sucrose

Values for yeast biomass components were taken from Lange and Heijnen [22] and estimations of the caloric content of protein, carbohydrate and lipid from Southgate and Durnin [23]. This allowed estimation of the caloric content

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Abbreviations: CI, confidence interval; DR, dietary restriction; SY, sugar yeast

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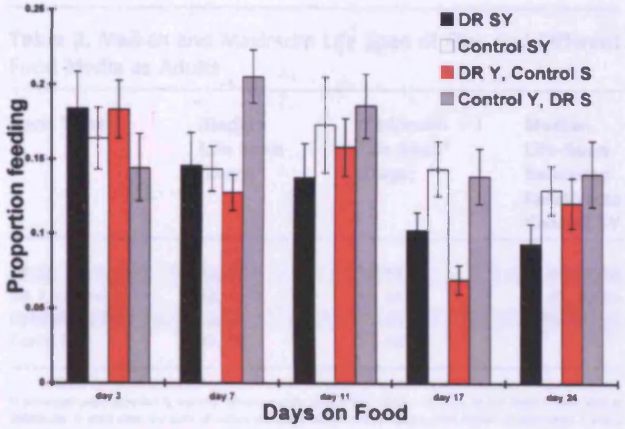

Figure 1. Feeding Rates of Female *Drosophila* on Food Media with Different Nutrient Concentrations

Feeding rates were recorded by direct observation as the proportion of time flies spent on the surface of the media with their proboscis extended and touching the food (*y*-axis). Replicate measurements of the proportion of females feeding versus those not feeding were recorded during a 2-h period on the days shown. No significant different was seen between flies fed different diets on days 3, 7, 11, and 24 as assessed by chi-squared tests ($p > 0.01$, Bonferroni correction for multiple comparisons). There was a significant difference in feeding rates on day 17 ($p = 0.0068$) with flies on the DR yeast/control sugar media eating less. These data show that *Drosophila* does not exhibit compensatory feeding behaviour for the DR regime imposed.

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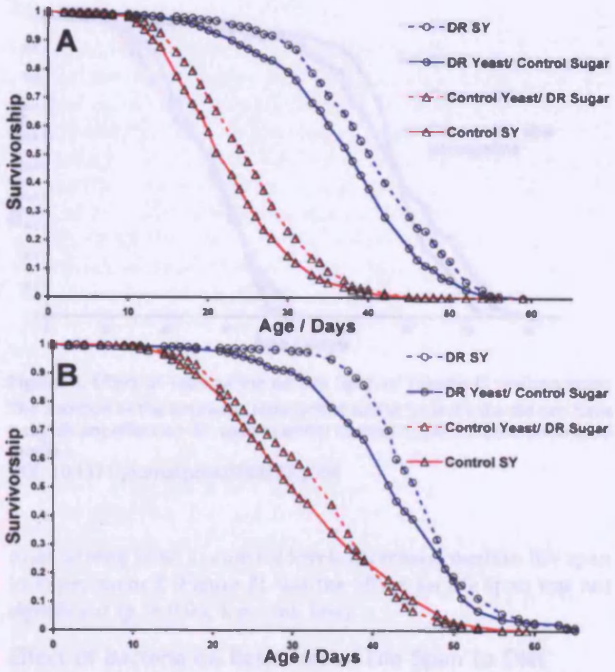

Figure 2. Survivorship (l_x) Analysis of Life Span of Female *Drosophila* on Different Food Regimes

Colour/Symbol of the curves shows yeast level while the line type represents sugar levels in the respective foods. (A) and (B) are independent repeats. In both cases, changing caloric content of the food by altering yeast levels had a much greater effect on life span than that seen when the same change in caloric content was brought about by manipulating sugar levels.

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per gram of sucrose and autolysed yeast powder, the only sources of nutrients in the *Drosophila* food medium. These values were 4 kcal/g sucrose and 4.02 kcal/g autolysed yeast powder. Since these values are virtually identical, changing either the sugar or yeast content of the foods between the DR and control concentrations generated food types with similar caloric values but with different nutritional compositions (see Table 1).

Life Span of Female *Drosophila* Given Foods of Different Caloric Value

Life span of female *Drosophila* was extended much more by reduction of yeast from control to DR concentration than by the equivalent reduction in sugar (Figure 2; Table 2), and median life span therefore did not correlate with caloric content of the food medium to which the flies were exposed (Figure 3). In two independent experiments, reducing yeast concentration from control to DR levels whilst keeping sugar levels constant significantly increased life span ($p < 0.0001$ in both cases, log-rank test). Lowering caloric content to the same extent by reducing sugar from control to DR levels increased life span at DR yeast levels in both experiments ($p < 0.0001$ in both cases, log-rank test), but the effect on median life span was much less than that of changing yeast levels (Figure 3; Table 2). Reducing sugar from control to DR concentrations whilst keeping yeast at control levels signifi-

Table 1. Nutritional Composition and Caloric Content of Experimental Food Types

Food Type	Nutritional Content (Grams of Components per Litre Water)	Estimated Protein (g/l)	Estimated Carbohydrate (g/l)	Estimated Lipid (g/l)	Estimated Caloric Content (kcal/l)
DR SY	65 g Y, 65 g S	27.755	89.96	5.59	521.17
DR yeast/control Sugar	65 g Y, 150 g S	27.755	174.96	5.59	861.17
Control yeast/DR Sugar	150 g Y, 65 g S	64.05	122.6	12.9	862.7
Control SY	150 g Y, 150 g S	64.05	207.6	12.9	1202.7

Food media were based on standard sucrose/yeast (SY) media as described in [9]. Y, autolysed yeast powder; S, sucrose. Estimations of yeast biomass components are taken from [22] and estimations of the calorific content of protein, carbohydrate, and lipids are taken from [23]. This gives values of 4 kcal/g sucrose and 4.02 kcal/g yeast.

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Table 2. Median and Maximum Life Span of Flies Fed Different Food Media as Adults

Food Type	Median Life Span (Days)	Maximum Life Span[a] (Days)	Median Life-Span Extension Relative to Control SY
DR SY	42, 48	54, 58	82.6%, 60.0%
DR Y, control S	38, 43	52, 56	65.2%, 43.3%
Control Y, DR S	25, 35	38, 48	8.7%, 16.7
Control SY	23, 30	37, 48	—

Y, autolysed yeast powder; S, sucrose. [a]Maximum life span is the median life span of the longest lived 10% of individuals. In each case, the pairs of values represent results of two independent repeats (experiments 1 and 2, respectively).

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cantly increased life span in experiment 1 ($p < 0.0001$, log-rank test), but again the effect on median life span was much less than that of changing yeast levels (Figure 3; Table 2). Reducing sugar from control to DR concentrations whilst

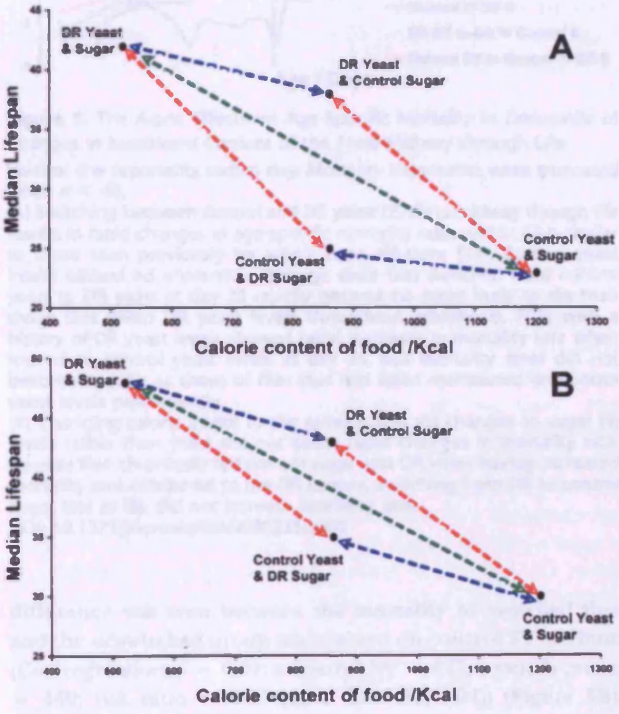

Figure 3. Plot of Median Life Span of Female *Drosophila* against the Estimated Caloric Content of the Food Medium

(A) and (B) represent independent repeats. Red arrows link pairs of food types where differences in caloric content are due to different yeast concentrations. Blue arrows link pairs of food types where differences in caloric content are due to different sugar concentrations. Green arrow links food types where differences in caloric content are due to both different sugar and yeast concentrations. Life span is extended to a greater extent per calorie by reducing yeast concentration from control to DR levels than by reducing sugar. This is in contrast to what would be predicted if calorie intake were the key mediator of life-span extension by DR in fruit flies.

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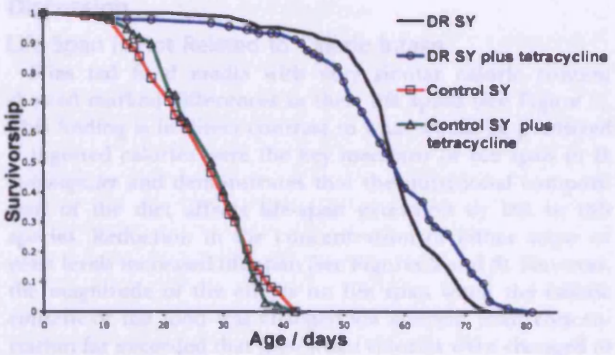

Figure 4. Effect of Tetracycline on Life Span of Female *D. melanogaster.*

The addition of the antibiotic tetracycline to the food media did not have a significant effect on life span at either control or DR concentration food media.

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maintaining yeast at control levels increased median life span in experiment 2 (Figure 3), but the effect on life span was not significant ($p > 0.05$, log-rank test).

Effect of Bacteria on Response of Life Span to Diet

To test if different levels of bacteria in the food medium could account for effects of nutrient composition on life span, we tested the effect of an antibiotic. The addition of the antibiotic tetracycline to the food media did not have a significant effect on life span on either control or DR food medium (Figure 4, $p > 0.05$; log-rank test in each case), and the life-span extension seen when sugar and yeast levels were reduced from control to DR concentrations was therefore not blocked or modified by the addition of antibiotic to the food medium.

Effects on Mortality of Switching Yeast and Sugar

The effect of DR on mortality in *Drosophila* is acute; within 48 h flies switched between DR and control diets adopt the mortality rates characteristic of flies chronically exposed to the nutritional regime that the switched flies have joined [24]. We therefore measured the acute effects on mortality of switching the yeast and sugar components of the diet separately. When yeast was switched, mortality rates responded similarly to the responses to switches between control and DR SY food medium. Forty-eight hours after being switched from control SY medium to DR yeast/control sugar medium at day 25, flies were no more likely to die than those maintained on DR yeast/control sugar medium throughout adulthood (Cox regression; $p = 0.22$; n DR yeast/control sugar chronic group = 626; n switch group = 475; risk ratio = 0.96 [95% confidence interval {CI}: 0.91, 1.02]) (Figure 5A). In the reciprocal switch, flies moved from DR SY medium to control yeast/DR sugar medium showed a rapid increase in mortality rate, although this did not quite reach the level seen in flies that had been on control yeast/DR sugar medium throughout adult life (Cox regression; $p < 0.05$; n control yeast/DR sugar chronic group = 480; n switch group = 668; risk ratio = 0.88 [95% CI: 0.83, 0.93]) (Figure 5A).

In contrast, switching of sugar had no significant effect on mortality. From 48 h after being switched from control SY medium to control yeast/DR sugar at day 25, no significant

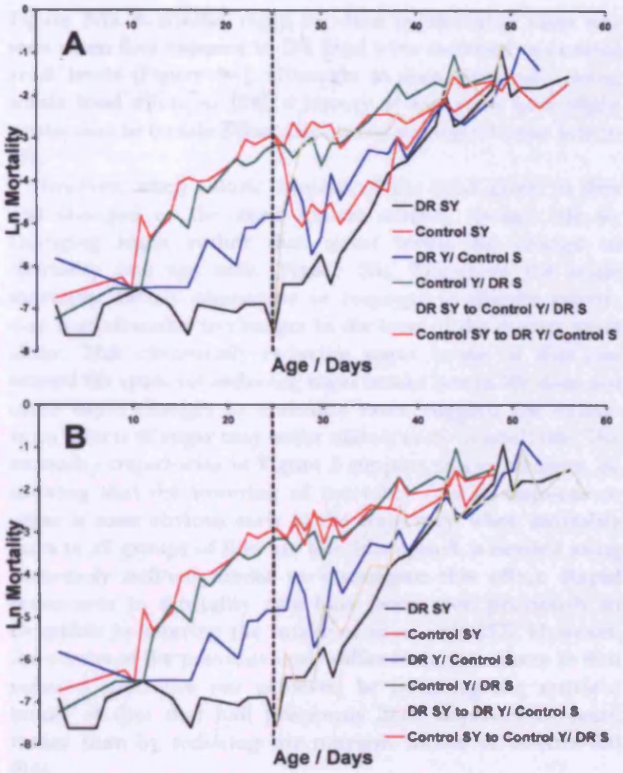

Figure 5. The Acute Effects on Age-Specific Mortality in *Drosophila* of Changes in Nutritional Content of the Food Midway through Life

Vertical line represents switch day. Mortality trajectories were truncated when $n < 40$.

(A) Switching between control and DR yeast (Y) diets midway though life results in rapid changes in age-specific mortality rates within 48 h similar to those seen previously for whole food dilutions [24]. Control yeast intake caused no irreversible damage since flies switched from control yeast to DR yeast at day 25 rapidly became no more likely to die than those flies given DR yeast levels throughout adulthood. Flies with a history of DR yeast levels showed rapid increases in mortality rate when moved to control yeast levels at day 25, but mortality rates did not become as high as those of flies that had been maintained on control yeast levels permanently.

(B) Changing caloric intake to the same extent via changes to sugar (S) levels rather than yeast did not cause rapid changes in mortality rate. Despite flies chronically fed control sugar and DR yeast having increased mortality rate compared to the DR control, switching from DR to control sugar late in life did not increase mortality rate.

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difference was seen between the mortality of switched flies and the unswitched group maintained on control SY medium (Cox regression; $p = 0.34$; n control SY = 427; n switch group = 440; risk ratio = 0.97 [95% CI: 0.91, 1.04]) (Figure 5B). Similarly, flies switched to DR yeast/control sugar from DR SY medium at day 25 did not show increased mortality in comparison to unswitched controls (Cox regression; $p = 0.41$; n DR group = 615; n switch group = 676; risk ratio = 0.98 [95% CI: 0.93, 1.03]) (Figure 5B). A second experiment that was terminated 4 d after the switch in diet gave the same result (see Figure S1). These data show that the rapid switch in mortality rates upon changes between DR and control food medium are overwhelmingly attributable to the yeast rather than to the sugar component of the diet.

Discussion

Life Span Is Not Related to Calorie Intake

Flies fed food media with very similar caloric content showed marked differences in their life spans (see Figure 3). This finding is in direct contrast to what would be predicted if ingested calories were the key mediator of life span in *D. melanogaster* and demonstrates that the nutritional composition of the diet affects life-span extension by DR in this species. Reduction in the concentration of either sugar or yeast levels increased life span (see Figures 2 and 3). However, the magnitude of the effects on life span when the caloric content of the food was changed via altering yeast concentration far exceeded that seen when calories were changed to the same extent via manipulation of sugar levels, suggesting that protein/lipid levels have a greater effect on *Drosophila* survival than does carbohydrate. The differing effect of sugar and yeast on mortality in *Drosophila* could occur if different pathways sense nutrients during DR, possibly with different outputs affecting life span. Sir2 [25,26], Rpd3 [27], the insulin/IGF-like signalling [28], and target of rapamycin pathways [29] have all been implicated in mediating the response of life span to DR in *Drosophila*, with the latter two suggested to interact in the fly to control growth in response to nutrient levels [30]. The role of these and other candidate pathways in mediating the response of life span to specific nutrients should be investigated further. Sugar and yeast could affect mortality rates differently if they differentially modulate metabolic or other processes that increase risk of death.

Experimentally increased reproduction has been shown to decrease life span in a variety of species [31–35] and the level of dietary yeast and egg production are positively correlated in *Drosophila* [8,9]. Therefore an obvious hypothesis as to why there is a greater response of life span in *Drosophila* to changes in yeast than in sugar is that the increased mortality on control yeast levels represents the cost of reproduction, which correlates with yeast intake and not with sugar. However, since life-span extension via DR in *Drosophila* occurs normally when egg production or vitellogenesis are blocked either by X-irradiation or genetically [36], the greater response of life span to changes in yeast is not directly attributable to the reduction of reproductive output. Furthermore, although the magnitude of the response to DR in male *Drosophila* is less than that of females [21], males do live longer if nutrient levels are reduced, and they show the same rapid changes in mortality as females when dietary regime is changed [24], yet they do not suffer the high costs of producing eggs on high yeast.

Rapid Changes in Mortality in Response to DR Are Attributable Solely to Yeast Content

DR acts acutely to extend life span in *Drosophila;* it does not slow the accumulation of irreversible damage with age [24]. Flies subjected to DR for the first time in midlife rapidly become no more likely to die than those that have been under DR throughout adulthood [24]. We investigated the roles of the sugar and yeast components of the diet in producing this rapid change in mortality rate in flies switched between DR and control conditions. When flies previously subjected to control SY food were switched to DR yeast levels, there was a rapid (within 48 h) drop in mortality rates to those seen in the flies chronically exposed to DR yeast/control sugar food (see

Figure 5A). A similar rapid increase in mortality rates was seen when flies exposed to DR food were switched to control yeast levels (Figure 5A), although, as seen previously using whole food dilutions [24], a history of low yeast gave slight protection to female *Drosophila* moved to control yeast late in life.

However, when caloric content of the food given to flies was changed to the same extent midway though life by changing sugar rather than yeast levels, no change in mortality rate was seen (Figure 5B). Therefore the acute mortality 'switch' phenotype in response to dietary restriction is attributable to changes in the level of the dietary yeast alone. That chronically reducing sugar intake of flies can extend life span, yet reducing sugar intake late in life does not cause rapid changes to mortality rates, suggests the deleterious effects of sugar may occur mainly early in adult life. The mortality trajectories in Figure 5 support this conclusion, by showing that the lowering of mortality rate in response to sugar is most obvious early in the trajectory, when mortality rates in all groups of flies are low. More work is needed using accurately defined media to investigate this effect. Rapid reductions in mortality rate have been seen previously in *Drosophila* by altering the intake of yeast only [37]. However, the results of the previous study differ from those here in that reduced mortality was achieved by *increasing* the nutrient intake of flies that had previously been deprived of yeast, rather than by reducing the nutrient intake of control-fed flies.

Feeding Rates of Flies on Different Food Types

Unlike in rodents, where DR can be achieved by directly reducing the quantity of food eaten in comparison to animals given ad libitum access [1], DR is achieved in *Drosophila* by reducing the quality (nutrient concentration) of the food given to the flies [9] with the quantity maintained in excess of that which they can consume. Despite the fact that fecundity correlates with food medium concentration [9], it has been suggested that flies may be able to compensate when faced with reduced nutrients by increasing feeding rates, and therefore they may not be dietarily restricted [38]. However, our results suggest that flies on low-quality media do not compensate by eating more, as measured by time spent on the food with the proboscis extended. It is possible *Drosophila* can alter the rate of food uptake per unit time that the proboscis is extended, in which case our indirect measurements would not detect these changes. More direct approaches to quantify feeding rates require radio-labelling the food [39] or the addition of coloured food dye [40], with uptake rates assessed upon short-term exposure to labelled food. However, our own unpublished observations show that flies moved to fresh food medium display elevated feeding behaviour that is unrepresentative of the steady-state situation and that leads to a highly nonlinear relationship between time and uptake of the food label. We hence used the behavioural measure described here, which better represents the normal feeding of the flies. Our feeding assay results, in combination with the reduced fecundity seen as food nutrient concentration is reduced, suggest that diluting the food medium results in a co-ordinate reduction in the intake of nutrients in *Drosophila* and therefore is a robust protocol for DR in this species.

Effect of Tetracycline on Life Span

It has been suggested that higher nutrient concentrations in fly food may lead to higher proliferation rates of bacteria on the media, which in turn could increase mortality of *D. melanogaster* in a mechanism that is unrelated to ingestion of different amounts of nutrients [38]. If this were the case then we would expect that (1) flies fed antibiotics would live longer, and (2) the life-span extension seen when nutrient concentration is reduced would be blocked when antibiotics are present. Tetracycline did not extend the life span of flies in our experiments, nor did it block the DR response, meaning either that reduced bacterial challenge is not the mechanism by which diluting food media extends life span in *Drosophila*, or that the relevant microorganisms are tetracycline resistant.

Conclusions

The response of *Drosophila* life span to nutrition is not governed by calories, but rather by specific nutritional components of the food. This finding represents a departure from the generally accepted model in rodents, where it has been suggested that the level of calorie intake per se, not the source of calories, is critical for life-span extension [1]. The apparent disparity between the factors in the diet that affect life span in fruit flies and rodents leads to two possible conclusions. First, the mechanisms by which these organisms respond to food shortage could be different. Second, the long-held view that calorie intake is the critical variable in the response of mammalian life span to DR may require further evaluation.

Despite some reports in the literature that DR did not extend life span [38,41,42], the overwhelming majority of data support the idea that DR in some form extends life span across diverse taxa. However, it is still unknown if life-span extension under DR is achieved through common mechanisms in different species. A case for conservation of the mechanisms by which DR extends life span can be made from evolutionary considerations. It has been suggested that, during times of famine, diversion of resources away from reproduction towards somatic maintenance will increase the chances of an organism surviving to more plentiful times and thus increase long-term reproductive success [43–46]. The selective advantage of shifting resources from reproduction to maintenance when food is restricted could be the "public" factor shared between diverse organisms. However, the mechanisms by which extension of life span is achieved could be an example of convergent evolution, producing the same plasticity of life span in response to food shortage through mechanisms at least to some extent specific to different organisms, dependent upon their diet, experience of food shortages, and life history. More work is needed to elucidate the precise relationship between the composition of the diet and life span in different organisms, including mammals. Our results suggest that it may be possible to obtain the full extension of life span by DR by reducing critical nutrients in the food without any reduction in overall calorie intake.

Materials and Methods

Fly stocks and husbandry. The wild-type stock used in all experiments was collected in Dahomey (now Benin) in 1970 and has since been maintained in large population cages with overlapping generations on a 12:12-h light:dark cycle at 25 °C. This culturing

Figure 5A). A similar rapid increase in mortality rates was seen when flies exposed to DR food were switched to control yeast levels (Figure 5A), although, as seen previously using whole food dilutions [24], a history of low yeast gave slight protection to female *Drosophila* moved to control yeast late in life.

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method has been shown to maintain life span and fecundity at levels similar to those in freshly collected flies [47].

Feeding rates of flies on different food types. To measure feeding rates in *Drosophila* we observed behaviour of age-matched, once-mated Dahomey females on each of the four food types. This approach was adopted in preference to direct quantification of ingested food [40] because DR flies transiently elevate their feeding rate following transfer onto new food (unpublished observations). In the present assay, 30 female flies were individually allocated to a vial containing either control SY, control Y/DR S, DR Y/control S, or DR SY and placed at 25 °C overnight to adopt their undisturbed pattern of feeding. The following day, 1 h after lights on, observations were taken for a 2-h period, and flies were scored as eating if they were on the food with their proboscis extended and touching the food surface. During this time, 360 observations of flies in each treatment were made (12 observations of 30 flies) except on day 24 when 18 observations were made of each treatment set. The final data are the proportion of flies feeding out of the feeding opportunities given (total observations). Differences between treatments at a given time point were assessed using the chi-squared test.

Effect of tetracycline on life span. Tetracycline is a general antibiotic that inhibits ribosomal translocation and acts on both Gram-positive and negative bacteria [48]. A tetracycline solution was made up in 70% ethanol and added to the food media after it had been boiled and cooled to 60 °C. The final concentration of tetracycline in the media was 0.025% weight/volume [49], five times more than that used when tetracycline resistance is utilised as a selectable marker for bacterial transformation [50]. The wild-type stock Dahomey is infected by the cytoplasmic bacteria *Wolbachia* (unpublished). A 0.025% tetracycline solution is sufficient to remove bacteria such as *Wolbachia* from *Drosophila* stocks if fed to larvae [49] and can suppress *Wolbachia* in other insects when fed to adults only [51]. Therefore flies fed tetracycline media as adults may not only have reduced exposure to external microorganisms on the food surface compared to controls, but may also have reduced *Wolbachia* infection. Seven millilitres of food was poured into 30-ml glass vials and the life span of flies measured with 92–101 flies per treatment and 10 flies per vial. Fresh food was prepared once a week and flies moved onto new media three times per week.

Life span experiments. Experimental flies were raised at a standard density of 400–450 eggs per 200-ml bottle [52] on standard SY medium (1,000 ml distilled water, 100 g autolysed yeast powder, 100 g sucrose, 20 g agar, 30 ml Nipagin (100 gl^{-1}), 3 ml propionic acid). Adults were collected over a 24-h period and transferred without anaesthesia to fresh SY food for 48 h and allowed to mate. Females were then collected using light CO_2 anaesthesia and assigned randomly to the food regimes (Table S1). All experiments were done with mated females. Flies were kept on 35 ml of food at an initial density of 100 individuals per 200-ml bottle and transferred without anaesthesia to fresh food every 2–3 d. Deaths were scored 5–6 d a week and initial sample sizes (n_0) were calculated as the summed death and censor observations over all ages. To minimise any density effects on mortality, two bottles within cohorts were merged when the density of flies reached 50 ± 10. To standardise the effects of parental age on offspring fitness [53], parents of experimental flies were of the same age and reared at a constant density.

Statistical analysis. Age-specific mortality (μ_x) was estimated as $\mu_x = -ln(p_x)$, where p_x is the probability of an individual alive at age $x-1$ surviving to age x [54]. log-rank tests [55] were used for survivorship analysis. All statistical analysis was performed using JMP. 5.0 statistical software (SAS Institute Inc., Cary, North Carolina, United States).

Supporting Information

Figure S1. The Acute Effects on Age-Specific Mortality in *Drosophila* of Changes in Nutritional Content of the Food Midway through Life

Vertical line represents switch day. Experiment was terminated 4 d after the switch.

(A) Similar to the experiment shown in Figure 5, switching between control and DR yeast (Y) diets midway though life results in rapid changes in age-specific mortality rates within 48 h similar to those seen previously for whole food dilutions [24].

(B) Changing caloric intake to the same extent via changes to sugar (S) levels rather than yeast did not cause rapid changes in mortality rate.

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Table S1. Sample Sizes and Treatments

These represent the number of flies switched between treatments (i.e., n_{25}) and were sampled from the original chronic controls (control SY or DR SY) and censored from the life-span data of these treatments at day 25.

Found at DOI: 10.1371/journal.pbio.0030223.st001 (27 KB DOC).

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Competing interests. The authors have declared that no competing interests exist.

Author contributions. LP and WM conceived and designed the experiments. WM performed the experiments and analyzed the data. LP, WM, and MDWP wrote the paper. ■

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Appendix 8

Piper, M. D., W. Mair and L. Partridge (2005). Counting the calories: the role of specific nutrients in extension of life span by food restriction. J Gerontol A Biol Sci Med Sci 60(5): 549-55.