UNIVERSITY COLLEGE LONDON

Department of Genetics, Evolution and Environment

Establishing a role for specific nutrients in *Drosophila* dietary restriction

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Abstract

Dietary restriction (DR), the reduction in food intake that falls short of starvation, has been shown to be the most robust and reproducible intervention to extend lifespan in diverse organisms ranging from yeast to mammals, including the fruit fly *Drosophila*. Despite over 70 years of research, primarily on rodents, the mechanisms by which DR extend lifespan in any organism are poorly understood, partially due to the variation in how DR is defined and applied between laboratories. Lifespan extension by DR commonly trade-offs with reduced fecundity, leading to evolutionary-based theories predicting that DR elicits an evolved response to food shortage in nature, through reallocation of resources away from reproduction and towards somatic maintenance, hence increasing the chance of survival until food supply becomes more abundant.

In *Drosophila*, DR is typically implemented by dilution of sucrose and yeast in an agar-based medium, with yeast being the key component regulating lifespan. Firstly, this thesis presents an investigation of the response of the model organism *Drosophila* to different DR diets and protocols, thereby creating one standardized and optimized DR diet for use. Secondly, using the optimized diet, this project investigates the role of specific nutrients mediating the effects of DR and the potential pathways controlling these effects. Essential amino acids were shown to directly regulate the trade-off between high fecundity and reduced lifespan observed with full feeding. However, methionine addition alone was necessary and sufficient to increase fecundity to levels seen with full feeding, without reducing lifespan, demonstrating that reallocation of nutrients cannot explain the DR responses. The results of this thesis highlight the importance for a standard DR protocol and suggest that in other organisms, including mammals, the beneficial effects of DR may be achieved without impairing fertility by using a suitable balance of nutrients in the diet.

Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, this has been duly indicated within the work.

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Richard Grandison

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Appendix 2…….

Grandison, R. C., R. Wong, T. M. Bass, L. Partridge and M. D. Piper (2009). "Effect of a standardised dietary restriction protocol on multiple laboratory strains of Drosophila melanogaster." PLoS ONE **4**(1): e4067.

Abbreviations

AAs	Amino acids
AP	Antagonistic pleiotropy
ASG	Cornmeal-based medium
CR	Caloric / calorific restriction
daGAL4	DaughterlessGAL4 driver
Dilps	*Drosophila* insulin-like peptides
DN	Dominant-negative
DR	Dietary restriction
EAAs	Essential amino acids
IGF-1	Insulin-like growth factor 1
IIS	Insulin / insulin-like signalling
dInR / InR	Insulin receptor
MA	Mutation accumulation
ml	Millilitre
mM	Milli molar
N-EAAs	Non-essential amino acids
PI3K	Phosphoinositide 3-kinase
RNAi	RNA interference
ROS	Reactive oxygen species
dS6K / S6K	S6 kinase
SOD	Superoxide dismutase
SY	Sugar-yeast medium
TOR	Target of rapamycin
UAS	Upstream activating sequence
wDahomey / Wdah	White Dahomey flies
wsp	*Wolbachia* surface protein
yw	Yellow-white flies

Chapter 1

General Introduction

1.1 Introduction to ageing

The concept of ageing is familiar to everyone, yet a precise definition is not easy to find. Applied to humans, ageing might be seen as the deterioration of the body over time, whether it be in the form of physical appearance such as greying hair or wrinkles or the onset of ageing-related diseases such as cancer or diabetes. However, when viewed in a different context, ageing does not necessarily incorporate only negative attributes. For example some types of cheese and wine are often left to mature to enhance their flavour and increase their value. Hence ageing can mean different things to different people and thus a universal definition might define ageing simply as all positive, negative and indifferent things that change with age (Ricklefs and Finch 1995). In biological terms, ageing can be defined as the age-related decline in fitness traits as a result of internal physiological deterioration (Rose 1991). This definition of ageing will be applied throughout this work.

Whilst ageing is almost universally observed across species, there are a few exceptions, suggesting that ageing may not be inevitable. For example, the simple fresh-water animals *Hydra* have been reported to show no signs of senescence when assayed over a four year period (Martinez 1998). Furthermore, almost all cells in the body are only able divide a finite number of times (Hayflick's limit) (Hayflick and Moorhead 1961; Hayflick 1965); however, germ line cells (Weismann 1893) and tumour cells (HeLa cells) appear to be exceptions to the Hayflick's limit and are therefore potentially non-ageing. Despite being ruled out by William Hamilton (1966), recent evidence has provided support for the existence of negative senescence (Vaupel *et al.* 2004). Negative senescence is defined as a decline in mortality with age following reproductive maturity, which is generally accompanied

by an increase in fecundity (Vaupel *et al.* 2004). Perhaps the strongest case for negative senescence was shown in three coral species whose mortality was inversely related to colony size and age, whilst total fecundity increased with age (Babcook 1991). Other species including molluscs, sea urchins, some fish and some reptiles are also thought to display signs of negative senescence (reviewed in Vaupel *et al*. 2004).

One of the most interesting phenomena of ageing is the sheer diversity of lifespans amongst different species in nature (Ricklefs and Finch 1995; Austad 1997). Bats, for example, are renowned for their exceptional longevity relative to their body size, living around 3 ½ times longer than rodents of the same size (Austad 2005), whilst naked mole rats are also unusually long-lived for their size (Sherman and Jarvis 2002). Lifespan can even vary amongst individuals with the same genotype. For example in eusocial insects such as ants, the queen lives significantly longer than any of her workers which serve to protect her from predation (Bourke and Franks 1995), and in some ant species this can be a remarkable 28 years (Keller and Genoud 1997).

1.1.1 Studying ageing

Research into ageing has undergone a massive rise in popularity over the last 10 years, attracting many researchers from adjacent fields. The sheer diversity of ageing research, ranging from biochemistry, cellular senescence and genetics all the way through to evolutionary analysis and demographic studies, has made it an extremely attractive field for a wide range of scientists to work in.

Since the discovery that a single gene mutation (*age-1*) can extend lifespan in the nematode worm *C.elegans* (Klass 1983; Friedman and Johnson 1988), the field of ageing has advanced significantly. The recent sequencing of genomes for model organism such as the fruit fly *D. melanogaster* (*Drosophila melanogaster* sequencing consortium 2000) and *C. elegans* (*C. elegans* sequencing consortium 1998), in addition to the numerous breakthroughs in molecular and genetic techniques such as RNA interference (RNAi) (Fire *et al.* 1998), have been pivotal in enhancing our understanding of the genetic and molecular foundations of ageing.

During the last 50 years, world life expectancy has risen by more than 20 years from 46 years to 67 years[1]. In the UK, life expectancy has shown a progressive increase in both males and females during the last 25 years and is expected to continue rising[2] (Figure 1.1). The increase in life expectancy is owed largely to improvements in health care, medicine and sanitation leading to a better quality of life. However, as life expectancy increases, the risk of developing chronic ageing-related diseases becomes greater.

As is the case for several biological fields, trying to uncover ways of increasing human longevity has attracted strong opinions from supporters (de Grey *et al.* 2002) as well as critics, who believe it to be ethically wrong (Kass 2001; Fukuyama 2002). However, increased age is associated with numerous pathologies including stroke, cardiovascular disease, cancer, arthritis, diabetes, dementia and neurodegenerative diseases, just to name a few. The goal of gerontologists is not solely to extend human longevity, but ultimately to increase the length of healthy life. After all, living longer will not improve human lives if this meant the probability of contracting terminal

[1] Institute national d'études démographiques, www.ined.fr
[2] National statistics online, www.statistics.gov.uk/cci/nugget.asp?ID=168

illnesses is increased or if the length of time humans endure these illnesses is prolonged. Hence interventions that can extend lifespan whilst improving health during ageing become invaluable.

Figure 1.1: UK life expectancy at birth from period life tables, 1980-82 to 2005-2007: Taken from National Statistics online[2]

1.1.2 Demographic measurements of ageing

Gerontologists characterise ageing in a population by measuring the rate of mortality. The mortality rate is defined as the probability of an individual who is alive at a given age to die during the following age interval. Mortality can be represented either by the fraction of a population surviving at a particular age or the as the mortality rate at a particular age (Figure 1.2). In most species, mortality rate increases exponentially with increasing time, a phenomenon known as the law of mortality (Gompertz 1825). As an individual gets older the probability of dying in the next time-frame increases. The gradient of the slope of log mortality against age is representative of rate of acceleration of mortality (Figure 1.2), also known as the Gompertz parameter.

The time required for the mortality rate to double is known as the mortality rate doubling time (MRDT), and can be used as an indicator of how fast a population ages. The smaller the MRDT, the faster organisms in the population are ageing. For example, humans have an MRDT of approximately eight years in contrast to laboratory mice which have an MRDT of three months and fruit flies whose mortality rate doubles every five to ten days (Ricklefs and Finch 1995). Interestingly, in humans above 90 years of age, the slope of the Gompertz curve decreases, indicating that the mathematical risk of dying may actually decrease when an individual reaches a certain, extremely high age (Ricklefs and Finch 1995; Vaupel 1997; Vaupel *et al.* 1998; Vaupel *et al.* 2004). A similar pattern is observed in Mediterranean fruit flies (medflies), where the mortality rate slows down drastically to a constant when around 90% of the population have died (Carey *et al.* 1992).

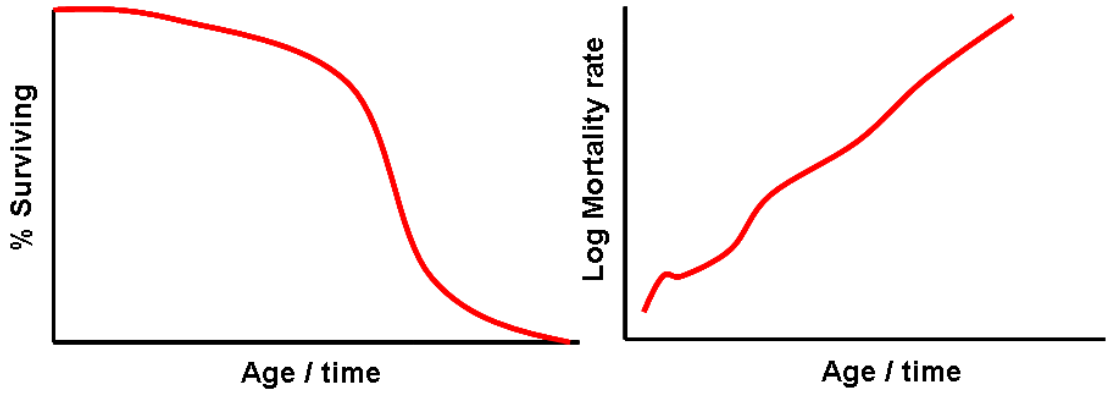

Figure 1.2: A diagram of typical survival (left) and mortality curves (right) over time of populations in protected environments.

1.2 Evolution of ageing / Evolutionary theories

The phenomenon of ageing has posed a paradox to evolutionary biologists. How is it that a process has evolved, which causes intrinsic decline in function leading to reduced survival and / or reduced fecundity, thereby reducing the ability of an individual to make a genetic contribution to the next generation? Ageing appears to be a disadvantageous trait, and natural selection should act to remove unfavourable genes that cause ageing. For over a century, biologists have been intrigued by this question and have formed various hypotheses as to why ageing may have evolved.

Theories of ageing primarily fall into two categories, the "how" and the "why" theories. The "how" theories (or mechanistic theories of ageing) try to explain the mechanisms that are causing ageing in organisms and will be discussed in more detail in section 1.3. The "why" theories (or evolutionary theories) try to account for the existence and prevalence of ageing in populations and will be discussed in the following sections.

1.2.1 Ageing for the good of species

The first evolutionary theories of ageing were proposed by scientists including August Weismann, Peter Medawar, J.B.S. Haldane, George Williams, William Hamilton and Ronald Fisher, amongst others. Over a century ago, it was believed that ageing may have evolved to benefit the group or population rather than to benefit the individual itself (so called ageing for the good of species). Alfred Russel Wallace suggested that ageing has evolved to prevent individuals that have already reproduced from consuming the resources of others, hence increasing the overall fitness of their successor(s) (published as footnote in Weissmann 1989). Later,

August Weismann proposed that ageing evolves to remove old and worn out individuals from populations to make way for the young. Weismann suggested that worn out individuals are not only valueless to the species, but can be harmful because they take the place of sound individuals (Weismann 1889). Upon first glance, the idea that ageing acts to remove older individuals in order to make way for new ones seems logical. However there are some flaws in this proposal. Firstly, both Weismann and Wallace's theories assume that ageing already exists. If ageing did not exist then there would be no advantage for older individuals to make way for new ones because the older individuals could continually reproduce. Therefore the idea of the old making way for the new does not serve as the original cause of ageing, but instead acts as a side-effect of the original cause, making the argument somewhat circular (Kirkwood 2005). Secondly, both hypotheses are confined to species living in family groups.

In 1941, J.B.S Haldane made a breakthrough in the understanding of why ageing exists by focusing on the prevalence of Huntingdon's disease. Huntingdon's is a late-onset neurodegenerative disease caused by a dominant mutation that exerts its phenotypes after the age at which most people have reproduced, usually between 30 and 50 years of age[3]. Haldane questioned why natural selection had not acted to remove this mutation from the population. His hypothesis was that ageing occurs as a result of late-acting deleterious mutations, and that the selection pressure to remove these mutations is weak because the effects are observed predominantly after reproduction (Haldane 1941).

[3] The Huntingdon's disease association, www.hda.org.uk

1.2.2 Mutation accumulation theory

The mutation accumulation (MA) theory was first proposed by Peter Medawar in 1952 (Medawar 1952), and followed on from Ronald Fisher's observations that the chance of individuals to contribute to the future ancestry of the population declined with age. Medawar suggested that ageing evolves as a side-effect of mutation pressure because of reduced force of natural selection to counteract its effects later in life. The force of natural selection is weak later in life because fewer bearers survive long enough to express late-acting mutations. Medawar demonstrated that even in a so called non-ageing population, death will still occur through extrinsic hazards such as disease, predation and accidents. Death rates will be constant with age and therefore the number of individuals alive will show a negative exponential decline (Figure 1.3). It has previously been argued that senescence is rarely observed in wild populations due to the high levels of extrinsic hazard (Comfort 1979). However, many studies have since reported ageing in nature, for example in mammals (Austad and Fischer 1991; Bronikowski *et al.* 2002) and also birds (Gustafsson and Part 1990).

Despite the mutation accumulation theory being an attractive hypothesis for the evolution of ageing, few experimental studies currently support it (Hughes and Reynolds 2005). However, there is some evidence for the existence of mutations with age-specific effects, as the MA theory would predict. In *Drosophila*, accumulation of mutations has been shown to have age-specific effects on lifespan, mating ability and fecundity, e.g. (Mack *et al.* 2000; Borash *et al.* 2007).

The MA theory makes two predictions about genetic variation in natural populations. Firstly, additive genetic variance (heritability) for survival and fecundity will

increase with age. This means that the parents and offspring should resemble each other more closely for survival and fecundity when they are both old compared to when they are both young because of an increase in genetic variants that they hold in common affecting later ages. However evidence from studies in *Drosophila* does not generally support this (Hughes and Charlesworth 1994; Promislow *et al.* 1996; Shaw *et al.* 1999). The second prediction is that inbreeding depression, which is the reduction in fitness of offspring of two parents that are more closely related than average for the population, will increase with age because more deleterious genetic variants are shared during later stages of life. Generally, experimental evidence from studies in *Drosophila* has supported this prediction (Charlesworth and Hughes 1996; Hughes *et al.* 2002); however an alternative explanation is that older individuals are weaker and more fragile, thus are likely to be more susceptible to the effects of inbreeding depression (Charlesworth and Hughes 1996). Furthermore, allowing real germ line mutations to accumulate over time, whilst protecting them from being removed by natural selection, revealed that most new mutations impair fecundity and/or survival and do so at several age-intervals; there is little evidence for specific mutational effects later in life (Pletcher *et al.* 1998; Pletcher *et al.* 1999).

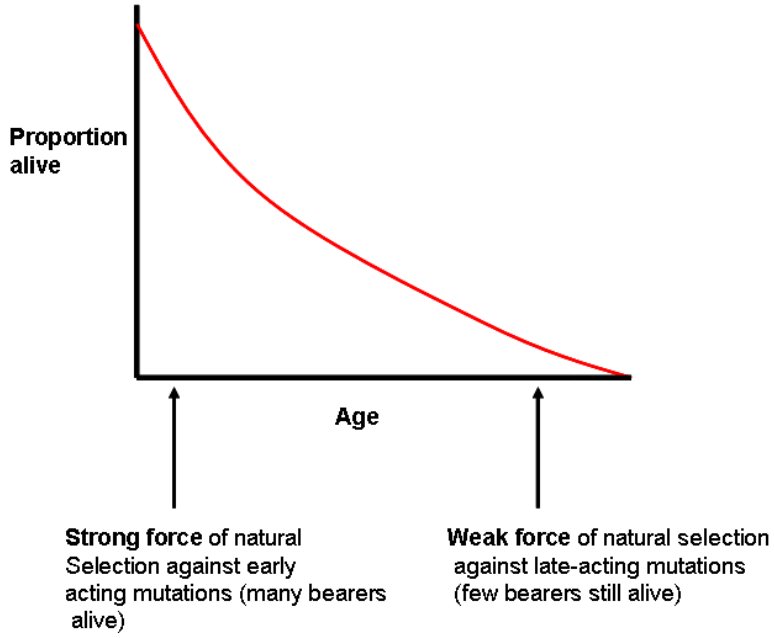

Figure 1.3: Diagrammatic representation of the force of natural selection on deleterious mutations over time in wild populations. Survival probability decreases exponentially with age. Early in life natural selection will act strongly to remove deleterious mutations from the population. However, later in life when there are fewer bearers the force of natural selection becomes weaker.

1.2.3 Antagonistic pleiotropy

In 1957, George Williams put forward the pleiotropy theory of ageing. The term antagonistic pleiotropy (AP) refers to expression of a gene which causes multiple competing effects, some which are negative and some which are positive. Williams proposed that due to the declining force of natural selection with age, gene mutations that are beneficial early in life but become costly later in life will be favoured by natural selection, because more individuals will survive to express the early benefit than will survive to experience the detrimental effects (Williams 1957). For example, in male humans the production of high levels of testosterone early in life can increase reproductive fitness, whereas later in life it can lead to increased risk of prostate cancer (Gann *et al.* 1996). Another example of AP might be the expression of the

P53 gene, which can help suppress cancer early in life, but also suppresses stem cells preventing efficient replacement of old, worn-out tissues (Rodier *et al.* 2007).

Additional support for the AP theory comes from the discovery of single gene mutations that extend lifespan. For example, the majority of *Drosophila* mutations that extend lifespan also cause a severe reduction in fecundity or even sterility (Lin *et al.* 1998; Rogina *et al.* 2000; Clancy *et al.* 2001; Tatar *et al.* 2001). However there are some exceptions, particularly in *C. elegans* where animals with specific mutations in *Age-1* (PI3K) or *Daf-2* (insulin receptor) display normal fecundity (Johnson *et al.* 1993; Kenyon *et al.* 1993; Gems *et al.* 1998).

1.2.4 Disposable soma theory

The disposable soma theory was proposed by Thomas Kirkwood and is a specific case of the antagonistic pleiotropy theory of ageing. This theory assumes that there are limited resources that can be allocated between somatic maintenance and repair on one hand and reproduction on the other (Kirkwood 1977; Kirkwood and Holliday 1979). Therefore the body must budget the amount of energy available to it. Natural selection will favour investing more heavily in reproduction to enhance the overall fitness of the individual. However, in times of food scarcity, the strategy changes and in such circumstances it is more optimal to invest the limited resources to somatic maintenance, thus extending the individual's chance of surviving until the food supply becomes more abundant and reproduction can successfully commence. The disposable soma theory thus predicts that the principal cause of ageing is a result of the accumulation of cellular and molecular damage, which arises due to evolved limitations in somatic maintenance and repair functions (Kirkwood 1977; Kirkwood and Holliday 1979). In the wild, 90% of mice are expected to live only one year due

to high extrinsic mortality rates (Berry and Bronson 1992) hence investing resources in somatic maintenance for the slim chance of living slightly beyond a year is not favoured (Kirkwood 2005).

Evidence for a lifespan / reproduction trade-off is strong. Data from historical records suggests that human life histories involve a trade-off between longevity and fertility whereby longevity is negatively correlated with the number of offspring but positively correlated with the age at first childbirth (Westendorp and Kirkwood 1998). In laboratory experiments, using *Drosophila*, selecting for increased longevity results in reduced fecundity and vice versa (Rose and Charlesworth 1981; Rose 1984; Fowler and Partridge 1992; Sgro and Partridge 1999). Furthermore, differences in mortality between lines selected for early and late reproduction are diminished in sterile flies (Sgro and Partridge 1999). In addition, dietary restriction, which robustly extends lifespan in diverse organisms, also causes reduced or delayed fertility (section 1.5). Hence dietary restriction could elicit an evolved response to food shortage, with a metabolic shift of resources away from reproduction to investment in repair and maintenance, thus increasing the probability of survival until the food supply becomes more abundant (Williams 1966; Kirkwood and Holliday 1979; van Noordwijk and de Jong 1986; Kirkwood and Shanley 2005) (section 1.5.5.1).

1.3 Mechanistic theories of ageing

Evolutionary theories attempt to explain why the phenomenon of ageing exists. However, a second group of theories has emerged, which try to explain the mechanisms behind the ageing process at the organismal level. It is thought that the number of proposed mechanistic theories of ageing has amassed to over 300 (Medvedev 1990). These range from primitive theories including, for example, Élie Metchnikoff's intestinal bacteria theory (which suggests that ageing occurs as a result of intestinal bacteria producing toxins that poison the body) through to more popular theories such as the oxidative theory of ageing (which predicts that the proximal cause of ageing is a build up of free radicals which cause oxidative damage to macromolecules) (Harman 1956). The lack of one universally agreed theory of ageing is not surprising because of the complex nature of this biological process. Ageing does not simply occur at the level of DNA, RNA or protein, nor does it occur solely at the level of tissues or organs. In fact ageing can occur at every level of organisation from the level of DNA all the way through to the organismal level. Another reason for the lack of a universal theory of ageing is the difficulty in experimentally testing theories and separating cause from effect. Hence, ageing is likely to be the result of a combination of several of the processes postulated by these theories, whilst many of these theories may simply describe by-products of ageing rather than the cause of the damage and loss of function that is ageing itself.

"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."

Brian Charlesworth, Evolutionary biologist

Whilst there is no central theory of ageing, a collection of damage-based theories have emerged that suggest ageing occurs as a result of the continuous accumulation of damage due to by-products of metabolism or inefficient repair mechanisms. The scope of this work does not allow an evaluation of the hundreds of theories proposed so the following sections will try to address some of the more plausible damage-based theories of ageing.

1.3.1 Rate of living theory

Live faster, die younger: This hypothesis states that smaller organisms have higher metabolic rates per unit mass than larger organisms and hence will die more rapidly (Figure 1.4). Ageing is inversely related to metabolic rate, as was demonstrated by Max Rubner in 1908 who studied five different mammalian species that had had a range of different lifespans but similar total metabolic output (energy consumed over a lifetime) per unit body mass (cited from Vijg 2007). In 1928, Raymond Pearl put forward the rate of living theory of ageing which states that "in general, the duration of life varies inversely with the rate of energy expenditure during its continuance. In short, the length of life depends on the rate of living" (Pearl 1928). This theory suggests that each organism has fixed energy expenditure per unit mass over lifetime and hence the longevity of the organisms can be determined by how quickly this energy potential is used up. Pearl's theory was proposed following Rubner's observations and work on *Drosophila* demonstrating that increasing the surrounding temperature resulted in reduced lifespan (Loeb and Northrop 1916; Loeb and Northrop 1917). In conjunction with another study in *Drosophila* (Miquel *et al.* 1976) these results demonstrated that higher temperature acted to accelerate all biological processes including ageing. Moreover, flies exposed to high temperatures

exhibit increased metabolic rate. Recent work assessing the mortality of flies exposed to higher temperatures confirms that the reduced lifespan is a result of accelerated ageing (Mair *et al.* 2003). Further support of the rate of living theory has come from evidence that short-lived shaker mutant *Drosophila* also have increased metabolic rates (Trout and Kaplan 1970).

Nonetheless, although there is a strong correlation between metabolic rate / body mass and lifespan (thereby supporting the rate of living theory) there are some exceptions including birds and bats, which exhibit remarkable longevity relative to their body size (Figure 1.4). One possible explanation for the long lifespans of bats and birds could be due to their ability to fly, making them well-equipped to avoid predation and disease, and subsequently experiencing reduced extrinsic hazard (Brunet-Rossinni and Austad 2004). Furthermore, tortoises, which are also very long-lived, have a thick shell to protect themselves from danger thus reducing risk of extrinsic hazard (Rose 1991). However, it is important to consider that birds, bats and tortoises are all long-lived in captivity as well as in nature.

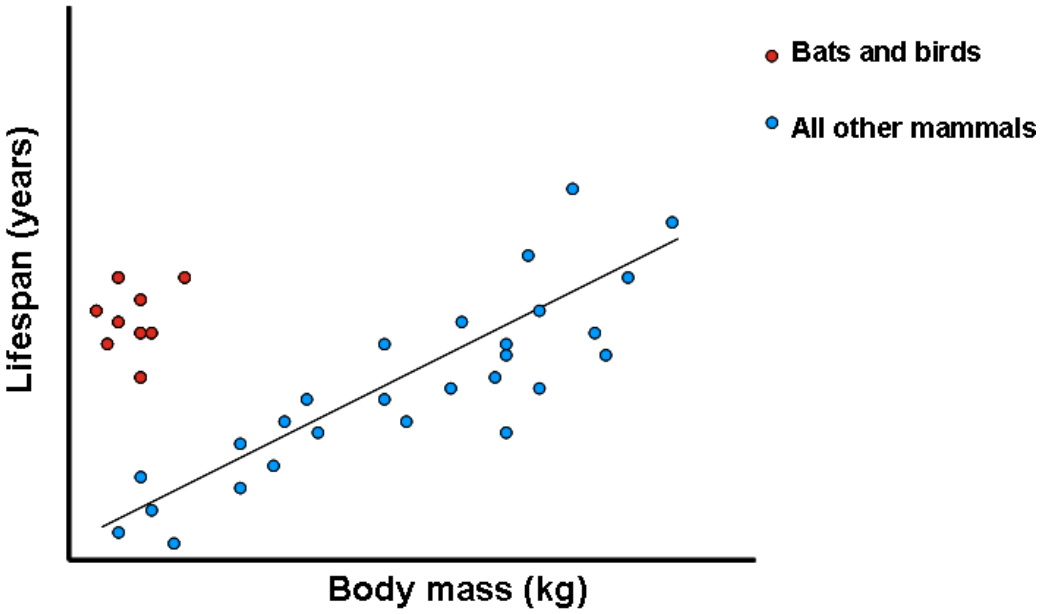

Figure 1.4: A schematic of the correlation between body mass and lifespan. The majority of mammals tend to conform to a strong positive correlation between increased body size and increased lifespan. However, birds and bats are exceptions to the correlation exhibiting remarkable lifespans relative to their body mass. Adapted from: Aging: a natural history (Ricklefs and Finch 1995).

1.3.2 Somatic mutation hypothesis

The somatic / spontaneous mutation hypothesis was first proposed by Leo Szilard in 1959. Szilard postulated that ageing was caused by accumulation of mutations leading to changes in the DNA of cells, which are passed on to the next generation of cells during cell division. Changes in DNA, as a result of somatic mutations can cause errors in protein structure and function, causing detrimental effects. This theory partially stemmed from observations that exposing mice to radiation results in somatic mutations, thereby causing phenotypes of ageing such as the premature appearance of grey hair. Furthermore, exposure to radiation shortens lifespan in both *Drosophila* (Lamb 1963; Lamb and Smith 1969) and mice (Lindop and Rotblat 1961). There also appears to be a general correlation between longevity and DNA repair, best illustrated by the enzyme poly (ADP-ribose) polymerase-1 (PARP-1),

which is involved in the cellular response to stress-induced DNA damage (Burkle 2001). Both longer-lived species and the longest lived individuals within the same species exhibit a positive increase in levels of PARP-1 (Grube and Burkle 1992).

In contrast, other studies have found little support for the somatic mutation hypothesis. One such study was conducted on haploid and diploid strains of the male parasitoid wasp *Habrobracon* (Clark and Rubin 1961). As would be expected, the lifespan of haploid males was significantly reduced compared to diploid males following exposure to radiation. Radiation caused a greater number of lethal mutations in the haploid species, which could not be rescued due to the lack of a second non-mutant copy, which is found only in diploid species. However, when no radiation was applied, negligible differences between the lifespans of haploid and diploid males were reported (Clark and Rubin 1961).

1.3.3 Oxidative damage / free radical theory of ageing

One of the most accepted theories of ageing to date is the free radical theory of ageing (also referred to as the oxidative damage theory), which was proposed by Denham Harman in 1956. Harman suggested that ageing was caused by free radicals formed as a by-product of oxidative phosphorylation (Harman 1956). Free radicals are molecules, ions or atoms that have one or more unpaired electrons. This makes them highly reactive species, with the ability to engage in a range of chemical reactions. Reactive oxygen species (ROS), which include free radicals, peroxides such as hydrogen peroxide, and oxygen ions, are produced predominantly in the mitochondria and can cause oxidative damage to DNA, RNA, lipids and proteins. Approximately 1% of the oxygen used for mitochondrial respiration forms superoxide radicals ($O_2^{\cdot -}$) (Boveris 1984). Naturally, the body is well equipped to

defend against ROS production with antioxidants such as superoxide dismutases (SOD), catalases and glutathione peroxidases in addition to non-enzymatic defences such as vitamin C and coenzyme Q.

Numerous experiments have been conducted to test the oxidative damage theory of ageing by looking at correlations between lifespan and oxidative stress as well as directly manipulating antioxidant defences, and have yielded conflicting evidence. The theory would predict that delayed senescence occurs as a result of reduced ROS production or an increased ability to remove ROS. It has been demonstrated that oxidative damage increases with age in different tissues and in different species (Sohal and Weindruch 1996). Furthermore, resistance to oxidative stress (in this case the chemical paraquat) was shown to be greater in *Drosophila* that had been selected for delayed ageing (Arking *et al.* 1991). In a study comparing ROS levels of seven different mammalian species, shorter-lived organisms exhibited higher levels of ROS production compared to longer-lived organisms (Ku *et al.* 1993). Moreover, pigeons, which are of a similar size to rats but live six times longer, display lower levels of ROS production than do rats (Ku and Sohal 1993). However, it is important to consider that these studies were performed using isolated mitochondria, which may not reflect the true situation *in vivo* (where making these measurements is extremely difficult).

Female mice that are heterozygous for a disruption in IGF-1 and Ames dwarf mice are both long-lived and have increased resistance to oxidative stress (Sanz *et al.* 2002; Holzenberger *et al.* 2003), whilst Ames dwarfs also exhibit higher levels of antioxidants (Brown-Borg *et al.* 1999; Brown-Borg and Rakoczy 2000). However, long-lived ant queens that can live up to 28 years were reported to have lower levels

of an antioxidant enzyme, CuZnSOD, than males (which live only a few weeks) or workers (which live between 1-2 years), indicating that SOD is not required for increased longevity (Parker *et al.* 2004). Furthermore, over-expression of different isoforms of SOD in *C.elegans* protected against oxidative damage, but had no effect on lifespan (Doonan *et al.* 2008). In general, long-lived species produce less ROS and have lower antioxidant defences (Barja 2002); however, fascinatingly, naked mole rats, which are extremely long-lived, show remarkably high levels of oxidative damage (Andziak *et al.* 2006).

Other studies have directly manipulated antioxidant defences, again yielding mixed results. Over-expression of antioxidant enzymes including superoxide dismutase (CuZnSOD) and catalase has been shown to increase lifespan in *Drosophila* (Orr and Sohal 1994; Parkes *et al.* 1998; Sun and Tower 1999). However, it was proposed that the lifespan extension reported in *Drosophila* may be as a result of artificially short-lived stocks (Spencer *et al.* 2003). Subsequently over-expression of CuZnSOD was performed in long-lived backgrounds and, although lifespan was still increased, the magnitude was significantly smaller than originally reported and was sex and genotype dependent (Spencer *et al.* 2003). Furthermore, the lifespan of long-lived *Drosophila* was not further extended by over-expression of antioxidant enzymes (Orr *et al.* 2003; Orr and Sohal 2003).

In mice, mitochondrial over-expression of human catalase increases lifespan (Schriner *et al.* 2005), whereas ubiquitous over-expression of SOD has no effect (Huang *et al.* 2000). In contrast, no lifespan extension was seen upon ectopic expression of catalase in *Drosophila* mitochondria (Mockett *et al.* 2003). However, the lifespan extension attributable to over-expression of human catalase in mice

mitochondria was reportedly diminished after back-crossing for nine generations and moving the mice to a new facility (Schriner *et al.* 2005), so this result should be treated with caution. *C.elegans* fed antioxidant mimetics were also thought to have increased lifespan (Melov *et al.* 2000). However, this observation could not be supported by a second study using *C.elegans* (Keaney and Gems 2003) or similar studies using mice (Perez *et al.* 2008) , the housefly *Musca domestica* (Bayne and Sohal 2002) or *Drosophila* (Magwere *et al.* 2006), which all reported no lifespan extension.

1.3.4 Mitochondrial damage theory

Mitochondria function as the powerhouse of all cells, providing the primary source of energy in the form of ATP. The production of free radicals during oxidative phosphorylation, coupled with the lack of defence mechanisms in the mitochondria, make this organelle highly susceptible to damage. The mitochondrial damage theory postulates that ageing results from a build up of damage to the mitochondria causing the organelles to lose their function. Once their function is lost, mitochondria cannot be replaced. Hence damage to mitochondria leads to gradual loss of energy and function in cells over lifetime. Indeed, evidence suggests a positive accumulation of mitochondrial DNA mutations with age (Wallace 1999), likely resulting in impaired ATP synthesis.

1.3.5 Error catastrophe theory

Leslie Orgel proposed that cellular ageing might occur through accumulation of defective proteins as a result of inherent inaccuracies in the protein translation machinery (Orgel 1963). The formation of defective proteins would result in positive

feedback leading to a further increase in translational errors and eventual catastrophe, namely breakdown of cellular information transfer. The theory predicts that there is an increase in the amount of defective proteins with age. Theoretical and experimental studies reveal evidence both for, e.g. (Holliday 1969; Lewis and Holliday 1970; Holliday and Tarrant 1972; Kirkwood 1977; Kowald and Kirkwood 1994) and against Orgel's theory, e.g. (Edelmann and Gallant 1977; Gallant and Palmer 1979; Harley *et al.* 1980; Mori *et al.* 1983; Goldstein *et al.* 1985).

One study in the fruit fly *Drosophila* sought to test the error catastrophe theory by examining whether feeding flies amino acid analogues, which inhibit protein function, could reduce lifespan. However, no effect on lifespan was reported (Dingley and Maynard Smith 1969). In contrast, artificially increasing translational errors in micro-organisms leads to the eventual death (after many cell generations) of the entire population (Holliday 1969; Lewis and Holliday 1970). Whilst the error catastrophe theory originally proved to be popular because it could be experimentally tested and verified, the theory now appears to have been widely disregarded.

1.3.6 Waste accumulation theory

The waste accumulation theory points to the fact that normal metabolic processes inevitably produce waste products. The build up of waste products will eventually interfere with the normal cell function and lead to cell death. Whilst it is evident that waste products do accumulate with age, not all waste products are likely to be harmful to cells. The most common waste product that accumulates in the cytoplasm is the yellow-brown granular pigment lipofuscin. Lipofuscin is found in almost all cells, although it is most abundant in cells which cannot divide or replicate, such as heart muscle and brain cells. Several studies have reported a direct correlation

between the accumulation of lipofuscin and ageing, e.g. (Reichel *et al.* 1968) and reviewed in (Gray and Woulfe 2005). The key to longevity may lie in the ability of cells to retain their capacity to repair DNA damage and reduce the rate of accumulation of waste products such as lipofuscin.

1.3.7 Cross-linking theory / glycosylation theory

The glycosylation theory was proposed by Johan Bjorksten in 1941, when he suggested that ageing was caused by intramolecular cross-links between proteins, nucleic acids and other molecular constituents of the cell, causing them to function less efficiently (Bjorksten 1941). The most common cross-links occur as a result of binding between proteins and glucose in the presence of oxygen (glycosylation), leading to the formation of advanced glycation end-products (AGE). Recently, treatments including aminoguanadine and carnosine have been reported to slow the formation of cross-links and even break existing cross-links (reviewed in (Hipkiss *et al.* 2002)).

Diabetics have been shown to exhibit an increase in the number of cross-linked proteins compared with non-diabetics of the same age, suggesting that diabetes may induce accelerated ageing (Sensi *et al.* 1995). Furthermore, glycation of collagen, the main protein of connective tissue in mammals, has been shown to increase with age, with diabetics showing a greater rate of increase in glycosylated collagen (Reiser 1991). Glycation of collagen can lead to a range of problems associated with age, including osteoarthritis and hardening of the arteries, thereby causing poor circulation, e.g. (Aronson 2003; DeGroot *et al.* 2004).

1.3.8 Hayflick's limit / replicative senescence theory

Prior to 1961, it was widely believed that all cells were potentially immortal. Evidence came from Alexis Carrel's experiments showing that chicken heart cells could be kept alive for almost 30 years *in vitro* (Carrel 1912), which is considerably longer than chickens themselves live, although this was never repeated. In 1961, it was shown that normal human fibroblast diploid cells lose the ability to divide after approximately 50 divisions, before entering a non-growth period (Hayflick limit or replicative senescence) (Hayflick and Moorhead 1961). The Hayflick limit of cells can vary both between species and differently aged cells of the same species. Furthermore, in a range of species, a strong positive correlation has been reported between lifespan and the Hayflick's limit of the species' cells cultured *in vitro* (Rohme 1981). Hayflick's limit is thought to be determined by the length of telomeres. Telomeres are specialised repetitive DNA sequences located at the end of chromosomes, which compensate for incomplete semi-conservative DNA replication and protect chromosome ends from recombination and fusion to other chromosomes. During each cell division, telomere length shortens due to a lack of telomerase activity, an enzyme which acts to maintain telomere length (Harley *et al.* 1990). However, despite telomere length decreasing with age, mice, which are considerably shorter-lived than humans, possess longer telomeres and unlike humans possess telomerase activity in somatic cells (Blasco *et al.* 1997).

Interestingly, the longevity of human fibroblast cells can be further extended by up to 10 divisions in the presence of the nutrient carnosine (McFarland and Holliday 1999). Furthermore, several studies have reported an even greater number of cell divisions in a range of cells in the presence of telomerase, e.g. (Bodnar *et al.* 1998). However, increasing the replicative capacity of cells beyond Hayflick's limit could

potentially lead to the development of cancer, which arises due to uncontrolled cell division. Hence Hayflick's limit may act as a barrier to enhanced longevity, but at the same time acts to increase fitness by reducing the likelihood of developing cancer (Campisi 2005). Until recently, the effects of telomerase on ageing had not been studied, primarily due to the cancer-promoting activity of telomerase. However, a recent study showed that the expression of telomerase reverse transcriptase (a component of telomerase) could extend the lifespan of cancer-resistant mice (Tomas-Loba *et al.* 2008).

In conclusion, it is evident that some of the more recent theories that have been proposed to explain how we age (mechanistic theories) have shown considerable development in logic and reasoning compared to some of the earlier theories. To date, perhaps the most popular single theories of ageing in the field are the free radical and the replicative senescence theories, both of which have received a lot of support from experimental studies. The difficulty in ascertaining the cause from effect of ageing means that finding one universal mechanistic theory of ageing is unlikely. Whilst the theories proposed differ from one another in terms of the exact cause of ageing, it is clear that the more popular and accepted theories of ageing are consistent with the notion that ageing is caused by the accumulation of cellular damage throughout life, coupled with the reduced efficiency of repair and cellular defence mechanisms over time. In my opinion, ageing occurs as a result of a combination of many of these theories due to the complex nature of ageing occurring at the level of macromolecules through to whole organs, although I believe production of free radicals is the most prominent contributor to ageing.

1.4 Using Model organisms to study ageing

Choosing a model organism to study a biological process such as ageing requires similar consideration to choosing a model to understand other biological processes such as development or physiology. The more related an organism is to humans, the more likely it is that the mechanisms uncovered will also be applicable to humans. However, a particularly important consideration when choosing an organism to study ageing is the length of time that it takes to perform a lifespan experiment. Primates such as rhesus monkeys may be one of the most evolutionarily related organisms to humans, making them ideal to study; however they can live around 40 years (Weindruch 2006), which makes lifespan studies extremely difficult and time consuming. Consequently, the majority of ageing studies are typically performed on four, fairly short-lived model organisms: budding yeast, nematode worms, fruit flies and mice, all of which have recently had their genomes fully sequenced. Although they are very different in complexity and lifespan, these organisms all exhibit similar survival kinetics to humans, whereby mortality of the population increases exponentially over time (Sinclair *et al.* 1998; Tissenbaum and Guarente 2002). Naturally, studying ageing in each model organism has advantages and disadvantages. Some of these strengths and weaknesses will be discussed below.

1.4.1 The budding yeast *Saccharomyces cerevisiae*

Budding yeast *Saccharomyces cerevisiae* are single-celled eukaryotes, measuring approximate 5-10μm in diameter. They have a very short generation time (1.5 – 2 hours at 31^{o}C) and are easily and economically cultured. In addition, *S. cerevisiae* are one of the most extensively studied eukaryotic models. They can grow and survive as diploid or haploid cells and can be easily genetically manipulated.

Budding yeast cells divide asymmetrically, resulting in a large mother cell and a smaller daughter cell. Yeast go through three distinguishable stages when cultured (Longo *et al.* 1999). The first stage is a period of logarithmic growth in which metabolism is predominantly glycolytic. The second stage commences when glucose becomes limiting, causing yeast cells to switch to respiratory growth. The final stage is called the stationary phase where cells stop dividing and become highly stress resistant (Tissenbaum and Guarente 2002).

Lifespan of yeast can be measured in two alternative assays: replicative and chronological lifespan. Replicative lifespan is a measure of the number of cell divisions a mother cell can undergo until it can no longer divide (Mortimer and Johnston 1959). In contrast, chronological lifespan is measured by the length of time non-dividing cells remain viable during the stationary phase (MacLean *et al.* 2001). Both methods are commonly adopted to determine whether a specific intervention can extend lifespan (Fabrizio *et al.* 2001; Kaeberlein *et al.* 2004a; Lin *et al.* 2004; Powers *et al.* 2006).

1.4.2 The nematode worm *Caenorhabditis elegans*

C.elegans were first used to study molecular and developmental biology by Sydney Brenner in the 1970s, and have since become a very popular model organism to work with (Brenner 1974). *C. elegans* are free-living nematodes, around 1mm in length that live in the soil and feed almost exclusively on bacteria (Caswell-Chen *et al.* 2005). Their short development time (egg to adult in 3 days) and short lifespan (18 days at 20^{o}C) make them an extremely useful model organism to study ageing (Vanfleteren and Braeckman 1999; Tissenbaum and Guarente 2002). In addition, *C. elegans* are extremely cheap and easy to maintain, whilst mutant stocks can be frozen

for long periods and remain viable when thawed. They exist as either males or self-fertilising hermaphrodites, allowing for simple genetics without the problems of inbreeding. A further advantage of using *C. elegans* is they are ideal for performing genetic screens and can be used simply and efficiently to perform RNA interference (RNAi) to knockdown expression of genes of interest. RNAi can be achieved by feeding the worms genetically transformed bacteria expressing double-stranded RNA (dsRNA) complimentary to the gene of interest (Carthew 2001) or injecting / soaking worms in a solution of dsRNA.

The life-cycle of *C. elegans* is divided into four post-embryonic larval stages of development. During stressful conditions such as nutrient deprivation or over-crowding, larvae can enter a separate stage called dauer larva (Cassada and Russell 1975). This is a non-ageing stage where larvae become highly stress resistant and can remain in this state for several months (Klass and Hirsh 1976). When environmental conditions return to being more favourable, dauer larvae resume the normal life-cycle and exhibit a normal lifespan. After development, nematodes undergo no further cell division; hence the ageing worm is post-mitotic. Interestingly, several gene mutations that have been shown to extend lifespan were first identified because of their role in development. Furthermore, the first single gene mutations to extend lifespan in any model organism were reported in *C. elegans*, originally screened by Michael Klass (Klass 1977). These findings contributed to the observation that a mutation in *age-1* (PI3 kinase) extended the lifespan in both males and hermaphrodites (Friedman and Johnson 1988).

1.4.3 Rodents

Studies on rodents have typically been confined to rats (*Rattus norvegicus*) and mice (*Mus musculus*), which are both commonly used laboratory animals whose genomes have been sequenced (Mouse sequencing consortium 2002 and Rat sequencing consortium 2004). In contrast to the invertebrate models discussed, rodents are more expensive to maintain and handle and require a large amount of space to house. More importantly, the typical lifespan of rodents is somewhere between three to five years (Weindruch and Walford 1988), resulting in lifespan experiments that take significantly longer than experiments in invertebrates. In addition, although rodents are fast reproducers, the sample sizes used in lifespan experiments are typically smaller than in invertebrate studies because of the expense and difficultly in producing large numbers, particularly of transgenic lines. However, studying ageing in rodents is important as a key to understanding mammalian ageing and hence human ageing, because almost all mouse genes have human homologues and their biology is more similar to that of humans. The use of rodents is essential when trying to confirm whether a single mutation that has been reported to extend lifespan in yeast, worms and flies is evolutionarily conserved in mammals and may therefore play an important role in human ageing (Partridge and Gems 2002; Tatar *et al.* 2003).

1.4.4 The fruit fly *Drosophila melanogaster*

Drosophila melanogaster are the most commonly used species of *Drosophila* in the laboratory. *Drosophila* are a useful model organism for ageing studies because they are cheap, easy to culture, they have a short generation time (10 days at 25^{o}C) and a short lifespan (2-3 months) relative to rodents. In addition, genetic manipulation of fruit flies is easy and there are a wide range of mutant stocks readily available.

Furthermore, their tissues are similar to mammals. They possess a heart, kidney and fat body (which is the fly equivalent of mammalian liver), and have a fully differentiated brain. *Drosophila* has been used extensively as a model organism since the pioneering genetic experiments of Thomas Morgan in the 1900s.

The main disadvantages of using *Drosophila* as a model organism are firstly, unlike *C.elegans,* the mutants stocks can not be frozen and hence require regular stock maintenance, which is time consuming. Moreover, performing homologous recombination and ubiquitous RNAi in flies is extremely difficult, whilst ubiquitous RNAi is toxic. In addition, although *Drosophila* have a short lifespan relative to mice and rats, they are considerably longer-lived than worms and yeast. In addition, *Drosophila* are commonly infected with the intracellular bacterium *Wolbachia,* which is maternally inherited and thought to have infected around 30% of the strains in the Bloomington stock centre (Clark *et al*. 2005). The presence of some *Wolbachia* strains has previously been reported to have effects on mutant phenotypes and fitness-related traits, including lifespan (Min and Benzer 1997; Fry and Rand 2002; Clark *et al.* 2005; Toivonen *et al.* 2007).

1.4.4.1 Drosophila life history

Drosophila life history is divided into four distinct morphological stages, hence periods of growth and development can be easily distinguished from sexual maturity and the adult phase (Figure 1.5). The development time from egg to adult is approximately 10 days at 25^{o}C. Once fertile eggs are laid, larvae start to emerge around 24 hours later and then enter three stages of growth or instars (L1, L2 and L3). L1 and L2 stages last for 24 hours each whereas the L3 stage lasts for 48 hours.

During the L2 stage, larvae start to become larger in size and switch from feeding on the surface of the food to burrowing down into the food. Feeding can last around 110 hours proceeded by the "wandering stage", during which larvae leave the food medium and crawl up the vial or bottle to find a suitable place to pupariate. Pupariation takes approximately four days, during which time pupae undergo metamorphosis before eclosion into adult flies. Adult flies consist almost entirely of post-mitotic, fully differentiated cells, with the exception of cells in the gonad and some cells in the gut which continue to divide (Bozuck 1972).

Freshly eclosed flies have shrivelled wings and have a pale complexion with a dark spot on their abdomen as a result of their last feed as L3 larvae (Greenspan 2004). Wings become expanded within an hour and pigmentation occurs shortly after this. Female flies will not mate within the first eight hours post-emergence (Greenspan 2004). After mating, females commence a heavy egg-laying period which peaks at around five days post-copulation. Virgin flies do lay eggs, although significantly less and in a different pattern from mated flies. Virgins are thought to live twice as long as continuously mated females (Smith 1958), potentially due to higher egg production and cost of mating in mated flies, both from physical damage and the transfer of seminal fluid from males which has been reported to reduce lifespan (Chapman *et al.* 1995)

Figure 1.5: The *Drosophila* life cycle. The development of a fertile egg to an adult fly over a 10 day period at 25°C. Following hatching of an egg, larvae go through three instars before reaching pupariation at which time metamorphosis takes place resulting in the emergence of an adult fly. Taken from: The Cell Cycle: Principles of Control: Online Resources by David Morgan (New Science Press)[4].

1.4.4.2 Nomenclature

Gene names are often descriptive of the gene function or mutant phenotype such as *Curly* (curly winged flies) or *chico* ("little boy" in Spanish), the latter describing the dwarf phenotype of a mutant in a gene encoding the insulin receptor substrate protein. Where a gene is an orthologue of a gene previously discovered in another organism, a "d" is added to the front of the gene name, for example *dFOXO* is an orthologue of the human FOXO transcription factor. The genotype, mutant and gene

[4] The cell cycle: Principle of control by David Morgan, www.new-science-press.com/info/illustration_files/nsp-cellcycle-2-4-2_13.jpg

name are always italicised. If the mutant phenotype is dominant to the wild-type then the first letter is capitalised, but not when it is recessive. If more than one mutant allele exists for a given gene then the gene name is followed by superscript numbers or letter, for example *dSir2*$^{4.5}$ and *dSir2*$^{5.26}$, describing two different alleles of *dSir2*, the *Drosophila* orthologue of mammalian SIRT1, a histone deacetylase. Protein products are written with the same name of the gene that encodes them (in capital letters), but are not italicised.

1.4.4.3 Potential pitfalls when using Drosophila for ageing studies

As is the case with all model organisms, along with the many advantages there are also some disadvantages that need to be considered when using *Drosophila* as a model for ageing studies. In *Drosophila*, inbreeding can be a problem, increasing the likelihood of the stock becoming homozygous. Fly stocks can accumulate deleterious mutations, which can result in artificially shortened lifespan. Using such an artificially short-lived stock can particularly be problematic when studying a specific mutation, because it could simply recover the artificially shortened lifespan back to normal, and hence be mistaken for a mutation that extends lifespan (Helfand and Rogina 2003). Therefore it is important to test the same mutation in an outbred stock or several other inbred stocks to determine whether lifespan extension is still observed. This problem was addressed by using the wild-type stock Dahomey for the majority of the experiments in this thesis. Dahomey is an outbred strain of *Drosophila*, maintained in population cages, resulting in adult survival rates comparable to those of stocks that had been freshly collected from the wild (Sgro and Partridge 2000; Sgro and Partridge 2001).

1.4.4.4 The importance of backcrossing

When assessing the effect of a single gene mutation on lifespan it is essential to perform a back-crossing regimen whereby the mutant is backcrossed for several generations into an outbred wild-type back ground (e.g. Dahomey). This ensures that the genetic background of the mutant is identical to the genetic background of the wild-type with the exception of the mutated gene of interest. Failure to backcross could result in an unclear lifespan effect if the mutant has come from a different background to the control because any lifespan difference could be associated with the genetic background as opposed to the mutation in question. It is preferable to backcross to an outbred strain because using an inbred strain, which can accumulate deleterious mutations, might lead to the false result of a mutation that is thought to extend lifespan but is simply recovering the artificially shortened lifespan of the inbred stock (Helfand and Rogina 2003). The longevity of *Drosophila* is reduced by inbreeding depression, but extended by heterosis (hybrid vigour) when separate inbred strains are crossed together (Swindell and Bouzat 2006). A recent study reported that heterosis was responsible for the originally reported long-lifespan of *Drosophila Indy* mutants because the lifespan extension phenotype diminished following extensive backcrossing to an outbred Dahomey strain (Toivonen *et al.* 2007).

In *Drosophila*, an extremely commonly used genetic tool is the GAL4/UAS system for targeted gene expression (Brand and Perrimon 1993; Duffy 2002). The system consists of two parts, the GAL4 gene, which encodes the yeast transcription factor (Gal4) and UAS (upstream activating sequence), a promoter region which Gal4 binds to and activates transcription of the gene of interest. Activation of transcription occurs when UAS flies carrying the gene of interest are crossed with flies carrying

the GAL4 gene. It is important, when using this system, to backcross both the GAL4 and UAS lines because failure to do so will likely result in an artificially long lifespan of the progeny due to heterosis (as opposed to the effect of the gene of interest). The short generation time of flies makes back-crossing easier and less time consuming than in, for example, rodents.

1.4.4.5 Measuring ageing in model organisms

Another problem, applicable to all model organisms, is how to measure a process like ageing, when it is already difficult to define (section 1.1). Typically one aspect of ageing is measured, for example lifespan. However measuring lifespan can create potential problems particularly when studying genetic interventions. For example, a mutation that reduces lifespan could do so through pathological reasons, unrelated to ageing itself (Helfand and Rogina 2003). Therefore the optimal approach is to look for interventions that extend lifespan rather than reduce it. However, an intervention that extends lifespan alone is not sufficient. If the same intervention results, for example, in flies becoming sick or incapacitated in some form (e.g. through immobility), then the intervention does not truly reflect a delay in the rate of ageing (Helfand and Rogina 2003). Therefore it is important to assay a secondary measure of health such as fecundity, metabolism or physical activity.

1.5 Dietary restriction

Dietary restriction (DR), a reduction in food intake that falls short of starvation or malnutrition, has been shown to be the most robust and reproducible intervention to extend lifespan in a diverse range of organisms (Mair and Dillin 2008). These include model organisms such as rodents (Yu *et al.* 1982; Bonkowski *et al.* 2006), *Drosophila melanogaster* (Chapman and Partridge 1996; Libert *et al.* 2007), *Caenorhabditis elegans* (Klass 1977; Panowski *et al.* 2007), *Saccharomyces cerevisiae* (Jiang *et al.* 2000; Kaeberlein *et al.* 2006b) and many organisms that are not commonly used in the laboratory including spiders (Austad 1989), medflies (Davies *et al.* 2005), grasshoppers (Hatle *et al.* 2006), rotifers (Fanestil and Barrows 1965), fish (Comfort 1960), dogs (Kealy *et al.* 2002), hamsters (Stuchlikova *et al.* 1975), and water fleas (Ingle 1933).

The first DR studies on non-human primates (rhesus monkeys and squirrel monkeys) commenced in the 1980s and are still ongoing. Although it is still too early to definitely tell whether DR will extend lifespan in monkeys, the DR cohorts are exhibiting many of the classical phenotypes associated with DR in rodents, including lower body weight and body fat and reduced blood glucose levels (Ramsey *et al.* 2000; Lane *et al.* 2004; Messaoudi *et al.* 2006; Mattison *et al.* 2007). Early indications of lifespan data have revealed only 13% of the DR-fed rhesus monkeys have died compared with 23% of the *ad libitum*-fed group (Lane et al. 2002). Another report looking at the effect of DR in squirrel monkeys has reported that the DR cohort had an extended median lifespan of up to seven years (Bodkin *et al.* 2003). However, these data should be considered preliminary because out of the 117 monkeys studied only 8 were dietarily-restricted (3 of which had died) compared

with 109 *ad libitum*-fed monkeys (of which 49 had died), making conclusions difficult because of the small sample sizes used in the DR cohort.

Although there is little evidence to date, it has been proposed that DR may have beneficial effects on humans (Fontana *et al.* 2004; Holloszy and Fontana 2007). Indeed, several ongoing studies are investigating biomarkers of DR in people partaking in strict caloric restriction diets (e.g. CALERIE[5], Biosphere 2[6] and the Calorie restriction society[7]). Additional support for the concept of dietary restriction also working in humans comes from the inhabitants of Okinawa, an island close to Japan. Okinawans not only have the greatest number of centenarians per 100,000 of the population compared with anywhere else in the world, but also remain remarkably healthy in their old age. These effects are thought to be accounted for by undergoing a mild form of prolonged DR for half their lives (Willcox *et al.* 2006; Willcox *et al.* 2007).

In this section, the effects of DR on four of the most commonly used model organisms for ageing studies will be discussed: the budding yeast *S. cerevisiae*, the nematode worm *C. elegans*, the fruit-fly *D. melanogaster* and the rodent models *Mus musculus* and *Rattus norvegicus*. These model organisms have been predominantly used to try and address the potential mechanisms involved in mediating lifespan extension by food restriction.

[5] Comprehensive assessment of long-term effects of reducing energy intake (CALERIE), http://calerie.dcri.duke.edu/
[6] Biosphere 2, www.b2science.org
[7] Calorie restriction society, www. calorierestriction.org

1.5.1 Investigating potential mechanisms / mediators of dietary restriction

Although many experiments have been conducted to try and assess whether specific components of various signalling pathways are required to observe the lifespan extension effects of DR (i.e. DR mediators), it is important to consider that many of interaction studies (between two interventions that extend lifespan) have been performed under a range of different experimental conditions and have largely shown confusing and conflicting data (Gems *et al.* 2002). Subsequently, several mechanisms proposed need to be interpreted with caution. In order to determine mechanistically whether a genetic mutation can cause an additive effect of lifespan to DR or blocks lifespan extension by DR, the responses of controls and mutant lines to a range of food concentrations need to be assessed (Figure 1.6) (Mair and Dillin 2008).

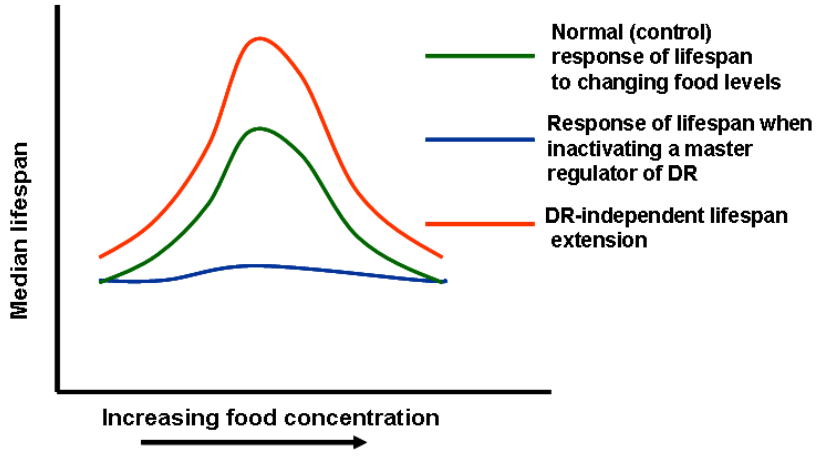

Figure 1.6: The response of lifespan to dietary restriction. Under normal circumstances (i.e. control or wild-type organisms), lifespan shows a tent-shaped response to changing nutritional levels (green line). Disabling a mediator or master regulator of DR will act to block the response of lifespan to changing nutrition, resulting in a flattening of the bell-shaped curve (blue line). An intervention which extends lifespan in a parallel pathway to DR will result in an additive increase in lifespan at all food concentrations. Adapted from (Mair and Dillin 2008).

1.5.2 Dietary restriction in rodents

The first reported study demonstrating lifespan extension by food restriction was performed by Clive McCay in 1935, where he showed that reducing the normal diet fed to rats by 60% increased lifespan by approximately 30% (McCay 1935). Since then, a whole range of studies has been performed on rodents documenting the effects of DR on lifespan and physiology (reviewed in (Weindruch and Walford 1988; Masoro 2002)). Whilst the majority of mice and rat studies have consistently reported lifespan extension by DR, a few reports suggest that not all laboratory strains show a lifespan response to DR, e.g. (Forster *et al.* 2003). In addition, no lifespan extension was reported in a study assessing the effect of DR in wild mice, despite the dietary restricted mice exhibiting other characteristic phenotypes of DR including reduced cancer incidence (Harper *et al.* 2006). This section will address some of the key findings of the numerous dietary restriction studies performed on rodents to date.

1.5.2.1 Methods of applying DR in rodents

DR in rodents is typically achieved through two different methods, both of which have been shown to extend the median and maximum lifespan of the DR cohort. The first and most common method of applying DR in rodents is by reducing the quantity of the chow diet given to the DR group. This is typically imposed by feeding the animals around 60-70% of the food they would normally eat if given unrestricted *ad libitum* access (Yu *et al.* 1985; Masternak *et al.* 2005; Bonkowski *et al.* 2006). Lifespan extension using this method of DR has also been reported by feeding the restricted cohort anywhere between 33 to 80% of the *ad libitum* control fed group (Weindruch and Walford 1988). Alternatively, as a variation to this method, the DR

group can be fed a smaller weight percentage of food than the control group (Merry and Holehan 1985).

The second method for achieving DR in rodents is through intermittent feeding, predominantly exercised in the form of every other day (EOD) feeding (Goodrick *et al.* 1982; Anson *et al.* 2005). DR though intermittent feeding can also be applied by feeding the restricted group 50% DR for three weeks followed by *ad libitum* feeding for three weeks. By alternating between these two feeding cycles, mice have been shown to adopt some of the phenotypes such as reduced tumour formation, shared with those seen using more common methods of DR (Cleary *et al.* 2007).

It had been believed for a long time that lifespan extension by DR in rodents is directly related to caloric intake and independent of the nutrients being restricted (Iwasaki *et al.* 1988; Weindruch and Walford 1988; Masoro *et al.* 1989; Masoro 2005). However, this view has since been challenged by several studies demonstrating that restriction of specific nutrients can also extend lifespan. For example, reducing the protein component of the diet has been shown to extend rat longevity (Yu *et al.* 1985). Lifespan extension has also been achieved by the restriction of either tryptophan (De Marte and Enesco 1986) or methionine (Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003; Miller *et al.* 2005), which are both essential amino acids for mammals. Moreover, reducing the protein concentration but not the fat or carbohydrate concentration in the diet reduced ROS production and oxidative damage, similarly to the effects of whole-food DR. These effects are believed to be the result of reduced methionine intake (Ayala *et al.* 2007). The term dietary restriction (DR) as opposed to the commonly

used terms caloric or calorific restriction (CR) will be used throughout this thesis because DR encompasses lifespan extension by reduced caloric and nutrient intake.

1.5.2.2 Biomarkers of DR in rodents

In addition to extending lifespan, DR induces several other phenotypes in rodents including a reduction in fecundity, body temperature, blood plasma glucose levels, insulin levels, body fat and weight (Holehan and Merry 1986; Weindruch and Walford 1988; Masoro 2002; Koubova and Guarente 2003; Selesniemi *et al.* 2008). In addition, DR-fed rodents appear leaner and younger for longer compared with age-matched *ad libitum*-fed controls (Masoro 2002). This has led to the suggestion that DR in rodents causes a reduced rate of ageing (delayed ageing), leading to a reduction in the trajectory of mortality slope (Figure 1.2). This hypothesis has been supported by analytical work on previous rodent studies (Holehan and Merry 1986; Pletcher *et al.* 2000). Holehan and Merry (1986) showed the mortality rate doubling time (MRDT; section 1.1.2) based on several DR experiments was 102 days for *ad libitum* groups and 203 days for DR groups. However, work on one strain of mice did not find a difference in the rate of age specific mortality between DR and *ad libitum*-fed cohorts (Weindruch *et al.* 1986). Weindruch *et al.* (1986) found that although DR delayed the start of senescence, it did not slow the process once it had begun. It remains unclear whether DR does slow ageing because there are a lack of mortality data available for different mice and rat strains, hindered in part because age-specific mortality data requires at least 100-500 individuals per treatment (Pletcher 1999), which is a difficult number of rodents to breed and maintain.

1.5.2.3 DR and metabolic rate

It was originally proposed that DR extended lifespan as a result of reduced metabolic rate (Sacher 1977)[8]. Sacher's hypothesis was based on observations that reduced food intake in humans lowered metabolic rate. This was an attractive mechanism, which fitted in with the oxidative damage theory of ageing and the hypothesis that metabolic rate strongly correlates with ROS production. Preliminary evidence suggested that DR may induce a lower metabolic rate (Weindruch and Walford 1988). However, when normalised for lean body mass, no differences in metabolic rate between DR and control-fed animals were detected (Masoro *et al.* 1982; McCarter *et al.* 1985). On the contrary, a slight increase was observed in the DR group (McCarter and Palmer 1992; Selman *et al.* 2005). Furthermore, a positive correlation between oxygen consumption (metabolic rate) and lifespan has been reported, and long-lived strains were also shown to have more uncoupled mitochondria (Speakman *et al.* 2004), which has been hypothesised to cause reduced ROS production and enhanced longevity (Brand 2000). This study argues against the likelihood that DR extends lifespan through reduced metabolism or through a reduced "rate of living" (Pearl 1928). However, controversy still remains as to what the best method for accurately measuring metabolic rate is (Greenberg and Boozer 2000), so that the possibility of a reduced metabolism in DR- treated organisms can perhaps not be discounted completely.

[8] Cited from Masoro 2005

1.5.2.4 DR and stress resistance

Another common phenotype coupled with lifespan extension under DR is increased resistance to different stresses, particularly heat and oxidative stress (Sohal and Weindruch 1996). In both rats and mice, DR results in decreased production of ROS (Sohal *et al.* 1994; Lopez-Torres *et al.* 2002). Furthermore, microarray analysis revealed a down-regulation of genes encoding inflammation and stress resistance in the brains of DR-fed rodents (Lee *et al.* 2000).

1.5.2.5 DR delays the onset of multiple ageing-related pathologies

The pioneering studies of Clive McCay showing that DR could extend the lifespan of rats, also revealed that this intervention could delay the onset of several ageing-related pathologies (McCay 1935). To date, beneficial effects of DR have been reported to postpone the effects of numerous pathologies (Weindruch and Walford 1988; Masoro 2002). These include, amongst many others, neuro-degenerative diseases (Mattson *et al.* 2001), cancer (Yu *et al.* 1982; Hursting *et al.* 1994; Berrigan *et al.* 2002; Cleary *et al.* 2007), auto-immune diseases (Fernandes *et al.* 1976; Kubo *et al.* 1984a; Kubo *et al.* 1984b), kidney diseases (Yu *et al.* 1982; Maeda *et al.* 1985), cataracts (Taylor *et al.* 1989), strokes (Stevens *et al.* 1998), and cardiovascular diseases (Koletsky and Puterman 1976; Maeda *et al.* 1985).

1.5.2.6 Does DR increase lifespan by retarding growth?

Clive McCay formulated the retardation of growth hypothesis, which suggests that DR extends lifespan by retarding growth (McCay 1935). This view became favourable and was modified to include the retardation of development because of the proposal that ageing is simply a continuation of development. However, this

hypothesis has been challenged by several studies showing that DR can extend lifespan when applied after the rapid growth phase (between 6 weeks and 6 months), but to a lesser extent than when initiated during or immediately after weaning. For example, male rats exhibit a 10-20% increase in lifespan when DR is applied at one year of age (Weindruch and Walford 1982). Furthermore, applying DR at 19 months has also been reported to extend the lifespan of one mouse strain (Dhahbi *et al.* 2004), although no lifespan extension was observed in a strain of rats when DR was applied at 18 or 26 months (Lipman *et al.* 1995). Another study has reported that when DR is applied after the rapid growth period, maximum lifespan (age of last 10% of population) was extended almost to the same degree as when DR was initiated at 6 weeks (Yu *et al.* 1985).

1.5.2.7 Does DR extend lifespan due to reduced body fat?

Another hypothesis proposed that DR extends lifespan due to a reduction in body fat, based on the assumption that increased body fat caused premature death in humans (Berg and Simms 1960). Indeed, DR has been shown to decrease body fat (Harrison *et al.* 1984; Weindruch *et al.* 1986; Masoro 2002), particularly visceral fat (Barzilai and Gupta 1999). However; no correlation was detected between body fat and lifespan of an *ad libitum*-fed rat strains (Bertrand *et al.* 1980). Moreover, the lifespan of a genetically obese mouse strain, ob/ob, could be extended despite these mice having more fat than shorter-lived *ad libitum* control-fed mice (Harrison *et al.* 1984).

1.5.2.8 Genetics of dietary restriction in rodents

Reports of single gene mutation that have extended lifespan in rodents have generally been restricted to one diet, usually *ad libitum*. However, lifespan studies involving mice with a mutation in the pituitary gland (prop1df; Ames dwarf mice) or growth hormone receptor knockout (Ghr/bp-/-; Laron dwarf or GHRKO) were conducted on four different diets (Bartke *et al.* 2001; Bonkowski *et al.* 2006). These diets included a standard lab diet, a casein diet with soy-derived components, and two soy-based diets (with high and low isoflavone content). Both Ames and Laron dwarfs lived longer than genetically-matched controls on all diets; however the magnitude of lifespan extension dramatically varied, indicating some interaction with nutrition. Interestingly, the long-lifespan of Ames dwarfs could be further extended by DR when applied at two months of age (Bartke *et al.* 2001). In contrast to work on Ames dwarfs, more recent work with Laron dwarf mice revealed no further increase in median lifespan when these mice were subjected to DR, and only a small increase in the maximum lifespan of females was detected (Bonkowski *et al.* 2006). These data suggest that the increased lifespans of Laron and Ames dwarf mice are mediated by different pathways. Alternatively, the dietary restriction regimen used in Laron mice may not have been sufficient to extend lifespan if for example these mice have different nutritional requirements. This could be tested by examining the response of Laron mice on a greater range of DR diets.

More recently, insulin receptor substrate 2 (IRS2) mutants have been reported to exhibit extended lifespan when fed a 9% fat diet (Taguchi *et al.* 2007), but not when fed a 5% fat diet (Selman *et al.* 2008). Furthermore, lifespan extension of growth hormone-deficient mice (Snell dwarf) is dependent on a 4% fat diet as opposed to a 7% fat diet (Flurkey *et al.* 2001). These results highlight an interesting interaction

between longevity, genotype and diet, with the majority of mutations having a greater effect on lifespan when mice are fed high calorie diets.

1.5.3 Dietary restriction in *Saccharomyces cerevisiae*

Yeast is commonly grown on a medium containing a relatively high concentration of glucose (2%) and a plentiful supply of amino acids. Reducing either the concentration of glucose (to 0.5% or even 0.05%) or the concentration of amino acids (or both simultaneously) extends both replicative and chronological lifespan of yeast (Jiang *et al.* 2000; Lin *et al.* 2000; Jiang *et al.* 2002; Anderson *et al.* 2003; Kaeberlein *et al.* 2004b; Reverter-Branchat *et al.* 2004; Fabrizio *et al.* 2005; Powers *et al.* 2006). In addition to inducing DR environmentally, two genetic models have been created to explain how glucose reduction could extend lifespan by blocking the uptake or metabolism of glucose. The first model emerged from data on gene mutations that reduce signalling through the cAMP-dependent protein kinase, PKA, which is activated by high levels of glucose (Lin *et al.* 2000). These include deletion of genes encoding the glucose sensing proteins *GPA2* or *GPR1*, and temperature-sensitive alleles of adenylate cyclase (*cdc35–1*) or the RAS-associated GTPase (*cdc25–10*) (Lin *et al.* 2000). The second model highlights that DR is achieved by deletion of *HXK2*, a gene encoding hexokinase, an essential enzyme for the initial utilisation of glucose by the yeast cell (Lin *et al.* 2000).

The extension of lifespan by glucose reduction was originally thought to occur through an increase in respiration (Lin *et al.* 2002) because deletion of *CYT1*, a gene encoding cytochrome C1, prevented these cells from exhibiting lifespan extension by DR (Lin *et al.* 2002). However, more recently it has been reported that by using a lower glucose concentration (0.05%) to induce DR, lifespan can be extended in

respiratory-deficient yeast (Kaeberlein *et al.* 2005a), thereby highlighting that other mechanisms may mediate the effects of lower glucose availability on lifespan in yeast.

1.5.3.1 Genetics of dietary restriction in yeast cells

The *SIR2* (silent information regulator 2) gene was first identified as a mediator of gene silencing in yeast (Rine and Herskowitz 1987). It has since been shown that *SIR2* encodes an NAD-dependent histone deacetylase (Imai *et al.* 2000). In yeast, Sir2 has been shown to be important in ageing because deletion of *SIR2* reduces replicative lifespan (Kennedy *et al.* 1995; Jiang *et al.* 2002), whereas over-expression of *SIR2* has been shown to extend lifespan (Kaeberlein *et al.* 1999). The absence of other members of the sirtuins, *SIR3* and *SIR4*, was also reported to shorten replicative lifespan (Kaeberlein *et al.* 1999).

The work on sirtuins has prompted investigations into whether dietary restriction is mediated by Sir2 in yeast (Guarente 2000; Guarente and Kenyon 2000). However, these experiments have yielded conflicting evidence (Lin and Guarente 2006; Kaeberlein *et al.* 2007; Kaeberlein and Powers 2007). It was first reported that certain strains of yeast did not respond to DR when *SIR2* had been deleted (Lin *et al.* 2000), and that DR extends lifespan by lowering levels of NADH, which is known to be a competitive inhibitor of Sir2 (Lin *et al.* 2004). This finding was supported by a genetic mimetic model of DR, whereby mutants with reduced PKA activity required the presence of Sir2 to extend lifespan (Lin *et al.* 2002). Moreover, the chemical compound resveratrol, which increases activity of the human homologue SIRT1 *in vitro*, could increase lifespan of yeast but had no additive effect on lifespan when

simultaneously applied with DR (Howitz *et al.* 2003). Furthermore, deletion of another histone deactylase, *RPD3*, extends the replicative lifespan of yeast (Kim *et al.* 1999; Jiang *et al.* 2002), but when *RPD3* mutants were subjected to DR no further lifespan extension was observed. These data suggest that in addition to Sir2, Rpd3 may be important in mediating the DR response in yeast cells (Jiang *et al.* 2002).

In contrast, other laboratories have provided evidence that deletions in *SIR2*, which normally shorten lifespan, can result in normal lifespan extension under conditions of reduced glucose (DR) (Jiang *et al.* 2002; Kaeberlein *et al.* 2004b; Fabrizio *et al.* 2005; Tsuchiya *et al.* 2006; Smith *et al.* 2007a). Kaeberlein *et al*. (2004) not only demonstrated that lifespan extension could still be achieved in yeast cells lacking *SIR2*, but also found that yeast cells with extra copies of *SIR2* showed an additive increase in replicative lifespan when subjected to DR, indicating that DR and Sir2 may in fact be acting in parallel pathways. One suggestion is that Sir2-independent lifespan extension by DR may be mediated through Hst2 (Lamming *et al.* 2005), a *SIR2* homologue that promotes the stability of repetitive ribosomal DNA (rDNA). However, more recent studies have shown that *HST2* mutants together with mutations in a range of other sirtuins still display extended lifespan in response to reduced glucose concentrations (Kaeberlein *et al.* 2006a; Tsuchiya *et al.* 2006; Smith *et al.* 2007a). An increase in rDNA circles, associated with lack of *SIR2*, has been shown to reduce the lifespan of wild-type yeast cells (Sinclair and Guarente 1997). The presence of these rDNA circles is exclusive to yeast and may be of limited importance when considering the role of Sir2 in terms of evolutionary conservation across a range of organisms.

The controversy surrounding DR in yeast has been source to great debate, firstly as to whether lifespan extension by DR is dependent on increased respiration and secondly whether the effects of DR are mediated by sirtuins, and particularly Sir2 (Lin *et al.* 2000; Lin *et al.* 2002; Fabrizio *et al.* 2005; Lamming *et al.* 2005; Kaeberlein *et al.* 2006a; Kaeberlein *et al.* 2006b; Lin and Guarente 2006; Sinclair *et al.* 2006; Tsuchiya *et al.* 2006; Smith *et al.* 2007a). Alternative pathways have been suggested to mediate the lifespan extension by DR. These include the Tor (target of rapamycin) and Sch9 pathways, both of which are involved in nutrient sensing (Kaeberlein *et al.* 2005b). The fact that two different main pathways (namely pathways via Sir2 or Tor/ Sch9) have been suggested as mediators of DR could be explained by differences in glucose concentrations used to implement DR by the Guarente (0.5%) and Kaeberlein / Kennedy laboratories (0.05%; Figure 1.7). This idea was proposed by Su-Ju Lin who suggested that the differences in glucose concentration may be prompting either fermentation or oxidation to take place. However, this model has been challenged by a recent independent study performed by another laboratory which showed that lifespan could be extended in *SIR2* mutant yeast cells when DR was applied using a 0.5% glucose medium (Smith *et al.* 2007a). In addition, the Kaeberlein / Kennedy laboratories have reported Sir2-independent lifespan extension using both 0.05% and 0.5% glucose concentrations (Tsuchiya *et al.* 2006).

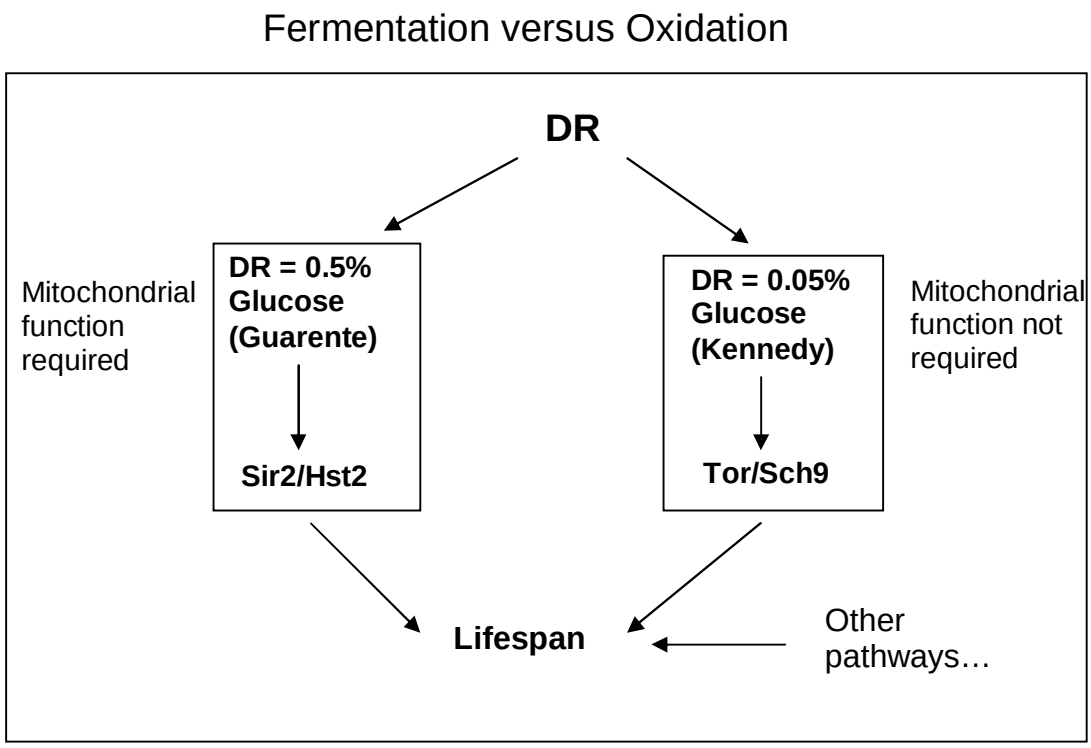

Figure 1.7: A model explaining potential mechanisms involved in lifespan extension of yeast using two concentrations of glucose. Su-Ju Lin's proposed model explaining differences seen between the Guarente and Kennedy laboratory for yeast cells lacking *SIR2* in response to DR (J.Toivonen personal communication adapted from Su-Ju Lin's presentation at the Gordon Biology of Aging conference Jan - Feb 2006). This model has since been disproved by two independent studies (Tsuchiya *et al.* 2006; Smith *et al.* 2007a).

1.5.4 Dietary restriction in the nematode worm *Caenorhabditis elegans*

Methods for applying DR in *C. elegans* are more diverse than in the budding yeast or rodents. There are thought to be up to 12 different methods of applying DR, adopted by laboratories (W. Mair, personal communication), many of which are small variations of more traditional DR protocols. It is thought that *C. elegans* in the wild feed exclusively on bacteria (Caswell-Chen *et al.* 2005). Indeed, in a laboratory, *C.*

elegans can be successfully maintained on a bacterial lawn (*E. coli*) layered on agar plates containing minerals, cholesterol and peptones (Brenner 1974).

DR in *C. elegans* is typically achieved through serial dilution of a bacterial source, usually *E. coli* on plates or liquid culture (Klass 1977; Houthoofd *et al.* 2003), which extends lifespan and reduces fecundity (Klass 1977; Bishop and Guarente 2007b; Bishop and Guarente 2007a). However, other methods that extend lifespan include complete removal of bacterial food (Kaeberlein *et al.* 2006b; Lee *et al.* 2006; Smith *et al.* 2008), altering the strain of bacteria in the worm diet (Garsin *et al.* 2001; Garsin *et al.* 2003) or using a synthetic axenic media in the absence of bacteria (Vanfleteren *et al.* 1998; Vanfleteren and Braeckman 1999; Houthoofd *et al.* 2005; Walker *et al.* 2005). Another DR method commonly adopted is through a genetic mutation called eat-2, which causes a defect in pharyngeal pumping and is thought to mimic DR (Lakowski and Hekimi 1998; Wang and Tissenbaum 2006; Hansen *et al.* 2008). However, *eat-2* mutants cultured on bacterially-diluted plates (DR) display a further extension of lifespan compared with wild type worms subjected to DR, suggesting that *eat-2* may extend lifespan through a different mechanism to DR itself ((Hansen *et al.* 2007); A.Brunet, personal communication). Finally, similarly to some of the genetic manipulations in yeast, lifespan can be extended by inhibiting specific nutrient transporters located in the gut of nematodes (Fei *et al.* 1998; Fei *et al.* 2004; Meissner *et al.* 2004).

Despite bacterial dilution being one of the most common methods of applying DR, it is complicated by the problem that *E. coli* is mildly toxic to nematodes (Gems and Riddle 2000; Garigan *et al.* 2002; Walker *et al.* 2005). This was demonstrated by growing nematodes on bacteria that had been killed by ultra violet (UV) radiation or

antibiotics, which was shown to extend lifespan (Gems and Riddle 2000; Garigan *et al.* 2002). Furthermore, wild-type nematodes exhibited extended lifespan when fed the Gram-positive bacterium *Bacillus subtilis* compared with *E. coli,* and the lifespan of *daf-2* (insulin receptor) mutants relative to controls was only fractionally extended when propagated on *B. subtilis,* which was in contrast to the remarkable lifespan extension observed when propagated on *E. coli* (Garsin *et al.* 2003).

1.5.4.1 Genetics of dietary restriction in C. elegans

Several studies in worms suggest that DR extends lifespan independently of the insulin / insulin-like signalling (IIS) pathway. *Daf-2* (insulin receptor) mutants fed a DR regimen exhibit a further extension of lifespan compared with *Daf-2* mutants fed a control diet (Lakowski and Hekimi 1998; Houthoofd *et al.* 2003; Wolff and Dillin 2006). Furthermore, DR has been shown to extend lifespan independently of the FOXO transcription factor DAF-16 (Lakowski and Hekimi 1998; Houthoofd *et al.* 2003; Kaeberlein *et al.* 2006b; Lee *et al.* 2006). However, using a similar method of bacterial dilution to extend lifespan, a recent report revealed that DAF-16 is required to observe the longevity phenotype under DR (Greer *et al.* 2007). Moreover, both lifespan extension by both reduced IIS signalling and DR (food deprivation method) appear to be dependent on the presence of heat shock factor 1 (Hsu *et al.* 2003; Steinkraus *et al.* 2008).

As is the case with yeast, numerous mechanisms have been proposed to explain lifespan extension by DR. One view was that DR extends lifespan by inducing a lower metabolic rate and hence reduced ROS production (Lakowski and Hekimi 1998). However direct measurements of the rate of oxygen consumption suggest that metabolic rate is similar between dietary-restricted and control-fed nematodes

(Houthoofd *et al.* 2002a; Houthoofd *et al.* 2002b). The *C. elegans* homologue of Sir2, *Sir-2.1* has also been investigated for its role in lifespan extension by DR. As is the case in yeast (Kaeberlein *et al.* 1999), over-expression of *Sir-2.1* also increases the lifespan of worms (Tissenbaum and Guarente 2001), and it had previously been suggested that SIR-2.1 is required for lifespan extension under DR (Wang and Tissenbaum 2006). However, more recent studies have failed to support this role for SIR-2.1 in mediating the response to DR (Kaeberlein *et al.* 2006b; Lee *et al.* 2006; Hansen *et al.* 2007). It is possible that the different methods of applying DR may explain differences in the proposed mechanisms mediating it.

Recently, a group of sirtuin activating compounds (STACs), which include resveratrol, a plant polyphenolic phytoallexin (anti-fungal defence compound) commonly found in the skin of grapes, were identified after they were found to activate production of the Sir2 protein (Wood *et al.* 2004). In *C. elegans*, resveratrol extends lifespan (Wood *et al.* 2004) and is thought to do so in a *Sir-2.1*-dependent manner (Viswanathan *et al.* 2005). However, more recent work has reported a negligible or no lifespan extension when feeding worms resveratrol in the diet (Bass *et al.* 2007b).

Another potential mechanism regulating the effects of DR is the TOR (target of rapamycin) pathway, which is involved in nutrient (particularly amino acid) sensing. Lifespan can be extended in mutants where TOR activity has been reduced (Vellai *et al.* 2003; Jia *et al.* 2004; Meissner *et al.* 2004; Henderson *et al.* 2006). Although little work has been carried out to date on the interaction between TOR and DR in worms, one study has reported that RNAi against TOR caused a further extension of lifespan of *eat-2* mutants, a genetic mimetic of DR (Henderson *et al.* 2006). In contrast, a

second study reported no further lifespan extension of *eat-2* mutants subjected to TOR RNAi (Hansen *et al.* 2007). Since it is still unclear whether DR and *eat-2* mutations extend lifespan through the same or alternative mechanisms, these results have to be interpreted carefully ((Hansen *et al.* 2007); A. Brunet, personal communication). It has also been proposed that autophagy is required for lifespan extension by DR. However, in these experiments DR was induced via the *eat-2* mutation, which also makes the results difficult to interpret (Jia and Levine 2007; Hansen *et al.* 2008).

Other notable genes and pathways that have been proposed to mediate the effects of DR include the energy sensing AMP-activated protein kinase (AMPK; (Greer *et al.* 2007), the mammalian orthologue of the Foxa family of transcription factors (PHA-4; (Panowski *et al.* 2007) and a homologue of the NF-E2-related transcription factors (SKN-1), which mediates DR in two neurons (Bishop and Guarente 2007b). The different mechanisms proposed may be the effect of a variety of DR protocols chosen. Nonetheless, PHA-4 appears to be a good candidate for the mediation of DR in *C. elegans*, since it mediates the response of lifespan to both bacterial dilution (tested over a range of concentrations) and *eat-2* mutations (Panowski *et al.* 2007).

1.5.5 Dietary restriction in the fruit fly *Drosophila melanogaster*

In the wild, *Drosophila* is commonly thought to feed on yeast and fungus growing on rotten and fermenting fruits (Spieth 1974). When maintained in a laboratory, *Drosophila* can be fed a range of diets varying from banana molasses, e.g. (Rose 1984) to a simple agar-based diet enriched with different nutritional components, e.g. (Mair *et al.* 2005). Perhaps the most commonly used diet is comprised of sucrose and

autolysed yeast powder dissolved in an agar gel, occasionally supplemented with cornmeal (Kapahi *et al.* 2004a). In contrast to methods of applying DR in rodents, *Drosophila* requires constant *ad libitum* access to food. DR is typically achieved in two ways. The most commonly used method involves the simultaneous dilution of sugar and yeast in an agar-based gel (Chapman and Partridge 1996; Mair *et al.* 2003). Alternatively, DR can be achieved by altering the availability of live yeast on the surface of the food (Chippindale 1993; Libert *et al.* 2007). More recently, it has been reported that the magnitude of lifespan extension observed when simultaneously diluting both sugar and yeast can be achieved solely by the reduction of the yeast component of the diet, whilst keeping the sucrose concentration fixed (Mair *et al.* 2005; Lee *et al.* 2008). Both methods of DR have been shown to be effective in extending the median and maximum lifespan of *Drosophila*. Attempts to reproduce the lifespan extension seen in rodents through intermittent feeding (Goodrick *et al.* 1982) in *Drosophila* have to date not been successful (Kopec 1928; Le Bourg and Medioni 1991), again indicating that *Drosophila* require constant access to food during DR studies.

1.5.5.1 Dietary restriction and reproduction

Lifespan extension by DR in many organisms is commonly expected to trade-off with reduced reproduction (Partridge *et al.* 2005a), and *Drosophila* is no exception (Chippindale *et al.* 1993; Chapman and Partridge 1996; Libert *et al.* 2007; Lee *et al.* 2008). As the concentration of food is increased, daily and lifetime fecundity increases progressively (Figure 1.8). In contrast, lifespan is reduced at high food concentrations. As the food becomes more dilute, lifespan is extended at an intermediate food concentration (DR) in conjunction with reduced fecundity (Figure

1.8). A more severe dilution of the food medium results in a further reduction in fecundity and a sharp decline in lifespan, indicating a starvation threshold. Egg production can be rapidly (within two days) induced / reversed with a change of nutritional intake (Chippindale 1993; Good and Tatar 2001).

The interaction between diet, reproduction and lifespan has formed the foundations of evolutionary hypotheses as an explanation for the mechanism(s) underlying lifespan extension by DR. These hypotheses predict that lifespan and fecundity are in competition with at least some of the same, limiting, nutrients (Charlesworth 1980) and DR regulates certain pathways causing a shift or resource allocation from reproduction and growth towards somatic maintenance and repair (Williams 1966; Kirkwood and Holliday 1979; van Noordwijk and de Jong 1986; Holliday 1989; Kirkwood *et al.* 2000; Kirkwood and Shanley 2005). The effects observed under conditions of DR could thus be an evolved response to food shortages in nature (Williams 1966; Kirkwood and Holliday 1979; van Noordwijk and de Jong 1986). However, a previous study has reported that DR also extends longevity of flies that have vitellogenesis blocked by the ovo^{D1} mutation and flies that have had their germ line removed by X-radiation (Mair *et al.* 2004). This suggests that reduced fecundity may not be essential for lifespan extension by DR or that the relevant aspects of reproduction lie further upstream of the interventions targeted (Mair *et al.* 2004).

Figure 1.8: The response of lifespan and egg production to a range of food concentrations in *Drosophila*. Open circles represent egg production and closed circles represent median lifespan. Increasing the concentration of sugar / yeast medium results in a continuous increase in lifetime fecundity. Lifespan peaks at an intermediate food concentration and is reduced at high concentrations as well as very low concentrations (starvation). Figure taken from (Chapman and Partridge 1996).

1.5.5.2 The importance of performing DR experiments in single-sex groups

As previously described, there is a strong interaction between diet, reproduction and lifespan whereby high food concentration causes increased fecundity, but reduced survival in female *Drosophila*. In addition, an interaction between diet and re-mating frequency in fruit flies has been reported (Harshman *et al.* 1988; Chapman and Partridge 1996), whereby an increase in the food concentration is directly correlated with an increase in re-mating frequencies (Figure 1.9). Furthermore, an increase in mating frequency has a knock on effect and reduces longevity in both males and females (Chapman and Partridge 1996; Partridge and Prowse 1997). Consequently, it

is imperative that lifespan experiments involving dietary manipulations are performed in single-sex groups, although this is not followed in all experimental set-ups (Chippindale 1993; Bradley and Simmons 1997; Rogina and Helfand 2004; Wood *et al.* 2004; Min and Tatar 2006b; Min and Tatar 2006a). Failure to control for mating status is also a problem when trying to indentify candidate genes or drugs treatments that mediate the response to of lifespan to diet (Piper and Partridge 2007).

Figure 1.9: The relationship between food concentration and re-mating frequency in female *Drosophila*. The re-mating frequency (% mating opportunities taken) of flies increases proportionally with increasing concentration of sugar / yeast diet. The data are representative of combined results of females that were continuously exposed or intermittently exposed to mating males. (Figure taken from (Chapman and Partridge 1996)

1.5.5.3 Compensatory feeding on DR diets?

Applying DR by food dilution, when the food in excess, has led to the suggestion that flies on a DR diet could compensate to lower nutrition levels by altering their feeding behaviour and increasing food intake (Cooper *et al.* 2004; Carvalho *et al.* 2005). The uptake of radio-labelled food was found to be higher in DR flies than fully-fed flies (Carvalho *et al.* 2005). However, by measuring steady state feeding

behaviour through proboscis extension (Mair *et al.* 2005) and calibrating these data with food intake of blue dye-labelled food (Wong *et al.* 2008), it has been argued that DR-fed flies and fully-fed flies consume a similar amount of food (Bross *et al.* 2005; Wong *et al.* 2008). In contrast, another study has reported that food uptake is up to four times greater in fully-fed flies than DR flies, providing further evidence that DR flies do not compensate by eating more (Min and Tatar 2006a). Furthermore, if compensatory feeding on a DR diet did occur and flies were ingesting a similar concentration of nutrients to full-fed flies, then one would expect differences in daily and lifetime fecundity to be negligible between the two groups, which is not the case (e.g. Figure 1.8).

1.5.5.4 Sex differences in response to DR

As well as extending the lifespan of female *Drosophila*, DR has also been reported to extend the lifespan of males (Magwere *et al.* 2004). However, the magnitude of the response appears to be much greater in females, which exhibited a 60% increase in lifespan relative to fully-fed controls, compared to a 30% increase in males (Magwere *et al.* 2004). Interestingly, in the same study, male lifespan was shown to peak at a lower food concentration than the peak lifespan of females. The explanation for the different responses of males and females to DR is not clear; however it is likely that females show a stronger response to nutrition due to high nutritional demands for egg production, supported by data suggesting that females exhibit higher feeding rates than males (R. Wong, unpublished data).

1.5.5.5 Dietary restriction and mortality rates

Analysing age-specific mortality allows for independent comparisons of vulnerability of death at different ages (Vaupel *et al.* 1998). Inducing chronic DR has been shown to cause a delay in the onset of a detectable ageing-related increase in mortality. However, once the increase has been detected, fully-fed and DR flies exhibit a similar rate of increase in mortality (Pletcher *et al.* 2002; Mair *et al.* 2003) (Figure 1.10). In contrast, reducing the temperature at which *Drosophila* are maintained, also extends lifespan but causes a reduction in the slope of mortality (Loeb and Northrop 1916; Loeb and Northrop 1917; Miquel *et al.* 1976). This indicates a slower accumulation of irreversible ageing-related damage (Pletcher *et al.* 2000; Mair *et al.* 2003; Magwere *et al.* 2004) (Figure 1.10). Mair *et al.* demonstrated that DR extends lifespan entirely by reducing the short-term risk of death as opposed to slowing the accumulation of ageing-related damage. By switching flies from a DR diet to a fully-fed diet (and vice versa) at different intervals throughout life; Mair *et al*. revealed that flies have no memory of their past feeding. Fully-fed flies switched to a DR diet within 48 hours are no more likely to die than aged-matched flies that have been exposed to DR throughout their entire lives (Mair *et al.* 2003). A similar shift in mortality was observed when DR flies were switched to full feeding within 48 hours.

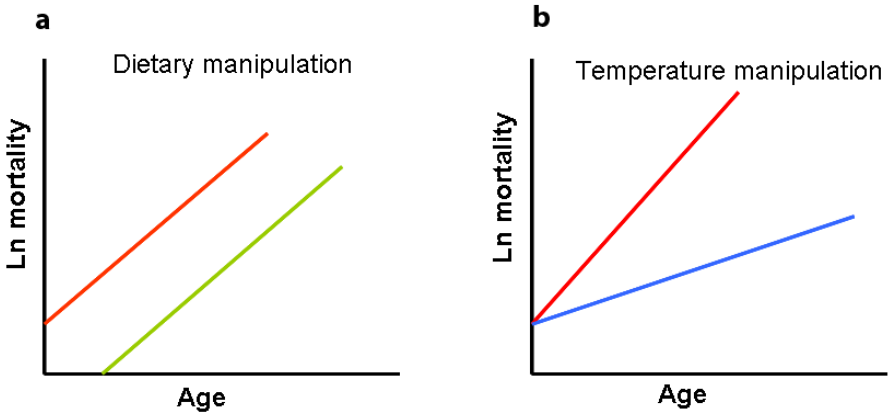

Figure 1.10: Different effects on mortality of dietary restriction and reduced temperature. (a) DR (green) induces a shift in the mortality trajectory and lowers the baseline mortality rate compared with full feeding (red). (b) Reducing the temperature (blue) lowers the mortality trajectory and delays the accumulation of irreversible ageing-related damage.

1.5.5.6 Genetics of dietary restriction in Drosophila

Studies over the last decade have attempted to shed light on the potential mechanisms underlying lifespan extension by DR in *Drosophila*, some of which appear to be conserved amongst yeast, worms, flies and mice. The insulin / IGF-like signalling pathways (IIS) and target of rapamycin (TOR) pathway are two of the most likely candidates to be involved in mediating the response to DR, predominantly due to their roles in insulin signalling and nutrient sensing respectively, in addition to growth and development.

Mutations in various components of the IIS signalling pathway have been shown to extend lifespan in mice, flies and worms (see Partridge *et al.* 2005 for detailed review). In *Drosophila*, a null mutation in the insulin receptor substrate protein CHICO, results in developmentally delayed dwarf flies which are sterile, long-lived and exhibit a range of stress resistant phenotypes (Bohni *et al.* 1999; Clancy *et al.* 2001). In a follow-up study, when subjected to a range of food concentrations, *chico*

homozygotes showed peaks in lifespan of similar magnitude to wild-type controls, but the lifespan of *chico* homozygotes peaked at a higher food concentration than that of control flies (Figure 1.11) (Clancy *et al.* 2002). Furthermore, the lifespan of *chico* homozygotes was reduced compared with controls at lower food concentrations. The fact that lifespan extension by DR could not be further increased by loss of CHICO indicates that lifespan extension by DR and reduced insulin signalling may be acting in the same pathway. In addition, this right-shift in the relationship between lifespan and nutrition (Figure 1.11) supports the idea that these two mechanisms may act in the same pathway, and suggests that the CHICO mutation induces a state similar to mild dietary restriction. However, an alternative explanation is that *chico* homozygotes eat less than controls, which is a distinct possibility due to their reduced body size and sterility.

Figure 1.11: The right-shift response of *chico* homozygotes to changing nutrition. *chico* homozygotes exhibit an increase in lifespan at higher food concentrations but reduced lifespan at lower food concentrations compared with control flies. Taken from (Clancy *et al.* 2002).

More recently, other long-lived *Drosophila* mutants have been tested for a possible interaction between DR and the IIS pathway. Over-expression of *dFOXO* in the fat body (Giannakou *et al.* 2004) and head fat body (Hwangbo *et al.* 2004) extends lifespan of flies fed a standard laboratory medium. Similarly to general consensus that DAF-16 is not required in worms (Mair and Dillin 2008), the presence of dFOXO is not required for lifespan extension by DR (Giannakou *et al.* 2008; Min *et al.* 2008). However, over-expressing *dFOXO* in the adult fat body (the fly equivalent of the mammalian liver) resulted in lifespan extension at high food concentrations (right-shifted response to nutrition), similarly to observations in *chico* homozygotes, that was not attributable to reduced food intake (Giannakou *et al.* 2008). In contrast, conflicting evidence suggests that only *dFOXO* over-expression in the adult fat body results in lifespan peaking at low food concentrations (left shift in response to nutrition), whereas over-expression of *dFOXO* in the head fat body resulted in lifespan peaking only at high food concentrations (Min *et al.* 2008). Min *et al.* also report that reduction in mRNA levels of one of the *Drosophila* insulin like peptides (*Dilps*), *Dilp5*, was associated with lifespan extension by DR, although knocking down expression of *Dilp5* with RNAi did not block the response to DR.

Components of the TOR pathway, including TOR, S6K, TSC1 and TSC2 play an important role in growth and body size in *Drosophila* (Montagne *et al.* 1999; Marygold and Leevers 2002). The TOR pathway is often viewed as a pathway parallel to the IIS pathway, although the two pathways are known to interact with each other (Marygold and Leevers 2002; Colombani *et al.* 2003). Similar to the deficiency of TOR activity in *C. elegans* (Vellai *et al.* 2003; Jia *et al.* 2004; Meissner *et al.* 2004; Henderson *et al.* 2006) and yeast (Kaeberlein *et al.* 2005b), reduced TOR signalling through over-expression of *dTsc1, dTsc2* or dominant negative forms of

dTOR / *dS6K* extends lifespan in *Drosophila* (Kapahi *et al.* 2004a). It was initially thought that down-regulation of *dTOR* activty solely in the adult fat body was sufficient to observe a longevity phenotype. However this claim has since been withdrawn (Kapahi *et al.* 2004b). Interestingly, flies over-expressing *dTsc2* show similar responses to nutrition as previously observed in *chico* homozygotes (Clancy *et al.* 2002) and flies over-expressing *dFOXO* in the fat body (Giannakou *et al.* 2008). In these flies, lifespan extension is greatest at the highest food concentrations, but is reduced at the lowest food concentration tested (Kapahi *et al.* 2004a). This again suggests that the TOR and IIS pathway interact closely with one another and may mediate lifespan extension by DR.

As previously reported in yeast and worms, over-expression of the *Drosophila* homologue of the histone deacetylase Sir2, *dSir2*, has also been shown to extend lifespan in both male and female *Drosophila* (Rogina and Helfand 2004). However, lifespan could not be further extended in *dSir2* over-expressers upon implementing DR. Furthermore, it was also reported that trans-heterozygous null mutations in *dSir2*, which lead to normal fecundity and normal or slightly reduced lifespan relative to controls (Newman *et al.* 2002; Astrom *et al.* 2003), abolish lifespan extension by DR. This suggests that *dSir2* mediates the responses of DR in *Drosophila* (Rogina and Helfand 2004). This is supported by work involving sirtuin activating compounds (STACs), including resveratrol, which activate Sir2-like proteins and extend lifespan in *Drosophila* without the cost of reduced fecundity (Bauer *et al.* 2004; Wood *et al.* 2004). Moreover, STACs did not extend lifespan of *dSir2* null mutants and no further lifespan extension was seen in flies fed STACs and a DR diet, again suggesting *dSir2* may mediate lifespan extension by DR (Wood *et al.* 2004). However, a more recent report has failed to support these findings,

demonstrating no lifespan extension with the addition of resveratrol in seven independent experiments, varying wild-type strain, diet and mating status of males and females (Bass *et al.* 2007b).

Deletion of *dRPD3,* a histone deactylase, which extends lifespan and mediates lifespan extension by DR in yeast cells (Kim *et al.* 1999; Jiang *et al.* 2002), has also been investigated in *Drosophila* (Rogina *et al*. 2002). Male and female flies heterozygous for a mutation in *dRpd3* exhibit an increased lifespan (Rogina *et al.* 2002). When subjected to DR, these flies showed no further extension of lifespan, suggesting that *Rpd3* and DR mediate lifespan extension through the same pathway (Rogina *et al.* 2002). However, it is important to consider that Rogina *et al*. (2002) only tested two food concentrations representing their DR and fully-fed conditions. It is possible, that the lifespan of *Rpd3* mutants may peak at a different DR concentration to controls, as seen with males (Magwere *et al.* 2004), *chico* homozygotes (Clancy *et al.* 2002) and flies with *dFOXO* over-expressed in the fat body (Giannakou *et al.* 2008). This is also a consideration when determining whether *dSir2* mediates the response of DR, as this study was limited to testing two food concentrations only (Rogina and Helfand 2004). It is therefore important to test the response of these mutants to a range of food concentrations before concluding whether *dRpd3* blocks lifespan extension by DR (Figure 1.12).

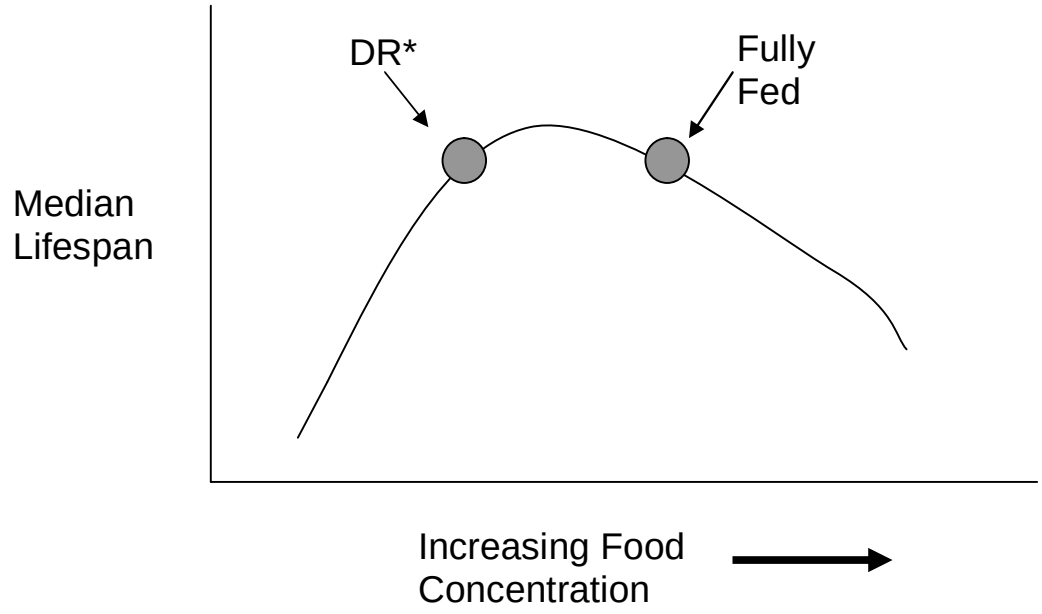

Figure 1.12: **Choosing only two food concentrations for DR and fully-fed conditions**. An illustration of the potential problems when choosing only two food concentrations to test whether DR extends lifespan, particularly when testing whether a specific pathway mediates the response to DR. * Condition that may have been chosen to represent DR.

Lifespan extension has also been reported in males and females heterozygous for a mutation in *Indy* ("I'm not dead yet"), which encodes a protein that is closely related to a mammalian sodium dicarboxylate co-transporter, involved in transporting Krebs' cycle intermediates (Rogina *et al.* 2000). *Indy* has been shown to be expressed in oennocytes, the fat body and the mid-gut, the latter two being the fly equivalent of the mammalian liver and white adipose tissue. In contrast to DR, mutations in *Indy* appear to extend lifespan by decreasing the slope of mortality (Figure 1.10), indicating a slower rate of ageing than control flies (Marden *et al.* 2003). Interestingly *Indy* mutants show normal or slightly elevated fecundity when fed a normal diet, but exhibit reduced fecundity when fed a low food diet, suggesting a conditional trade-off between lifespan fecundity dependent upon levels of nutrition (Marden *et al.* 2003). However, a more recent study has demonstrated that the

original extension of lifespan of *Indy* mutants diminished following extensive backcrossing into several different genetic backgrounds. (Toivonen *et al.* 2007).

Partial-loss-of function mutation in *methuselah* was the first gene mutation to be shown to extend lifespan in *Drosophila* (Lin *et al.* 1998). Similarly to the earlier reports on *Indy* mutants, *methuselah* appears to extend lifespan by lowering the slope of mortality trajectory, suggesting that this mutation is reduces the rate of ageing, although no interaction with diet has been tested to date. Lifespan can also be extended by two dominant negative forms of the *Drosophila* homologue of the mammalian tumour suppressor p53 (*Dmp53*), but the lifespan of *Dmp53* is not further extended by DR (Bauer *et al.* 2005).

Another pathway that has been proposed to be important in regulating lifespan is the olfactory pathway. Microarray analysis of dietary restricted flies compared with fully-fed flies has previously revealed changes in expression levels of genes encoding odourant-binding proteins both with nutrition and age (Pletcher *et al.* 2002). Furthermore, flies that were exposed to the odour of live yeast, without actually being able to feed on it, showed a significant reduction in lifespan when fed a DR diet but not a fully-fed diet, without any clear effect on fecundity or feeding behaviour (Libert *et al.* 2007). This led to the suggestion that certain sensory receptors may be involved in mediating lifespan in *Drosophila*, as previously observed in *C.elegans*, via mutations in the sensory cilia or sensory-signal transduction, which both significantly extend lifespan (Apfeld and Kenyon 1999). Libert *et al*. targeted one of the 62 proposed odorant receptors in *Drosophila*, *Or83b*, which is highly conserved between insect species and ubiquitously expressed in olfactory neurons (Neuhaus *et al.* 2005). Previous work has demonstrated that loss of

function of *Or83b* disrupted several behavioural and physiological responses to odours (Larsson *et al.* 2004). Libert *et al*. showed that flies either heterozygous or homozygous for $Or83b^2$ exhibit extended lifespan in both sexes. However, the magnitude of lifespan extension was greatest in homozygous females, again with no noticeable effects on fecundity or feeding. Furthermore, lifespan of $Or83b^2$ mutants could be extended on a range of food concentrations, indicating that *Or83b* is not required for lifespan extension by DR (Libert *et al.* 2007).

1.5.5.7 Phenotypes of DR

In addition to extending lifespan and reducing fecundity, DR causes several other physiological changes and induces some of the stress resistance phenotypes previously observed in rodents. One of the most prominent stress resistant phenotypes is increased resistance to starvation in dietary-restricted flies (Bradley and Simmons 1997; Burger *et al.* 2007). However, the starvation resistance of DR flies was only observed early in life, whilst later in life DR induced starvation sensitivity (Burger *et al.* 2007). Flies, similarly to mammals, have the ability to store excess nutrients predominantly in the form of glycogen and triglycerides (TAG). Interestingly, fat storage (TAG) was shown to be inversely related with increased yeast concentration, but positively correlated with increased sucrose concentration in the diet (Bradley and Simmons 1997; Skorupa *et al.* 2008). In contrast, protein storage is increased as a result of increased dietary yeast but not sucrose (Skorupa *et al.* 2008). Activity levels (fly movements) have also been shown to vary with nutrition levels, whereby reduced activity appears to correlate with increased food concentration (Bross *et al.* 2005).

Microarray analysis of wild-type flies has revealed a correlation between increased age and elevated expression of numerous innate immunity-related transcripts, which become delayed in dietary restricted flies (Pletcher *et al.* 2002). However, despite this finding, the same group reported no increase in pathogenic resistance in dietary-restricted flies; but enhanced pathogenic resistance was detected in long-lived *chico* mutants (Libert *et al.* 2008). In contrast, another study has reported that DR causes some positive effects on immunity later in life but not early in life when flies are challenged with different strains of bacteria (Burger *et al.* 2007).

As is the case in rodents (Masoro *et al.* 1982; Selman *et al.* 2005) and worms (Houthoofd *et al.* 2002a; Houthoofd *et al.* 2002b), DR also has no effect on oxygen consumption and heat production, suggesting that DR does not extend lifespan by lowering metabolic rate (Hulbert *et al.* 2004). Furthermore, DR flies do not exhibit increased resistance to oxidative stress, providing evidence against the oxidative damage theory of ageing (Burger *et al.* 2007).

1.5.6 A role for protein synthesis in ageing

Over the last 20 years, research on model organisms has demonstrated that mutations in certain components of the insulin /insulin-like signalling (IIS) and target of rapamycin (TOR) pathways can extend the lifespan of yeast, worms, flies and mice (Partridge *et al.* 2005a; Mair and Dillin 2008). These pathways are also involved in a whole range of biological processes including growth, reproduction, metabolism and stress response. Protein synthesis is a regulated cellular process that links nutrients in the environment to growth and development of an organism. Protein synthesis is essential for all biological processes and it is unsurprising that it plays an important role in ageing. Activity of key mRNA transcription factors has been shown to

decrease with age, resulting in a reduction in total protein synthesis (Makrides 1983). Until recently, few studies have tested a direct role for protein synthesis in ageing because protein synthesis is essential for growth and development, hence manipulating general mRNA translation is likely to have widespread effects which are difficult to dissociate from the effects on ageing.

Three recent reports in *C.elegans* have highlighted a specific role for protein synthesis in ageing, by demonstrating that inhibition of different genes encoding translation-initiation factors can extend lifespan (Hansen *et al.* 2007; Pan *et al.* 2007; Syntichaki *et al.* 2007a). These include depletion or inhibition of the eukaryotic mRNA initiation factor 4E (eIF4E), ifg-1 and rsks-1 the worm homologues of eIF4G and S6 kinase respectively. Interestingly, of the five *C.elegans* isoforms of eIF4E, which are predominantly expressed in germline, only depletion of IFE-2 (which is expressed in the somatic cells) reduced protein synthesis, extended lifespan and protected against oxidative damage (Syntichaki *et al.* 2007a). These data suggest that reduction of protein synthesis specifically in the soma is required for lifespan extension. Furthermore, the IFE-2 mutants exhibited an additive increase in lifespan when crossed with long-lived *eat-2* mutants (which induces DR), indicating that *eat-2* and *ife-2* mutants extend lifespan by different mechanisms. Similarly, despite reduced TOR activity causing a general decrease in protein synthesis and up-regulation of autophagy, long-lived TOR mutants also showed a further increase in lifespan when crossed with *ife-2* mutants. In addition to work in *C.elegans*, the lifespan of yeast cells can be extended following treatment with the antibiotic erythromycin, which decreases protein synthesis (Holbrook and Menninger 2002).

1.5.6.1 Methionine restriction extends lifespan and reduces oxidative damage in rodents

In addition to lifespan extension by whole food DR, restriction of the protein portion of the diet has been shown to extend median and maximum lifespan of rats (Yu *et al.* 1985). Furthermore, restriction of the essential amino acid methionine can extend the lifespan of both mice and rats and lead to other characteristic phenotypes of whole food DR including lowered body mass, glucose, insulin and IGF-1 levels and a reduction in some ageing-related pathologies including some cancers and cataracts (Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003; Miller *et al.* 2005; Komninou *et al.* 2006).

Methionine is one of two sulphur containing amino acids, the other being cysteine, which is a non-essential amino acid. Methionine constitutes 2% of amino acid residues in proteins (McCaldon and Argos 1988) and is the start codon for all protein-encoding mRNA sequences. It is readily converted to methionine sulfoxide by physiological oxidants (Vogt 1995), which is thought to interfere with the biological activity of proteins. However, methionine sulfoxide can be converted back to methionine by the enzyme methionine sulfoxide reductase (Brot and Weissbach 2000). Furthermore, reduced levels of methionine sulfoxide reductase are associated with reduced lifespan in rats (Moskovitz *et al.* 2001), whilst over-expression of this enzyme in the nervous system extends *Drosophila* lifespan and increases resistance to oxidative damage (Ruan *et al.* 2002), thus highlighting a potentially important role for methionine in the ageing process.

Inadequate dietary methionine slows growth and development in young mammals (Galiani *et al.* 2006). The importance of methionine in the diet early in life is

supported by the finding that methionine restriction in the first year of life caused a large number of deaths in mice (Miller *et al.* 2005). However, excessive levels of methionine have been shown to be extremely toxic in both young and old mammals, more so than any other amino acid (Harper *et al.* 1970). For example, doubling the concentration of methionine causes growth retardation, anaemia, vascular damage and kidney and liver damage (see Troen *et al.* 2007). Furthermore, some sulphur containing intermediates of methionine metabolism e.g. homocysteine are reactive and potentially toxic.

A few studies have recently set out to determine the mechanism of lifespan extension by methionine restriction. One of the most attractive candidates is through a reduction in oxidative damage and a suppression of ROS production. Reducing the concentration of protein and not fat or carbohydrates decreased ROS production and oxidative damage, which was attributed to reduced methionine intake (Ayala *et al.* 2007). Long-term dietary restriction of rats reportedly decreased oxidation of protein and DNA damage (Lopez-Torres *et al.* 2002), whilst methionine restriction reduced mitochondrial ROS production as well as oxidative damage to mitochondrial DNA and caused a marked reduction protein oxidation similarly to whole food DR (Sanz *et al.* 2006). Moreover, methionine restriction caused an elevation in antioxidant defences, for example an increase in glutathione levels, and increased the efficiency of mitochondrial respiration (Richie *et al.* 1994; Zimmerman *et al.* 2003; Pamplona and Barja 2006).

1.5.6.2 Why might reduced protein synthesis extend lifespan?

Inhibition of genes encoding translation initiation factors can extend the lifespan and reduced oxidative damage in the nematode worm *C.elegans*. Likewise, methionine restriction has also been shown to decrease oxidative damage, increase antioxidant defences and increase lifespan in rats and mice. Due to methionine encoding the start codon for all mRNA protein sequences, these results may suggest that extension of lifespan in by methionine restriction may be partially explained by reduction in protein synthesis, similarly to the effects of inhibiting translation machinery (Hipkiss 2007; Hipkiss 2008). Messenger RNA translation is the most error prone step in gene expression, with approximately 3 in 10,000 codons being mis-translated (Kirkwood *et al.* 1984), resulting in the production of erroneous proteins which are normally rapidly degraded by intracellular proteases. One suggestion is that severe methionine restriction leads to a reduction in mRNA translation initiation, as observed with the *C.elegans* mutants with depleted eIF4E, and subsequently a decrease in overall protein synthesis. A reduction in protein synthesis is thus likely to reduce the amount of erroneously produced proteins, potentially freeing-up more proteases and chaperones to break down polypeptides that have been modified by ROS and glycosylation (Hipkiss 2008).

Another explanation for why a reduction in protein synthesis may extend lifespan is because mRNA translation is one of the most energy consuming cellular processes (Proud 2002). Hence reducing protein synthesis will inevitably reduce energy expenditure and will also lower the production of toxic metabolic derivatives (Syntichaki *et al.* 2007b). The excess energy conserved by reducing protein synthesis could then be allocated towards somatic maintenance and repair, contributing to extended longevity (Kirkwood 1977; Kirkwood and Holliday 1979).

1.6 Thesis outline

Dietary restriction (DR) has been shown to robustly extend lifespan in a diverse range of organisms, from yeast to mammals (Mair and Dillin 2008). However, despite over 70 years of research, the mechanisms underlying lifespan extension by DR in any organism remain to be fully elucidated. Despite the majority of DR studies having been carried out in rodents, invertebrate model organisms including yeast, flies and worms have recently been adopted to try and uncover the genetics of DR. An important recent discovery has been that the mechanisms that influence lifespan are conserved during evolution over great evolutionary distances between yeast, multicellular invertebrates and mammals (Partridge and Gems 2002; Tatar *et al.* 2003; McElwee *et al.* 2007). Using *Drosophila melanogaster* as a model organism, this thesis sets out to highlight the need for optimisation of DR protocols for studies using *Drosophila*. Further, it highlights the roles of individual nutrients in the diet in mediating the effects of DR as well as the potential mechanisms involved.

1.6.1 Chapter 3: Factors affecting the responses to dietary restriction in *Drosophila*

In all model organisms, the beneficial effects on lifespan can be achieved through multiple methods of applying DR. However, subtle differences in specific nutrients can lead to discrepancies between potential candidate pathways that mediate the effects of DR, pointing to a need for laboratories to work with a common DR protocol particularly when working with the same model organism. In *Drosophila*, DR is typically implemented by the dilution of sucrose and yeast in an agar-based gel. However, different laboratories use different sources of yeast and different concentrations of ingredients, which can potentially result in conflicting results. As

part of a larger study of optimising dietary restriction and lifespan protocols in *Drosophila* (Bass *et al.* 2007a), chapter 3 sets out to investigate different factors affecting the responses of lifespan and fecundity during *Drosophila* DR. This includes testing whether intermittent feeding can extend *Drosophila* lifespan, as observed in rodents (Goodrick *et al.* 1982). In addition, the responses of a range of commonly used wild-type strains to DR will be investigated, with particular emphasis on the possible role of the intracellular bacterium *Wolbachia* in mediating the effects of DR.

1.6.2 Chapter 4: Identifying specific nutrients mediating the responses of lifespan and fecundity in *Drosophila* dietary restriction

Lifespan extension by DR is commonly expected to trade-off with a reduction in fecundity / reproduction (Partridge *et al.* 2005a), prompting the suggestion that DR is an evolved response to food shortages in nature (Williams 1966; Kirkwood and Holliday 1979; van Noordwijk and de Jong 1986; Holliday 1989; De Jong 1993). This prediction assumes that both reproduction and somatic maintenance are in competition for at least some of the same limiting nutrients (Charlesworth 1980), and thus DR induces a metabolic shift of resources away from reproduction and towards somatic maintenance and repair. In *Drosophila*, dilution of the yeast component of the diet alone is sufficient to account for almost the entire lifespan extension observed when simultaneously diluting yeast and sucrose, suggesting that specific nutrients as opposed to calories may be mediating the responses to DR in flies (Mair *et al.* 2005). The work presented in Chapter 4 investigates the role of different nutritional groups present in dietary yeast, using an optimised DR protocol (Bass *et al.* 2007a), to determine whether the increased fecundity and reduced lifespan with full feeding are regulated by the same or different nutrients.

1.6.3 Chapter 5: Uncoupling the responses of lifespan and fecundity in *Drosophila* dietary restriction

Despite the classical view in rodents that DR extends lifespan as a result of reduced caloric intake independent of nutrients, several studies have reported lifespan extension of a similar magnitude to whole food DR when either the protein portion of the diet (Yu *et al.* 1985) or specific essential amino acids such as methionine are restricted (Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003; Miller *et al.* 2005). The data shown in Chapter 5 follow on from the findings presented in Chapter 4 and discusses whether specific amino acids are also important in regulating *Drosophila* lifespan. Furthermore, this chapter investigates two candidate pathways, the IIS and TOR pathways, which may potentially mediate the effects of DR in flies.

Chapter 2

General materials and methods

2.1 *Drosophila melanogaster* stocks

2.1.1 Dahomey wild-type flies

The majority of experiments were performed using the wild-type, outbred strain Dahomey unless otherwise stated. Flies were originally collected in 1970 from Dahomey (now Benin) and have since been maintained in stock cages at 25°C at 65% humidity under a 12 hour light / 12 hour dark cycle with overlapping generations. Cages contained around 12 bottles of the standard sugar yeast food medium (section 2.2.1), which were replaced periodically. This method of stock culturing ensures that adult survival rates are comparable to those of stocks that had been freshly collected from the wild (Sgro and Partridge 2001).

2.1.2 Other wild-type stocks

Other wild-type stocks included: white Dahomey (wDahomey), Oregon-R, Canton-S, W1118 and yellow-white (yw). wDahomey were produced by backcrossing the white gene from W1118 into the Dahomey background, and these stocks were maintained in population cages as described for Dahomey (section 2.1.1). All other stocks were maintained at 18°C in glass vials or bottles containing the standard sugar yeast medium (section 2.2.1) or a cornmeal-based diet (section 2.2.2) and were transferred to fresh medium every generation.

2.1.3 Insulin receptor dominant-negative flies (dUAS-*InRDN*)

The dUAS-*InRDN* transgene has an amino acid substitution in the kinase domain (arginine 1409 replaced by alanine, R1409A) of the insulin receptor (InR). This results in dominant negative behaviour of the protein (Wu *et al.* 2005). Expression of

dUAS-I*nRDN* was driven by the ubiquitous and constitutive driver daughterlessGAL4 (daGAL4). Both the dUAS-*InRDN* and daughterlessGAL4 lines were obtained from the Bloomington stock centre[9] and were extensively backcrossed into wDahomey background[10]. Flies were maintained in vials containing a cornmeal-based diet at 25°C (section 2.2.2) and transferred to fresh medium approximately every two weeks.

2.1.4 S6 kinase dominant negative flies (dUAS-*S6K^{KQ}*)

The dUAS-*S6K* dominant-negative (dUAS-*S6K^{KQ}*) line was a kind gift from Mary Stewart, North Dakota State University, USA. The dUAS-*S6K^{KQ}* flies were generated by mutating a conserved lysine (K_{100}) in the ATP binding site of S6K1 and replacing it with glutamine (Q) (Barcelo and Stewart 2002). Expression of dUAS-*S6K^{KQ}* was driven by the ubiquitous daughterlessGAL4 driver (daGAL4). Both lines were backcrossed into wDahomey background[11]. Flies were maintained in vials containing a cornmeal-based diet at 25°C (section 2.2.2) and transferred to fresh medium approximately every two weeks.

2.2 *Drosophila* food media

2.2.1 Sugar yeast medium (SY)

Standard SY medium (Ashburner 1989) comprised 967ml of distilled water, 100g autolysed Brewer's yeast powder, 50g sucrose, 15g agar , 30ml nipagin (100 g/L methyl 4-hydroxybenzoate in 95% ethanol), 3ml propionic acid per litre of prepared

[9] Bloomington stock centre, http://flystocks.bio.indiana.edu/
[10] daGAL4 and dUAS-*InRDN* lines were backcrossed at least five times prior to the start of lifespan experiments. Backcrossing was performed by T. Ikeya
[11] dUAS-*S6K^{KQ}* and daGAL4 lines were backcrossed by C. Slack for at least five times prior to the start of lifespan experiments.

food. Nipagin and propionic acid were added as preservatives and anti-fungal reagents.

For preparation of the medium, 700ml of distilled water was poured into a saucepan in addition to the agar. The agar was brought to the boil at which point the yeast and sucrose were added and the mixture was stirred thoroughly. The medium was left to cool and the remaining 267ml of water were added to the food. The medium was allowed to cool to 60°C before the nipagin and propionic acid were added. The medium was dispensed into vials or bottles in aliquots of 7ml or 60ml respectively using a liquid dispenser and left to air overnight before being plugged with cotton wool and stored at 4°C. Fresh food was prepared approximately every three weeks.

2.2.2 Cornmeal-based medium (ASG)

All stocks were maintained on an ASG medium consisting of yeast, sugar and maize, supplemented with some live yeast granules. This medium has a softer texture than SY medium, which can aid development of mutant stocks that show high larval lethality. The ASG medium was prepared by combining 10g agar, 85g sucrose, 20g autolysed yeast, 60g maize and 1000ml distilled water in a saucepan and bringing the mixture to the boil, stirring regularly. The ingredients were allowed to simmer for five minutes and were then returned to the boil, before being taken off the heat. The medium was allowed to cool to 60°C at which point 25ml nipagin (100 g/L) was added. Food was dispensed into glass vials in 7ml aliquots and left to air overnight before each vial was plugged with cotton wool prior to storing at 4^{o}C.

2.2.3 Grape juice medium

Grape juice medium consisted of 500ml distilled water, 25g agar, 300ml grape juice, 50ml extra water and 21ml nipagin (100 g/L). Water and agar were brought to the boil, at which point the grape juice was added and the mixture was returned to the boil. The medium was then taken off the heat and allowed to cool to 60°C, at which point the extra water was added. The nipagin was stirred in when the medium had cooled to below 60°C and the medium was poured into large plastic Petri dishes to set. This volume was sufficient to fill around 18-20 large dishes.

2.2.4 Starvation medium

To test the response of flies to starvation, a 1% agar medium was prepared (Clancy *et al.* 2001; Broughton *et al.* 2005). This contained 10g agar and 1000ml of distilled water. No nipagin or propionic acid was added. 1% agar medium was chosen, as opposed to an empty vial, because it provides flies with a water source and therefore avoids the problem of desiccation. Due to the absence of antifungal agents, fresh starvation medium was prepared every week.

Ingredient	Supplier
Brewer's yeast	MP Biomedicals, Solon, OH (USA)
Sucrose	Tate and Lyle sugars, London, UK
Agar	Sigma, Dorset, UK
Nipagin	Clariant UK Ltd., Pontypridd, UK
Ethanol	Sigma, Dorset, UK
Propionic acid	Sigma, Dorset, UK
Cornmeal	B.T.P Drewitt, London, UK
Live yeast granules	DCL Yeast Ltd, Alloa, UK
Concentrated Grape juice	Solvino Ltd. UK
Baker's yeast	B.T.P Drewitt, London, UK
All nutrient add-back reagents (Table 4.1)	Sigma, Dorset, UK

Table 2.1: Supplier names for ingredients used to make different types of food media throughout this thesis.

2.3 Fly husbandry and culturing

2.3.1 Separating males and females

The sex of flies can be easily distinguished, whilst anaesthetised with CO_2, under a light microscope. Males are typically much smaller than females and possess tarsal sex combs on their front pair of legs, which are used during courting. Males also possess a much rounder abdomen that has a darker pigmentation on the posterior portion of the dorsal side (Greenspan 2004). Sex determination can be more difficult within the first few hours of emerging as adults, and the presence of sex combs in males is perhaps the most reliable marker of successfully sexing *Drosophila* (Greenspan 2004). Males and females were carefully sorted using a very fine paintbrush do avoid causing any damage to the flies.

2.3.2 Virgin collection

Female *Drosophila* will not mate within eight hours of emerging as adults at 25°C (Greenspan 2004). Therefore, to ensure only virgin flies were collected, all adult flies were removed from the bottles or vials and any emerging flies within a six hour window were collected as virgins. Female virgins were separated from males during this time frame using ice anaesthesia and a very fine paintbrush. CO_2 anaesthesia should be avoided where possible because flies are very sensitive to gas within the first few hours of emergence. Excess CO_2 exposure at a young age can cause bloated abdomens, resulting in flies dying within a few days. Virgin females were maintained in glass vials containing standard SY medium (refer to section 2.2.1) at a density of 20 females per vial for 48 hours, to ensure that no larvae appeared on the medium. The appearance of larvae would indicate that one or more of the females had mated and the vial of flies should be discarded. To further identify that flies had not mated, virgins can be distinguished under a light microscope by their pale complexion and a dark spot on their abdomen from their last feed as L3 larvae (Greenspan 2004). Freshly eclosed virgins also develop shrivelled wings, which expand out within a couple of hours.

2.3.3 Standard larval density

2.3.3.1 Preparing the larvae

Maintaining standard density in larvae is extremely important particularly when studying fitness-related traits such as longevity (Zwaan *et al.* 1991; Priest *et al.* 2002). Throughout this thesis, all experiments were performed with flies that had been cultured at standard density for two generations (parental and experimental). The most accurate method of standardising density is to pick 1st instar larvae into

culture vials (section 2.3.3.2); however, this can be extremely time consuming especially when experiments require large numbers of flies to be reared. An alternative method for achieving constant larval density was described by Clancy and Kennington and was used for almost all experiments described in this thesis (Clancy and Kennington 2001). Parental flies in population cages were allowed to lay eggs on Petri dishes containing grape juice medium (refer to section 2.2.3) supplemented with a globule of live yeast paste for a maximum of 22 hours, but ideally for a four to eight hour period during the day. The collection of eggs over a shorter time frame ensured that the majority of flies emerged at the same time, which is an important aspect to consider when collecting virgins.

After a sufficient number of eggs were laid, the yeast paste was removed from the grape medium and eggs were washed off with phosphate buffer saline (PBS) into a 50ml Falcon tube with the aid of a soft brush to carefully dislodge the eggs from the surface of the medium. Eggs were allowed to settle for a few minutes and the excess supernatant was poured away. If any yeast paste had dissolved in the PBS a series of washes would be performed until the solution was clear. Finally, 18µL of eggs were aspirated from the solution using a 200µL Gilson pipette with a cut pipette tip to allow the maximum number of eggs to be taken up. Eggs were squirted into 200ml bottles containing 70ml standard SY medium, resulting in a standard density of approximately 300-350 eggs per bottle.

2.3.3.2 Picking individual larvae

The standard larval density technique is ideal for culturing large numbers of flies, particularly wild type flies. However, from previous experience in the laboratory, some of the dwarf mutants, for example the insulin receptor substrate protein mutant

chico, do not have optimal viability when using this culturing technique. To increase viability of such mutants, 1st instar larvae (L1) can be individually picked one day after eggs have been laid on grape plates and transferred carefully onto fresh 1.0 SY media at a density of 40 larvae per vial until adults emerge.

2.4 Experimental procedures

2.4.1 Once-mated females

All experiments throughout this thesis (unless otherwise stated) were conducted on females that had been allowed to mate with males for a period of 48 hours after eclosion, ensuring that all females had mated at least once. This was achieved by transferring newly emerged flies to bottles containing fresh SY medium and allowing them to mate, before discarding the males. These flies will be referred to as once-mated flies from hereon.

2.4.2 Lifespan assays

Experimental flies were reared at standard density (Clancy and Kennington 2001); section 2.3.3) in glass bottles containing 70ml of the standard SY medium. Emerging flies were transferred without CO_2 anaesthesia into bottles containing fresh medium and were allowed to mate for a period of 48 hours. Females were separated from males under light CO_2 anaesthesia and placed into glass vials containing 4ml of food medium at a density of 10 females per vial. All lifespan experiments were performed with 100 flies per genotype or treatment, unless stated otherwise. Flies were transferred to fresh medium at least three times per week and the number of deaths was recorded on these days. Deaths were recorded when flies were motionless and

had their legs curled and their bodies shrivelled. A fly was censored from the experiment in the event it had escaped from a vial, had been accidentally damaged during the transfer to fresh media or was stuck to the food despite clearly moving.

2.4.3 Fecundity assays

Experimental flies were maintained in their vials for between 18-24 hours and then transferred into fresh vials. The number of eggs in the vacated vials were counted by hand under a light microscope and calculated as the number of eggs laid per female over a 24 hour period. Deaths and censors occurring throughout the duration of the experiments were taken into account when calculating the mean number of eggs laid per female during this time frame. Fecundity measurements were performed at regular intervals throughout life until egg-laying reached a minimum later in life. The data represents an index of lifetime fecundity, which is the sum of eggs laid by an average female during 24 hours on the days the assay was performed. Assays were carried out during the period of heaviest egg-laying, which provides an indication of relative lifetime fecundity (Chapman and Partridge 1996).

2.4.4 Feeding assays

Once-mated female flies were randomly assigned to different dietary treatments at a density of five flies per vial (10 vials per treatment) and maintained at 25°C throughout life. To ensure steady state observations of feeding , flies were transferred to fresh food the evening before the assay and vials were then coded by another lab member and placed in a randomised order in rows on viewing racks at 25°C overnight to avoid any bias (Wong *et al.* 2008). Observations were carried out the next day, starting one hour after lights-on for a period of 90 minutes. In turn, each

vial was observed for no longer than one second during which the number of flies feeding was noted. Feeding behaviour was scored when a fly had its proboscis extended and touching the food surface while performing a bobbing motion. Once all vials in the experiment had been scored in this way, a second round of observations was carried out in the same order as the first. Repeated observations were made every five minutes, allowing for 19-20 observations to be made for each vial during the 90 minute period. At the end of the assay, the vial labels were decoded and the data was presented as the proportion of flies feeding on a specific diet on a given day This was calculated as the sum of scored feeding events divided by total number of feeding opportunities, where

Total feeding opportunities = number of flies in vial x number of vials in the group.

2.4.4.1 Calibration of feeding behaviour

In order to determine whether the feeding observations correspond to actual food consumption, the feeding behaviour of flies on different diets had to be calibrated. This was achieved by transferring seven-day-old flies to their respective diets, containing 2.5% blue dye (FD&C Blue No.1 (Wong *et al.* 2008)) at a density of five flies per vial and 10 vials per treatment. Proboscis extension on blue-labelled food was examined over a 30 minute period, at which point the amount of dye accumulated in the fly reflects feeding rate alone. Following a longer time frame of feeding, blue dye is already excreted in the faeces (Wong *et al.* 2008). After 30 minutes, flies were immediately transferred without CO_2 to Eppendorf tubes and frozen in liquid nitrogen. Samples were then homogenised in 1ml of distilled water using a pestle before being filtered through a 0.22µm Millipore filter in order to remove any debris and lipids. Absorbance of the liquid sample was measured using a

Hitachi U-2001 spectrophotometer (Lambda Advanced Technology Ltd., UK; 629nm). Readings for flies in all 10 vials on each treatment were taken and the amount of food ingested was calculated from a standard curve. Background absorbance was corrected for by measuring absorbance of extracts of control flies not exposed to blue-labelled food.

2.4.5 Removal of *Wolbachia* from wild-type populations

In chapters 3 and 5, the effects of the intracellular bacteria *Wolbachia* in response to different food treatments are examined. *Wolbachia* infection was removed by treating wild-type populations for three generations with food medium containing 25µg/ml of the antibiotic tetracycline. Experimental flies were obtained more than 10 generations after tetracycline treatment to allow sufficient time to recover from the treatment.

2.4.6 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is an invaluable molecular technique in molecular biology which allows to the amplification of specific regions of DNA. PCR was used to detect the presence or absence of *Wolbachia* in *Drosophila* populations by using primers to detect and amplify the gene for *Wolbachia* surface protein (wsp) in *Drosophila* (Braig et al. 1998; Zhou et al. 1998; Toivonen et al. 2007).

2.4.6.1 DNA extraction

Genomic DNA was isolated from single flies according to a standard protocol (Gloor and Engels 1992). Flies were anaesthetised under light CO_2 and placed individually into 0.5ml Eppendorf tubes. 50µl of "squishing buffer" (10mM Tris-HCL (pH 8.3),

1mM EDTA, 25mM NACL, 200µg/ml proteinase K) was drawn up into a pipette tip and the fly was squashed thoroughly, without expelling the liquid. After squashing the fly, the squishing buffer was expelled from the pipette tip. The resulting mixture was incubated in a 37°C water bath for 30 minutes, followed by an incubation of 90 seconds at 95°C to inactivate the proteinase K. The extracted DNA was stored at -20°C until used in a PCR reaction.

2.4.6.2 PCR reaction

The PCR reaction mixture consisted of forward and reverse primers, Milli Q water (Millipore^{TM}), DNA and a PCR mastermix (2.5x, Eppendorf®). The Eppendorf® mastermix contains Taq DNA polymerase (62.5 U/ml), 125mM KCL, 75mM Tris-HCL (pH 8.3), 3.75mM $Mg(OAc)_2$, 0.25% lgepal® –CA630, 500µM of each dNTP and stabilisers. The primers used to amplify the wsp gene were wsp81F (5'TGG TCC AAT AAG TGA TGA AGA AAC) and wsp691R ((5' AAA AAT TAA ACG CTA CTC CA) as previously described (Braig *et al.* 1998; Zhou *et al.* 1998). Wsp primers were a kind gift from Greg Hurst.

A single PCR reaction contained 1µL forward primer (20µM), 1µL reverse primer (20µM), 11µL MilliQ water, 10µL Eppendorf® mastermix and 1µL DNA extract. When multiple reactions were performed, a large volume of PCR stock including all components but the different DNA extracts was prepared. Subsequently 24µL of the PCR mix were added to 1 µL of the respective DNA extracts. In all PCR experiments a negative control lacking any DNA was included. The PCR tubes were gently vortexed and centrifuged briefly before being placed into the PCR machine (Gene Amp PCR System 2700, Applied Biosystems).

The following cycling conditions were used for the PCR reaction:

Initial melting step:	95°C for 15 minutes	(1 cycle)
Melting step:	95°C for 30 seconds	(30 cycles)
Annealing step:	55°C for 30 seconds	
Elongation step*:*	72°C for 2 minutes	
Final elongation step:	72°C for 7 minutes	(1 cycle)

Completed PCR reactions were stored at -20°C.

2.4.6.3 Agarose gel electrophoresis

A 1% agarose gel was prepared by dissolving 1g agarose in 99ml of 1x TAE buffer and heating the mixture. Once the agarose (Sigma, UK) had fully dissolved and the solution had cooled slightly, 1µL of ethidium bromide (Sigma, UK) was added. The gel was poured into a prepared gel tank and an appropriate comb was inserted. Bubbles on the surface of the gel were removed using a pipette tip. The gel was allowed to set for between 30 minutes or one hour depending on the size.

Once the gel had set, the comb was removed and the gel was covered in 1x TAE buffer. 3µL of O'GeneRuler™ 100bp DNA ladder (Fermantas) were used as a molecular weight marker. The PCR samples, loading dye (6x loading dye: 60% glycerol (w/v), 0.05% bromophenol blue (w/v)) and ladders were kept on ice during this time. 2µL aliquots of loading dye were mixed with 10µL of each PCR sample. 10µL of the mixture were loaded onto the gel. Electrophoresis was carried out at 80-

100V for approximately 40 minutes, depending on the size of the gel. Following electrophoresis, DNA bands were visualised using a UV transilluminator (Syngene) and the Gene Snap imaging program. Images were printed using a Sony Digital Graphic Printer (UP-D895).

2.5 Statistical analysis

2.5.1 Median lifespan

The median lifespan refers to the age of the cohort when the population has reached 50% of its original size. Gerontologists tend to use median lifespan as opposed to mean because it is less sensitive to highly skewed distributions.

2.5.2 Maximum lifespan

The term maximum lifespan refers to the 95^{th} percentile of the surviving population. This definition of maximum lifespan is used as opposed to the longest lived individual because the latter is highly dependent on the sample size (Masoro 2005). Furthermore, the longest-lived individual could be an anomalous result that does not reflect the longevity of the population as a whole.

2.5.3 Survivorship analysis

Survivorship (L_x) refers to the probability at birth of an individual surviving to a given age (x). L_x can be simply calculated by dividing the number of individuals alive at time x (N_x) by the number of individuals that were alive at the start of the experiment (N_o). However, when individuals are censored from a population, L_x should be calculated using the following formula:

$$L_x = \prod_{o}^{x} p_x$$

where p_x is the probability of surviving from age tx-1 to age tx (=1 – qx)

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

Statistical differences in survivorship between two groups were analysed using the non-parametric Log-rank test (Mantel-Cox test) (Mantel 1966; Peto and Peto 1972).

2.5.4 Fecundity analysis

The non-parametric Wilcoxon test was used to analyse differences in egg-laying between genotypes or treatments. This non-parametric test was used because egg-laying data is not normally distributed.

2.5.5 Feeding analysis

Feeding behaviour was analysed using a generalised linear model with quasibinomial error distribution to compare the range of values for the proportion of flies feeding in each vial during the assay on a given day. Differences in the uptake of food (blue dye) were analysed using the non-parametric Wilcoxon test.

2.5.6 Statistics software

All statistical analyses on survivorship and fecundity data were performed using JMP 5.0 JMP (version 5.1) software (SAS institute, Cary, NC). Feeding data were analysed using R, v2.2.1 (Gentleman and Ihaka 2005).

Chapter 3

Factors affecting the responses to dietary restriction in *Drosophila*

3.1 Introduction

Lifespan extension by some form of food restriction has been known about for over 70 years; however the mechanisms which regulate this process in any organism remain unclear. During the last 10 years, research has focused in particular on using model organisms such as yeast, worms, flies and mice to try and understand the genetic and molecular mechanisms leading to lifespan extension by dietary restriction (DR). The IIS pathway, TOR pathway, SIR2, PHA4 and AMPK are all candidates that have been reported to mediate lifespan extension by DR (section 1.5). However, several reports have shown conflicting evidence, even within the same species.

One of the major problems when determining whether DR acts through evolutionary conserved pathways is the variability in how DR is defined and applied. For example, in rodents, DR is typically achieved through either a reduction in the quantity of chow diet fed to the calorically-restricted cohort (usually between 60-70% of the *ad libitum* cohort) (Merry and Holehan 1985; Yu *et al.* 1985; Weindruch and Walford 1988; Masternak *et al.* 2005; Bonkowski *et al.* 2006) or through an intermittent feeding regimen, usually implemented through every other day feeding (EOD) of the calorically-restricted group (Goodrick *et al.* 1982; Anson *et al.* 2005). Both methods have been shown to extend the medium and maximum lifespan of rodents (Piper and Bartke 2008).

In the nematode worm *C. elegans*, methods for applying DR are more diverse. There are thought to be up to 12 different methods of applying DR, adopted by various laboratories (W. Mair personal communication). DR in *C. elegans* is typically

achieved through serial dilution of a bacterial source, usually *E.coli* (Klass 1977; Houthoofd *et al.* 2003). However, other methods that extend lifespan include; complete removal of bacterial food (Kaeberlein *et al.* 2006b; Lee *et al.* 2006), altering the strain of bacteria in the worm diet (Garsin *et al.* 2001; Garsin *et al.* 2003), using a synthetic axenic medium (Vanfleteren *et al.* 1998; Vanfleteren and Braeckman 1999; Walker *et al.* 2005), or a genetic mimetic of DR induced by a mutation in the eat-2 gene, which causes a defect in pharyngeal pumping (Lakowski and Hekimi 1998). In worms, strong evidence suggests that DR extends lifespan independently of the IIS pathway, because *daf-2* mutants fed a DR regimen showed a further extension of lifespan than mutants on a control diet (Lakowski and Hekimi 1998; Houthoofd *et al.* 2003; Hansen *et al.* 2005b). Additionally, DR has been shown to extend lifespan independently of the FOXO transcription factor DAF-16 (Houthoofd et al. 2003); however, DAF-16 was required for lifespan extension when DR was induced using a slightly different bacterial dilution protocol (Greer *et al.* 2007). The conflicting results of Greer *et al.* point to the need for a standardised protocol when using the same model organism for dietary restriction studies.

Drosophila DR is typically implemented by reducing all the ingredients present in a gelled medium containing predominantly sucrose and yeast, occasionally supplemented with cornmeal (Kapahi *et al.* 2004a), with the food always in excess. Dilution of these nutrients to a DR level causes an increase in median and maximum lifespan coupled with a reduction in daily and lifetime reproduction in females (Chapman and Partridge 1996). However, it has been reported that lifespan extension by DR can be achieved almost entirely by reducing only the yeast component of the diet to a DR level (Mair *et al.* 2005).

Although, lifespan extension under DR is thought to occur as a result of reduced nutrition, it is equally possible that the explanation could simply be through a relief from toxicity (Longo and Finch 2003; Prentice 2005; Piper and Partridge 2007). Elements of the food could be toxic and hence diluting the food could extend lifespan by diluting potential toxins. Determining whether DR extends lifespan through reduced toxicity or due to reduced nutrition is difficult; however looking at parallel effects of the diet such as reproductive output can give an indication of health and nutritional status (Piper and Partridge 2007). Increasing nutrition has been shown to have a positive effect on both mating rates and egg production (Chippindale *et al.* 1993; Chapman and Partridge 1996). Hence, a reduction in lifespan as a result of increased nutrition should be coupled with an increase in both daily and lifetime fecundity as observed in rodents (Holehan and Merry 1986; Weindruch and Walford 1988), *Drosophila* (Chippindale *et al.* 1993; Chapman and Partridge 1996; Libert *et al.* 2007) and *C. elegans* (Klass 1977; Bishop and Guarente 2007b). If higher food concentrations contain a higher concentration of toxins, it is likely that lifespan would decrease in conjunction with no increase in fecundity or even reduced fecundity. It is therefore essential when performing DR experiments with new ingredients and protocols to fully establish whether fully-fed diets cause an increase in fecundity as well as a decrease in lifespan relative to DR diets.

Different fly ageing laboratories use different sources of yeast and different concentrations of sucrose, yeast and agar (Chippindale *et al.* 1993; Chapman and Partridge 1996; Kapahi *et al.* 2004a; Bross *et al.* 2005), hence it is difficult to determine whether all laboratories are studying the same DR effects, particularly when trying to determine the mechanisms involved. Not all laboratories have tested reproductive output as a measure of nutrition in response to their food, thus it

remains unclear whether different diets may reduce lifespan through toxicity. Furthermore, it is essential to perform lifespan and fecundity experiments over a range of food concentrations, using the chosen fly diet, in order to determine the food concentration that gives rise to the DR and fully-fed conditions respectively (Clancy *et al.* 2002; Gems *et al.* 2002). Graphical representation of the lifespan response to changing food levels results in a tent shape (Chapman and Partridge 1996; Magwere *et al.* 2004). The potential problem with choosing only two food concentrations (Rogina *et al.* 2002; Rogina and Helfand 2004) for DR and fully-fed conditions is that these may represent two points at opposite sides of the tent, and hence not accurately reflect lifespan changes in response to variation in the diet (Figure 1.12).

These differences in DR methods and the proposed mechanisms mediating DR even within one model organism highlight the absolute requirement for standardised DR protocols to be established and applied. As part of a larger study of optimising dietary restriction and lifespan protocols in *Drosophila* (Bass *et al.* 2007a), this chapter sets out to investigate different factors affecting the responses to DR in *Drosophila*. These include testing whether intermittent feeding can be utilised as a method of DR in *Drosophila*, as previously shown in rodents (Goodrick *et al.* 1982; Anson *et al.* 2005), and whether variation in genetic background and culturing conditions cause commonly-used laboratory wild-type populations to respond differently to changing nutrition levels. Finally, the role of the intracellular cytoplasmic bacterium *Wolbachia*, which is thought to have infected anywhere between 25-70% of insects (Kozek and Rao 2007), will be investigated to determine if *Wolbachia* infection partially mediates the phenotypic effects observed during DR. Although the relationship between *Wolbachia* and *Drosophila* is thought to be

symbiotic, some strains of *Wolbachia* have been reported to effect fitness-related traits including lifespan (Min and Benzer 1997; Fry and Rand 2002).

3.2 Methods

3.2.1 Testing a range of food concentrations

For the wild-type comparison and *Wolbachia* / tetracycline treatment experiments, the response of flies to a range of food concentrations was examined. Five food concentrations (0.1 – 2.0 SY) were tested whereby only the concentration of yeast was varied, whilst the sucrose and agar concentrations were kept constant (Table 3.1). The concentrations of sucrose and agar, in addition the source of yeast (Brewer's yeast), had previously been optimised for lifespan extension by DR and high fecundity with full feeding (Bass *et al.* 2007a). Food media were prepared as outlined in section 2.2.1. Flies were transferred to fresh medium every two days and the number of deaths was scored on these days.

Treatment (SY)	Agar (g)	Sucrose (g)	Yeast (g)
0.1	15	50	**10**
0.5	15	50	**50**
1.0	15	50	**100**
1.5	15	50	**150**
2.0	15	50	**200**

Table 3.1: Food recipes used for experiments involving a range of food concentrations. Only the concentration of dietary yeast was manipulated. All quantities represent the number of grams added to distilled water to produce a final volume of one litre of food medium. 30ml nipagin and 3ml propionic acid were added once the food had cooled to 60^{o}C. Media were prepared as described in section 2.2.1.

3.2.2 Vial orientation experiment

Once-mated males and females were separated under light CO_2 anaesthesia and allocated into vials containing 4ml of 1.0 SY medium (Table 3.1). Vials were

orientated either horizontally or vertically in plastic trays throughout the duration of the lifespan. Deaths and censors were recorded at least every two days.

3.2.3 Testing the effect of providing flies with a separate water supply

One possible explanation for the detrimental effects on lifespan of high food concentrations could be due to water availability, because the food is the only source of water for flies. The effect of providing a separate water supply in the vials was tested to establish whether the addition of water can overcome the adverse effects of high nutrition. A solution of 1% agar was made up with the addition of 30ml nipagin and 3ml propionic acid (preservatives). The agar solution was poured into 200µl pipette tips and allowed to set. The pipette tips were trimmed to a length that brought the agar close to the surface of the food once the tips had been inserted into the food. Pipette tips with no agar were placed in the food as a control. To avoid trapping of flies, the opening of the tip was filled with cotton wool. Tips were replaced with fresh ones every two days when flies were transferred to fresh medium.

3.2.4 Testing Intermittent feeding as a method of DR in *Drosophila*

Experiments were performed in one litre population cages that had been modified to have two horizontal openings for vials to be securely placed. One vial contained fresh water, plugged with cotton wool, used as a water supply. The second vial contained 2.0 SY medium that had been dispensed horizontally into the vials to enable flies to have easier access to the food medium. Since the aim was to investigate intermittent feeding as a possible method of applying DR without an

interaction with a known DR protocol, 2.0 SY medium was chosen as opposed to 1.0 SY because the latter represented our standard DR treatment.

Dahomey wild-type flies were cultured at standard density (see section 2.3.3) for parental and experimental generations. Females from the experimental generation were separated under light CO_2 anaesthesia and were randomly allocated to population cages for three different dietary regimens (see section 3.2.4.1) at a density of 100 flies per cage (n = 500 per treatment). The experiment was set-up in the afternoon and flies on all treatments were provided with food for the first 18 hours of the experiment, until the following morning when the intermittent feeding treatments commenced (see section 3.2.4.1). Flies in all cages had constant access to a water supply.

3.2.4.1 Experimental treatments:

The following treatments were used during this experiment.

1) **No starvation / continuous access to food**: - Flies had constant access to food throughout life with no periods of starvation
2) **Three hours starvation**: - Flies had access to the food for a period of 21 hours per day, with a starvation period lasting three hours.
3) **Six hours starvation**: - Flies had access to the food for a period of 18 hours per day, with a starvation period lasting six hours.

The flies on the non-starvation treatment were provided with fresh medium at the same time each morning. At this time, the food vials in the cages containing flies on both the three and six hour starvation regimen were replaced with an empty vial containing no food, the start of the starvation period. The empty vials were replaced

with vials containing fresh food medium after three and six hours respectively. This procedure was applied seven days a week throughout life. Dead flies were counted daily and carefully removed from their cages.

3.2.5 Comparing the response of different wild-type laboratory strains to DR

The response to DR of six different *Drosophila* wild-type strains commonly used in laboratories was assessed. These included: Dahomey, white Dahomey (wDahomey), Oregon-R, Canton-S, W1118 and yellow-white (yw). Dahomey and wDahomey stocks were maintained in population cages at 25^{o}C with overlapping generations and fed the standard 1.0 SY laboratory diet (section 2.2.1). wDahomey stocks were produced by backcrossing the white gene from W1118 flies into a Dahomey background. Oregon-R, W1118, Canton-S and yw flies have been maintained in the laboratory for several years under a range of different culturing conditions. More recently, these fly stocks have been maintained at 18^{o}C in several glass bottles or vials containing 1.0 SY medium or a cornmeal-based diet (ASG, section 2.2.2) and transferred to fresh medium every generation.

For all wild-type strains with the exception of yw, the responses of lifespan and fecundity in once-mated females were assessed on 0.1, 0.5, 1.0, 1.5 and 2.0 SY diets (see Table 3.1). Due to difficulties in culturing yw stocks, resulting in limited numbers of female adults, experiments were only performed on 0.5, 1.0 and 2.0 SY diets with this strain.

3.2.6 Removal of *Wolbachia* infection

Wolbachia was removed from the infected populations through tetracycline treatment, as described in section 2.4.5. All strains were allowed to recover for at least 10 generation on standard food (without tetracycline) before lifespan experiments commenced. Removal of *Wolbachia* was verified by PCR using primers to detect the gene for *Wolbachia* surface protein (wsp) (Braig *et al.* 1998; Zhou *et al.* 1998; Toivonen *et al.* 2007) (section 2.4.6).

3.3 Results

3.3.1 Effects of vial orientation on female and male *Drosophila*

Based on previous observations, it was clear that the texture of the fly food becomes stickier as flies get older. Subsequently, flies begin to stick to the food. This results in flies having to be censored from the experiment despite being alive or not having died of natural causes. The severity of this problem appeared to be greater in males and some of the known long-lived insulin signalling pathway mutants, such as *chico* homozygotes, that are both small and either infertile or less fecund. When flies begin to age they tend to reduce their activity and spend large proportions of time standing on the food, hence later in life flies maintained in vertical-orientated vials will always be exposed to the food surface and often stick to it. To try and overcome this problem, it was chosen to investigate whether males and females that were maintained in vials with a horizontal orientation would be less prone to sticking to the food later in life than flies in vials orientated vertically. By orientating the vials horizontally, flies will be able to stand on the side of the glass and can access the food by walking to it. However, the possible beneficial effects of reducing the number of flies sticking to the food when orientating the vials horizontally may be cancelled out by potential adverse effect on lifespan or fecundity due to difficulty in accessing the food later in life.

In agreement with a previous study (Magwere *et al.* 2004), female *Drosophila* were significantly longer lived than males, independent of vial orientation (Figure 3.1a). Females maintained in vials orientated horizontally (median, 64 days) showed no significant differences in lifespan compared with females maintained in vertically-orientated vials throughout life (median, 64 days) (Figure 3.1a). In addition, both

daily and lifetime fecundity of females remained unaffected by horizontal vial orientation (Figures 3.1b and c). In contrast, males maintained in horizontally-orientated vials exhibited a longer lifespan (median, 53 days) than males maintained in vertically orientated vials (median, 46 days; Figure 3.1a). Furthermore, the vial orientation had a large effect on the number of males that had to be censored as a result of sticking to the food during this experiment. Almost half of the initial population of males (46) were censored in the vertically-orientated vials, whereas considerably fewer (29) had to be censored in the horizontally-orientated vials. Hence, orientating vials horizontally resulted not only in an extended lifespan compared to males maintained in vertically-orientated vials but also, as a result of fewer censors, produced considerably smoother survival curves (Figure 3.1a).

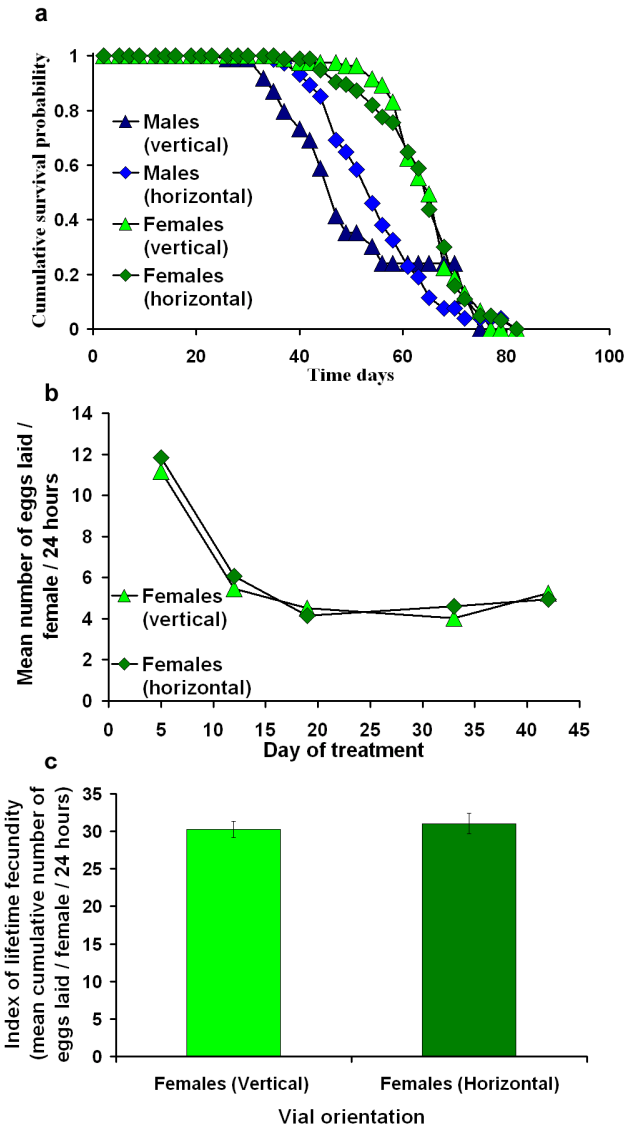

Figure 3.1: The effects of vial orientation on fecundity and lifespan of *Drosophila.* (a) Maintaining vials on their side (horizontal) throughout the course of the experiment resulted in a significant increase in male lifespan (blue) compared with males maintained in vertically-orientated vials (P = 0.006, log-rank). Lifespan of females (green) was unaffected by vial orientation (P = 0.69, log-rank). (b) No clear differences were observed between the average numbers of eggs laid on any given day by females maintained in horizontal compared with vertical vials. (c) Furthermore, no significant differences were observed in cumulative fecundity throughout life as a result of vial orientation (P = 0.76, Wilcoxon). Fecundity assays were performed on days: 5, 12, 19, 33 and 43 of treatment. Error bars represent ± s.e.m.

3.3.2 An optimised dietary restriction protocol for *Drosophila* (Bass *et al.* 2007a)

The experiments conducted throughout this thesis were performed using a dietary restriction protocol optimised for *Drosophila* in our laboratory (Bass *et al.* 2007a). The experiments in this study were designed and analysed by Richard Grandison, Tim Bass, Matthew Piper, Richard Wong and Linda Partridge. The majority of experiments were performed by Tim Bass, with the exception of the experiment investigating the effect of water on the response to dietary restriction, which was performed by Richard Grandison and has been included in this thesis (Figure 3.2). The findings and implications of the paper by Bass *et al.* are briefly summarised below.

The study by Bass *et al.* firstly investigated the effect of a range of sucrose concentrations on lifespan and fecundity of Dahomey flies. Although it has previously been reported that reducing the yeast component of the diet extends lifespan to a greater magnitude than reducing the concentration of sucrose in an otherwise iso-caloric diet (Mair *et al.* 2005), it is possible that higher sucrose concentrations (than those previously used by Mair *et al.*) may reduce lifespan and affect fecundity. Keeping the concentration of yeast (Baker's yeast) fixed at 150 g/L, Bass *et al*. demonstrated that increasing the concentration of sucrose beyond 50 g/L had a detrimental effect on both daily and lifetime fecundity. Interestingly, *Drosophila* had no dietary requirement for sucrose in terms of fecundity; however, the presence of sucrose was essential for healthy lifespan.

It has previously been suggested that DR might extend lifespan simply by a reduction of toxicity as opposed to a reduced nutrition (Longo and Finch 2003; Prentice 2005).

It is possible that the reduced lifespan observed when increasing the concentration of dietary yeast could be caused by reduced availability of the nutrients in the food (increased food density) or as a result of reduced water availability. To test this hypothesis, Bass *et al.* investigated the effect of increasing the agar concentration, whilst keeping sucrose and yeast concentrations fixed at 50 g/L and 200 g/L (2.0 SY; Brewer's yeast) respectively. This work demonstrated that increasing the agar concentration mimics to some extent the effect of reducing the concentration of dietary yeast (DR). Generally, increasing the concentration of agar extended median lifespan, but reduced fecundity. These data are consistent with agar controlling food availability in a non-detrimental way and therefore the reduction in lifespan with full feeding cannot be explained by reduced availability of nutrients on high food.

Finally, Bass *et al*. studied the effects of dietary restriction using four commonly-used yeast sources (obtained from different yeast suppliers). The source of yeast and the variability in yeast quality is likely to be an important consideration for DR experiments because dietary yeast has been shown to be the key component of the diet influencing *Drosophila* lifespan (Mair et al. 2005). Keeping the sucrose fixed at 50 g/L, Bass *et al.* demonstrated that of the four yeast diets tested, only one yeast source (Brewer's yeast) was suitable to use for DR experiments. Flies fed a Brewer's yeast diet exhibited elevated fecundity with each increase of yeast concentration tested. Furthermore, median lifespan peaked at an intermediate food concentration (1.0 SY) and was reduced at higher concentrations (coupled with increased fecundity) and lower concentrations (presumably through malnutrition). In contrast, the Baker's yeast and Torula yeast appeared optimal in terms of lifespan, characterised by the tent-shaped graphs; however fecundity did not increase beyond a plateau reached at 1.5 SY, despite lifespan showing a further decline beyond this

point. Yeast extract appeared to be the least optimal diet for DR studies due to the detrimental effects of both lifespan and fecundity at higher food concentrations, indicating dose-dependent toxicity.

As a result of this study (Bass *et al.* 2007a), Brewer's yeast was chosen as the yeast source to be used for all experiments throughout this thesis. Furthermore, having ascertained the responses of female *Drosophila* to a range of yeast concentrations using Brewer's yeast (Bass *et al.* 2007a), 100 g/L (1.0 SY) yeast will be used as the DR condition and 200 g/L (2.0 SY) will be used for the fully-fed condition for all experiments (refer to Table 3.1). These concentrations were chosen because median lifespan showed the greatest peak on this yeast at 1.0 SY, coupled with reduced fecundity, whereas 2.0 SY caused maximised fecundity in conjunction with reduced lifespan. The agar and sucrose concentrations for 1.0 SY and 2.0 SY were fixed at 15 g/L and 50 g/L respectively.

3.3.3 Reduced water availability does not explain life-shortening effects associated with increased nutrition

The sugar yeast medium not only provides a food source to flies, but is also the only source of available water. Therefore, a potential explanation for the life-shortening effects associated with more concentrated yeast diets could be as a result of reduced water availability, potentially due to the more dense texture of these diets. To rule out the possibility that the fully-fed condition (2.0 SY, Brewer's yeast) shortens lifespan due to reduced water availability, a separate water supply in form of an agar-filled pipette tip was provided (section 3.2.3). The addition of a separate water supply to the food did not affect the response of flies to DR (Figure 3.2). In the

presence or absence of a separate water supply, flies exhibited increased fecundity coupled with reduced lifespan on a high yeast diet (2.0 SY, fully-fed) compared with the lower fecundity but extended lifespan of flies fed a DR diet (1.0 SY). Furthermore, no significant effect on lifespan or fecundity was detected in either DR or fully-fed flies in the presence of a water supply. This result indicates that accessibility to water cannot explain the life-shortening effects associated with higher levels of nutrition.

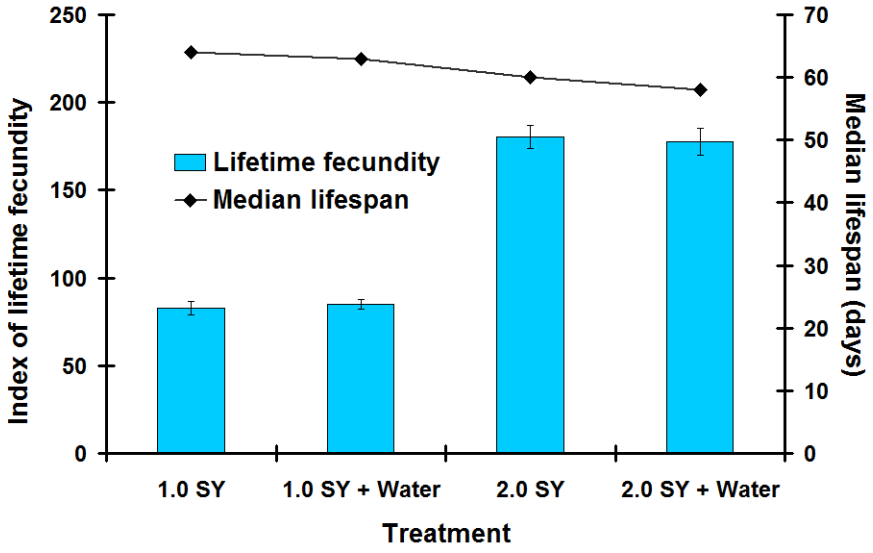

Figure 3.2: The effect of water addition on the response of females to dietary restriction using Brewer's yeast. Free access to water was provided by inserting a pipette tip containing 1% agar into the food medium. Flies exhibited increased fecundity and reduced lifespan on a fully-fed diet (2.0 SY) both in the presence or absence of a separate water supply. The addition of water had no effect on fecundity at an intermediate (1.0 SY; $P = 0.7$, Wilcoxon) or high concentration of yeast (2.0 SY; $P = 0.65$). Median lifespan was also unaffected by the presence of water on both yeast diets. Fecundity assays were performed on days: 3, 6, 10, 13, 17, 26, 31 and 38 of treatment. Error bars represent ± s.e.m. Adapted from Bass *et al*. 2007a, experimental work performed by R. C. Grandison.

3.3.4 Testing intermittent feeding as a method of applying DR in *Drosophila*

In rodents, intermittent feeding is used as a DR method, resulting in an extension of lifespan (Goodrick *et al.* 1982; Anson *et al.* 2005). In order to assess whether it can also be used as a suitable method to extend lifespan in *Drosophila*, the effect of restricted access of females to food for three or six hours per day throughout life was examined[12]. Interestingly, reducing the length of time flies had access to food throughout life had no effect on lifespan in the intermittent feeding regimens tested (Figure 3.3). Flies with continuous access to food throughout life (0 hours starvation; median, 51 days) had almost identical lifespans compared with flies that had no access to food for either a three hour (median, 51 days) or a six hour period per day (median, 51 days), throughout the duration of their lives.

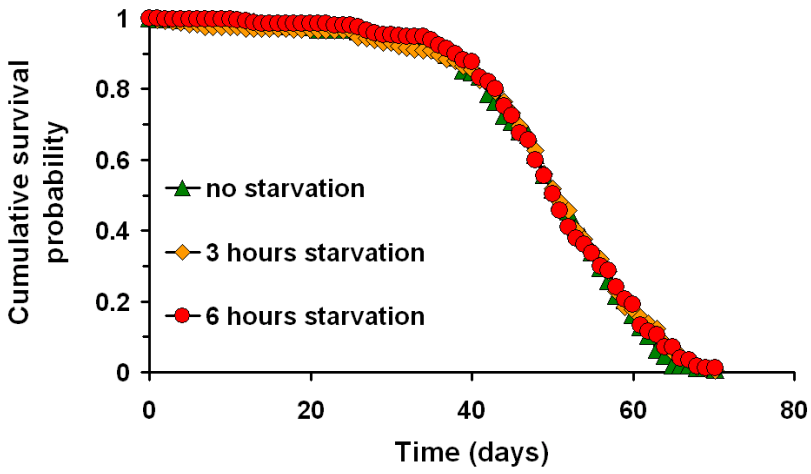

Figure 3.3: An intermittent feeding regimen that does not extend lifespan in *Drosophila*. Female flies were given access to either a continuous food supply (no starvation), or a continuous food supply for 21 or 18 hours each day (3 or 6 hour starvation) daily. Applying either 3 or 6 hour periods of starvation each day throughout life had no significant effect on lifespan compared with the continuously fed control group ($P \geq 0.38$, log rank). Figure adapted from (Grandison *et al.* 2009).

[12] Experimental work investigating intermittent feeding was equally contributed to by R. C. Grandison, M. W. Piper, R. Wong and T. Bass

3.3.5 Comparison of the DR responses between different wild-type laboratory strains of *Drosophila*

Previous experiments in this chapter have been performed using the outbred laboratory strain Dahomey. This strain has been maintained on an SY diet for over 30 years in large population cages with overlapping generations; a culturing method which ensures that adult survival rates are comparable to those of stocks that had been freshly collected from the wild (Sgro and Partridge 2000; Sgro and Partridge 2001). The majority of other wild-type laboratory strains are housed in small numbers in individual containers and fed a cornmeal-based diet. Maintaining stocks in this way can select for early reproduction and reduced adult lifespan (Luckinbill *et al.* 1984; Rose 1984; Sgro and Partridge 1999). As a result, it was of interest to test whether different wild-type laboratory strains would respond to the optimised Brewer's yeast diet. If different responses are detected between some of the strains, this may provide further insight into potential mechanisms involved in DR and highlight whether DR studies in different strains can be compared[13].

By examining the response of lifespan and fecundity to a range of food concentrations, it was evident that all wild-type strains exhibited a classical DR response, whereby lifespan peaked at an intermediate food concentration and decreased at very low and higher food concentrations (Figure 3.4a). Due to the limited number of adult females available for the yw strain, resulting in only three concentrations being tested, the response was less clear, although lifespan was extended at 0.5 SY and significantly reduced at 1.0 SY. Furthermore, all strains displayed a progressive increase in fecundity with each increase in food

[13] Experimental work on the effect of dietary restriction on a range of wild-type strains was equally contributed to by R. C. Grandison, R. Wong and M. D. Piper. PCR analysis was performed by R. C. Grandison

concentration, with the exception of Oregon-R. Fecundity increased in the Oregon-R strain when the food concentration was increased from 0.1 to 1.5 SY; however, fecundity showed a small decline at the highest food concentration (2.0 SY), despite lifespan being decreased at this concentration. Hence the associated lifespan decrease from 1.5 to 2.0 SY was not accompanied by increased intake of biologically valuable nutrition and therefore could be due to a non-specific detrimental effect of high food affecting this strain.

Despite all strains generally showing a classical DR response, the exact nature of these responses varied amongst strains (Figure 3.4a). The median lifespan peaks differed between the various wild-type strains. Dahomey (73 days), wDahomey (73 days) and yw (69 days) all peaked at 0.5 SY whereas Canton-S (57 days) and W1118 (57 days) peaked at a slightly higher food concentration (1.0 SY). Oregon-R appeared less sensitive to intermediate levels of nutrition because they exhibited the same median lifespan at 0.5 and 1.0 SY (53 days). Dahomey and wDahomey showed similar peaks in fecundity (Dahomey compared with wDahomey, $P = 0.97$, Wilcoxon) and displayed the highest egg-laying capacity of all strains, peaking at 2.0 SY (Figure 3.4a; $P \leq 0.003$, Wilcoxon, relative to the peak fecundity of any other strain). In contrast, Oregon-R and yw appeared to exhibit the lowest egg production overall.

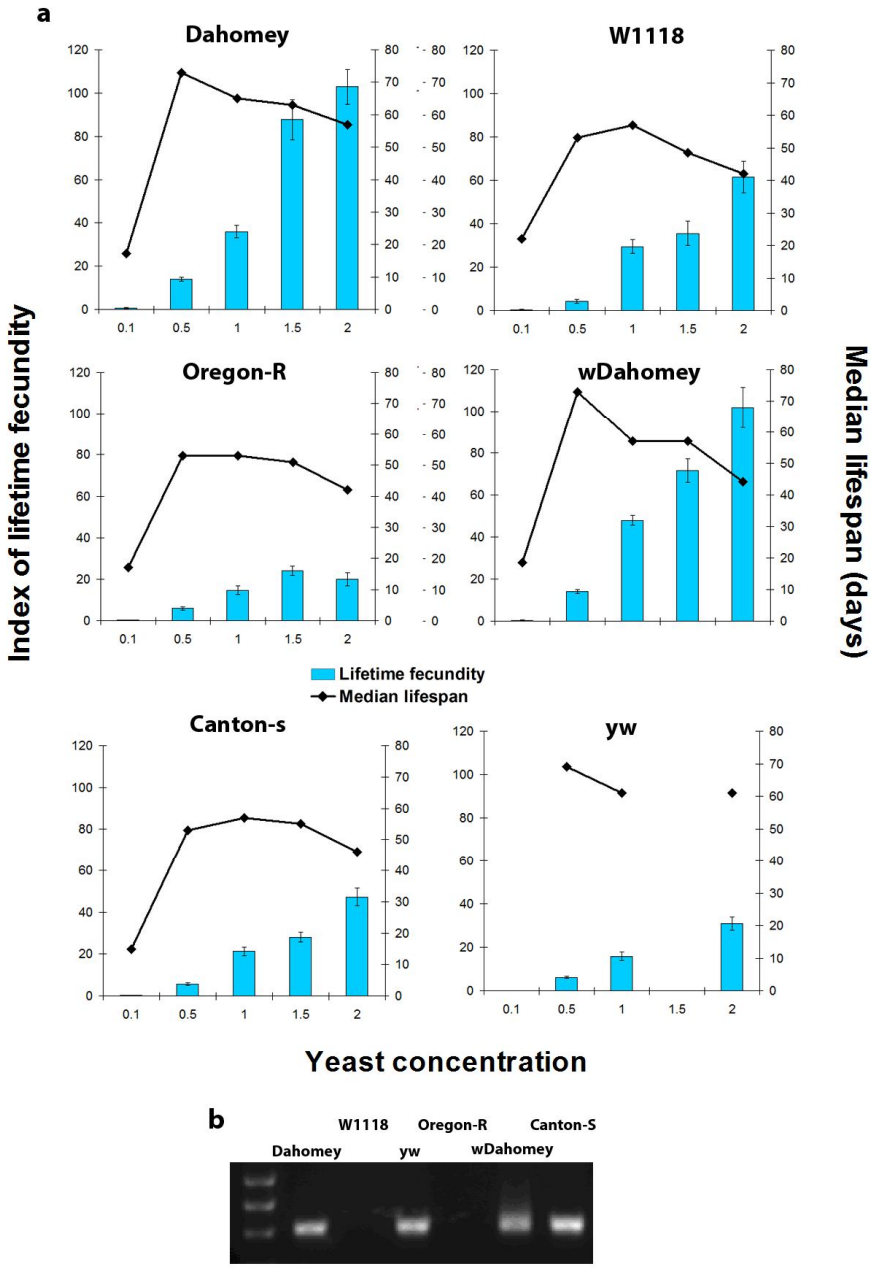

Figure 3.4: Comparing the responses of different wild-type laboratory strains to DR. (a) The responses of lifespan and fecundity in five different wild-type strains to a range of yeast concentrations. All strains generally exhibited a classical response to changing nutrition levels: fecundity increased at higher food concentrations, whereas lifespan peaked at an intermediate food concentration and was reduced at high and very low food concentrations. (b) PCR analysis revealing the presence or absence of the intracellular bacterium *Wolbachia*. Dahomey, wDahomey, yw and Canton-s strains were all found to be infected with *Wolbachia* whereas W1118 and Oregon-R were not infected. Fecundity assays were performed on days 4, 11, 18, 33 and 45 of treatment. Error bars represent ± s.e.m. Figure adapted from (Grandison *et al.* 2009).

3.3.6 Testing the effects of tetracycline treatment on DR responses

Drosophila play host to a range of bacteria and microbes. One of the most well known is the intracellular cytoplasmic bacterium *Wolbachia,* which is thought to have infected around 30% of the strains in the Bloomington Stock centre (Clark *et al.* 2005). The interaction between *Wolbachia* and host species is thought to be symbiotic; however, a virulent strain of *Wolbachia* has been shown to reduce *Drosophila* lifespan (Min and Benzer 1997) whereas another strain of *Wolbachia* caused positive and negative effects on lifespan depending on the genetic background of the flies used (Fry and Rand 2002). More recently, it was shown that extensive backcrossing and removal of *Wolbachia* by tetracycline treatment entirely abolished the lifespan extension phenotype of an *Indy* mutant stock (Toivonen *et al.* 2007). Moreover, interactions between *Wolbachia* and viability of the c*hico*^{2} mutant stocks have been uncovered, whereby the viability of c*hico*^{2} was reduced upon *Wolbachia* removal (Clark *et al.* 2005). However, the effect on viability of the c*hico*^{2} stock was later found to be linked to another, unmapped locus rather than *chico* itself. Based on these observations, it was important to determine whether *Wolbachia* or other bacterial infections may be partially mediating the effects of DR in *Drosophila*[14].

Firstly, PCR analysis with primers encoding stretches of *Wolbachia* surface protein (wsp) cDNA was performed on all wild-type strains to test for the presence or absence of *Wolbachia* infection (Braig *et al.* 1998; Zhou *et al.* 1998; Toivonen *et al.* 2007). PCR analysis revealed that all strains with the exception of W1118 and

[14] Experimental work investigating the effect of dietary restriction on tetracycline-treated wild-type strains was contributed to equally by R. C. Grandison, R. Wong and M. D. Piper. PCR analysis was performed by R. C. Grandison.

Oregon-R were infected with *Wolbachia* (Figure 3.4b). The fact that all strains exhibited a DR response suggested that *Wolbachia* infection *per se* cannot account for the lifespan and fecundity responses to changing levels of nutrition (Figure 3.4a). However, wild-type strains could be infected with different strains of *Wolbachia* or other bacteria which may cause an interaction with DR. To test this possibility, three different strains (the *Wolbachia*-infected Dahomey and Canton-S strains and the non-infected Oregon-R strain) were treated with tetracycline for two generations, before recovering for at least 10 generation on standard SY food without tetracycline. PCR analysis on the experimental generation confirmed that the tetracycline treatment had been effective in removing *Wolbachia* from the originally infected strains, Dahomey and Canton-S (Figure 3.5a).

All three tetracycline-treated strains retained their response to changes in food concentration (Figures 3.5b-d). Median lifespan for Dahomey (70 days), Oregon-R (67 days) and Canton-S (57 days) all peaked at 1.0 SY and decreased with increasing food concentration. Fecundity for each strain peaked at 2.0 SY. Interestingly, in the Oregon-R strain, tetracycline treatment resulted in a progressive increase in fecundity at each increasing food concentration including from 1.5 SY to 2.0 SY, which had not been evident in the untreated line (Figure 3.4a). Furthermore, the fecundity of tetracycline-treated Oregon-R (Figure 3.5d) appeared universally higher at all food concentrations compared with the non-tetracyline-treated strain (Figure 3.4a), whilst our laboratory control strain Dahomey retained a similar magnitude of fecundity before and after tetracycline treatment. Again, lifespan and fecundity were greatest in the Dahomey strain.

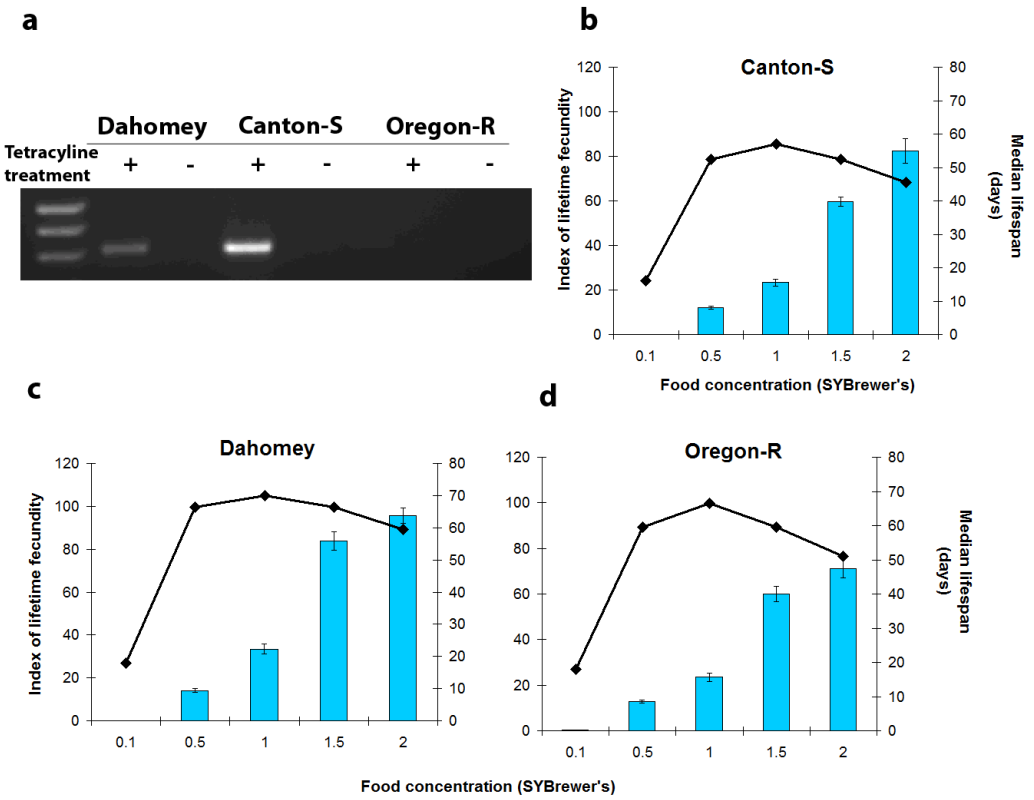

Figure 3.5: Response of tetracycline-treated wild-type strains to changing levels of nutrition. (a) PCR analysis confirming the removal of the intracellular bacterium *Wolbachia* from the infected Dahomey and Canton-S lines following tetracycline treatment (+ refers to non-tetracycline treatment and – refers to lines that had been treated with tetracycline treatment). (b, c and d) The responses of median lifespan and fecundity to changing food concentration of three wild-type strains following tetracycline treatment. Wild-type strains were allowed to recover for at least 10 generations before lifespan experiments were performed. All strains exhibited a DR response following tetracycline treatment. Fecundity assays were performed on days 5, 15, 22, 29, 36 and 43 of treatment. Error bars represent ± s.e.m. Figure adapted from (Grandison *et al.* 2009).

3.4 Discussion

3.4.1 Vial orientation affects male but not female lifespan

When choosing a model organism to study biological processes such as ageing, behaviour or development it is important to ensure that the experimental set-up has been optimised. In *Drosophila*, lifespan experiments are typically performed in three different environments; cages, vials or bottles. The majority of experiments in our laboratory are currently performed in glass vials containing between 4-7ml of a sugar yeast medium, with the vials orientated vertically in plastic trays. However, one problem when performing lifespan experiments under these conditions is that when flies age they have reduced mobility and are likely to become susceptible to bacterial infection, a hypothesis that is supported by microarray analysis showing an increase in numerous innate immunity-related transcripts with increasing age of wild-type flies (Pletcher *et al.* 2002). Furthermore, older flies exposed to a septic bacterial challenge exhibit a higher level of anti-microbial peptides than younger flies; however, when challenged with killed bacteria older flies show reduced efficiency in producing anti-microbial peptides (Zerofsky *et al.* 2005). This suggests that flies, like mammals, show signs of immunosenescence, as evident by reduced functional capacity of the innate immune system with age (Zerofsky *et al.* 2005). The bacterial infection present in older flies appears to spread onto the food causing it to become moist and sticky. Consequently, older flies are susceptible to stick to the food, despite still being alive, resulting in these flies being censored from the experiment. This is particularly an issue in experiments involving males, where the food becomes sticky due to bacterial presence. Females are less prone to this, likely as a result of the antibiotic effects of their eggs.

In an attempt to try and alleviate the problem with censoring flies toward the end of the experiment due to stickiness, vials were orientated horizontally (as well as vertically) in plastic trays. The results shown demonstrate that orientating vials horizontally instead of vertically presents a better solution to this problem. In experiments involving male *Drosophila,* optimisation of lifespan and survival curves was achieved when vials were kept horizontally. In the case of females, neither lifespan nor fecundity was affected by vial orientation, so that negative effects of changes in vial orientation can be ruled out. Consequently, experiments should be carried out in vials orientated horizontally to obtain ideal survival curves particularly for male flies and when comparing differences between the two sexes.

3.4.2 The importance of optimising ingredients used for dietary restriction experiments

Drosophila in the wild are thought to consume fungi and yeast growing on rotten fruits (Spieth 1974). In the laboratory, they can be maintained on a diet comprising water, sucrose and autolysed yeast in an agar gel. Previous work has demonstrated that dietary yeast as opposed to sucrose is the key ingredient in the diet influencing the lifespan of *Drosophila* (Mair *et al.* 2005). However, DR experiments in *Drosophila* are often still applied by the simultaneous dilution of both the sucrose and yeast in the diet. This does not take into account the possibility that sucrose may influence lifespan if the concentration is high enough. Moreover, dietary yeast can be produced in many different ways and from different sources. Several different types of yeast are utilised for *Drosophila* dietary restriction experiments in laboratories around the world. Dietary restriction in multiple organisms conforms to the paradigm that lifespan and fecundity should trade-off with one another. Hence reduced lifespan

with full feeding should be accompanied by increased fecundity as a result of greater nutrition (Partridge *et al.* 2005a; Mair and Dillin 2008; Piper and Bartke 2008). This has been reported in rodents (Holehan and Merry 1986; Weindruch and Walford 1988; Selesniemi *et al.* 2008), flies (Chippindale *et al.* 1993; Chapman and Partridge 1996; Libert *et al.* 2007; Lee *et al.* 2008) and worms (Klass 1977; Bishop and Guarente 2007b).

Work carried out in our laboratory investigated the responses of Dahomey wild-type flies to a range of food concentrations using four different commonly-used yeast diets (Bass *et al.* 2007a). Flies fed three of the four yeast diets (Baker's, Brewer's or Torula yeast) exhibited a classical tent-shaped response to increasing nutrition, whereby lifespan peaked at an intermediate food concentration and decreased at higher concentrations or at the lowest concentration (starvation). However, only one yeast diet, Brewer's yeast, caused a progressive increase in fecundity with each increasing concentration tested (Bass *et al.* 2007a). These data suggest that Torula and Baker's yeasts could be mildly toxic at higher concentrations or that the nutritional composition of these yeasts at this concentration could account for detrimental effects to lifespan, for example through reduced access to food. Furthermore, a fourth yeast diet (yeast extract) tested by Bass *et al.* (2007a) caused lifespan to peak at the lowest concentration tested and progressively decreased with each increase in concentration. In contrast, fecundity peaked at an intermediate yeast concentration and was reduced at higher concentration. As opposed to lifespan extension through reduced nutrition, it appears flies fed this yeast source display extended lifespan due to relief from a dose-dependent toxicity at higher yeast concentrations.

The study by Bass *et al.* also investigated the effect of a range of sucrose concentrations on lifespan and fecundity, increasing sucrose to a higher concentration than had previously been used (Mair *et al.* 2005). In agreement with Mair *et al.* (2005), the level of sucrose in the diet had a negligible effect on lifespan, although the absence of sucrose shortened lifespan. However, Bass *et al.* reported that the presence of sucrose was not required for maximum fecundity and increasing the concentration of sucrose beyond 50 g/L had detrimental effects on fecundity, potentially due to unfavourable effects on fly physiology. These data suggest that *Drosophila* generally have a low requirement for dietary sucrose, which is supported by evidence in the wild that rotting bananas contain sugar levels of no more than 20 mM or 4.5 g/L sucrose (Omura and Honda 2003).

These observations highlight the importance of measuring fecundity in addition to lifespan before choosing the food conditions on which to perform DR experiments. The concentration of sucrose and source of yeasts may also be an important consideration when studying *Drosophila* physiology and behaviour, since they may be influenced by different dietary conditions. As a result of the study by Bass *et al.*, all experiments presented in this thesis were performed using the optimised Brewer's yeast and a concentration of sucrose fixed at 50 g/L. Hence for DR experiments, only the concentration of yeast was varied.

3.4.3 Reduced lifespan on fully-fed diets cannot be explained by food hardness or water availability

It is generally assumed that lifespan extension of *Drosophila* under DR conditions is as a result of reduced nutrient intake following food dilution (Piper and Partridge 2007). However, it could also be that the shortened lifespan of flies on food

containing higher yeast concentrations is due to increased density and thus hardness of the food, thereby limiting availability. Alternatively, because the food medium provides the only source of water to flies, the increased yeast concentration with full feeding may restrict access to water, thereby causing a detrimental effect on survival.

Dehydration or restricted availability of water could not explain the reduced lifespan with full feeding (using Brewer's yeast). Providing flies with a separate, excess, water supply in the form of a pipette tip containing 1% agar did not rescue the reduced lifespan of fully-fed flies. In addition, the study by Bass *et al.* (2007a) examined the effects of manipulating the concentration of agar (to test food hardness) used in the fully-fed diet (2.0 SY). Increasing the agar concentration resulted in decreased lifetime fecundity but increased median lifespan, mirroring to some extent the typical DR response observed when reducing the yeast concentration (Bass *et al.* 2007a). In conjunction, these results confirm that DR extends lifespan in *Drosophila* through reduced nutrient availability. They also demonstrate that DR does not lead to longer lived flies by rescuing them from detrimental or toxic effects of full feeding, a view which had previously been put forward (Longo and Finch 2003; Prentice 2005).

3.4.4 Lifespan is not affected by an intermittent feeding regimen in *Drosophila* females

In rodents, DR is typically achieved by feeding the restricted group around 60-70 % of the food that they could eat if given *ad libitum* access (Yu *et al.* 1985; Weindruch and Walford 1988; Masternak *et al.* 2005; Bonkowski *et al.* 2006). However, another method which has been shown to extend lifespan is through intermittent feeding, whereby the DR group is given *ad libitum* access to food but only on every other day

(EOD) (Goodrick *et al.* 1982; Anson *et al.* 2005). Two previous studies have tested whether a similar regimen can extend lifespan in *Drosophila* (Kopec 1928; Le Bourg and Medioni 1991). The results of the first study suggested that there may be a beneficial effect on lifespan when flies are given access to food for only 18 hours, followed by 6 hours with only water (Kopec 1928), although overall there was no clear correlation between intermittent feeding and lifespan extension. Le Bourg and Medioni reported no positive effect on lifespan when assessing several different time periods of feeding / starvation regimens (Le Bourg and Medioni 1991). However, it is important to consider that the regimens used by Le Bourg and Medioni were only implemented on five out of seven days of the week. The two days of the week where flies were provided constant access to food may have masked any beneficial effects the periods of intermittent feeding had.

In the current study, a food source optimised for fecundity with full feeding was used (Bass *et al.* 2007a) to assess whether the laboratory wild-type strain Dahomey would respond to an intermittent regimen applied every day throughout the duration of the lifespan. In agreement with the previous studies (Kopec 1928; Le Bourg and Medioni 1991), no effect on lifespan was observed when applying either a three or six hour starvation period daily. In rodents, lifespan extension can also be achieved by feeding animals a measured amount of food that is completely consumed before the next meal. However, similar studies on house flies and medflies indicate that lifespan extension using this method of DR may also be unique to rodents (Carey *et al.* 2002; Cooper *et al.* 2004). One explanation for these differences in the ways that lifespan extension can be achieved in rodents and flies could be that the fly DR protocols are simply not optimised to achieve the same outcome observed in rodents. Alternatively, it is also possible the mechanisms of lifespan extension by DR in

mammals and flies may be different. Differences in body size and the ability to store energy in tissues may account for the differences observed between species. However, before it is possible to conclude whether or not intermittent feeding extends lifespan in *Drosophila*, a greater range of starvation intervals would need to be studied, including a pre-longed starvation interval that shortens lifespan. The longest starvation period tested in the current study was only six hours, which did not prove to be either beneficial or detrimental to lifespan.

Neither the studies of Kopec, Le Bourg and Medioni or this current study have examined the effect of intermittent feeding on daily or lifetime fecundity. Lifespan extension by DR should be accompanied by a reduction in fecundity (Partridge *et al.* 2005a; Piper and Partridge 2007) and it would be interesting to see whether flies exposed to periods of starvation display reduced fecundity or similar fecundity to continuously-fed flies. If lifetime fecundity was similar between continuously and intermittently-fed groups this may help to explain why no lifespan extension was observed. Another possibility of why this method of applying DR may not extend lifespan is because flies on intermittent feeding regimens could compensate by eating more upon return of the food supply, a hypothesis that has been put forward upon applying DR by food dilution (Carvalho *et al.* 2005). However, difficulties in ascertaining feeding rates and direct food intake make this hypothesis a difficult one to test (Carvalho *et al.* 2005; Wong *et al.* 2008). Unfortunately, the experimental set-up discussed did not allow accurate measurements of fecundity for two reasons. Firstly, the experiments were performed in one litre cages containing 100 females and one vial of food, which can result in over 20,000 eggs in one vial during the start of a new experiment. Such numbers cannot be counted accurately, so that false results would have been generated. Secondly, during the starvation period of three or

six hours, flies not only had no food supply but also no egg-laying site. Flies will refrain from egg-laying when there are no appropriate sites to lay eggs and will select for optimal egg-laying sites (Richmond and Gerking 1979; Yang *et al.* 2008). This results in the intermittently-fed group having a significantly shorter time frame in which they can lay eggs compared with the continuously-fed cohort.

A perhaps more suitable experimental protocol would therefore be to set-up a smaller scale experiment in vials, at a density of 10 females per vial, where flies are transferred to vials containing starvation medium (1% agar; section 2.2.4) during the periods of starvation. This would not only make egg counts more manageable, but would also provide flies on intermittent regimens a suitable, moist, egg-laying surface during periods of starvation.

3.4.5 DR extends lifespan of five different wild-type populations

Fly stocks in laboratories tend to be maintained in small numbers in vials or bottles containing a sugar/yeast medium or a cornmeal-based diet, and are transferred to fresh medium every generation. For ease of handling, stocks are often stored at lower temperatures, usually 18^{o}C, to reduce development time and hence reduce the frequency that flies are transferred to fresh medium. These environmental conditions can exert strong selection pressures that may subsequently affect fitness-related traits such as lifespan and fecundity. One example of such mechanisms is the fact that rearing flies at lower temperatures can lead to selection for larger body size (e.g. (Anderson 1966; Partridge *et al.* 1994). Another problem is that frequent transfer of stocks to fresh medium every generation potentially leads to selection for early reproduction. This in turn has been shown to correlate with reduced lifespan of adult *Drosophila* (Fowler and Partridge 1992), and hence can severely affect lifespan

studies. Furthermore, it has been reported that flies maintained under routine conditions (i.e. flies maintained in relatively low numbers) exhibit reduced lifespan similarly to flies that have been selected for early reproduction (Linnen *et al.* 2000). In contrast, flies collected from the wild exhibit significantly longer lifespans, similarly to flies that had been maintained in the laboratory and selected for late reproduction (Linnen *et al.* 2000).

The wild-type stock Dahomey is an outbred stock, and has been maintained in the laboratory for several in large population cages at 25^{o}C with overlapping generations. Maintaining flies in large population cages appears to result in similar adult survival rates to flies that have been freshly caught from the wild (Sgro and Partridge 2000; Sgro and Partridge 2001). The data from the current DR experiment support this notion because Dahomey and wDahomey flies were not only the most fecund but also exhibited the longest lifespans compared with other wild-type flies that had been maintained using routine stock handling conditions (Figure 3.4a). These data imply that some of the life-history characteristics of wild-flies can be preserved by maintaining flies in large population cages, because this method of culturing does not select for early reproduction. Differences in fecundity between the wild-type strains are likely to be explained by the different genetic backgrounds, body and ovary sizes and culturing conditions.

Despite differences in stock handling conditions and feeding regimens between the different wild-type stocks, potentially leading to different selection pressures, importantly all wild-type flies displayed a characteristic response to DR in terms of lifespan and fecundity. Interestingly, lifespan and fecundity peaks for the different wild-type populations were not all the same, again indicating that differences in stock

culturing or genetic background may cause differences in fitness-related traits. Previous work on long-lived mutants has already highlighted that genotype can affect the interaction between lifespan and diet (Clancy *et al.* 2002; Giannakou *et al.* 2008; Min *et al.* 2008). Oregon-R flies appeared to behave slightly differently from the other groups because in this experiment fecundity showed a small decrease from 1.5 SY to 2.0 SY, coupled with a decrease in lifespan. This was in contrast to all other groups which displayed a progressive increase in fecundity with increasing food concentration. One possibility is that Oregon-R flies are more sensitive to certain nutrients (found in higher concentrations in 2.0 SY) than other wild-type flies. Alternatively, Oregon-R may have difficulty in extracting nutrients from the food, particularly at the highest food concentration. The second explanation may be supported by the fact that Oregon-R generally exhibited the lowest fecundity of all groups, indicating that their efficiency of food uptake could be lower. This hypothesis could be investigated by measuring feeding behaviour and food uptake of Oregon-R flies compared to other wild-type populations on several different food concentrations, although as previously discussed feeding can be difficult to measure accurately.

A recent study reported that wild mice when subjected to DR did not exhibit an increase in median lifespan compared with a continuously-fed cohort, leading to the suggestion that DR could be an artefact of laboratory selection (Harper *et al.* 2006). In contrast, another study showed that by using bacteria deprivation as a method of applying DR (Kaeberlein *et al.* 2006b; Lee *et al.* 2006; Smith *et al.* 2008), the lifespan of five independent wild-derived *C.elegans* could be extended (Sutphin and Kaeberlein 2008). In *Drosophila*, the response of wild-caught flies to DR has yet to be investigated; however the strong response of our wild-type strain Dahomey,

which is maintained in population cages with overlapping generations producing similar lifespans to wild flies (Sgro and Partridge 2000; Sgro and Partridge 2001), suggests that wild-caught *Drosophila* are also likely to respond to DR. A possible explanation for the failure to detect lifespan extension in wild mice (Harper *et al.* 2006) is that the authors of this study only implemented two levels of feeding regimens, *ad libitum* and DR (60% of *ad libitum*). It is possible that wild mice, due to their lower body mass compared with laboratory-reared mice (Harper *et al.* 2006), have different energy or feeding demands, and would therefore respond differently to the standard laboratory food concentrations used.

3.4.6 Lifespan extension by DR not mediated by *Wolbachia*

The presence of *Wolbachia* is unique to arthropods and nematodes and anywhere between 25-70% of insects are thought to be infected with this bacterium (Kozek and Rao 2007). The interaction between *Wolbachia*, genotype and lifespan in *Drosophila* appears to be a complicated one. For example, evidence from a recent study suggests that the lifespan extension of an originally long-lived male *Indy* mutant line is abolished after backcrossing and removal of *Wolbachia* infection (Toivonen *et al.* 2007). Furthermore, insulin receptor (*InR*) dominant-negative flies display a significant extension of lifespan compared with controls in a *Wolbachia* background; however this appears to be completely diminished upon curing the lines of *Wolbachia* with tetracycline treatment (Ikeya et al. unpublished data). If *Wolbachia* and Dahomey have been co-evolving for a long time, one distinct possibility is that removing *Wolbachia* makes the flies sick to some extent. Future work will also need to examine whether the lifespan extension reported in other known long-lived

mutants including *chico*, *InR*, *TOR*, etc, is repeatable when differences in cytoplasmic background have been corrected for.

It has been proposed that increased nutrition may be associated with higher proliferation rates of bacteria, which in turn might explain the reduced survival rates of fully-fed flies (Cooper *et al.* 2004). Based on this hypothesis and the findings that at least part of the lifespan extension phenotype of a long-lived *Indy* mutant line and *InR* dominant-negative flies is attributable to *Wolbachia* infection, it was chosen to investigate whether lifespan extension by DR is simply a phenomenon caused by the presence of *Wolbachia*. If this were the case, then tetracycline treatment of flies to remove the *Wolbachia* infection should block any DR effect observed. Importantly, following tetracycline treatment of two infected populations, Dahomey and Canton-S, and a non-infected population (Oregon-R), all three strains retained their response to DR. Interestingly, however, the fecundity of tetracycline-treated Oregon-R flies showed a marked increase at all food concentrations compared with the non-tetracycline-treated line, suggesting that another bacterial infection other than *Wolbachia* may be present in Oregon-R flies, which acts to restrict their egg-laying capacity. These results demonstrate that *Wolbachia* infection and/or other bacterial infections removed by tetracycline cannot account for the effects of DR. Our data is also in agreement with previous work that demonstrated that DR still extended lifespan when experimental flies were exposed to tetracycline treated food to remove bacterial infection (Mair *et al.* 2005).

Interestingly, lifespan for all three wild-type populations following tetracycline treatment peaked at 1.0 SY; however, in the non-tetracycline treated Dahomey population, lifespan peaked at 0.5 SY (Figure 3.4a). This raises the possibility of a

small interaction between *Wolbachia* and nutrition in certain genotypes. However, a more likely explanation is that there is seasonal variation in the nutritional content of yeast due to differences in production and the quality of its feedstock. Indeed, the experiments conducted by Bass *et al*. (2007a), where lifespan was measured over a range of concentrations using Brewer's yeast, yielded a lifespan peak of 1.0 SY. Ideally, it would have been optimal to perform parallel experiments investigating the effects of DR on *Wolbachia* infected strains compared with the same strains that had been cured of *Wolbachia* infection. The differences in lifespan peaks due to seasonal variation also point to the need for a standard defined diet, particularly when assessing lifespan extension by DR and potential mechanisms regulating this process.

3.4.7 Concluding remarks

The short lifespan and generation time, in conjunction with the ability to use and large population sizes make *Drosophila* a commonly used model organism for ageing studies. However, experimental conditions often vary greatly between different laboratories and even individual researchers within the same group. The results of this chapter in conjunction with a recent paper by Bass *et al.* (2007a) highlight the need to use optimised DR and lifespan protocols, to achieve consistent and more importantly comparable results.

In addition, the work reported here reveals that DR can extend lifespan in *Drosophila* independent of the presence of *Wolbachia* infection or the type of wild-type strain used. Furthermore, the data highlight the importance of measuring the response of fecundity and lifespan over a range of food concentrations. Although all wild-type strains exhibited a strong response to DR, the food concentration which lifespan

peaked at differed, suggesting a possible interaction between genotype and nutrition. In contrast to rodents, evidence appears to suggest that intermittent feeding may not extend lifespan in *Drosophila.* However, future work on intermittent feeding, using a wider range of starvation intervals, will prove more conclusive.

Chapter 4

Identifying specific nutrients mediating the responses of lifespan and fecundity in *Drosophila* dietary restriction

4.1 Introduction

Chapter 3 together with the recent report by Bass *et al.* (2007a) discussed the importance of a common dietary restriction (DR) protocol being used by different laboratories working with the same model organism for studies investigating the effects of diet on ageing. Over the last ten years, several different mediators or master regulators of DR have been discovered; however, the lack of a common protocol or defined diet makes it difficult to draw comparisons between the results of the different laboratories.

One of the major questions when addressing lifespan extension by some form of food restriction is whether it is independent of or dependent on caloric intake. In rodents, DR is often referred to as caloric or calorific restriction (CR) because it was thought that a reduction in calories alone can account for the extension of lifespan independent of which nutrients are being restricted (Iwasaki *et al.* 1988; Weindruch and Walford 1988; Masoro *et al.* 1989). Masoro *et al.* (1989) suggested that lifespan can be extended by restricting calories without reducing protein intake; hence concluding that protein restriction does not play a significant role in lifespan extension by CR. However, the conclusions of this study do not appear to fully support the results because protein restriction almost totally prevented the progression of chronic nephropathy and extended lifespan to a similar magnitude observed with regular CR (without protein restriction). Hence, the role of protein does appear to be important in accounting for some of the effects of CR. The same group also reported that the lifespan of rats cannot be extended by a reduction of either fat or minerals in an otherwise iso-caloric diet. Nonetheless, the onset of chronic nephropathy was retarded by fat restriction (Iwasaki *et al.* 1988). Other

studies have since reported strong evidence that restriction of specific nutrients can indeed cause an extension of lifespan in rodents. For example, the lifespan of rats can be extended by a reduction of the protein concentration in the diet (Yu *et al.* 1985). Moreover, the restriction of methionine, one of the essential amino acids, was shown to increase the lifespan of both rats and mice (Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003; Miller *et al.* 2005). In addition, mice fed a restricted diet containing reduced amounts of another essential amino acid, tryptophan, showed increased longevity compared with control-fed mice (De Marte and Enesco 1986), thereby further highlighting a possible role for specific individual nutrient components in mediating dietary restriction.

In *Drosophila* it appears that lifespan extension by DR is independent of caloric intake (Mair *et al.* 2005; Bass *et al.* 2007a). Mair *et al*. independently varied the sucrose and yeast concentration from a control (fully-fed) level to a DR level. Reducing either the sucrose or yeast concentration resulted in an increase in lifespan. However; reducing the yeast concentration increased lifespan to a greater magnitude than reducing sucrose, despite the relative caloric content of autolysed yeast powder and sucrose being almost identical (4.02 kcal/g autolysed yeast versus 4.0 kcal/g sucrose) and feeding rates of flies being similar on all diets (Mair *et al.* 2005). These data suggest that specific nutrients in dietary yeast are likely to play an important role in *Drosophila* ageing, as opposed to caloric intake *per se*. A study by Min and Tatar (2006) investigated caloric flux in *Drosophila* fed different diets. Caloric flux relates to the energy in the diet that is physiologically utilised by the organism during a given period of time (Piper *et al.* 2007). Min and Tatar proposed a strong correlation between reduced caloric flux and increased lifespan, leading to their conclusion that calorie intake could be important for lifespan extension by DR (Min

and Tatar 2006a). However; the authors measurements were only performed on the first five days of adulthood and no measurements were made on respiration (a key energy expenditure effecting caloric flux) because dietary-restricted and fully-fed flies had previously been reported to have similar metabolic rates (Hulbert *et al.* 2004). An important consideration is that Hulbert *et al.* measured resting metabolic rates as opposed to energy expenditure under experimental conditions. Energy expenditure through respiration is likely to differ between flies on different dietary treatments, for example through differences in physical activity, which has been previously been shown to be greater in dietary restricted *Drosophila* (Bross *et al.* 2005). Hence, the measurements of caloric intake or caloric flux by Min and Tatar were not sufficient for the authors to conclude that DR extends lifespan because flies on DR diets assimilate fewer calories (Piper *et al.* 2007).

Despite early work in the mid 1900s on characterising nutritional requirements of *Drosophila* larvae (Sang 1956; Sang 1959) and female adults (Sang and King 1961), the roles of different nutrient components on lifespan in *Drosophila* have been less well documented than in rodents. Previous studies have shown that reducing the concentration of the phosphoprotein casein causes an extension of lifespan in adult flies (Hollingsworth 1970; Van Herrewege 1974). However these studies did not measure the response of mortality or fecundity to varying casein diets as parallel measures of health (Piper and Partridge 2007). Hence reduced toxicity cannot be ruled out as an explanation for lifespan extension at lower casein concentrations.

In addition to extending lifespan, DR generally lowers fecundity, for instance in *Caenorhabditis elegans*, *Drosophila* and rodents (Partridge *et al.* 2005a). This finding has led to the suggestion that DR elicits an evolved response to food

shortages in nature, with reallocation of nutrients away from reproduction to somatic maintenance and repair, thus increasing probability of survival until reproduction can be recommenced more successfully with the return of the food supply (Williams 1966; Kirkwood and Holliday 1979; van Noordwijk and de Jong 1986; Holliday 1989; De Jong 1993; Kirkwood and Shanley 2005). When food is abundant (full feeding), an organism will favour maximising its fitness by investing resources heavily in reproduction. This hypothesis thus predicts that survival and reproduction are in competition with each other for at least some of the same limiting nutrients (Charlesworth 1980) and that the DR responses occur because high survival and high reproductive rate are mutually exclusive (Figure 4.1). This trade-off is partially supported by previous work in *Drosophila*, which revealed that increasing the concentration of live yeast reduced both lifespan and somatic storage (in terms of carbohydrate and lipid storage), but increased fecundity (Bradley and Simmons 1997). However, an important consideration is that this study was conducted in mixed sex groups. Re-mating frequency has previously been shown to increase with higher nutrition levels (Chapman and Partridge 1996) (Figure 1.9), thus the reduced lifespan of mixed sex flies maintained on concentrated live yeast diets could partially be explained by increased mating frequency as opposed solely to effects on ageing caused by increased nutrition.

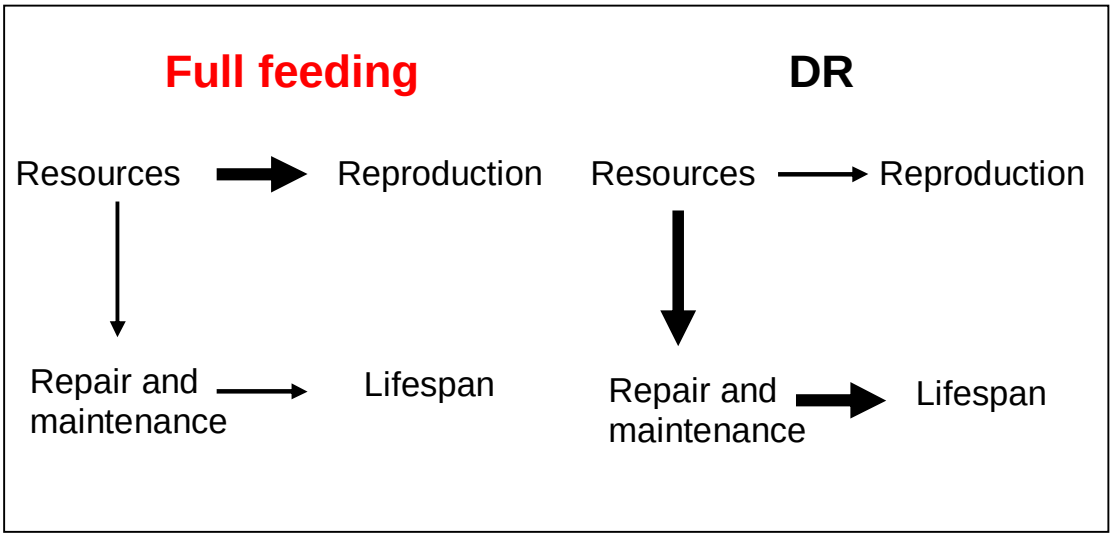

Figure 4.1: A trade-off model of lifespan extension under DR as a result of reduced reproduction. When provided with a nutrient rich diet (full feeding), an organism is likely to invest resources more heavily in reproduction to increase its fitness. As a consequence, fewer resources are directed towards somatic maintenance and repair and subsequently lifespan. In contrast an organism provided a DR diet displays reduced reproduction and is predicted to invest limited resources more heavily into repair and maintenance, extending lifespan until a more plentiful food supply returns. Adapted from (Partridge *et al.* 2005a).

This chapter sets out to test the reallocation hypothesis in *Drosophila* DR in more detail using a semi-defined diet approach to determine which specific nutrients present in dietary yeast might account for the reduced lifespan but increased fecundity of fully-fed female *Drosophila* compared with flies fed a restricted diet. Importantly, this approach enables flies to have access to a basal level of nutrients in the yeast that are essential for supporting and maintaining healthy lifespan (Sang and King 1961); M. Piper unpublished results), yet allows assessment of the effects that individual nutrients have on lifespan and fecundity. Experiments were performed initially on female flies because the magnitude of lifespan extension by DR appears to be greater in this sex (Magwere *et al.* 2004) and fecundity could be used as an

indicator of health on the different diets (Bass *et al.* 2007a; Piper and Partridge 2007). Importantly, the yeast diet had previously been optimised for lifespan extension under DR and fecundity with full feeding (Bass *et al.* 2007a).

4.2 Methods

4.2.1 Preparation of the add-back solutions

In order to determine the concentration of nutrients to add-back to the DR diet (1.0 SY) (Table 4.1) and to establish the ratios which the nutrients are present in yeast, a chemical analysis of the Brewer's yeast was obtained from the supplier (Bass *et al.* 2007a). Since the availability of free nutrients to the flies is likely to be greater than when they are present in larger molecules and in whole yeast, firstly the effect on fecundity of adding back all nutrients, in the ratio in which they occurred in yeast (determined by chemical analysis (Bass *et al.* 2007a)) at a few different concentrations was measured. The concentration of nutrients that gave rise to the same increase in fecundity observed with full feeding (2.0 SY) was used for further experiments (Figure 4.2a). Solutions containing vitamins, amino acids or carbohydrates were made up prior to media preparation. The individual ingredients were weighed out and dissolved in distilled water to make up a stock solution for each nutrient add-back group, as outlined (Table 4.1). The lipid add-back was prepared by dissolving 0.25g of phosphatidylcholine in 50ml of 100% ethanol. Phosphatidylcholine was chosen as the lipid source because it is the major phospholipid found in eukaryotic cells and contains choline and fatty acids, both thought to be essential for *Drosophila*. In addition it can be easily hydrolyzed by triglyceride lipase, found in the fat body of insects (Arrese *et al.* 2006).

Add-back group	Individual nutrients added to make stock solution	Quantity of nutrient used to make stock solution (g)	Biologically-available nitrogen concentration (mM)
Amino acids (final volume of stock solution = 100ml)	L-arginine HCL*	0.85	4.0
	L-histidine*	0.42	2.7
	L-isoleucine*	0.68	5.2
	L-leucine*	0.95	7.2
	L-lysine HCL*	1.03	5.7
	L-methionine*	0.2	1.4
	L-phenylalanine*	0.52	3.1
	L-threonine*	0.73	6.2
	L-tryptophan*	0.18	0.9
	L-valine*	0.8	6.8
			Total = 43.2mM
	L-alanine	0.85	9.5
	L-asparagine	0.54	4.1
	L-aspartic acid	0.54	3.5
	L-cysteine HCL	0.2	1.3
	L-glutamic acid	0.83	5.7
	L-glutamine	0.83	5.7
	Glycine	0.67	8.9
	L-proline	0.4	3.5
	L-serine	0.58	5.6
	L-tyrosine	0.53	2.9
			Total = 50.6mM
Carbohydrates (final volume of stock solution = 600ml)	Lactose	11.7	
	Sucrose	1.6	
	Glycogen	14.07	
	Trehalose	9.38	
Vitamins (final volume of stock solution = 500ml)	Biotin	0.0021	
	Ca pantothenate	0.20	
	Nicotinic acid	0.067	
	Pyridoxine	0.83	
	Riboflavin	0.04	
	Thiamine-HCl	0.25	
Folate (dissolved in 500ml distilled water)	Folate	0.5	
Lipids (dissolved in 50ml Ethanol (100%)	Phosphatidylcholine	0.25	

Table 4.1: Preparation of nutrient add-back stock solutions. Individual nutrients for each diet were weighed out in the quantities indicated and dissolved in the specified volume of distilled water or ethanol (in the case of the lipid add-back) to make a stock solution. The biological available nitrogen concentration is based on the theoretical nitrogen yield that would be available if each amino acid was fully catabolised. This concentration represents the molar concentration of each amino acid, based on the quantities of each amino acid added back to the DR diet. * Denotes amino acids that are thought to be essential for *Drosophila* (Sang and King 1961).

4.2.2 Starvation assays

Once-mated females and males were divided by sex and allocated to their respective diets for a period of seven days prior to starvation. On day seven of treatment, flies were then transferred to a starvation media (1% agar, section 2.2.4) and deaths were scored 4-5 times per day.

4.3 Results

4.3.1 Identifying the major nutritional group(s) regulating fecundity and lifespan

The aim of this work was to determine which nutrients present in yeast contribute to the high fecundity of fully-fed flies in order to determine whether any of these same nutrients decrease lifespan, as predicted by the reallocation hypothesis (Figure 4.1). Initially four major nutritional groups: amino acids, vitamins, lipids and carbohydrates were targeted. These nutritional groups were individually added back to the DR diet, and the effects on lifespan and fecundity were measured in order to determine whether any of these nutrient groups can account for the increased fecundity when adding back all nutrients or with full feeding. Each nutrient addition will be referred to as an add-back.

Adding back lipids, vitamins or carbohydrates to DR had no significant effect on fecundity, demonstrating that these nutrients are not limiting for fecundity in the DR diet (Figure 4.2a). In contrast, the addition of amino acids to the DR diet accounted for the entire increase in fecundity observed when either adding back all nutrients or with full feeding. Furthermore, adding back amino acids also caused a significant reduction in lifespan compared with DR, mirroring the effect of adding back the combination of all nutrients (Figure 4.2b). However, this reduction in lifespan was greater than the reduction observed with full feeding, potentially due to an increased availability of free amino acids in the add-back diets, compared with full feeding, which cannot be utilized for further increasing fecundity. Adding back vitamins, lipids or carbohydrates alone did not affect lifespan, thereby providing further evidence that lifespan extension by DR in *Drosophila* is independent of calories.

These data are in agreement with a reallocation of amino acids from reproduction towards somatic maintenance and repair. This model could hence provide a mechanism responsible for lifespan extension under DR. However, an alternative explanation could be that different amino acids mediate the lifespan and fecundity responses. Such a model would imply that the addition of all amino acids results in the up-regulation of fecundity and shortening of lifespan via independent pathways.

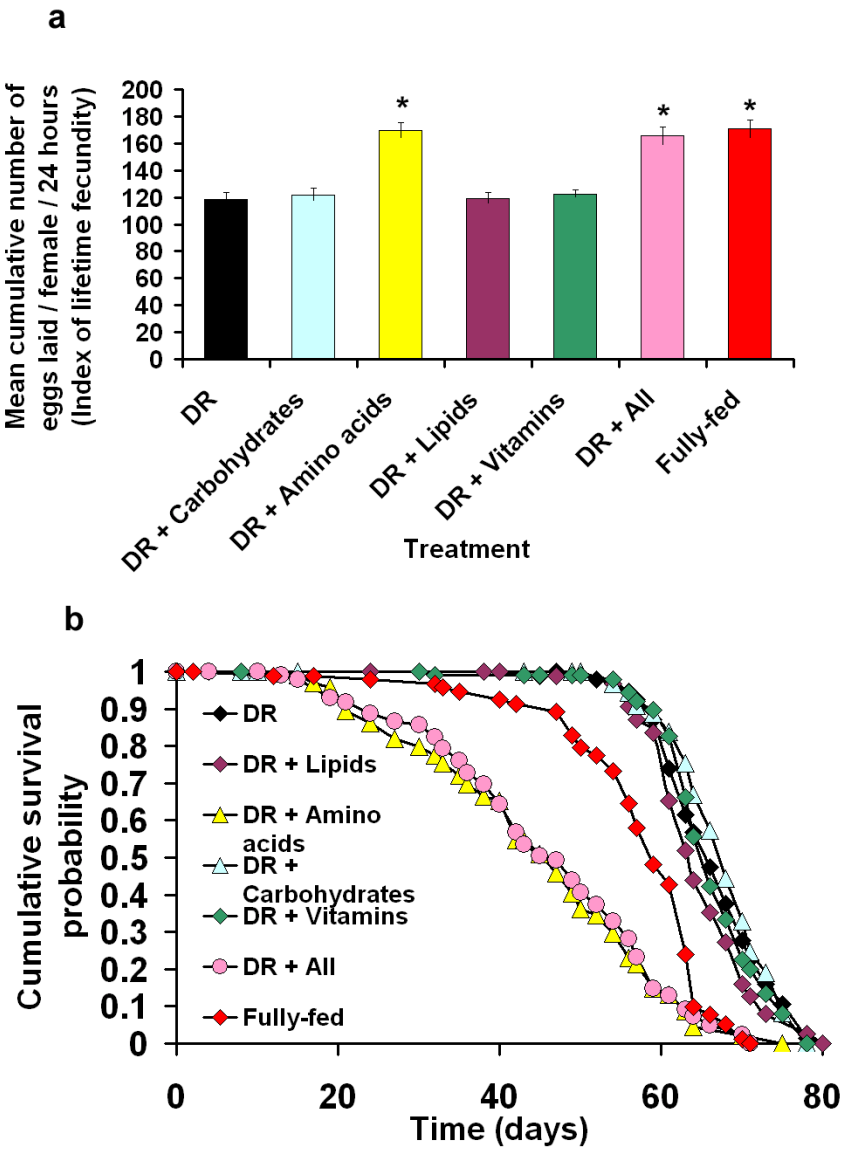

Figure 4.2: Identifying the major nutritional groups that mediate the responses of lifespan and fecundity to DR. (a) Adding back all nutrients to DR caused an increase in fecundity, to a similar extent to adding back yeast itself (full feeding; $P = 0.706$, Wilcoxon). Furthermore, the increase in fecundity was entirely attributable to the amino acids added back (amino acids compared with all nutrients, $P = 0.94$, Wilcoxon). (b) Adding back amino

acids or all nutrients together significantly shortened lifespan compared with DR controls ($P < 0.0001$, log-rank). In contrast, no difference in fecundity or lifespan was detected when adding back vitamins, lipids or carbohydrates ($P = 0.2$-0.75, Wilcoxon; $P = 0.1$-0.7, log-rank for fecundity and lifespan respectively). Fecundity assays were performed on days 6, 9, 12, 15, 18, 21, 28, 35 and 42 of treatment. Error bars represent ± s.e.m. * Indicates a significant increase in fecundity ($P < 0.0001$) when compared with DR (black) using the non-parametric Wilcoxon test.

4.3.2 The life-shortening effect of amino acids is not due to a reduction in water availability or an increase in osmotic pressure

In order to rule out the possibility that adding back amino acids was simply shortening lifespan either due to reduced water availability or due to an increase in osmotic stress, flies were provided with access to a separate water supply by placing an agar tip in the food (section 3.2.3). This method of providing water has previously been adopted to determine whether reduced lifespan with full feeding can be reversed upon providing flies with a separate water supply (section 3.3.3) (Bass *et al.* 2007a). The life-shortening effect of amino acids was not reversed upon the addition of a water supply (Figure 4.3a). In addition, no effect of water itself was observed in DR control-fed flies as previously reported (Figure 3.2) (Bass *et al.* 2007a). To demonstrate the efficacy of using an agar tip, the effect of adding salt to DR, which is a known osmotic stressor, was investigated[15]. Supplementing the diet with 0.8% salt caused a significant shortening of lifespan (Figure 4.3b), similarly to the effect of adding back amino acids. However, this life-shortening effect was completely reversed upon the addition of an agar tip to the food.

[15] Experimental work investigating the effect of salt on lifespan in the presence or absence of an agar-filled tip was performed by M. D. Piper

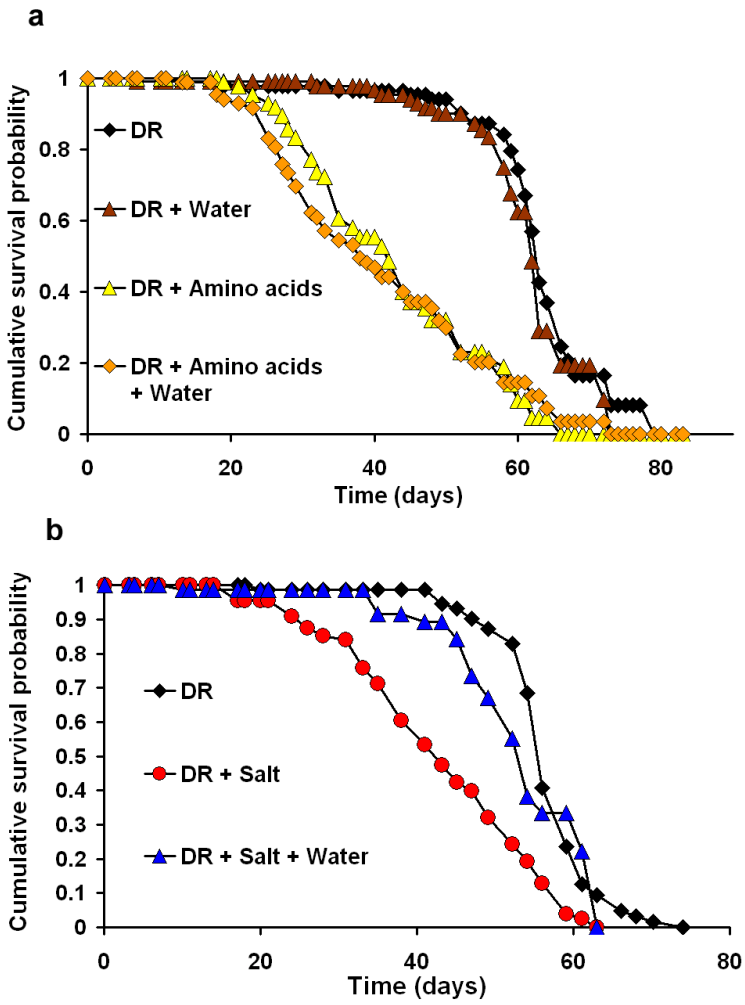

Figure 4.3: Amino acids do not reduce lifespan by reducing water availability. Free access to water was provided in the form of a 1% agar tip placed into the food. (a) The life shortening effect caused by adding back amino acids to DR was not rescued by the addition of a water supply ($P = 0.37$, log-rank) and no effect was observed in DR controls when water was provided ($P = 0.28$, log-rank). In contrast, adding 0.8% salt to dietary yeast (b) resulted in a significant shortening of lifespan ($P < 0.0001$, log-rank), which was completely rescued by water (DR + salt + water compared to DR, $P = 0.16$, log-rank), demonstrating the efficacy of this method of water supply. Experimental work for the salt addition experiment was performed by M. D. Piper.

4.3.3 Regulation of lifespan and fecundity independent of nitrogen concentration

To determine whether the increase in fecundity and decrease in lifespan attributed to amino acids in the diet (Figure 4.2) were due to an increase in nitrogen concentration or due to the effect of specific amino acids, the amino acids were categorised into essential and non-essential, which are thought to be the same for *Drosophila* as they are for mammals (Sang and King 1961); Table 4.1). Adding back the 10 non-essential amino acids (N-EAAs) had no effect on fecundity relative to the DR control treatment (Figure 4.4a). In contrast, adding back the 10 essential amino acids (EAAs) caused a significant increase in fecundity, accounting for the full effect observed with both full feeding and with adding back all 20 amino acids together. Despite having no effect on fecundity, adding back N-EAAs caused a small but significant decrease in lifespan later in life (Figure 4.4b). In contrast, adding back EAAs caused a much greater reduction in lifespan, decreasing it to a similar magnitude observed with full feeding. To determine whether the increase in fecundity and decrease in lifespan attributed to the EAAs could be explained by differences in the nitrogen levels in the diet, the relative nitrogen concentration provided by N-EAA and EAA add-back diets was calculated. The theoretical biologically available nitrogen was greater in the N-EAA (50.6mM) compared with the EAA add-back treatment (43.2mM, Table 4.1), suggesting that the concentration of available nitrogen cannot account for the differences.

In order to ensure that the reduction in lifespan attributed to adding back essential amino acids (EAAs) was not simply due to extreme acidity or alkalinity, the pH of the EAA stock solution compared with distilled water alone (DR control) was measured. Although, the addition of EAAs increased the pH slightly from pH 5.8

(distilled water) to pH 6.9, this pH is neutral and hence is unlikely to be detrimental to flies.

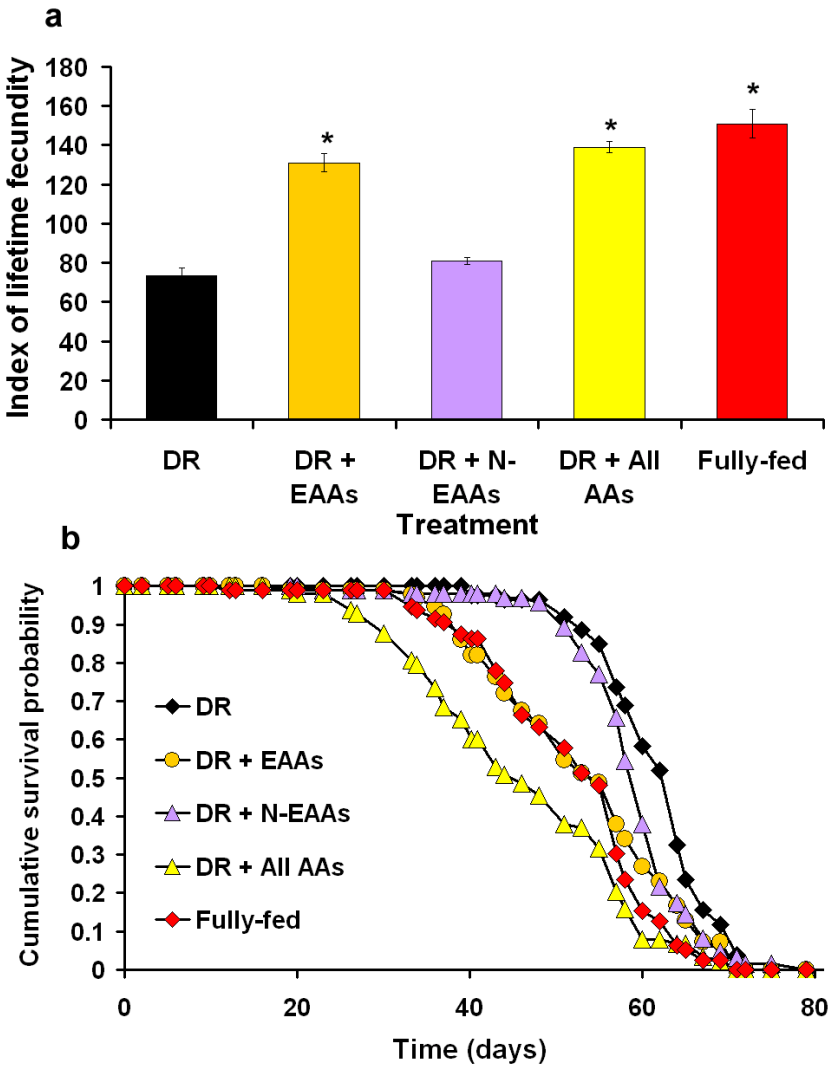

Figure 4.4: Distinguishing the effects of essential and non-essential amino acids. (a) Adding back the 10 essential amino acids (EAAs) accounted for the whole increase in fecundity observed in flies fed a DR diet supplemented with all 20 amino acids (10 EAAs compared with all 20 amino acids (all AAs), $P = 0.05$, Wilcoxon). (b) EAAs also decreased survival to a similar magnitude seen in fully-fed flies (EAAs compared with fully-fed, $P = 0.17$, log-rank). Non-essential amino acid supplementation (N-EAAs) had no effect on fecundity, but caused a small, but significant decrease in lifespan compared with DR ($P = 0.011$, log-rank). Fecundity assays were performed on days 5, 9, 12, 19, 26, 33 and 40 of treatment. * Denotes a significant increase in fecundity ($P < 0.0002$) when compared with DR (black) using the non-parametric Wilcoxon test.

4.3.4 Essential amino acids regulate the lifespan / fecundity trade-off in virgin females

To determine whether the response of lifespan and fecundity to EAAs is dependent upon mating status, the effect of adding back EAA and N-EAAs on non-mated virgin flies was assessed relative to the response of dietary-restricted controls. In virgins, adding back N-EAAs once again had no effect on fecundity (Figure 4.5a), and caused a small but significant decrease in lifespan compared with DR control-fed virgins, mirroring the effects observed in once-mated flies. Adding back all 20 amino acids caused a drastic increase in fecundity, which, as was the case in once-mated flies, was entirely attributable to the EAAs. Furthermore, the increase in fecundity associated with adding back EAAs caused a significant decrease in lifespan. However, in contrast to once-mated flies, no further reduction of lifespan was seen when adding back all AAs compared with adding back EAAs alone.

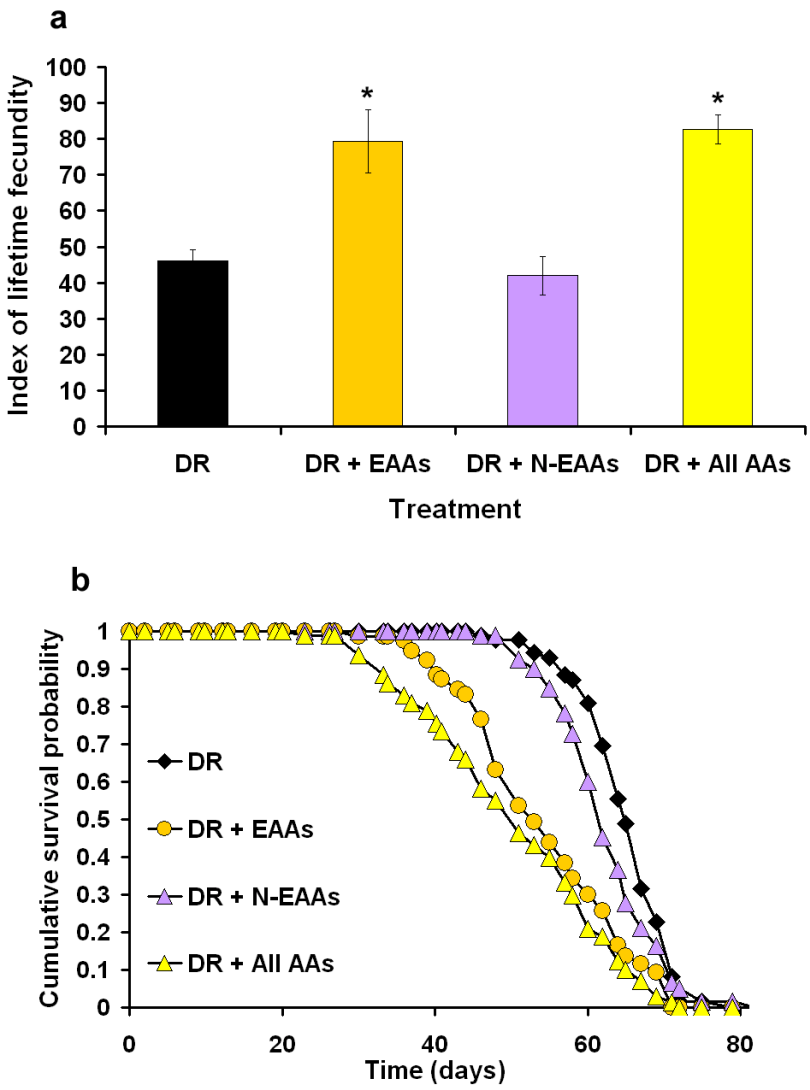

Figure 4.5: Essential amino acids mediate lifespan / fecundity trade-off in virgin females. (a) Supplementing the DR diet with non-essential amino acids (N-EAAs) had no effect on the fecundity of virgin females (P = 0.51, Wilcoxon). In contrast, adding back essential amino acids (EAAs) caused a significant increase in fecundity, similarly to adding back all 20 amino acids (all AAs; P = 0.51, Wilcoxon). (b) Adding back N-EAAs caused a subtle but significant decrease in lifespan (P = 0.03, log-rank), but not to magnitude seen with adding back EAAs (EAAs compared with N-EAAs, P < 0.0001, log-rank). No further reduction in lifespan was observed when adding back all 20 AAs (EAAs compared with all AAs, P = 0.1, log-rank). Fecundity assays were performed on days 5, 9, 12, 19, 26, 33 and 40 of treatment. * Indicates a significant increase in fecundity (P < 0.0001) when compared with DR (black) using the non-parametric Wilcoxon test.

4.3.5 Examining the effects of increasing the concentration of essential amino acids in the diet

EAAs have been shown to account for the entire increase in fecundity and a similar reduction in lifespan to full feeding (Figure 4.4). However, the question remains whether it is possible to observe a continual trade-off between lifespan and fecundity when increasing the concentration of EAAs added back to the DR diet beyond the concentration previously used (43mM). If EAAs are the only nutritional group that regulate fecundity in this context, one might predict that a further increase in the concentration of EAAs should result in a further increase in fecundity. To test this hypothesis, EAAs were added back to the DR diet at two and four times the original concentration used.

Supplementing the DR diet with an increasing range of EAAs from 43mM to 172mM resulted in a progressive increase in fecundity with each increasing concentration (Figure 4.6a). Adding back twice the concentration of EAAs (86mM) significantly increased fecundity compared with adding back the originally used concentration of 46mM. When adding four times this amount (i.e. 172mM) an even greater increase in fecundity was observed. As the reallocation hypothesis would predict, lifespan continued to trade-off with fecundity upon increasing the dose of EAAs added back (Figure 4.6b). Lifespan significantly decreased upon doubling the concentration of EAAs, and a further reduction was detected when quadrupling the concentration. These data further highlight that in *Drosophila*, essential amino acids play a major role in the regulation of both lifespan and fecundity.

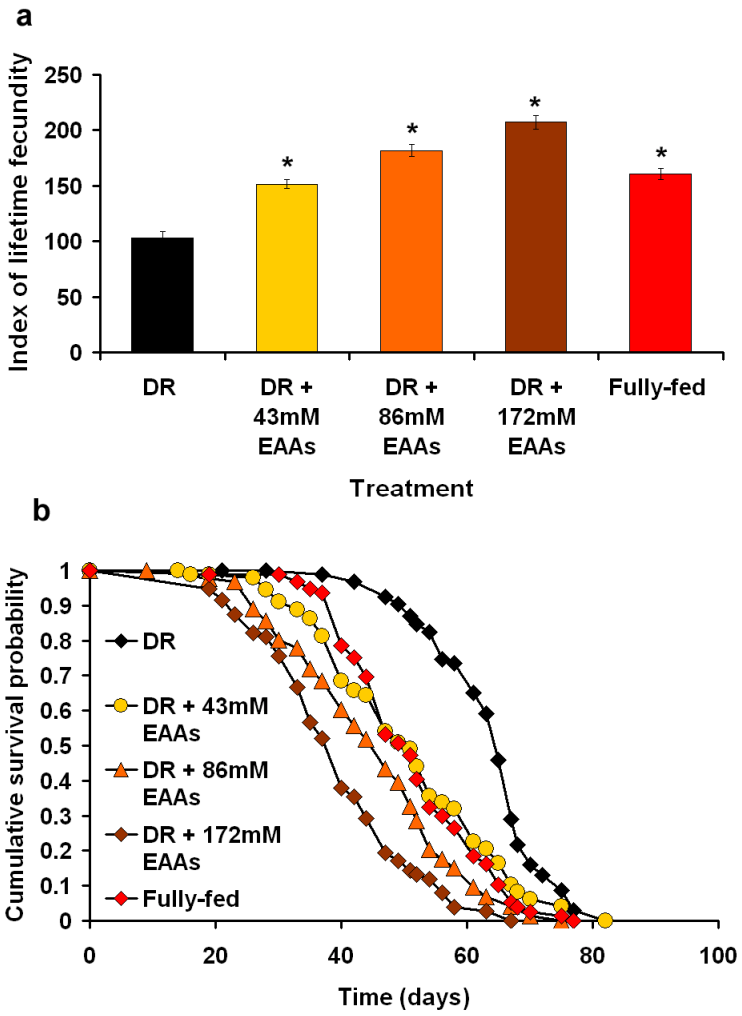

Figure 4.6: Continual trade-off of lifespan and fecundity with increasing concentrations of essential amino acids. (a) Increasing the concentration of essential amino acids (EAAs) added-back caused a progressive increase in fecundity at each increasing concentration (43mM compared with 86mM EAAs, $P = 0.0009$; 86mM compared with 172mM EAAs, $P = 0.008$, Wilcoxon). (b) In contrast, lifespan progressively decreased with each increasing concentration of EAAs added-back (43mM compared with 86mM EAAs, $P = 0.009$; 86mM compared with 172mM EAAs, $P = 0.0015$, log-rank). Fecundity assays were performed on days 6, 9, 13, 16, 20, 27, 34, 43 and 50 of treatment. Error bars represent ± s.e.m. * Denotes a significant increase in fecundity ($P < 0.0002$) when compared with DR (black) using the non-parametric Wilcoxon test.

4.3.6 Effects of manipulating larval diet on adult lifespan

It has been suggested that the environment which *Drosophila* are exposed to as larvae can have dramatic effects on adult fitness (Luckinbill and Clare 1986; Zwaan *et al.* 1991; Zwaan *et al.* 1992; Sorensen and Loeschcke 2001; Tu and Tatar 2003; Zwaan 2003; Baldal *et al.* 2005). To determine whether the reduced lifespan observed with increasing the concentration of EAAs added back to the diet was solely confined to the adult diet, 1st instar (L1) larvae were individually picked and reared at standard density (see section 2.3.3.2) on either control DR and fully-fed diets or DR diets supplemented with 43, 86 or 172mM EAAs throughout their development until adults emerged. At this point the lifespan of once-mated females from all juvenile treatments was measured on a standard 1.0 SY diet.

Exposure to different concentrations of EAAs as juveniles had no effect on the subsequent lifespan of adult flies (Figure 4.7). Larvae which had been reared under DR conditions had a similar adult median lifespan (62 days) as larvae reared on any of the EAA supplemented diets (62-65days). In addition, no effect on lifespan was observed in flies that had been reared on a fully-fed diet as larvae (median: 62 days; Figure 4.7)

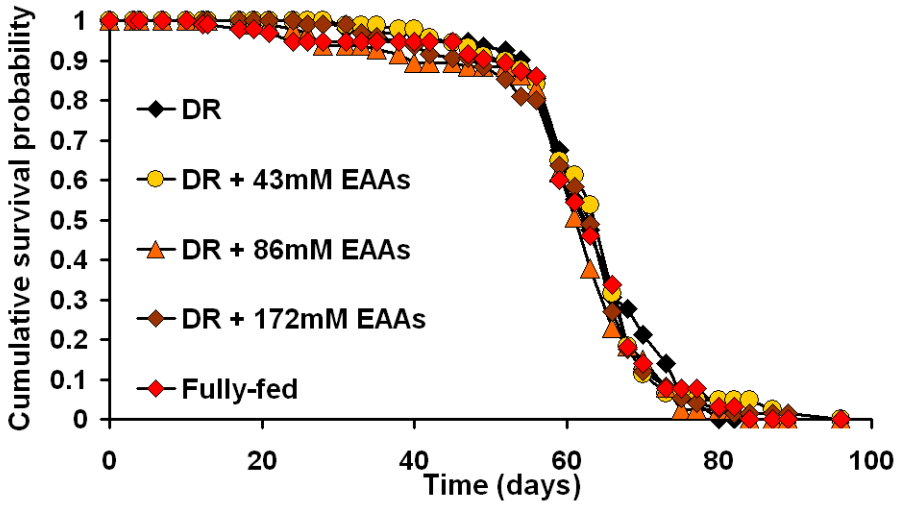

Figure 4.7: Adult lifespan not affected by juvenile diets. L1 larvae were reared on five different diets until adults emerged and then all females were switched to a standard 1.0 SY medium for the duration of their lifespan. Larvae that had been exposed to a DR diet supplemented with either 43, 86 or 172mM showed no difference in lifespan compared with larvae reared on a DR control diet (P = 0.85 - 0.2, log-rank). In addition, no significant difference was observed in flies that had been subjected to a fully-fed yeast diet as larvae (P = 0.76, log-rank).

4.3.7 Sex specific nutritional effects in adults

In females, supplementing the DR diet with amino acids during adulthood accounted for the whole increase in fecundity seen with full feeding and also significantly shortened lifespan, suggesting that amino acids are the key component of the diet that regulate a trade-off between lifespan and fecundity. Although DR has been reported to extend lifespan in males, the magnitude of response is greater in females (Magwere *et al.* 2004). This raises the possibility that the nutrients that influence lifespan in females are not the same in males.

To establish whether amino acids had a similar effect on the lifespan of males or whether indeed other nutrients played a role, the effects of adding back the same concentration of nutritional groups previously applied to females (Table 4.1) were subsequently tested in males. Adding back yeast itself (full feeding) had no effect on male lifespan compared with DR control-fed flies (Figure 4.8). Furthermore, adding back all nutrients together or amino acids alone, both of which caused a significant reduction to female lifespan, had no significant effect on the lifespan of male flies. In addition, adding back neither vitamins nor lipids had any deleterious effects to male lifespan. Interestingly, supplementing the DR diet with carbohydrates caused an increase in the median lifespan of males compared with DR (57 and 51 days respectively), but the differences amongst the two populations were not statistically significant.

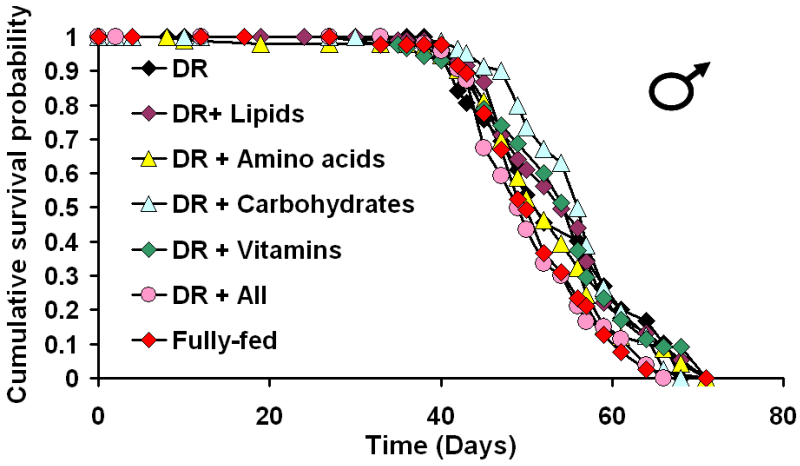

Figure 4.8: The lifespan of male *Drosophila* is not mediated by amino acids in the diet. Dietary restriction did not significantly extend the lifespan of males compared with fully-fed controls (P = 0.15, log-rank). Supplementing the DR diet with amino acids had no effect on lifespan compared with DR (P = 0.62, log-rank). Furthermore, no differences in lifespan were observed when adding back lipids (P = 0.5, log-rank), carbohydrates (P = 0.27, log-rank) or vitamins (P = 0.57, log-rank) compared with DR.

4.3.8 Effect of exposure to different add-back diets on subsequent starvation resistance

In addition to lifespan extension and reduced fecundity, another phenotype commonly associated with DR in *Drosophila* is starvation resistance early in life (Chippindale *et al.* 1993; Burger *et al.* 2007). The fact that amino acids increase fecundity and shorten lifespan similarly to full feeding leads to the question whether the starvation resistance of DR flies can be explained by the presence of fewer amino acids in the diet. This hypothesis was tested by maintaining adult flies (males and females) on different add-back diets for seven days before being switch to starvation media of 1% agar.

As previously reported (Chippindale *et al.* 1993; Burger *et al.* 2007) females subjected to a DR diet prior to starvation (section 2.2.4) proved to be starvation resistant compared to fully-fed controls (Figure 4.9a). The effect of adding back all nutrients appeared to mirror the starvation sensitivity of fully-fed flies, suggesting that one or a combination of nutrients may be responsible for starvation sensitivity caused by a fully-fed diet. Further analysis revealed that starvation sensitivity was attributable to amino acids only. Adding back amino acids reduced the starvation resistance of DR flies to a similar magnitude observed with full feeding or adding back all nutrients. Exposure to add-back diets containing vitamins, lipids or carbohydrates, prior to being switched to starvation medium, had no significant effect on starvation response compared to pre-treatment with DR.

In contrast to female flies, males subjected to either full feeding or to an amino acid add-back diet did for seven days prior to being transferred to starvation media did not show any sensitivity to starvation (Figure 4.9b). Furthermore, DR did not induce

starvation resistance in male flies. Interestingly, males fed a DR diet supplemented with carbohydrates demonstrated a small but significant resistance to starvation compared with DR controls. No other single nutrient add-back diet affected the subsequent response of flies to starvation.

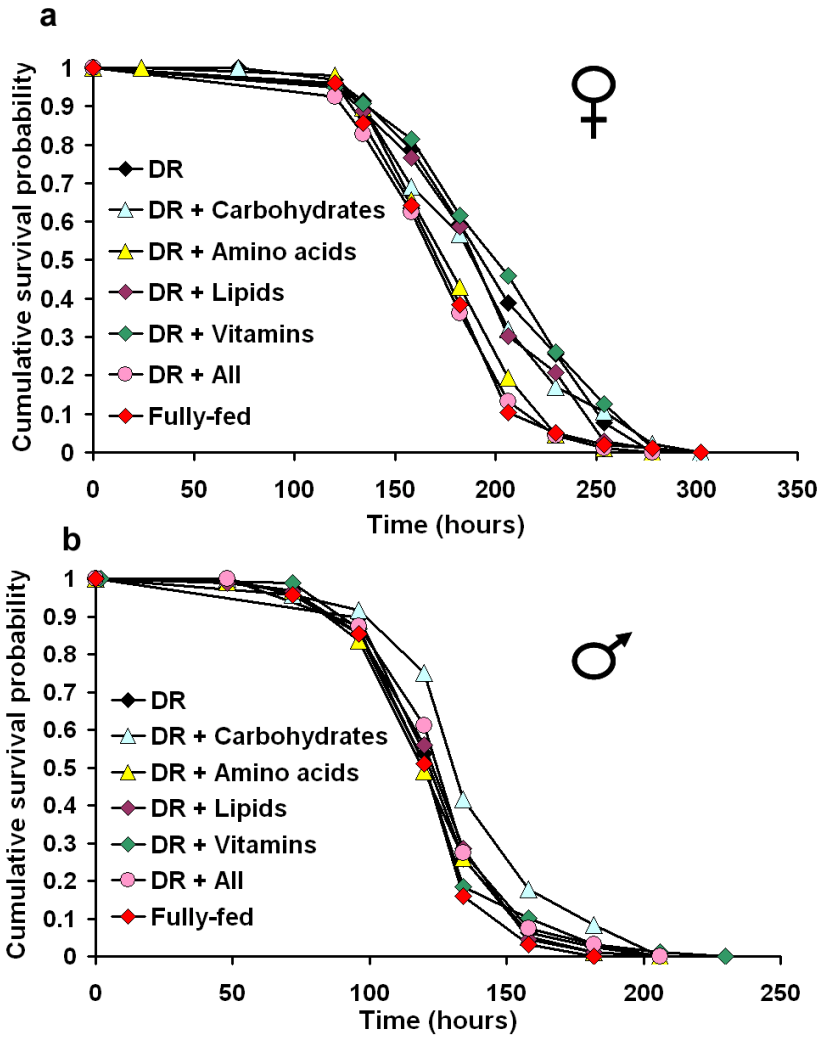

Figure 4.9: Starvation responses of flies exposed to different nutritional diets for seven days prior to starvation. Flies were maintained on their respective diets for a period of seven days before being switched to a starvation media containing 1% agar. (a) Females previously fed a DR diet were more resistant to starvation than fully-fed flies ($P < 0.0001$, log-rank). Adding back all nutrients to DR reduced the starvation resistance seen with DR alone ($P < 0.0001$, log-rank), to a similar extent seen with full feeding ($P = 0.72$, log-rank). The starvation sensitivity of fully-fed flies and flies on a DR diet supplemented with all nutrients was entirely attributable to amino acids ($P = 0.27$ - 0.43, log-rank). No significant effects on starvation were observed when flies were previous fed DR diets supplemented with lipids, carbohydrates or vitamins ($P = 0.31 – 0.58$, log-rank). (b) In males, DR did not

increase starvation resistance compared with fully-fed males (P = 0.11, log-rank). Furthermore, no differences in starvation responses were observed in flies fed a DR diet with all nutrients (P = 0.58, log-rank) or only amino acids (P = 0.5, log-rank) added back compared with DR. Interestingly, flies fed an add-back diet containing carbohydrates displayed increased starvation resistance compared with DR controls (P < 0.002, log-rank). No effect was observed when adding back vitamins or lipids (P = 0.75 – 0.95, log-rank).

4.4 Discussion

4.4.1 Amino acids and not calories regulate lifespan in *Drosophila*

In contrast to the conflicting evidence for and against caloric intake regulating lifespan extension by DR in rodents (Yu *et al.* 1985; Iwasaki *et al.* 1988; Weindruch and Walford 1988; Masoro *et al.* 1989; Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003; Miller *et al.* 2005), evidence based on experiments in *Drosophila* strongly suggests that restriction of nutrients as opposed calories regulate longevity (Mair *et al.* 2005; Bass *et al.* 2007a; Lee *et al.* 2008; Skorupa *et al.* 2008). A reduction in the concentration of yeast in the diet alone, whilst keeping the sucrose concentration fixed, is sufficient to extend lifespan of female *Drosophila* (Mair *et al.* 2005; Bass *et al.* 2007a; Lee *et al.* 2008). By breaking down the different nutritional components of the yeast and assessing their effects on both lifespan and fecundity, the current study confirms recent reports that DR extends *Drosophila* lifespan independent of calories (Mair *et al.* 2005; Bass *et al.* 2007a; Lee *et al.* 2008; Skorupa *et al.* 2008). If an increase in calories accounted for a decrease in lifespan, supplementing the DR diet with any type of nutrients would decrease lifespan. Instead, only amino acids caused a significant reduction to lifespan, coupled with increased fecundity, when added back to the DR diet. Amino acids make up approximately 45% of the nutritional content of dry yeast (Bass *et al.* 2007a), hence the fact that they are the key component of the *Drosophila* diet to regulate lifespan and fecundity is perhaps not too surprising. Furthermore, the role of amino acids in the reduction of lifespan is supported by studies in flies, worms and yeast showing that reduced target of rapamycin (TOR) signalling can extend lifespan (Vellai *et al.* 2003; Jia *et al.* 2004; Kapahi *et al.* 2004a; Meissner *et al.* 2004; Kaeberlein *et al.* 2005b; Henderson *et al.* 2006). The TOR pathway is involved in nutrient

(particularly amino acid) sensing, hence reduced TOR activity to some extent mimics the effect of reduced intake of amino acids.

Since the work presented in this chapter was performed, another study has also reported that restriction of amino acids can extend lifespan in *Drosophila* (Min and Tatar 2006b). Using casein as a source of amino acids, the authors of this study demonstrated that lifespan can be increased by decreasing casein concentration, a finding that had also been previously reported in earlier studies (Hollingsworth 1970; Van Herrewege 1974). However, in contrast to the effects of manipulating the concentration of dietary yeast (Chippindale *et al.* 1993; Chapman and Partridge 1996; Bass *et al.* 2007a; Lee *et al.* 2008), no clear increase in fecundity was associated with increasing casein concentrations. Flies on the highest casein concentration had similar survivorships patterns to flies on 0% casein (starvation), but a drastic reduction in fecundity compared to the 10% yeast control-fed flies, which were significantly longer lived. This study by Min *et al.* does not reflect DR as observed when restricting dried yeast because the trade-off between lifespan and reproduction was not clearly observed (Partridge *et al.* 2005a; Piper and Partridge 2007). Instead, their results are likely to be explained by toxicity or reduced accessibility to nutrients at higher concentrations due to the viscous nature of casein. Furthermore the diet used in the study by Min *et al.* comprised only casein and sucrose. This means that other nutrients essential for supporting healthy lifespan (Sang and King 1961) were missing, which might cause false results. The interpretation of the results of the casein study is further complicated by the fact that males and females were maintained in mixed sex groups throughout the course of the lifespan experiments. Although this approach attempts to mimic the situation in the wild, performing DR experiments with mixed-sexed groups should be avoided

because higher levels of nutrition result in increased re-mating frequencies (section 1.5.4.2) (Harshman *et al.* 1988; Chapman and Partridge 1996). This in turn reduces lifespan, potentially due to a higher cost of mating or a cost of reproduction (Chapman and Partridge 1996; Partridge and Prowse 1997).

In the current study, the negligible effects of supplementing the DR diet with vitamins, lipids or carbohydrates on lifespan and fecundity suggest that these nutrients are not important in influencing ageing or reproduction in the context examined. Previous work in *Drosophila* has shown that a deficiency of the vitamin biotin shortens lifespan and reduces fertility (Landenberger *et al.* 2004). However, a more recent study reported that culturing flies for multiple generations on a biotin-deficient diet before being switched to a standard diet results in up to 30% lifespan extension (Smith *et al.* 2007b). It is unclear from the add-back studies whether biotin, which formed part of the vitamin add-back diet, would have any effect on lifespan because the DR diet itself contained basal levels of all nutrients, including biotin. Whether or not biotin plays a specific role in lifespan regulation would therefore have to be established in a food environment completely devoid of this vitamin. In addition to a potential role of biotin in the regulation of lifespan, vitamin E has been shown to have variable effects on *Drosophila* longevity. Intermediate concentrations of vitamin E lifespan resulted in lifespan extension, whereas high concentrations caused a reduction of lifespan (Driver and Georgeou 2003). As with biotin, the semi-defined diet used in this work is likely to contain sufficient amounts of vitamin E. The effects of individual vitamins would therefore have to be investigated in a defined diet where precise regulation of all nutrients present can be achieved.

In agreement with carbohydrate supplementation having no effect on lifespan, a recent paper has reported that glucose restriction in *Drosophila* using a defined diet approach has very little effect on lifespan (Troen *et al.* 2006). This is also consistent with other reports demonstrating that manipulating carbohydrate concentration in the form of sucrose only moderately affects longevity (Mair *et al.* 2005; Bass *et al.* 2007a; Lee *et al.* 2008).

4.4.2 Do amino acids shorten lifespan as a result of nutrition or toxicity?

Chapter 3 together with the findings of Bass *et al.* (2007a) highlighted the importance of measuring fecundity when working with different diets, to ascertain whether the life-shortening effects of a given diet are not simply due to toxicity. A reduced lifespan as a result of increased toxicity would be expected to be accompanied by reduced fecundity. Hence, although directly testing toxicity is difficult, measuring fecundity throughout life is an important way of testing toxicity because it provides a parallel indicator of health and nutritional status (Piper and Partridge 2007). This means that if adding back amino acids (AAs) to the DR diet was simply resulting in a toxic effect and thereby shortening lifespan, one would expect to observe no increase or even a large decrease in fecundity. However, the fecundity of flies fed a DR diet supplemented with AAs was greatly elevated compared with DR controls.

Further evidence that the addition of amino acids results in a nutritional as opposed to a toxic effect is demonstrated by the fact that providing flies with a separate water supply did not alter the effects observed. It might have been possible that reduced water availability or osmotic stress could cause or contribute to the reduction of

lifespan attributed to AAs. Providing excess water was not able to rescue the reduced lifespan caused by AAs. However, supplementing the DR diet with 0.8 % sodium chloride (salt), a known osmotic stressor, caused a similar reduction in lifespan to AAs. In contrast to the addition of amino acids, this effect could be completely reversed upon addition of a separate water supply. These data suggest that the life-shortening effect of amino acids is unlikely to be a result of reduced water availability or increased osmotic stress.

Indirect evidence which suggests that AAs are shortening lifespan due to nutritional and not toxic effects is the fact that females maintained on a DR diet supplemented with AAs for seven days before being switched to starvation media display starvation sensitivity similarly to flies previously exposed to a fully-fed diet. Adding back vitamins, lipids or carbohydrates did not cause subsequent starvation sensitivity, suggesting that AAs are mediating a specific nutritional response as opposed to a toxic one.

The majority of the experiments to test the effects of nutrients on lifespan and fecundity were performed in female flies. However, the effect of adding back the major nutritional groups was also performed in male flies. If AAs were simply shortening lifespan as a result of increased toxicity in females, one might predict that the lifespan of males would also be reduced. However, supplementing the DR diet with the same concentration of AAs had no significant effect on male lifespan compared to DR.

Whilst these data suggest that the supplementation of amino acids to the DR diet provide a nutritional explanation for reduced lifespan as opposed to a toxic one, the

further reduction of lifespan when adding back all AAs compared with full feeding (without a further increase in fecundity), suggest the increased availability of some of the nutrients in this diet may compromise survival without benefiting fecundity. In addition, the effect of adding back all AAs or all nutrients together appears to result in a slightly different shaped survival curve to that of the control groups (Figure 4.2b), potentially due to some non-ageing related damage from excess nitrogen in the diet. The N-EAAs may account for a large part of this effect because, when added back separately from the EAAs, they caused a small but significant reduction in lifespan without any positive effect on fecundity. Future work on developing ageing markers in *Drosophila* will be required to shed further light on whether adding back amino acids to the diet is causing similar cellular and molecular phenotypes of ageing to full feeding.

4.4.3 Essential amino acids mediate the lifespan / fecundity trade-off

The addition of amino acids to a DR diet proved to be the only single nutrient addition tested that caused an increase in fecundity. To establish whether nitrogen or specific AAs were important for the increase in fecundity, the 20 AAs were divided into AAs that are thought to be essential (EAAs) and non-essential amino acids (N-EAAs) (Sang and King 1961). Interestingly, the biological available nitrogen concentration, based on the theoretical nitrogen yield that would be available if each amino acid was fully catabolised, was greater in the group of N-EAAs (50.6mM) compared with the group of EAAs (43.2mM). These differences suggest that nitrogen concentration is not important in the regulation of fecundity in this context because adding back N-EAAs had no effect on fecundity, despite providing more

available nitrogen. Instead, the increase in fecundity observed when adding back all 20 AAs or with full feeding was entirely attributable to the EAAs.

The elevated fecundity of flies fed a DR diet supplemented with EAAs also resulted in a marked reduction in lifespan, mimicking fully-fed flies. This result suggests that the lifespan / fecundity trade-off under DR is mediated predominantly by EAAs in the diet. Hence, it is conceivable that DR extends lifespan through reallocation of EAAs away from reproduction and towards maintenance and repair mechanisms, with the reverse scenario occurring upon full feeding or an EAA-enriched diet. An alternative explanation for the observed effects could be explained by a direct cost of high reproductive output, for example through physiological damage to the fly (Barnes and Partridge 2003; Partridge *et al.* 2005b). These two mechanisms need not to be mutually exclusive and could thus occur simultaneously. Further evidence that either or both of these mechanisms may explain lifespan extension by DR or reduced lifespan with full feeding was seen when the concentration of EAAs added back to the DR diet was increased. Increasing the concentration of EAAs from 43mM to 86mM caused an even greater increase in fecundity and decrease in lifespan. Furthermore, the fecundity of flies could be elevated even further when increasing the concentration to 172mM EAAs. This was accompanied by a further reduction in lifespan.

The addition of N-EAAs to the DR diet caused a small, but significant decrease in lifespan but had no effect on fecundity, suggesting that adding back N-EAAs in this context may result in mild toxicity. The small life-shortening effects attributed to N-EAAs might be explained by metabolic costs associated with removal of excess nitrogen or consequent damage, for instance to the excretory malphigian tubules, the

fly equivalent of the mammalian kidney (Wessing 1978). This may also be the cause of the further reduction in lifespan compared to full feeding when all 20 AAs were added back.

Interestingly, the responses of virgin females to adding back EAAs and N-EAAs were almost identical to the responses of once-mated females. In virgins, EAAs caused an increase in fecundity coupled with a marked reduction in lifespan, where as N-EAAs had no effect on fecundity but caused a fractional decrease in lifespan. This would suggest that the nutritional requirements of females for increased egg production are independent of their mating status. The production of fertile eggs (in once-mated females) appeared to come at a negligible cost on lifespan when compared with the production of non-viable eggs (in virgin flies).

4.4.4 Different nutritional demands of male *Drosophila*

The dietary restriction protocols used throughout these experiments had been optimised for female flies in terms of their effects on lifespan and fecundity (Bass *et al.* 2007a). In our laboratory, no work had previously been carried out to investigate the response of male *Drosophila* to a range of yeast concentrations when using the optimised Brewer yeast. Previous work had used a Baker's yeast diet (Magwere *et al.* 2004). Using the same yeast concentration for DR and fully-fed conditions as for females, male flies did not exhibit a significant response to DR, which is in contrast to the results of the previous study using Baker's yeast (Magwere *et al.* 2004). Such a result does not seem surprising, when taking into account that there are examples of several mutations in components of signalling pathways which robustly extend female lifespan but often have a negligible effect or even reduce the lifespan of

males. Examples include the insulin receptor (*InR*) and insulin receptor substrate (*chico*) mutants (Clancy *et al.* 2001; Tatar *et al.* 2001).

Males portray a reduced feeding rate compared to females (R. Wong, unpublished data). This in turn may mean that males require a different nutritional range, and may therefore explain the lack of response to DR. The concentration chosen for DR may have been too high or alternatively the concentration chosen for full feeding was too low. Ideally, the response of male lifespan should be tested across a range of yeast concentrations because it is also possible that the two concentrations picked were to the left and right of the concentration at which male lifespan peaks (Figure 1.12). Another reason for the lack of a male response to DR may be that, in contrast to females, sucrose plays a role in regulating lifespan in males. In the previous DR study with males, both the concentration of Baker's yeast and sucrose was simultaneously diluted (Magwere *et al.* 2004). Although sucrose has a negligible effect on female lifespan (Mair *et al.* 2005; Bass *et al.* 2007a; Lee *et al.* 2008), it is possible that it may be important in influencing male lifespan.

Despite no evidence of lifespan extension in males when reducing the yeast concentration (DR), the effect of adding back amino acids (AAs), carbohydrates, vitamins or lipids on lifespan was tested to assess whether individual nutrients have any effect on male lifespan when added in isolation. In contrast to females, adding back AAs had no effect on male lifespan. Only carbohydrates showed any effect on lifespan. Fascinatingly, supplementing a DR diet with carbohydrates caused an increase in median lifespan compared with DR control-fed flies. This result would suggest that, similarly to females, male lifespan is not regulated by the caloric content of the diet. The carbohydrate add-back contained four different components;

glycogen, trehalose, lactose and sucrose, with the predominant carbohydrate present being glycogen and the least abundant being sucrose (Table 4.1). The addition of these carbohydrates to the food may have extended lifespan because they provide a plentiful energy supply, supporting the high physical activity of male flies. Dissecting the different components of the carbohydrate add-back diet may reveal whether specific carbohydrates account for the differences in lifespan observed.

4.4.5 Increased starvation resistance under DR is attributable to fewer amino acids in the diet

Previous reports have suggested that flies subjected to a low yeast diet (DR) show resistance to starvation when switched from their food media to a starvation media (Chippindale *et al.* 1993; Burger *et al.* 2007); however, the resistance to starvation of DR-fed flies is only evident early in life (Burger *et al.* 2007). In the current study, young dietary restricted flies also exhibited a stronger resistance to starvation compared with fully-fed flies. In order to try and understand the mechanisms behind starvation resistance in dietary-restricted flies, specific nutrients that may induce starvation resistance on DR food or starvation sensitivity with full feeding were tested. Female flies previously exposed to carbohydrate, vitamin or lipid add-back diets displayed no starvation sensitivity compared with DR controls. In contrast, females exposed to an amino acid add-back diet prior to starvation displayed starvation sensitivity that mimicked the response of flies previously exposed to full feeding. These results mirrored the lifespan effects observed with the add-back diets, where only the addition of amino acids led to a reduction in lifespan. It thus seems that amino acids can account for the full differences between DR and fully-fed conditions. One possible explanation for this is that flies on fully-fed or an amino acid add-back diet, allocate amino acids heavily into reproduction and not somatic

maintenance. Hence when a starvation period begins, DR flies are more resistant because they have been investing their resources into repair and maintenance and are more adapted to survive the starvation period. It was noticeable that up to the first two days on starvation media, fully fed flies and flies on the AA add-back diets continued to invest resources heavily into reproduction, despite being exposed to starvation media.

The responses of males to starvation differed from the responses of females, and once again similar trends in results were observed to the lifespan effects of the different diets. Prior exposure to the amino acid add-back diet or full feeding had no effect on subsequent starvation response compared with the effect of DR. Only supplementing the DR diet with carbohydrates evoked a starvation response, whereby males subsequently displayed starvation resistance. Thus once again the increased lifespan is associated with an increased starvation resistance. The different responses of males and females to starvation suggest that different mechanisms may be involved. Females appear to be only sensitive to starvation when they have been fed a diet enriched with amino acids whereas males display starvation resistance upon supplementing the diet with carbohydrates. Another interesting consideration is that DR protects against starvation only early in life, and has a negative effect later in life (Burger *et al.* 2007). This aspect was not explored in these studies, but provides an interesting basis for further investigation of the effects of different individual nutrients on starvation resistance in flies. Fecundity is known to decrease with age (Hamilton 1966; Rauser *et al.* 2003; Burger *et al.* 2007), therefore fully-fed flies are unable to produce the quantities of eggs observed in the first few weeks and hence later in life may be able to invest more nutrients towards somatic maintenance and

repair than DR flies can, potentially increasing their resistance to starvation. These results further highlight the complex sex specific interaction with different nutrients.

Similarly to DR extending lifespan and inducing starvation resistance, albeit in young flies, mutations in genes including *chico* (Clancy *et al.* 2001) and *methuselah* (Lin *et al.* 1998) both extend lifespan and increase starvation resistance. In addition, ablation of the *Drosophila* insulin-like peptide producing cells (Broughton *et al.* 2005) and over-expression of the *Drosophila* homologue of apolipoprotein D (Walker *et al.* 2006) lead to similar lifespan extension and starvation resistant phenotypes. These data suggest that starvation resistance may serve as a good screen for detecting lifespan extending interventions or mutations, with the added advantage that starvation assays yield results in a very short time frame (Rose *et al.* 1992; Wang *et al.* 2004). However, an interesting exception to this hypothesis has been reported whereby a negative correlation between lifespan and starvation resistance in five wild-caught *Drosophila simulans* populations was reported (Ballard *et al.* 2008). Further work would be required to dissect further the fascinating interaction between longevity and starvation resistance.

4.4.6 Regulation of lifespan by amino acids is confined to adult flies

The environmental conditions in which larvae are reared are known to play a role in effecting development time, body size and adult longevity (Luckinbill and Clare 1986; Zwaan *et al.* 1991; Zwaan *et al.* 1992; Sorensen and Loeschcke 2001; Tu and Tatar 2003; Zwaan 2003; Baldal *et al.* 2005). One intervention that delays development time and reduces body size is dietary manipulation (Zwaan *et al.* 1991;

Tu and Tatar 2003). However, the effects of manipulating juvenile diets on adult lifespan are less clear. Reports as to whether there is a correlation between reduced food intake as larvae and extended lifespan as adults are conflicting (Zwaan *et al.* 1991; Tu and Tatar 2003). In this study, the effect of DR and in particular increasing concentration of amino acids added back to DR was investigated to determine whether extreme changes in diet which larvae are exposed to could cause changes to adult lifespan. In agreement with Tu and Tatar (2003), no effect on adult lifespan was seen when larvae were fed a dietary restricted diet. Furthermore, adult lifespan was unaffected by the exposure of larvae to an increasing concentration range of EAAs, suggesting that the regulation of lifespan by EAAs is confined solely by the adult diet.

4.4.7 Concluding remarks

The work carried out in this chapter set out to address which nutrients present in the dietary yeast can account for the high fecundity of flies maintained on a fully-fed diet, and whether some of these same nutrients reduce lifespan as predicted by the reallocation of resources hypothesis (Figure 4.1). By adding back different nutrient groups to a DR diet, it was shown that essential amino acids mediate the trade-off between lifespan and fecundity observed when manipulating the concentration of dietary yeast in the food, with other nutrient having negligible effects. Reallocation of essential amino acids away from reproduction and towards somatic maintenance and repair could hence be the mechanism that extends lifespan under DR in *Drosophila*. However, another explanation could be that a higher concentration of essential amino acids in the diet increases fecundity, which shortens lifespan due to damage inflicted by elevated fecundity. Alternatively, different essential amino acids

could be mediating the responses of lifespan and fecundity. The role of specific essential amino acids and possible signalling pathways involved in the response to these amino acids will be discussed in chapter 5.

Chapter 5

Uncoupling the responses of lifespan and fecundity in *Drosophila* dietary restriction

5.1 Introduction

In *Drosophila*, the effects of dietary restriction (DR) have been shown to be regulated by the concentration of the yeast in the diet as opposed to the concentration of sucrose, independent of caloric intake (Mair *et al.* 2005; Bass *et al.* 2007a; Lee *et al.* 2008). The data presented in chapter 4 clearly revealed that essential amino acids (EAAs) are the key component of dietary yeast mediating a direct trade-off between lifespan and fecundity. One interpretation of these data could be that DR induces a reallocation of EAAs from reproduction and towards somatic maintenance and repair, as an evolved response to food shortages in nature (Williams 1966; Kirkwood and Holliday 1979; van Noordwijk and de Jong 1986; Holliday 1989; De Jong 1993; Kirkwood and Shanley 2005). The observed reduction of lifespan by increasing the amount of EAAs present in the diet might also be explained by direct damage inflicted by higher reproductive output (Barnes and Partridge 2003; Partridge *et al.* 2005a). However, an alternative hypothesis is that individual EAAs regulate lifespan and fecundity through independent mechanisms.

To test which of these hypotheses may apply in *Drosophila*, the role of the individual EAAs in regulating lifespan and fecundity would need to be explored more carefully to test whether supplementing the DR diet with specific EAAs to the food can account for the phenotypes observed in flies fed a diet supplemented with all EAAs or with full feeding. In rodents, lifespan can be extended by the restriction of either methionine (Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003; Miller *et al.* 2005) or tryptophan (De Marte and Enesco 1986), both of which are essential amino acids for mammals and *Drosophila* (Sang and King 1961). It is therefore important to determine whether extension of lifespan by methionine

restriction, for example, is evolutionary conserved between rodents and *Drosophila* or whether this is a "private" mechanism unique to mammals.

The second part of this chapter will highlight the investigation of potential mechanisms involved in the responses of flies to EAA-supplemented diets. Some of the likely candidate pathways include the TOR/S6K pathway and the IIS pathway. Previous work has demonstrated that down-regulation of various components of the TOR pathway can extend lifespan in flies, worms and yeast (Vellai *et al.* 2003; Jia *et al.* 2004; Kapahi *et al.* 2004a; Meissner *et al.* 2004; Kaeberlein *et al.* 2005b; Henderson *et al.* 2006). The TOR pathway is involved in nutrient (particularly amino acid) sensing. Hence loss of TOR function may mimic amino acid deprivation. In mammals mTOR negatively regulates protein synthesis and positively up-regulates autophagy under conditions of reduced nutrients, such as amino acid limitation. In *Drosophila* it appears that the longevity of flies with over-expression of *dTsc2* , a negative regulator of TOR, is dependent on the level of nutrition, with lifespan only being extended on higher food concentrations (Kapahi *et al.* 2004a).

A second candidate pathway involved in the regulation of the effects of amino acids in the diet is the IIS pathway. The insulin receptor is activated by *Drosophila* insulin-like peptides (DILPs), which in larvae are primarily secreted in response to increased food (particularly carbohydrates) uptake (Brogiolo *et al.* 2001; Colombani *et al.* 2003). However, studies have shown that the IIS pathway plays an important role in regulating growth, development, stress resistance, lifespan and reproduction (Giannakou and Partridge 2007). The three latter phenotypes are also affected by EAAs in the diet, hence a connection between amino acids in the diet and the IIS pathway seems possible. In *Drosophila,* lifespan can be extended by ablation of the

median neurosecretory cells (mNSC) (Broughton *et al.* 2005) which produce DILPs, over-expression of *dFOXO* in the adult fat body (Giannakou *et al.* 2004) and the head fat body (Hwangbo *et al.* 2004), mutations in the insulin receptor substrate protein CHICO (Clancy *et al.* 2001; Libert *et al.* 2008), and a specific heteroallelic combination resulting in loss of the insulin receptor (Tatar *et al.* 2001). Furthermore, a complex interaction between diet and the longevity of IIS pathway mutants suggests that lifespan extension of some of these mutants is dependent on either higher levels of nutrition (Clancy *et al.* 2002; Giannakou *et al.* 2008; Min *et al.* 2008) or lower levels of nutrition (Min *et al.* 2008).

Both the TOR/S6K and IIS pathways will be investigated for their roles in the response to the presence of EAA-supplemented diets to determine whether the fecundity increase and lifespan shortening traits observed when adding back all EAAs are regulated by either of these pathways.

5.2 Methods

5.2.1 Nutrient add-back diets

All nutrient additions were prepared as described in chapter 4 (section 4.2.1).

5.2.2 Removal of *Wolbachia* infection

Wolbachia was removed from the infected Dahomey population through tetracycline treatment, as outlined in section 2.4.5, and was verified by PCR using primers to detect the gene for *Wolbachia* surface protein (wsp) (Braig *et al.* 1998; Zhou *et al.* 1998; Toivonen *et al.* 2007).

5.2.3 Direct feeding observations

Feeding assays were performed on the following days: 6, 9, 16, 20, 28, 36 and 43. The data presented represents the proportion of flies feeding as a percentage of the total number of feeding opportunities (total observations) on each day the assay was performed. Detailed methods on feeding behaviour and calibration by measuring blue dye uptake are described in sections 2.4.4 and 2.4.5.

5.2.4 Insulin receptor dominant negative flies (section 2.1.3)

The dUAS-*InRDN* transgene has an amino acid substitution in the kinase domain (arginine 1409 replaced by alanine, R1409A) of the insulin receptor (InR). This results in dominant negative behaviour of the protein (Wu *et al.* 2005). Expression of dUAS-I*nRDN* was driven by the ubiquitous and constitutive driver daughterlessGAL4 (daGAL4). Both the daGAL4 driver and the effector (UAS-*dInRDN*) lines had previously been backcrossed extensively into the white Dahomey

(Wdah) background to avoid heterosis[16] (section 1.4.4.4). Parental flies were reared for one generation at standard density (section 2.3.3) on 1.0 SY medium. Virgin females of the Wdah and daGAL4 lines were collected over ice. All lines were infected with the intracellular bacteria *Wolbachia*. The following crosses were setup:

1) UAS– *dInRDN* ♂ x daGAL4 ♀ (virgins) = UAS– *dInRDN* / daGAL4
2) Wdah ♂ x daGAL4 ♀ (virgins) = daGAL4 / +
3) UAS- *dInRDN* ♂ x Wdah ♀ (virgins) = UAS- *dInRDN* / +
4) Wdah ♂ x Wdah ♀ = + / +

The cross for the experimental line (1) was set up two days prior to the other three crosses because, similarly to other insulin signalling pathway mutant flies (Bohni *et al.* 1999; Tatar *et al.* 2001), *dInR* dominant-negative flies are developmentally delayed by approximately two days. The first cross was performed with daGAL4 femlaes and UAS- *dInRDN* males as opposed to the reciprocal cross to try and ensure that was no leaky expression of the driver. The second and third crosses (2 and 3) were performed because the driver and UAS lines are homozygous and hence need to be crossed to wild type flies (Wdah) to ensure they are heterozygous like the experimental line (1). Wild type controls (4) are required to ensure there are no insertional effects of the driver. In addition to being developmentally delayed, *dInR* dominant-negative flies are also small (dwarf flies) and have very low fecundity compared to controls. These phenotypes are also associated with other insulin pathway mutants, including *chico* homozygotes and some insulin receptor mutants (Bohni *et al.* 1999; Clancy *et al.* 2001; Tatar *et al.* 2001).

[16] Backcrossing of daGAL4 and UAS-dInRDN lines performed by T. Ikeya

All crosses were set up in small cages containing a grape plate supplemented with a spot of hydrated live yeast. Parents were allowed to lay eggs for a period of eight hours before being discarded. L1 larvae from each cross were picked 24 hours later and carefully placed into vials containing 1.0 SY medium at a density of 40 larvae per vial (section 2.3.3.1). Emerging experimental flies were tipped into fresh bottles of 1.0 SY medium and allowed to mate for 48 hours. Females were collected under light CO_2 anaesthesia and allocated to either a DR control diet, a DR diet supplemented with 1.4mM methionine or a DR diet supplemented with all 10 EAAs.

5.2.5 S6 kinase dominant negative flies (section 2.1.4)

The dUAS-*S6K*^{KQ} flies were generated by mutating a conserved lysine (K_{100}) in the ATP binding site of S6K1 and replacing it with glutamine (Q) (Barcelo and Stewart 2002). Expression of dUAS-*S6K*^{KQ} was driven by the ubiquitous daughterlessGAL4 driver (daGAL4). The daughterlessGAL4 driver (daGAL4) and effector (UAS-*dS6K*^{KQ}) lines had previously been backcrossed into white Dahomey[17]. All lines had previously undergone tetracycline treatment, at least five generations before experiments began, to remove the intracellular bacterium *Wolbachia* from the population (section 2.4.5). Parental flies were reared for one generation at standard density (section 2.3.3) on 1.0 SY medium. Wild type white Dahomey (Wdah) and daGAL4 female virgins were collected over ice. Crosses were set up as outlined below in 1 litre population cages. Eggs were laid over an eight hour period and reared at standard density in bottles containing SY medium. Emerging flies were transferred to fresh medium and allowed to mate for 48 hour. Females were collected over light CO_2 anaesthesia and randomly allocated to either the control DR diet, a

[17] Backcrossing of daGAL4 and UAS-*dS6K*^{KQ} performed by C. Slack

DR diet supplemented with 1.4mM methionine, or a DR diet supplemented with all 10 EAAs.

1) UAS–*dS6K*^{KQ} ♂ x daGAL4 ♀ (virgins) = UAS– *dS6K*^{KQ} / daGAL4
2) Wdah ♂ x daGAL4 ♀ (virgins) = daGAL4 / +
3) UAS– *dS6K*^{KQ} ♂ x Wdah ♀ (virgins) = UAS– *dS6K*^{KQ} / +
4) Wdah ♂ x Wdah ♀ = + / +

Ectopic expression of the wild-type UAS-dS6K in the dorsal compartment of the wing imaginal disc, under the control of ap-GAL4 driver, causes adult wings to bend downwards, suggesting that dorsal surface of the wing is increased due to increased growth (Montagne *et al.* 1999; Barcelo and Stewart 2002). In contrast, due to the role of S6K in growth, a dominant-negative form of *dS6K* when expressed in the dorsal wing should have the opposite effect and cause the wings to bend up due to decreased growth in the dorsal surface (Barcelo and Stewart 2002). Crossing dUAS-*S6K*^{KQ} flies with the ap-GAL4 driver caused the wings to bend upwards slightly, thus suggesting that these flies were *dS6K* dominant-negative[18].

[18] Crossing of dUAS-*S6K*^{KQ}flies with ap-GAL4 flies to confirm dominant negative activity was performed by C. Slack.

5.3 Results

5.3.1 Restriction of specific essential amino acids (EAAs)

The results of chapter 4 highlighted a role for EAAs in mediating both the increase in fecundity and reduction in lifespan observed with full feeding. Two of the amino acids which make up the EAAs are methionine and tryptophan, which are particularly interesting because restriction of either of these two EAAs has been shown to extend the lifespan of rodents (De Marte and Enesco 1986; Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003; Miller *et al.* 2005). Using the same methodological approach adopted in chapter 4, the effect of adding back all EAAs with the exception of either methionine (methionine restriction) or tryptophan (trytophan restriction) was assessed to determine whether omission of these specific EAAs from the EAA add-back diet could recover the reduced lifespan observed when adding back all 10 EAAs. This study enables us to determine whether restriction of these amino acids can extend lifespan in *Drosophila*, as previously observed in rodents, hence establishing whether the lifespan extending phenotype of restricting these EAAs is evolutionary conserved between flies and rodents. In addition, the effect of methionine and tryptophan restriction on fecundity was tested to establish whether the presence of either of these two EAAs is essential for the increased fecundity observed on the all EAA add-back diet or under full feeding conditions. Adding back all EAAs except histidine (histidine restriction) was chosen as a control, because it is from a structurally different class of amino acids (basic polar) compared to methionine and tryptophan (neutral non-polar), yet forms part of the EAA pool.

As previously observed, flies fed a DR diet supplemented with all 10 EAAs showed a marked increase in fecundity compared with DR controls, similarly to the effects of full feeding (Figure 5.1a). In contrast, adding back all EAAs except methionine, tryptophan and histidine had no effect on fecundity, suggesting that a combination of these three EAAs was required for the increased fecundity observed with full feeding. Restriction of either tryptophan or histidine alone restored the full increase in fecundity seen when adding back all 10 EAAs. Surprisingly, restriction of methionine did not increase fecundity beyond the level seen with DR control flies. This suggests that the presence of methionine is essential for the elevated fecundity observed when adding back all EAAs together and with full feeding.

The reduced lifespan observed when adding back all EAAs was partially rescued by removal of methionine, histidine and tryptophan simultaneously (Figure 5.1b). Removal of the amino acids individually showed that this effect was entirely attributable to methionine restriction. Removal of methionine alone extended lifespan to the same magnitude observed when restricting these three EAAs simultaneously. Interestingly, methionine restriction extended lifespan to the same extent as the DR control diet, indicating that methionine restriction produced a complete rescue in lifespan from the effects of adding back all EAAs. In contrast, removal of neither tryptophan nor histidine could rescue the life shortening effects of adding back all 10 EAAs.

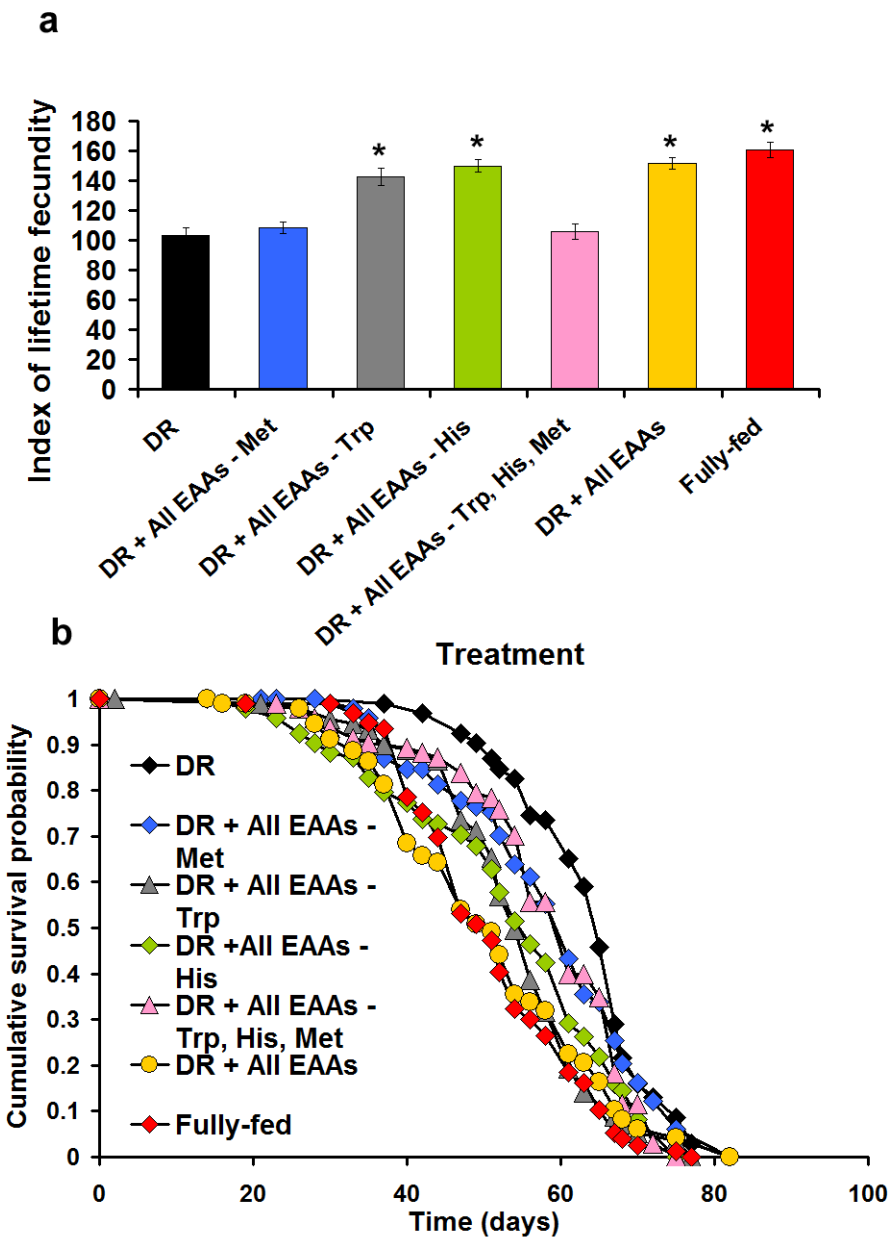

Figure 5.1: Methionine restriction reduces fecundity and extends lifespan in *Drosophila*. (a) Adding back all essential amino acids (EAAs) again caused a significant increase in fecundity, to a similar level with full feeding (P = 0.2, Wilcoxon). Adding back all EAAs except methionine, tryptophan and histidine resulted in fecundity being reduced down to the level seen with DR alone. Furthermore, this reduction in fecundity was entirely attributable to methionine restriction (-met) which had no significant effect on fecundity compared with DR (P = 0.29, Wilcoxon). In contrast, adding back all EAAs except tryptophan (- trp) or histidine (- his) resulted in an increase in fecundity to the level seen with supplementation with all EAAs or full feeding (- trp compared with EAAs, P = 0.26; - his compared with EAAs, P = 0.65, Wilcoxon). Fecundity assays were performed on days: 6, 9, 13, 16, 20, 27, 34, 43 and 50 of treatment. (b) Adding back all EAAs except methionine (-

met) rescued the reduction in lifespan caused by all 10 EAAs (- met compared with all EAAs, $P < 0.0015$, log-rank). Methionine restriction could fully recover lifespan to the same magnitude as DR flies ($P = 0.088$, log-rank). Conversely, tryptophan (- trp) or histidine (- his) restriction was not able to rescue the life shortening effects of adding back all EAAs (- trp or - his compared with all EAAs, $P \leq 0.29$, log-rank).* Indicates a significant increase in fecundity ($P < 0.0001$) when compared with DR (black) using the non-parametric Wilcoxon test. Error bars represent ± s.e.m.

5.3.2 Supplementing the DR diet with methionine

Adding back all EAAs except methionine (methionine restriction) was shown to have no effect on fecundity and could rescue the lifespan shortening effects of adding back all 10 EAAs. These results imply that reallocation of methionine away from reproduction towards somatic maintenance and repair could be the mechanism behind lifespan extension by DR in *Drosophila*. To determine whether methionine alone was sufficient for the entire increase in fecundity seen with adding back all 10 EAAs, the DR diet was supplemented with 1.4mM methionine (Table 4.1) in the absence of all other EAAs (other than those present in the yeast base diet).

Methionine supplementation (1.4mM) caused a significant increase in fecundity compared to DR. This increase reached the same magnitude seen when adding back all 10 EAAs and under full feeding conditions (Figure 5.2a). Adding back a range of methionine concentrations (0.7mM to 13mM) revealed that methionine could increase fecundity at each concentration tested (Figure 5.2b). However, no clear trend of an additional increase in fecundity was observed with increasing methionine concentrations, as had been previously detected when the concentration of all 10 EAAs was increased (Figure 4.6), suggesting a further increase in fecundity requires the addition of other EAAs that may at this point be limiting in the food. Interestingly, only 0.7mM, which represents half the concentration of methionine

originally used, was required to produce the full increase in fecundity observed when adding back all 10 EAAs.

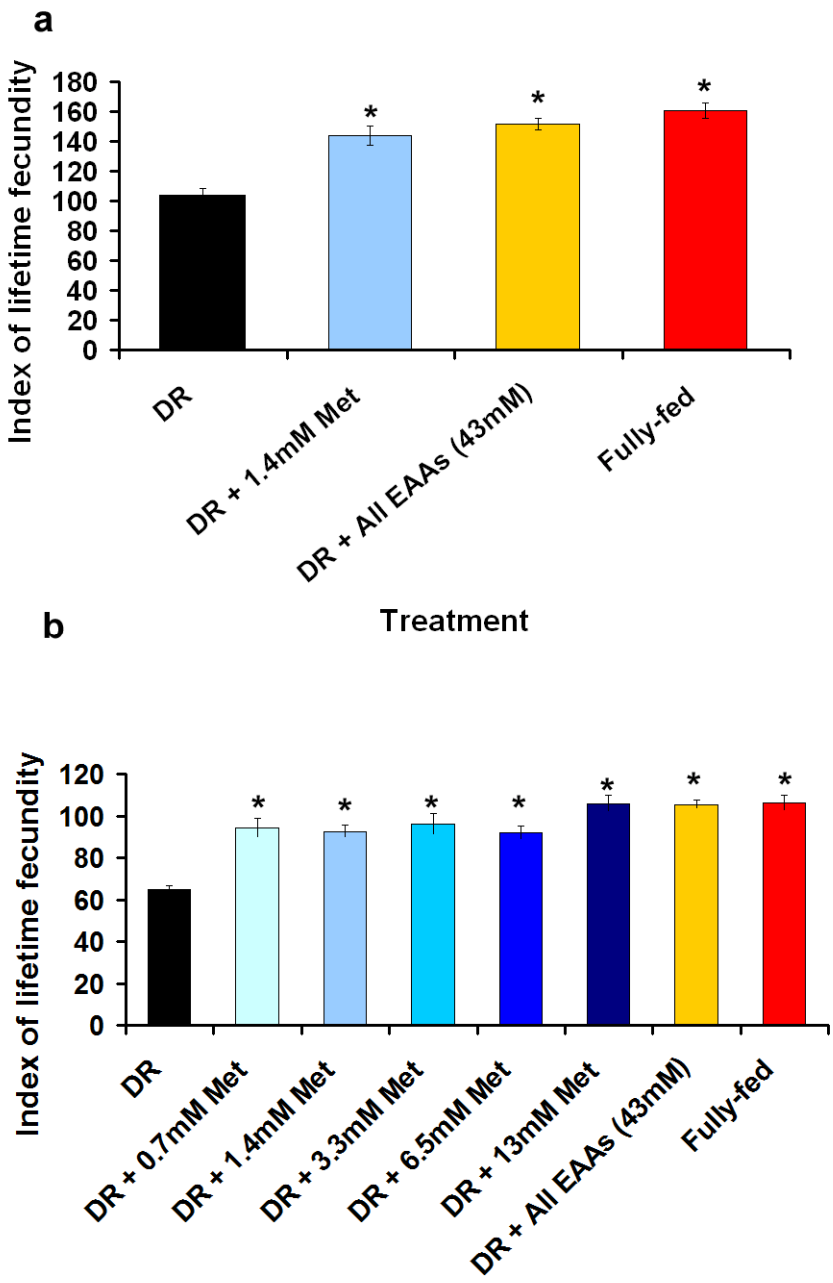

Figure 5.2 Supplementation of the DR diet with methionine entirely accounts for the higher fecundity of fully-fed flies. (a) Adding back methionine (met) alone at the concentration present in the all EAA add-back (1.4mM) increased fecundity to the levels seen with all EAAs and full feeding (+ met compared with all EAAs, P = 0.41; + met compared with fully-fed, P = 0.10, Wilcoxon). Fecundity assays were performed on days 6, 9, 13, 16, 20, 27, 34, 43 and 50 of treatment. (b) Adding back methionine in a range of concentrations from 0.7 to 13mM caused a significant increase in fecundity at all

concentrations compared with DR. No further increase in fecundity was observed when adding back higher concentrations of methionine. Fecundity assays were performed on days 5, 13, 20, 27, 34 and 41 of treatment. * Indicates a significant increase in fecundity ($P < 0.0001$) when compared with DR (black) using the non-parametric Wilcoxon test. Error bars represent ± s.e.m.

These data reveal that methionine supplementation is sufficient for the increase in fecundity observed with full feeding and therefore should trade-off with a reduction in lifespan. However, remarkably, the increased fecundity observed upon methionine supplementation did not come at a cost of reduced lifespan (Figure 5.3a). No differences in lifespan were observed between flies maintained on a DR diet supplemented with methionine and DR control-fed flies. In contrast, fully-fed flies or flies fed a DR diet supplemented with all 10 EAAs showed a marked reduction in lifespan, despite exhibiting an increase in fecundity to a similar magnitude observed with methionine supplementation alone. Furthermore, no significant decrease in lifespan was observed upon supplementing the DR diet with a range of methionine concentrations (Figure 5.3b), again despite these additions all significantly increasing lifetime fecundity.

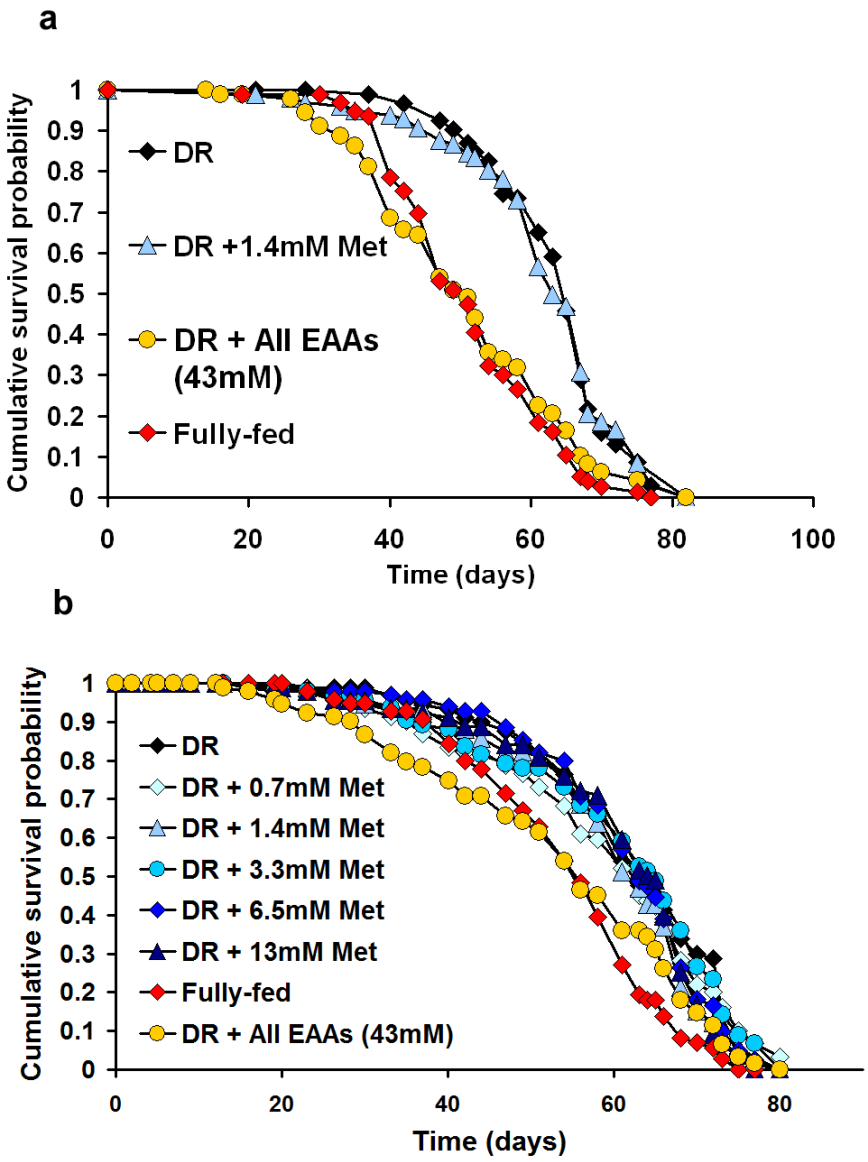

Figure 5.3: Methionine supplementation has no effect on lifespan. (a) Adding back methionine (met) at the concentration found in the all essential amino acid (EAA) add-back diet (1.4mM) caused no significant effect on lifespan compared with DR (P = 0.89, log-rank). As previously observed, adding back all essential amino acids (all EAAs) significantly reduced lifespan (P < 0.0001, log-rank) to a similar extent to full feeding (P = 0.63, log-rank). (b) No significant decrease in lifespan was detected in flies fed DR diets supplemented with a range of methionine concentrations (0.7 to 13mM) compared with DR control flies ($P \geq 0.25$, log-rank).

5.3.3 A further increase in fecundity requires the presence of other EAAs

Despite methionine supplementation alone causing a remarkable rise in fecundity, increasing the concentration of methionine further (than the initially used concentration) did not increase fecundity beyond a threshold (Figure 5.2a). This suggests that methionine is limiting in the DR diet for fecundity as opposed to acting as a signal regulating fecundity. In contrast, increasing the concentration of all EAAs added back was sufficient to cause a dose-response effect on both fecundity and lifespan (Figure 4.6). Therefore, the addition of other, now limiting EAAs should result in a further fecundity increase in flies fed methionine-supplemented diets, which proved to be the case. Increasing the concentration of all EAAs, whilst keeping the concentration of methionine fixed at 1.4mM, caused a further increase in fecundity (Figure 5.4a). However a plateau was reached at a higher concentration of EAAs (84mM) whereby methionine was once again limiting. Supplementing the DR diet with a higher concentration of methionine (6.5mM) followed by increasing the concentration of all other EAAs lead to a progressive increase in fecundity with each concentration increase. As previously observed, when increasing the concentration of all 10 EAAs (Figure 4.6), a further increase in fecundity was coupled with a greater decline in survival at both methionine concentrations tested (Figure 5.4b).

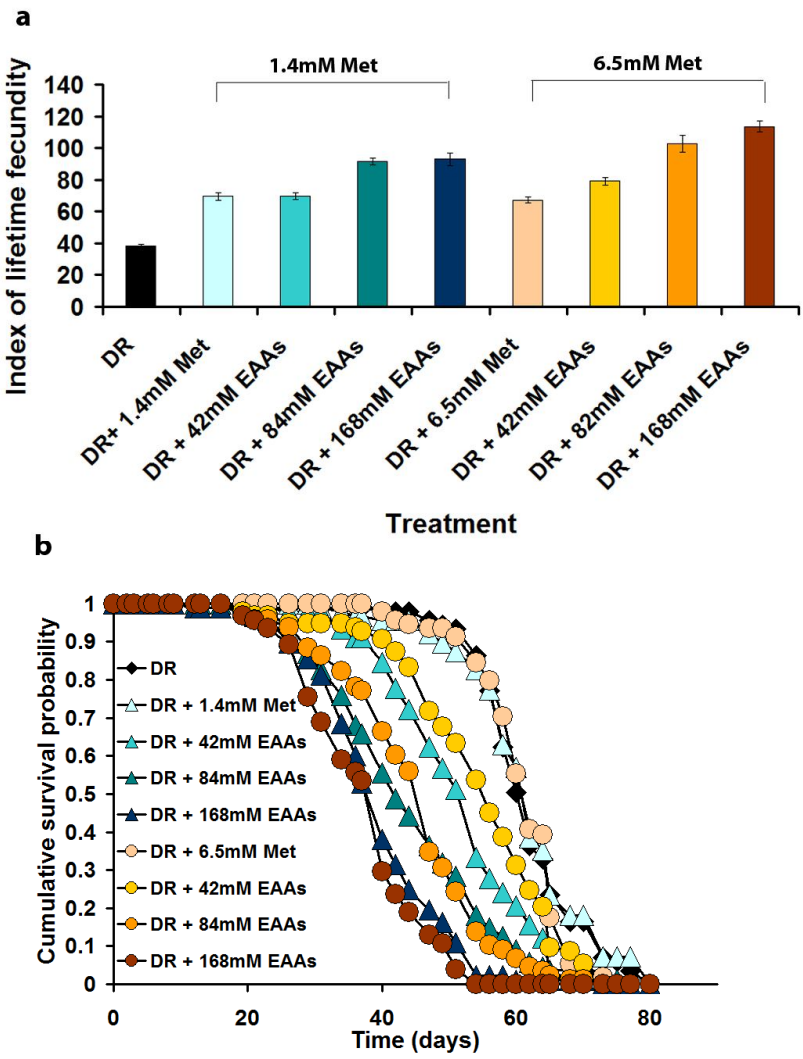

Figure 5.4: Increasing fecundity associated with higher concentrations of essential amino acids depends upon sufficient methionine concentration in the diet. (a) In the presence of a low concentration (1.4mM) of methionine (met), increasing the concentration of all other essential amino acids (EAAs) increased fecundity until a plateau was reached (84mM EAAs) after which no further effect on fecundity was observed upon the addition of increased concentration EAAs (84mM compared with 168mM, $P = 0.82$, Wilcoxon). Adding back a higher concentration of methionine (6.5mM) and increasing the concentration of all other EAAs to 168mM resulted in a further increase in fecundity compared with flies fed the same concentration of EAAs with only 1.4mM methionine added back ($P = 0.0015$, Wilcoxon). (b) Lifespan decreased with each increasing EAA concentration added back in the presence of low (triangles) and higher (circles) concentrations of methionine. Fecundity assays were performed on days 6, 9, 13, 20, 27, 37 and 41 of treatmen. Error bars represent ± s.e.m.

5.3.4 Methionine supplementation does not alter feeding behaviour

One possible explanation for the increased fecundity of flies fed a DR diet supplemented with methionine could be elevated feeding in response to methionine. To test this hypothesis, direct feeding observations were performed by examining proboscis extension (Mair *et al.* 2005) at intervals throughout life (section 2.4.4). Direct feeding observations revealed that there was no trend towards elevated feeding of flies fed DR diets supplemented with either methionine or all EAAs on any given day the assay was performed (Figure 5.5). As previously reported using this method (Mair *et al.* 2005; Wong *et al.* 2008), DR control flies did not compensate by increasing their feeding rate compared with fully-fed yeast controls.

In order to suitably calibrate feeding behaviour with the rate of food intake, the feeding behaviour of flies on blue dye labelled food was measured for a period of half an hour (on day seven on treatment) prior to measuring absorbance of blue dye in the flies (section 2.4.4.1). This method of calibration has previously been adopted in the assessment of the food intake of dietary-restricted and fully-fed flies (Wong *et al.* 2008). No significant differences were detected in feeding behaviour on DR diets supplemented with either methionine or all EAAs compared with DR and fully-fed controls on blue-labelled food (Figure 5.6a). In agreement with the assessment of proboscis extension, no significant differences were detected in blue dye uptake between DR control flies and fully-fed controls, or flies fed DR diets supplemented with either methionine or all EAAs (Figure 5.6b). These results imply that the differences in fecundity between DR control-fed flies and flies fed DR diets supplemented with methionine or all EAAs can not be attributed to an increase in feeding frequency or food uptake.

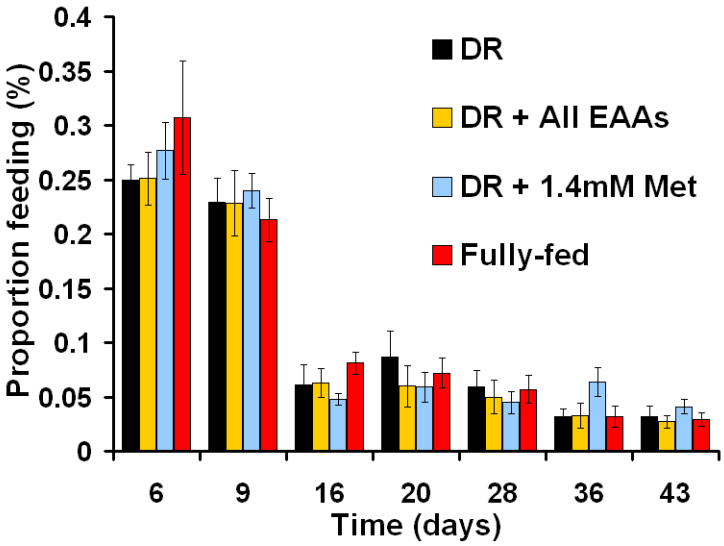

Figure 5.5: Feeding behaviour is unaffected by methionine supplementation. Adding back 1.4mM methionine to the DR diet had no significant effect on feeding behaviour, as assessed by proboscis extension assays, on any given day compared with flies maintained on DR diet ($P \geq 0.07$). In addition, no significant differences were detected between fully-fed flies or flies fed a DR diet with all essential amino acids (all EAAs) added back compared with DR controls on any given day, with the exception of day 16 where a small but significant increase in feeding behaviour was detected ($P = 0.036$). Feeding assays were performed on days 6, 9, 16, 20, 28, 36 and 43 of treatment. Error bars represent ± s.e.m.

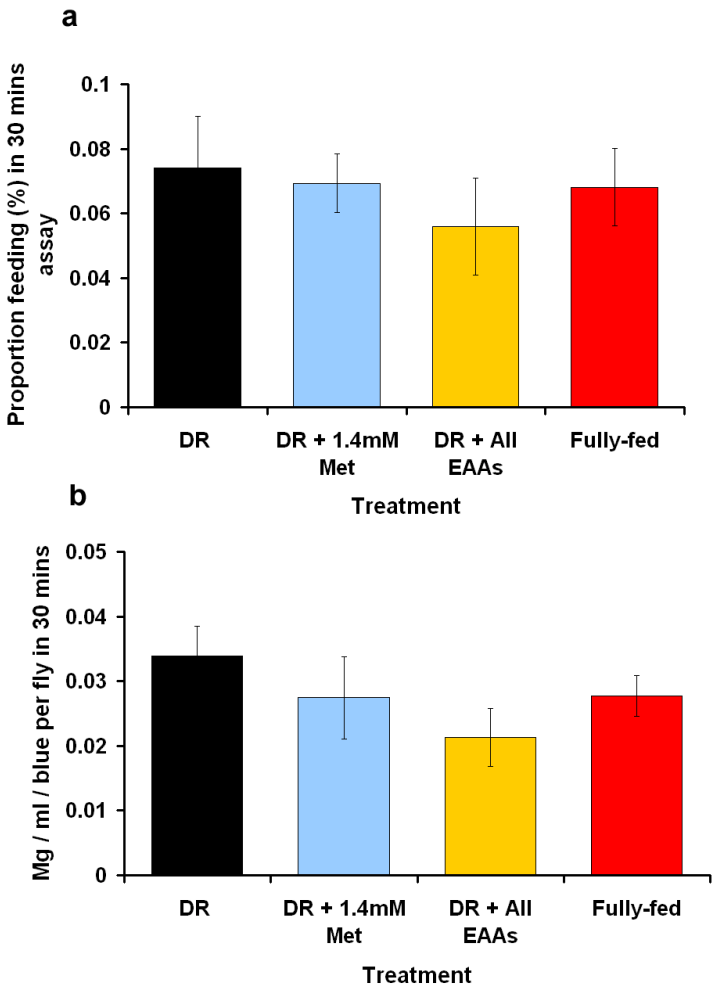

Figure 5.6: Methionine supplementation has no effect on food uptake.

(a) To calibrate feeding behaviour by rate of dye uptake, flies were maintained on their respective food types for seven days and subsequently transferred to their respective diet containing 2.5% blue dye. Feeding behaviour was observed during a 30 minute assay. No significant differences were observed in feeding behaviour between flies fed the DR diet and DR diets supplemented with either methionine (P = 0.78), all essential amino acids (EAA; P = 0.42) or fully-fed control flies (P = 0.69). (b) Following the 30 minute feeding behaviour assay on blue labelled food, blue dye uptake was measured by homogenising the flies in 1ml distilled water and measuring the absorbance of the filtered liquid sample with a spectrophotometer (see supplementary methods). The amount of food ingested was calculated from a standard curve. No significant differences were detected in blue food uptake by flies fed the DR diet and DR diets supplemented with either methionine (P = 0.34), all EAAs (P = 0.14) or fully-fed control flies (P = 0.39). Error bars represent ± s.e.m.

5.3.5 Effects of methionine supplementation cannot be explained by the presence of *Wolbachia*

To assess whether *Wolbachia*, or another intracellular bacterium, may be mediating the increased fecundity associated with methionine supplementation in the diet, fecundity and lifespan were measured in Dahomey flies from which the *Wolbachia* infection had been removed by treatment with tetracycline (refer to section 2.4.5).

Firstly, *Wolbachia* removal from the experimental generation was verified by PCR (Figure 5.7a) using primers to detect the gene for *Wolbachia* surface protein (wsp) (Braig *et al.* 1998; Zhou *et al.* 1998; Toivonen *et al.* 2007). As previously observed in a *Wolbachia*-infected background (Figure 5.2), adding back methionine resulted in an increase in fecundity mirroring the increased observed when adding back all 10 EAAs (Figure 5.7b). Furthermore, the increase in fecundity with methionine supplementation was not accompanied by a decrease in lifespan (Figure 5.7c). In contrast, adding back all 10 EAAs once again resulted in a significant reduction in lifespan compared with DR control-fed flies. In conclusion, the presence of *Wolbachia* had no effect on the responses of flies to methionine or EAA add-back diets.

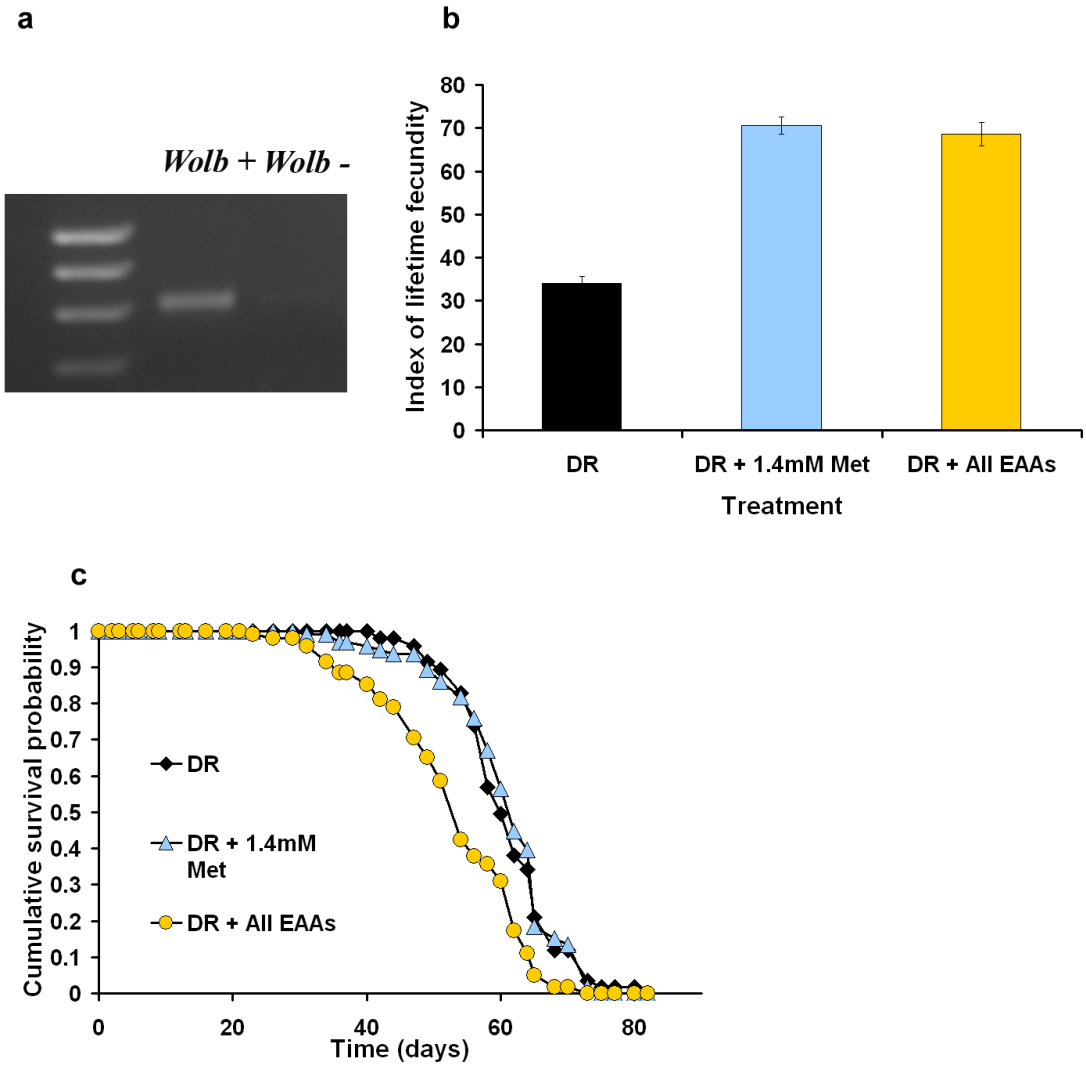

Figure 5.7: Longevity and fecundity phenotypes due to methionine supplementation not explained by presence of *Wolbachia*. *Wolbachia* infection was detected by using primers specific to *Wolbachia* surface protein (wsp). (a) The original Dahomey stock is infected with *Wolbachia* (left lane), which was removed permanently following two generations of tetracycline treatment (right lane). Adding back methionine resulted in a significant increase in fecundity compared with DR in *Wolbachia*-free flies ($P < 0.0002$, Wilcoxon), to a similar magnitude seen with the addition of all essential amino acids (EAAs) ($P = 0.5$, Wilcoxon). Fecundity assays were performed on days 6, 9, 13, 20, 27, 37 and 41 of treatment. (b) No decrease in lifespan was observed when methionine as added-back compared with DR (c; $P = 0.67$, log rank), but a significant reduction was detected in flies fed an add-back diet containing all EAAs ($P < 0.0001$, log-rank). Error bars represent ± s.e.m.

5.3.6 Assessing the effects of individual essential amino acids on lifespan

Supplementing the DR diet with methionine alone caused elevated fecundity (up to the magnitude observed with full feeding and with adding back all 10 EAAs) without any detrimental effects to lifespan. This poses the question as to which EAAs are causing the life-shortening effects seen when adding back yeast itself (full feeding) or all 10 EAAs together. To address this question, each EAA was added back to DR individually at the same concentration that was present when adding back all 10 EAAs together (Table 4.1).

Flies showed no differences in fecundity in response to adding back any of the EAAs individually, with the exception of methionine as previously reported (Figure 5.8a). By plotting lifetime fecundity against the biologically available nitrogen added back, it is clear that the increase in fecundity associated with methionine supplementation can not be explained by an increase in available nitrogen concentration, relative to the other EAAs. Adding back leucine provided the highest concentration of biologically available nitrogen (7.2mM), whilst adding back tryptophan (0.9mM) and methionine (1.4mM) provide the least. Adding back each EAA individually also revealed no significant effect on lifespan compared with DR, indicating that none of the EAAs alone could be responsible for the life-shortening effects observed when adding back all 10 EAAs together (Figure 5.8b). Methionine supplementation once again revealed that fecundity can be increased without any detrimental effects on lifespan. These data suggest that the life-shortening effects of full feeding or adding back all 10 EAAs are attributable to an increase in the presence of several EAAs in the diet.

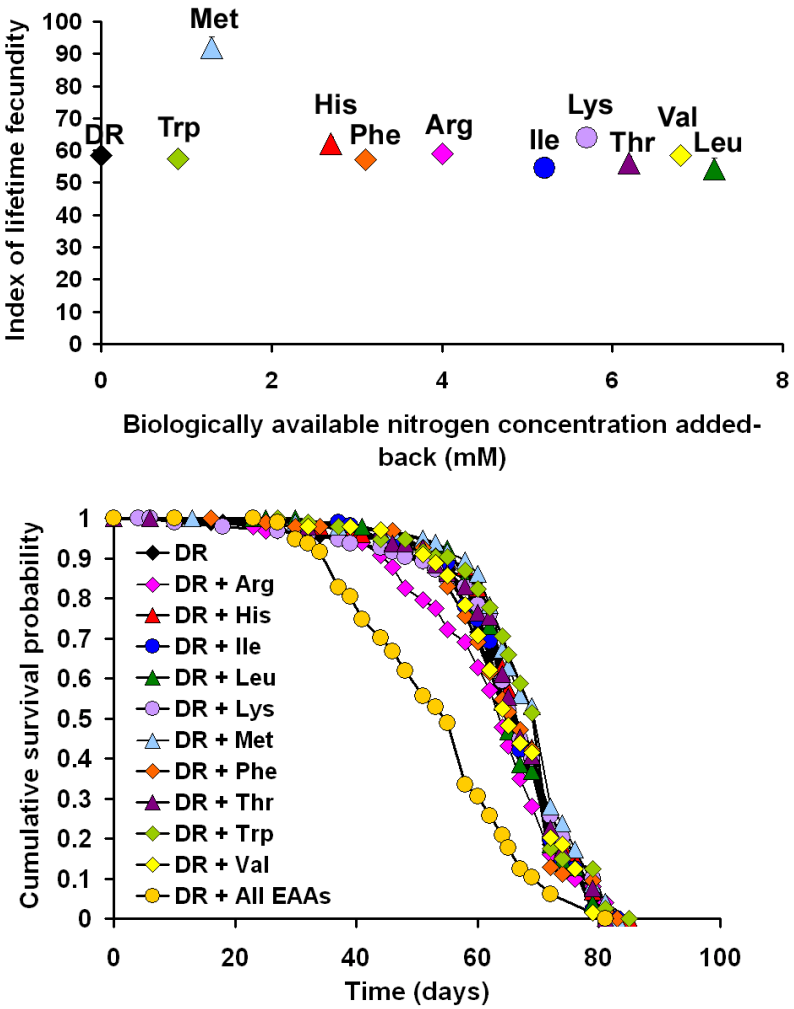

Figure 5.8: Effect of adding back individual essential amino acids on lifespan and fecundity. (a) Fecundity plotted against the concentration of biologically available nitrogen added-back to the DR diet arising from single EAA additions (see Table 4.1). Only methionine addition (+ met) significantly increased fecundity compared with DR (+ met compared with DR, P = 0.0001, Wilcoxon) and this was not attributable to a higher concentration of biologically available nitrogen compared with the addition of other EAAs. No other single EAA addition significantly increased fecundity compared with DR ($P \geq$ 0.27, Wilcoxon). (b) Supplementation of the diet with each essential amino acid singly had no significant effect on lifespan ($P \geq 0.3$, log-rank), whereas adding back all EAAs together significantly shortened lifespan ($P < 0.0001$). Fecundity assays were performed on days 6, 10, 17, 24, 30, 37 and 44 of treatment.

Earlier in this chapter it was shown that adding back all EAAs except methionine (methionine restriction) rescues the reduced lifespan of adding back all 10 EAAs (Figure 5.1b). Although methionine supplementation alone does not reduce lifespan, it is apparent that, in the presence of an excess of other EAAs, methionine is playing a key role in regulating lifespan. To investigate this role further and to assess the effect of increasing the number of amino acids, EAAs were added back in groups of 2, 4, 6, 8 and 10 with methionine always being present. The EAAs that made up each group were chosen alphabetically to ensure the groups consisted of random structural and functional amino acids. For example, the 2 EAA add-back contained arginine and methionine, the 4 EAA add-back contained arginine, histidine, isoleucine and methionine, the 6 EAA add-back contained arginine, histidine, isoleucine, leucine, lysine and methionine, etc.

Adding back an increasing number of EAAs produced a partially graded response, whereby lifespan began to decrease upon adding back 6 EAAs (Figure 5.9). Lifespan decreased to a greater extent upon adding back 8 EAAs and a further decrease was seen with 10 EAAs. In contrast, no significant effect on lifespan was observed with the addition of 2 or 4 EAAs to the DR medium. These results suggest that the life-shortening effects with full feeding are not attributable to specific amino acids, but are a result of a combination of all EAAs.

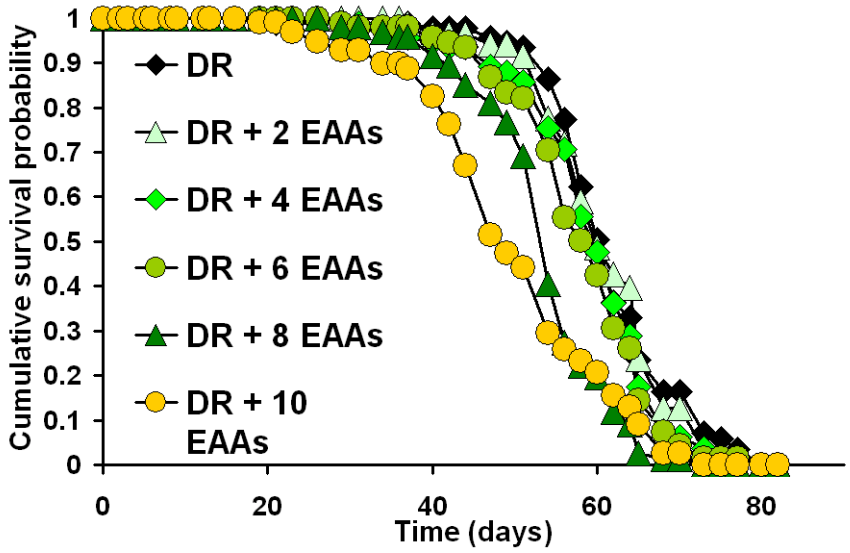

Figure 5.9: Effect on female lifespan of increasing the number of EAAs added back to the DR diet. In the presence of excess methionine (1.4mM), increasing the number of EAAs added back to the DR diet produced a partially graded effect of lifespan, with lifespan being reduced upon the addition of 6 or more EAAs to DR ($P \leq 0.02$, log-rank). No significant differences were observed between flies fed DR diets with 4 or fewer EAAs added back ($P \geq 0.11$, log-rank).

5.3.7 Investigating potential mechanisms mediating responses to EAAs and methionine supplementation

To investigate possible mechanisms involved in regulating the different responses of lifespan and fecundity to adding back all EAAs and methionine alone, two candidate pathways were targeted, the insulin / IGF-1 (IIS) signalling pathway and the TOR pathway. These pathways were chosen because down-regulation of several components of both the IIS and TOR pathway has been shown to extend lifespan in *Drosophila* and other model organisms (Friedman and Johnson 1988; Clancy *et al.* 2001; Holzenberger *et al.* 2003; Vellai *et al.* 2003; Kapahi *et al.* 2004a; Kaeberlein *et al.* 2005b; Taguchi *et al.* 2007). Moreover, in *Drosophila*, lifespan extension of some

of these mutants is conditional on diet (Clancy *et al.* 2002; Kapahi *et al.* 2004a; Giannakou *et al.* 2008; Min *et al.* 2008).

Insulin receptor dominant-negative flies (*dInRDN*) were chosen because although they exhibit reduced fecundity, they are not sterile like for example *chico* homozygotes (Bohni *et al.* 1999; Clancy *et al.* 2001) and some insulin receptor trans-heterozygotes (Tatar *et al.* 2001). Therefore, *dInRDN* flies could be used to determine whether down-regulation of the IIS pathway can block the elevated fecundity caused by the adding back methionine or all 10 EAAs to the DR diet. Furthermore, *dInRDN* flies also exhibit a substantial and reproducible lifespan extension phenotype on standard food (T. Ikeya, unpublished data), characteristic of several other mutant flies with reduced IIS signalling (Clancy *et al.* 2001; Tatar *et al.* 2001; Giannakou *et al.* 2004; Hwangbo *et al.* 2004; Broughton *et al.* 2005). To test the effect of mutants with reduced TOR signalling, *dS6K* dominant-negative flies were chosen. These have previously been reported to be long-lived (Kapahi *et al.* 2004a), although no lifespan experiments have been performed in our laboratory using these flies on our optimised diet (Bass *et al.* 2007a). Kapahi et al. (2004) also reported lifespan extension in a range of other flies with down-regulated TOR activity; including *dTsc1*, *dTsc2* over-expressers and *dTOR* dominant-negative flies. However, our laboratory has failed to confirm lifespan extension of any of these flies on a range of diets and hence these flies were not suitable for experiments assessing the role of the TOR pathway in the lifespan and fecundity effects mediated by amino acid add-back diets (T. Bass, unpublished data).

5.3.7.1 dS6 kinase dominant-negative flies exhibit increased fecundity in the presence of amino acids

In conflict with a previously published study reporting that that *dS6K* dominant-negative flies (UAS– $dS6K^{KQ}$ / daGAL4) are long lived (Kapahi *et al.* 2004a), no lifespan increase was detected on any of the diets tested (Figure 5.10a). Adding back all 10 EAAs shortened lifespan in the three control lines as well as the *dS6K* dominant-negative experimental line, all to a similar magnitude. As previously reported in Dahomey populations, supplementing the DR diet with methionine had no effect on the control lines or the experimental line.

In contrast to the absence of lifespan effects of the add-back diets on *dS6K* dominant negative flies, interesting changes in fecundity were observed. Adding back methionine or all EAAs caused a significant increase in fecundity in all lines compared with the respective DR control lines (Figure 5.10b). However, the increase in fecundity from adding back methionine in this study was not quite to the same magnitude observed when adding back all EAAs, as had previously been observed in Dahomey flies (Figure 5.2a). Fascinatingly, the fecundity of the *dS6K* dominant-negative flies fed an EAA add-back diet was significantly higher than any of the three control lines fed the same diet.

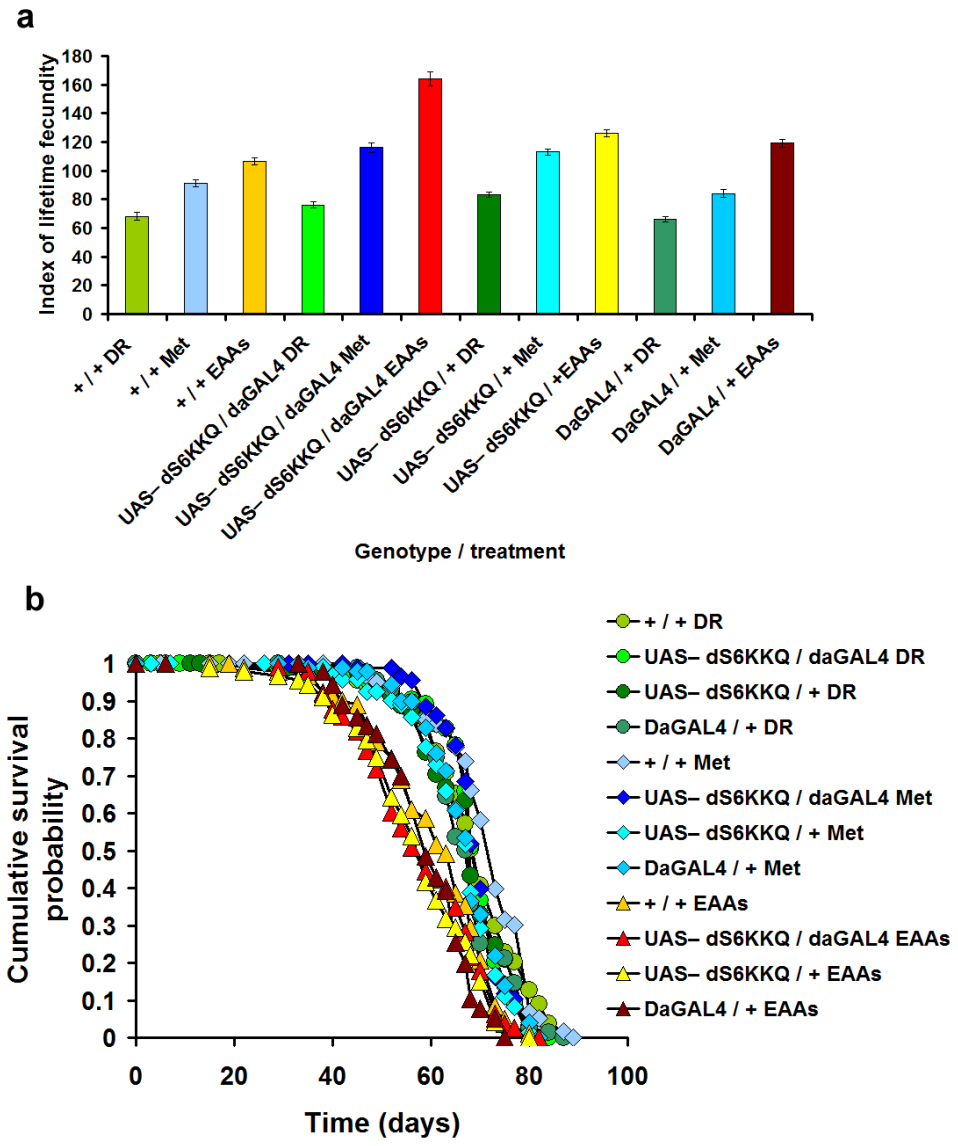

Figure 5.10: The effects of different diets on flies with reduced TOR activity. (a) The effect of fecundity of control lines and *dS6K* dominant-negative flies (UAS-dS6K^{KQ} / DaGAL4) to a control DR diet and DR diets supplemented with either methionine (Met) or all EAAs. Fecundity was increased in all lines upon supplementation with methionine ($P \leq$ 0.0012, Wilcoxon) or EAAs ($P \leq$ 0.0002, Wilcoxon), relative to DR. *dS6K* dominant-negative flies fed EAA add-back diets exhibited an increase in fecundity to a greater magnitude compared to control lines fed the same diet ($P <$ 0.0005, Wilcoxon when compared to the most fecund EAA control line). (b) No significant increase in lifespan was detected in *dS6K* dominant-negative flies on any food treatment, relative to controls ($P \geq$ 0.1, log-rank). The addition of EAAs to the DR diet significantly shortened lifespan in all lines compared with DR ($P <$ 0.0001, log-rank for all comparisons). In contrast, methionine addition had no significant effect on lifespan in any of the lines ($P \geq$ 0.17, log-rank). Fecundity assays were performed on days 5, 13, 17, 24, 31, 38 and 45 of treatment. Error bars represent ± s.e.m.

5.3.7.2 Insulin receptor dominant negative (InRDN)

As previously observed in other IIS pathway mutant flies including *chico* homozygotes, mNSC-ablated flies and some insulin receptor mutants (Clancy *et al.* 2001; Tatar *et al.* 2001; Broughton *et al.* 2005), *dInRDN* (UAS-*dInRDN* / daGAL4) flies also exhibited a severe reduction in fecundity on all diets, but were not completely sterile (Figure 5.11a). Supplementing the DR diet with all 10 EAAs or methionine alone was sufficient to cause a drastic increase in fecundity in all three control lines compared with DR. However, similarly to the effect in *dS6K* dominant-negative experiment, the magnitude of fecundity increase in control lines was marginally greater in the presence of all EAAs compared with methionine. Interestingly, supplementation of methionine did not increase the low fecundity of *dInRDN* flies (P = 0.45, Wilcoxon), and only a small but significant increase in fecundity was observed when these flies were fed a diet supplemented with all 10 EAAs (P = 0.004, Wilcoxon). Furthermore no significant differences were detected between fecundity of *dInRDN* on methionine and EAA-supplemented diets (P = 0.07, Wilcoxon) suggesting that *dInRDN* can almost entirely block the increase in fecundity in response to methionine or all EAAs.

Lifespan of all control lines fed diets supplemented with all EAAs was reduced (Figure 5.11b). Again, methionine supplementation, despite increasing fecundity in these lines, had no significant effect on lifespan ($P \geq 0.59$, log-rank). *dInRDN* flies exhibited a further extension of lifespan on all three diet, although the magnitude observed differed dramatically. *dInRDN* flies fed a control DR diet displayed a further increase in lifespan compared to controls (16-33% extension of median lifespan; $P < 0.0001$, log-rank). A similar extension of lifespan was detected in *dInRDN* fed a methionine-supplemented diet (16-33% extension of median lifespan;

$P < 0.0001$, log-rank). However *dInRDN* flies fed a diet supplemented with all 10 EAAs exhibited an extension of median lifespan between 67-95% compared with controls fed EAA-supplemented diets ($P < 0.0001$, log-rank), indicating that the life-shortening effect of EAAs was at least partially rescued in *dInRDN* flies. However, the lifespan of *dInRDN* flies fed the EAA add-back diet was not quite extended to the magnitude seen with the even longer lived *dInRDN* flies on a DR diet or on a methionine-supplemented diet, with flies on these treatments being 9% longer-lived $P < 0.0001$, log-rank).

Despite the fact that both the daughterless GAL4 and UAS-*dInRDN* lines had been extensively backcrossed into the white Dahomey wild-type background, the daughterless GAL4 heterozygous control line appeared to be slightly longer-lived than the wild-type and UAS-*dInRDN* control lines. However, importantly the *dInRDN* experimental lines were still significantly longer lived than the daughterless GAL4 (longest lived control) on all three diets tested, validating the lifespan extension of the *dInRDN* flies.

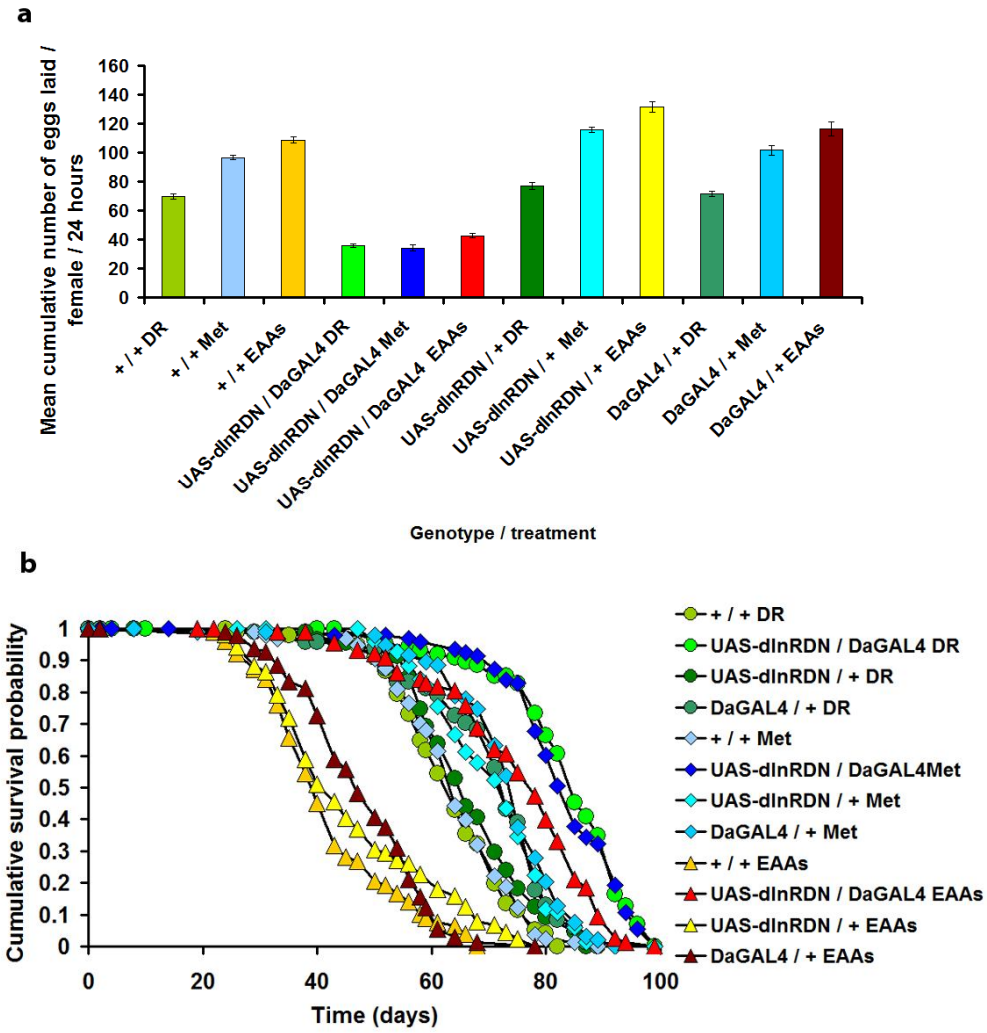

Figure 5.11: The effects of different diets on flies with reduced IIS signalling. (a) Fecundity of control lines and *dInRDN* flies (UAS- *dInRDN* / DaGAL4) fed a control DR diet or a DR diet supplemented with either methionine (Met) or all EAAs. All control lines exhibited elevated fecundity in response to adding back methionine ($P \leq 0.0002$, Wilcoxon) or all EAAs ($P \leq 0.0001$, Wilcoxon) compared to flies fed DR diets. *dInRDN* flies on all diets showed a marked reduction in fecundity relative to controls, and either no increase or a negligible increase in fecundity was seen in these flies upon supplementing the DR diet with methionine or EAAs. (b) *dInRDN* flies were longer lived on all diets compared with control lines; however, the magnitude of lifespan extension was greatest when *dInRDN* flies were fed a DR diet supplemented with EAAs (red triangle line). Fecundity assays were performed on days 4, 8, 15, 22, 24, 29 and 36 of treatment. Error bars represent ± s.e.m.

5.4 Discussion

5.4.1 Extension of lifespan by methionine restriction might be evolutionary conserved

In rodents, dietary restriction can extend lifespan through intermittent every other day (EOD) feeding and through a reduction in the quantity of food provided compared to *ad libitum*-fed cohorts. Despite earlier evidence suggesting that calories are the key component of the diet that regulate ageing in rodents (Iwasaki *et al.* 1988; Weindruch and Walford 1988; Masoro *et al.* 1989), other studies investigating the effects of specific amino acids have suggested this may not be the case. For example, restriction of a single essential amino acid, methionine, can extend the median and maximum lifespan of both rats (Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003) and mice (Miller *et al.* 2005). In addition, maximum but not median lifespan of male mice can be extended by restriction of another essential amino acid, tryptophan (De Marte and Enesco 1986).

Using the add-back approach, the data presented demonstrate that methionine restriction extends both median and maximum lifespan in *Drosophila*. Adding back all EAAs except methionine significantly rescued the reduced lifespan observed when adding back all ten EAAs, and resulted in a lifespan of similar magnitude as DR. Moreover, the mechanism of lifespan extension by methionine restriction in *Drosophila* might have been explained by the reallocation of resources hypothesis because methionine-restricted flies also displayed reduced fecundity, similarly to DR flies. In contrast flies fed an add-back diet containing all EAAs displayed significantly higher fecundity coupled with reduced lifespan, resembling fully-fed yeast control flies. Hence, from the results of this experiment, it appears that lifespan

extension under DR may occur due to reallocation of methionine from reproduction and towards somatic maintenance.

In contrast, adding back all EAAs except tryptophan or histidine did not affect the high fecundity observed when adding back all 10 EAAs. Interestingly, early work from Sang and King (1961) on creating an axenically defined diet for *Drosophila* adults revealed that although omission of each individual EAA caused a significant reduction in egg-laying, removal of methionine, histidine or arginine for up to eight days did not completely arrest egg-laying. Conversely, removal of any of the other seven EAAs for this duration of time caused egg-laying to cease (Sang and King 1961). Hence, the results from Sang and King (1961) would suggest that methionine, in the context of a defined diet, is not as important for egg-laying as several of the other EAAs. However, although methionine does not regulate egg-laying *per se*, its presence is clearly essential for elevated fecundity in the context of the *Drosophila* diet used in these experiments when adding back all other EAAs.

Since performing these add-back experiments, another paper has also reported, using a defined diet approach, that *Drosophila* lifespan can be extended by methionine restriction (Troen *et al.* 2006). However, this study did not test the response of fecundity to the chemically defined diets. In addition, no dietary yeast control conditions were used, which their defined diets were based on, hence any potential differences in lifespan could have arisen as a result of toxicity. Excess methionine intake has been shown to be extremely toxic in mammals, with its toxicity being higher than that caused by excessive intake of any other amino acid (Harper *et al.* 1970). The concentration of methionine used by Troen *et al.* (Troen *et al.* 2006) in the high methionine diet represented an extremely high concentration at 4.05 g/L

(0.405%), and lifespan was shown to be extended when this concentration was reduced to 1.35 g/L (0.135%), but no further extension of lifespan was detected when methionine concentration was reduced to 0.45 g/L (0.045%). Using the add-back approach, the addition of the equivalent molar concentration of adding back all 10 EAAs (43mM) suggests that reducing the concentration of methionine from 4.05g/L (Troen *et al.* 2006) could have extended lifespan through relief from toxicity. Adding back 43mM (equivalent to 6.433 g/L), which represented a higher dose than used by Troen *et al.*, resulted in a drastic reduction in lifespan and decrease in fecundity (Figure 5.12), indicating severe toxicity at this level similar to the effect of excess methionine intake in mammals (Harper *et al.* 1970). It is possible that the toxic effects begin at 4 g/L or slightly lower, and only through measuring daily and lifetime fecundity would the nature of lifespan extension seen by Troen *et al.* (2006) be more conclusive.

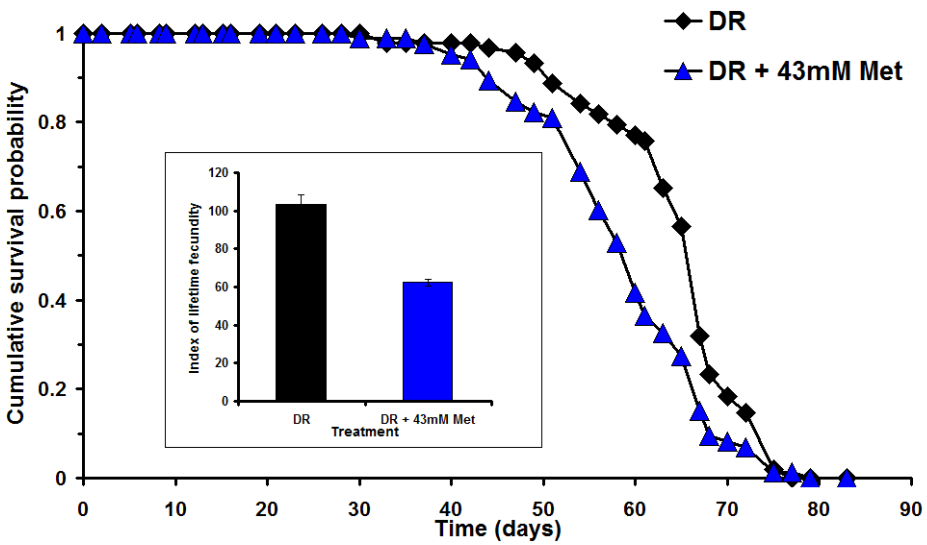

Figure 5.12: The adverse effects of high concentrations of methionine in the diet. Supplementing the DR diet with 6.4g (43mM) methionine (Met) results in a significant reduction in lifespan ($P < 0.0001$, log-rank), coupled with a marked reduction in fecundity (inset; $P < 0.0001$, Wilcoxon).

Lifespan extension as a result of methionine restriction in rodents and *Drosophila* may be explained by a reduction in protein synthesis, which is known to decline with age (Makrides 1983). The start codon of all protein-encoding mRNA sequences codes for methionine and hence reduced methionine intake could potentially cause either a slower turn over of proteins or simply reduced production of proteins. However, from extensive work, particularly on rats, unexpectedly protein synthesis and turnover appears to be greater in dietary/calorically-restricted animals (Lewis *et al.* 1985; Merry and Holehan 1985; Merry and Holehan 1991; Ward and Richardson 1991). Nonetheless, support for the hypothesis that reduced protein synthesis can extend lifespan is evident in three recent studies on *C.elegans* (Hansen *et al.* 2007; Pan *et al.* 2007; Syntichaki *et al.* 2007a). These studies demonstrated that inhibition of different genes in the translation initiation complex, including the initiation factor eIF4E (IFE-2), a principle regulator of protein synthesis, and reducing the levels of S6K extended the lifespan of nematodes (Hansen *et al.* 2007; Pan *et al.* 2007; Syntichaki *et al.* 2007a). Further analysis into mechanisms underlying lifespan extension by methionine restriction in rodents and *Drosophila* should shed more light on whether reduced protein synthesis is responsible.

In contrast to rodents (De Marte and Enesco 1986), no lifespan extension was detected when adding back all EAAs except tryptophan (tryptophan restriction). However, it is important to consider that the magnitude of lifespan extension observed in rodents by tryptophan restriction was negligible, with only the maximum lifespan being significantly extended (Figure 5.13a) (De Marte and Enesco 1986). This is in contrast to the magnitude of lifespan extension reported with methionine restriction in rodents, which extends both median and maximum lifespan (e.g. Figure 5.13b) (Orentreich *et al.* 1993; Richie *et al.* 1994; Zimmerman *et al.* 2003; Miller *et*

al. 2005). Although tryptophan restriction cannot explain lifespan extension by DR when reducing the yeast concentration, the presence of tryptophan in the diet may influence ageing when found at higher concentrations. This could be tested by using a range of tryptophan concentrations in a synthetically defined diet.

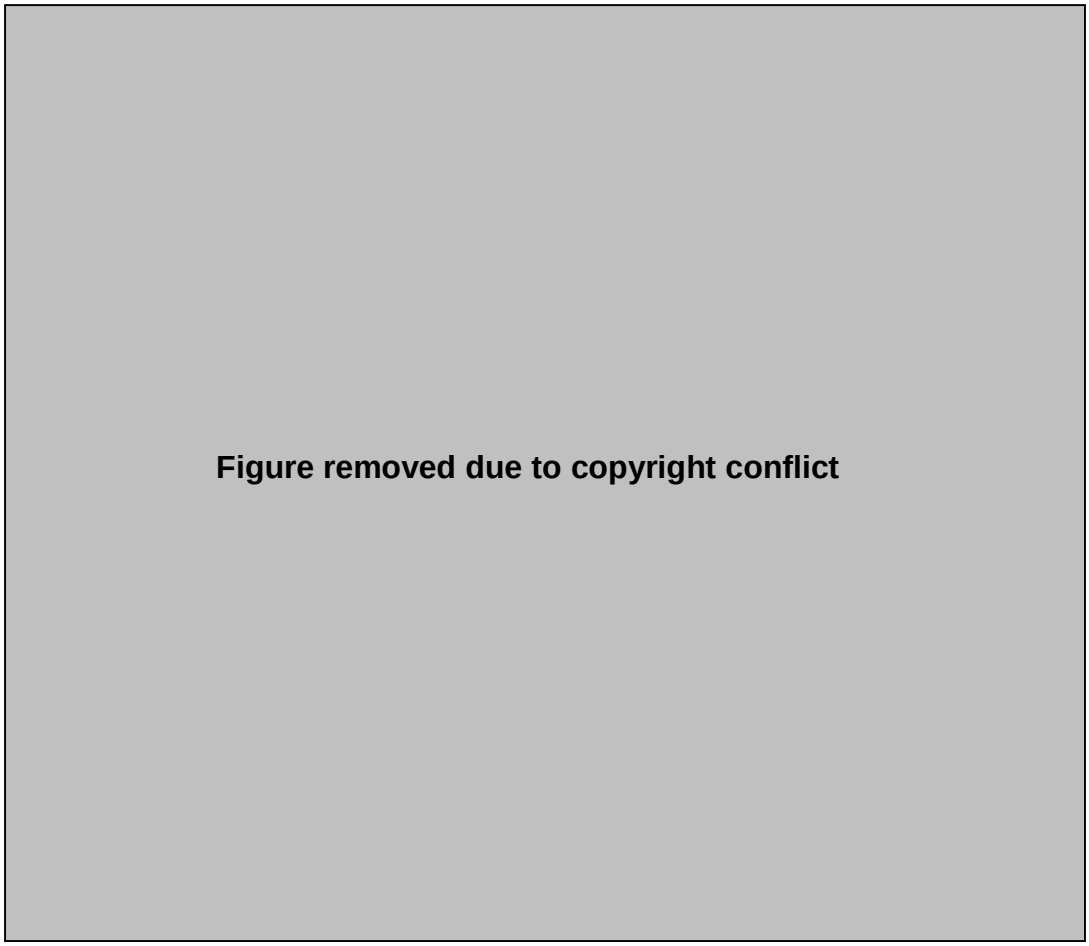

Figure 5.13: A comparison of the effects of tryptophan and methionine restriction in male rodents. (a) Restriction of tryptophan (trp rest.) causes negligible lifespan extension in male mice compared with a control-fed cohort (26% ptn). (Taken from De Marte and Enesco 1986). (b) Methionine restriction (0.17%) in male rats causes a substantial increase in both median and maximum lifespan compared with mice fed control levels of methionine (0.86%). (Taken from Zimmerman *et al*. 2003).

Histidine restriction, similarly to tryptophan restriction in this context, had no effect on lifespan. Although no previous work has pointed to a link between histidine

restriction and lifespan extension in any organism, histidine was an interesting candidate not only because of its structural difference to methionine and tryptophan (Figure 5.14), but also because of previous work performed on *slimfast*, a known cationic amino acid transporter in insects (Bradley and Leevers 2003; Colombani *et al.* 2003; Attardo *et al.* 2006). Down-regulation of *slimfast* specifically in the larval fat body has been shown to cause a ubiquitous growth defect (Colombani *et al.* 2003), similarly to rearing larvae in a nutritionally-poor environment. This makes *slimfast* a potential candidate to extend lifespan in *Drosophila* because mutations in several of the components of the IIS or TOR pathways also lead to growth defects and extend lifespan (Bohni *et al.* 1999; Clancy *et al.* 2001; Tatar *et al.* 2001; Marygold and Leevers 2002; Kapahi *et al.* 2004a).

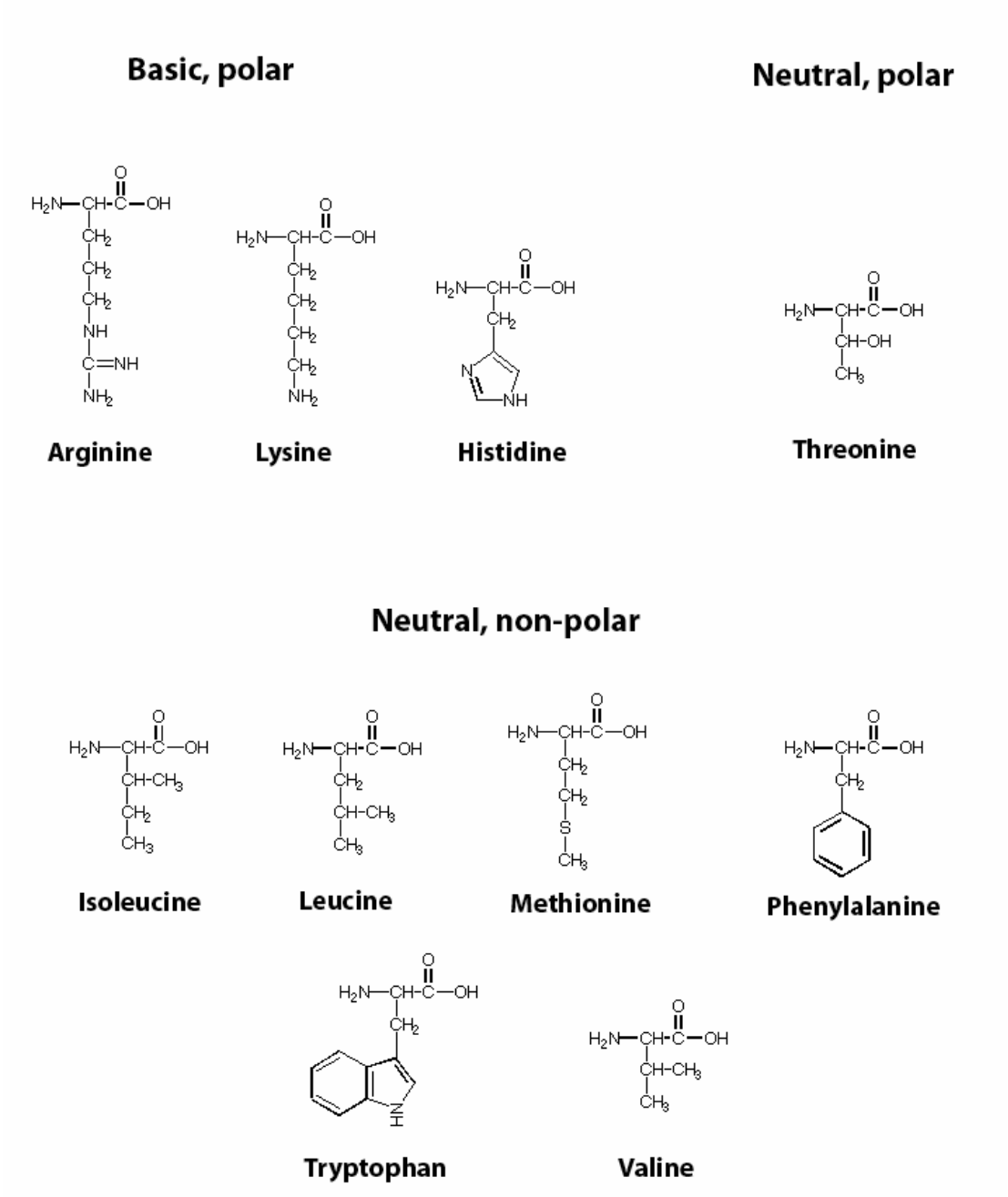

Figure 5.14: Chemical structures of the essential amino acids. Methionine and tryptophan are both neutral and non-polar whereas histidine is structurally and chemically different (basic and polar). Methionine is notably the only sulphur-containing EAA. Figures adapted from: A review of amino acids[19].

[19] A review of amino acids. Curtin University of Technology, http://www.biomed.curtin.edu.au/biochem/tutorials/AAs/AA.html

5.4.2 Lifespan extension by DR cannot be explained by reallocation of resources

All previous experiments performed in chapters 3, 4 and 5 have pointed to a strong trade-off between lifespan and fecundity when applying DR in female *Drosophila*. By determining the nutrients that regulate lifespan and fecundity during DR, it was apparent that amino acids and particularly essential amino acids were mediating this trade-off. Furthermore, adding back all EAAs except methionine reduced fecundity to the level observed with DR-fed flies and extended lifespan compared with the high fecundity and reduced lifespan observed in flies fed a fully-fed yeast diet or an add-back diet with all EAAs. These data suggest that reallocation of methionine from reproduction to lifespan may be the mechanism that extends lifespan by DR in *Drosophila*. However, addition of methionine alone to the DR diet could account for the entire increase in fecundity observed with full feeding and adding back all EAAs, but in contrast has no significant effect on lifespan. This was also observed when adding back a greater range of methionine concentrations to the diet which all caused a marked increase in fecundity without any cost in terms of reduced lifespan.

These data not only suggest that methionine is limiting for fecundity in the DR diet, but more importantly highlight that high fecundity and extended lifespan need not be mutually exclusive. Furthermore, these results demonstrate that the reduction in lifespan upon full feeding is not the result of reallocation of nutrients from somatic maintenance to reproduction, because the only nutrient required for the increase in fecundity, in this case methionine, did not reduce lifespan. Additionally, the decrease in lifespan upon full feeding does not appear to be a consequence of damage inflicted by reproduction (Tatar and Carey 1995; Barnes and Partridge 2003; Partridge *et al.* 2005a), since this hypothesis would also predict that high fecundity and increased

survival would be mutually exclusive. Support for this finding that DR does not extend lifespan either through reallocation of resources or due to reduced damage inflicted by reproduction is limited. However, DR has previously been reported to still extend lifespan in both flies that have vitellogenesis blocked by the *ovo*^{D1} mutation and flies that have had their germ line removed by X-radiation (Mair *et al.* 2004).

Furthermore, a recent paper also suggests that DR in *Drosophila* not extend lifespan through reallocation of resources from reproduction towards somatic maintenance and repair (O'Brien *et al.* 2008). The authors labelled carbon and nitrogen in yeast with stable isotopes to try and determine how carbon and nitrogen are distributed upon DR and full feeding. As would be expected, more nitrogen, carbon and EAAs were allocated to eggs with full feeding. However, fully-fed flies also allocated more nitrogen, carbon and EAAs to somatic tissue than DR flies, although resource allocation to somatic tissue relative to eggs was greater with DR. O'brien *et al.* concluded that DR may extend lifespan through somatic investment relative to damage from increased reproduction. However, from the current add-back study, supplementing the DR diet with methionine resulted in elevated fecundity without any cost of lifespan. If increased nutrition shortens lifespan as a result of increased fecundity leading to reproductive damage (Tatar and Carey 1995; Barnes and Partridge 2003; Partridge *et al.* 2005b) then supplementing the DR diet with methionine should also shorten lifespan, which was not the case.

One of the potential explanations why methionine supplementation could increase fecundity to the magnitude seen with full feeding or supplementation of all EAAs is as a result of increased feeding rate. However, through a combination of direct

feeding behaviour assays throughout life and calibration of food uptake using blue-dye labelled food, it was apparent that flies fed DR diets supplemented with either methionine or all EAAs did not adjust their feeding behaviour or increase rate of food uptake. Hence increased feeding could not account for the fecundity increase on either of these diets. It is important to consider in this instance that even if flies had altered feeding behaviour in response to a methionine-supplemented diet, accounting for elevated fecundity, this cannot explain why the increased fecundity did not trade-off with reduced lifespan.

Measuring feeding behaviour accurately in *Drosophila* can be subject to many technical difficulties, especially because flies are thought to eat only around 5µl of food per day (Ja *et al.* 2007). Several different methods have been proposed for measuring behaviour, including labelling food with non-absorbable dyes (Edgecomb *et al.* 1994; Wood *et al.* 2004; Bross *et al.* 2005; Min and Tatar 2006a; Wong *et al.* 2008) or radioactive isotopes (Brummel *et al.* 2004; Carvalho *et al.* 2005; Carvalho *et al.* 2006), or using a capillary feeder (CAFE) (Ja *et al.* 2007). Despite one study suggesting that DR flies might compensate by eating more food than fully-fed flies (Carvalho *et al.* 2005), greater evidence suggests that DR flies do not compensate (Bross *et al.* 2005; Min and Tatar 2006a; Wong *et al.* 2008). Furthermore, if flies were compensating by increasing feeding rate on DR then one would expect fecundity, a parallel measure of nutrition (Piper and Partridge 2007), to also be increased on DR food, to a similar magnitude seen with full feeding; however, this is not the case (Chippindale *et al.* 1993; Chapman and Partridge 1996; Bass *et al.* 2007a; Libert *et al.* 2007; Lee *et al.* 2008). The method used for measuring feeding in the current experiments was chosen as opposed to radio-labelling food because it allows steady-state feeding observations to be made in an undisturbed environment.

In chapter 3, it was shown that *Wolbachia* or other bacterial infections removed by tetracycline treatment could not account for the response of flies to DR. The striking phenotypes of long lifespan coupled with increased fecundity due to methionine supplementation could also not be explained by the presence of *Wolbachia* in the wild-type Dahomey strain. Flies which had been cured of *Wolbachia* infection also exhibited increased fecundity with no cost of reduced lifespan when methionine was added-back to the DR diet.

Another possible explanation for the increased fecundity attributed to methionine in the diet is that methionine is one of two sulphur-containing amino acids (Figure 5.14). The second amino acid which contains sulphur is the non-essential amino acid cysteine, which yields a very similar concentration of biologically available nitrogen to methionine (1.3mM and 1.4mM respectively). Although the addition of cysteine to the DR diet alone was not tested, no effect on fecundity was observed by the addition of all N-EAAs. If the presence of sulphur-containing groups was responsible for the fecundity increase observed with methionine supplementation then it is likely that adding back all N-EAAs would result in elevated fecundity, which was not observed.

5.4.3 Decreased lifespan with full feeding may be caused by an imbalance of amino acids in the diet

Supplementation of the methionine to the DR diet could account for the increase in fecundity but not the reduction of lifespan observed with full feeding. However, despite methionine alone not being important in regulating lifespan it clearly plays an influential role in conjunction with other amino acids because adding back all EAAs except methionine could almost entirely reverse the reduced lifespan when adding back all EAAs. The reduced lifespan with full feeding could also not be explained by

a single EAA because adding back each EAA individually caused no significant effect on lifespan compared with DR. Nor can it be attributed to unidentified toxins in the yeast because the yeast diet had previously been optimised for increased lifespan with DR and high fecundity with full feeding (Bass *et al.* 2007a). Instead, lifespan was reduced upon the addition of an increasing number of EAAs in the diet as long as methionine was present.

The results of this study demonstrate that there is an imbalance in the ratio of amino acids present in the yeast diet compared with what is required for flies to achieve maximum fecundity. This imbalance of amino acids, particularly essential amino acids, is likely to account for the reduced lifespan associated with full feeding. All the amino acids with the exception of methionine contributed to the reduced lifespan without having any positive effect on fecundity. Reduced lifespan with full feeding could be caused by metabolic costs involved in removing these amino acids not required for reproduction, for example through damage inflicted to the excretory malphigian tubules, the fly equivalent of the mammalian kidneys (Wessing 1978). Nutrient imbalance in the diet may also account for the responses of lifespan and fecundity to DR in other organisms, including mammals, if specific nutrients in their diet are also limiting for full physiological function. An important recent discovery has been that the mechanisms that influence lifespan are conserved during evolution over the great evolutionary distances between yeast, multicellular invertebrates and mammals (Partridge and Gems 2002; Tatar *et al.* 2003; McElwee *et al.* 2007).

5.4.4 Uncoupling the lifespan / fecundity trade-off during dietary restriction

Fecundity and lifespan are commonly expected to trade-off with one another. Examples from laboratory experiments using fruit flies have revealed that selection for high reproduction or early reproduction reduces lifespan (Rose and Charlesworth 1981; Rose 1984; Fowler and Partridge 1992; Sgro and Partridge 1999). In addition, ablation of the germ line in the latter part of development or very early in adulthood can extend *Drosophila* lifespan (Flatt *et al.* 2008), replicating the findings of a similar, earlier study in *C.elegans* (Hsin and Kenyon 1999) Furthermore, mutations which extend lifespan often impair, delay or abolish reproductive function in flies, worms and rodents (Klass 1983; Friedman and Johnson 1988; Brown-Borg *et al.* 1996; Lin *et al.* 1998; Tissenbaum and Ruvkun 1998; Bohni *et al.* 1999; Rogina *et al.* 2000; Clancy *et al.* 2001; Flurkey *et al.* 2001; Tatar *et al.* 2001; Flurkey *et al.* 2002; Giannakou *et al.* 2004; Broughton *et al.* 2005). However, some isolated examples have been reported whereby mutations that extend lifespan do not affect fecundity. For example, in *C.elegans*, mutations in certain components of the IIS pathway including *age-1* (PI3K) and *daf-2* (insulin receptor) give arise to worms with extended lifespan but normal fecundity (Johnson *et al.* 1993; Kenyon *et al.* 1993; Gems *et al.* 1998). Moreover, it appears the timing requirements for IIS on fecundity and lifespan in *C.elegans* are different (Dillin *et al.* 2002). Dillin *et al.* (2002) demonstrated using RNAi that reducing the activity of *daf-2* in the adult worm was sufficient to extend lifespan without a cost of fecundity. However, reduced *daf-2* expression during the pre-adult period did not extend lifespan but caused a severe reduction in fecundity.

In *Drosophila*, *Indy* ("I'm not dead yet") mutants are reportedly longer-lived and have normal fecundity or slightly elevated fecundity compared with controls on a standard food diets. Conversely, their fecundity is reduced when fed a low food diet (Rogina *et al.* 2000), suggesting a conditional trade-off between lifespan and fecundity (Marden *et al.* 2003). However, the originally reported lifespan extension phenotype of *Indy* mutants has since been negated by a recent study which found no lifespan extension when the genetic and cytoplasmic background effects had been corrected for (Toivonen *et al.* 2007). Over-expression of *dFOXO* in the fat body and head fat body has also been suggested to extend lifespan, without a reduction in fecundity (Hwangbo *et al.* 2004; Giannakou *et al.* 2007). Adding sirtuin activating compounds (STACs) to the fly diet also appears to represent an exception to trade-off theory, since they extend lifespan without altering fecundity (Bauer *et al.* 2004; Wood *et al.* 2004). In rodents, long-lived IGF-1 receptor heterozygote-null mice and FIRKO mice (deletion of insulin receptor in the adipose tissue) exhibit normal reproductive phenotypes to control mice (Bluher *et al.* 2003; Holzenberger *et al.* 2003; Partridge *et al.* 2005a).

In contrast to a few examples of mutations which increase lifespan with no apparent cost of fecundity, dietary restriction consistently extends lifespan and reduces fecundity in flies, worms and rodents (Klass 1977; Holehan and Merry 1986; Weindruch *et al.* 1986; Chapman and Partridge 1996; Bishop and Guarente 2007b; Libert *et al.* 2007; Selesniemi *et al.* 2008). However, the results of the add-back experiments have revealed that the full benefits of lifespan extension by DR can be achieved, without any cost of reduced fecundity compared with full feeding, solely by supplementing the DR diet with methionine (Figure 5.15). The results of these experiments imply that in mammals it may also be possible to obtain the benefits of

DR for health and lifespan without impairing fecundity and without the need for DR itself, by a suitable balance of nutrients in the diet.

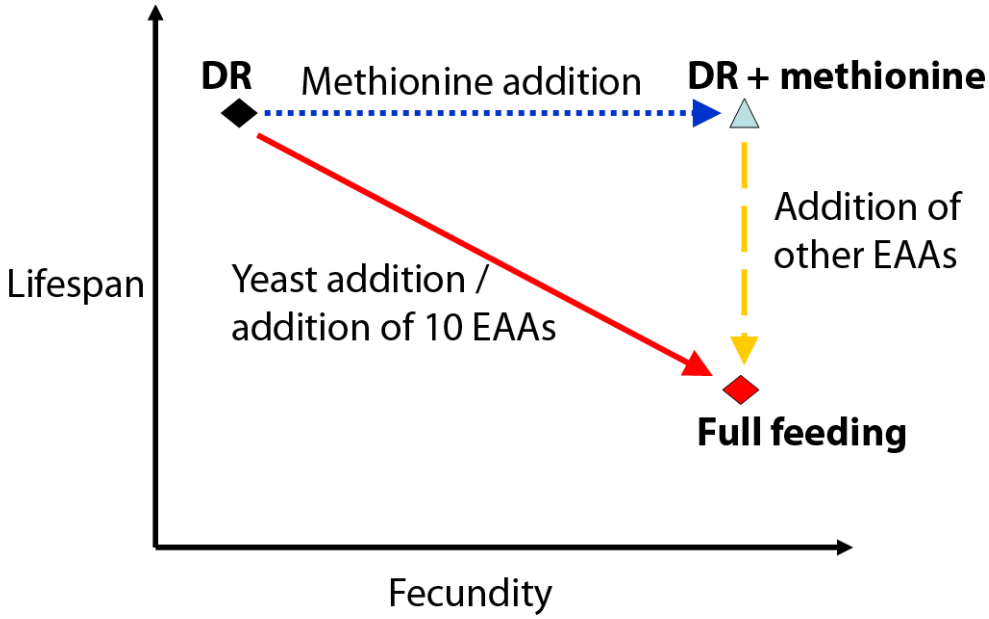

Figure 5.15: Uncoupling the lifespan / fecundity trade-off during dietary restriction. DR flies exhibit extended lifespan coupled with reduced fecundity, which is reversed upon the addition of increased dietary yeast (fully-fed) or the addition of 10 EAAs leading to increased fecundity but reduced lifespan. However, adding methionine to the DR diet increases fecundity to the level of all EAAs / fully-fed but has no effect on lifespan compare to DR.

5.4.5 dS6 kinase may negatively regulate high fecundity in *Drosophila*

The TOR / S6 kinase pathway (Figure 5.16) has previously been shown to play an important role in regulation of growth, body size, development and longevity (Montagne *et al.* 1999; Barcelo and Stewart 2002; Marygold and Leevers 2002; Colombani *et al.* 2003; Kapahi *et al.* 2004a). Loss of function of *dS6K* results in around 75% larval lethality whilst the remaining viable flies exhibit an extreme

developmental delay, severe growth reduction, female sterility and premature death, often within two weeks (Montagne *et al.* 1999).

In contrast, the *dS6* kinase dominant-negative females used in this study appeared normal in body size and were not developmentally delayed. Furthermore, these flies exhibited normal fecundity compared with controls lines when fed a standard DR diet. The dominant-negative activity of *dS6K* appeared to be verified by crossing dUAS-$S6K^{KQ}$ flies with the ap-GAL4 driver (refer to section 5.2.5) causing the wings to bend upward slightly (Barcelo and Stewart 2002), although, as previously published, the phenotype was quite weak (C. Slack, personal communication). Fascinatingly, despite exhibiting normal fecundity when fed a standard DR diet, *dS6K* dominant-negative flies fed a DR diet supplemented with all EAAs exhibited a significant increase in fecundity of beyond that observed in control lines fed an EAA-supplemented diet. The further increase in fecundity of *dS6K* dominant-negative flies fed an EAA add-back diet did not result in a further reduction in lifespan compared with control lines fed the same diet. This provides further evidence that high fecundity and reduced survival do not always trade-off. These data suggest that under normal dietary conditions, the dominant-negative form of *dS6K* does not influence fecundity; however when flies are fed a higher concentration of EAAs, *dS6K* may act to buffer some of the EAAs being invested in reproduction.

In mosquitoes, nutrient-dependent TOR and S6K signalling has been reported to control egg development (Hansen *et al.* 2005a). Hence *dS6K* appears to act as a negative regulator of high fecundity, with the excess amino acids potentially being utilised for production of other proteins. In Chinese hamster ovary (CHO) cells, S6K

has been shown to be directly regulated by amino acids in the cell medium (Wang *et al.* 1998). Furthermore, in CHO cells, amino acid deprivation causes inactivation of p70 S6 kinase and subsequently increased binding of the inhibitory protein 4E-BP1 to cap-binding translation factor eIF4E (eukaryotic initiation factor), which can be reversed upon the return of an amino acid-enriched medium (Wang *et al.* 1998). Future work in *Drosophila* might focus on the interaction between EAAs and translation factors including *4E-BP* (*Thor*), which has shown to play an important role in cell growth (Miron *et al.* 2001), immune response (Bernal and Kimbrell 2000; Levitin *et al.* 2007), and starvation and oxidative stress resistance (Tettweiler *et al.* 2005).

It had previously been reported that down-regulation of the TOR pathway through over-expression of *dTsc1* and *dTsc2* or dominant-negative forms of *dTOR* and *dS6K* flies extended *Drosophila* lifespan (Kapahi *et al.* 2004a), a finding which is supported by data in yeast and worms showing that mutations in genes that modulate TOR pathway activity can increase lifespan (Vellai *et al.* 2003; Jia *et al.* 2004; Meissner *et al.* 2004; Kaeberlein *et al.* 2005b; Henderson *et al.* 2006). However, in the current experiments, no extension in lifespan was observed in *dS6K* dominant-negative flies on any of the three diets tested. One of the reasons for the lack of lifespan extension may be that S6K is not only regulated by TOR but also by other proteins such as the kinase PDK1 (Rebholz *et al.* 2006). Thus removing S6K function may result in more widespread effects that do not mimic those seen in mutations affecting solely the TOR pathway.

Kapahi *et al.* (2004) demonstrated that the longevity of flies over-expressing *dTsc2* was conditional on a high food concentration, similarly to observations in *chico* homozygotes and flies over-expressing *dFOXO* in the adult fat body (Giannakou *et al.* 2008) and head fat body (Min *et al.* 2008). However, this finding may be explained by *dTsc2* over-expressing flies being more resistant to toxicity compared to controls at higher food concentrations because, as reported in Bass *et al*. (2007a), the diet used by Kapahi *et al*. (consisting of yeast extract supplemented with cornmeal) induces dose dependent toxicity in wild-type flies, causing reduced lifespan and reduced fecundity at higher food concentrations (Bass *et al.* 2007a). It is possible that the lifespan extension of *dS6K* dominant-negative flies may also be dependent on the diet.

An alternative explanation for the conflicting data may be the different temperatures that the lifespan studies were performed at. The current experiments were performed at 25^{o}C whereas Kapahi *et al.* performed their experiments at 29^{o}C. Although GAL4 enhancer traps are thought to produce stronger effects at higher temperatures (Seroude *et al.* 2002), performing lifespan experiments at 29^{o}C severely shortens lifespan (Miquel *et al.* 1976; Mair *et al.* 2003) and could be inducing thermal stress in flies. Therefore, any lifespan extension observed at 29^{o}C could be as a result of increased resistance to thermal stress of *dS6K* dominant-negative flies, a phenotype previously observed in long-lived flies with over-expression of human superoxide dismutase (*SOD*) (Spencer *et al.* 2003) or apolipoprotein D (*ApoD*) (Walker *et al.* 2006).

Figure 5.16: An overview of the *Drosophila* IIS / TOR pathway. *Drosophila* have a single insulin receptor (dINR) which is activated by *Drosophila* insulin-like peptides (DILPs). Upon activation, the dINR recruits the catalytic subunits of PI3 kinase (Dp110 –Dp60) directly or indirectly via the insulin receptor substrate protein CHICO. PI3 kinase converts phosphatidylinositol (4,5)-bisphosphate [PIP2] to phosphatidylinositol (1,4,5)-trisphosphate [PIP3]. dPTEN antagonises the actions of PI3K which degrades PIP3 to PIP2. PIP3 activates kinases including dPDK1 and PKB, which subsequently phosphorylate the transcription factor FOXO, causing it to become inactivated and migrate and translocate from the nucleus and into the cytoplasm. The TOR pathway is involved in amino acid sensing through the amino acid transporter slimfast. TOR kinase activates S6K which in turn phosphorylates the small ribosomal subunit S6. Phosphorylation by TOR inhibits 4EBP (eukaryotic initiation factor 4E-binding protein which causes mRNA cap-binding protein eIF4E (eukaryotic initiation factor 4E) to bind mRNAs, resulting in increased translation. The IIS and TOR pathways interact through PKB which can phosphorylate dTSC2 (in addition to FOXO), which negative regulates TOR. Green arrows represent activation, red lines represent inhibition. Figure adapted from (Giannakou and Partridge 2007).

5.4.6 Down-regulation of IIS signalling partially blocks lifespan and fecundity effects of EAAs

In *Drosophila*, studies assessing the effects of diet on the longevity of known long-lived IIS mutants have revealed a complex interaction between nutrition and lifespan.

Both *chico* homozygotes and flies with *dFOXO* over-expressed in the fat body have been reported to exhibit a right-shift response to nutrition (Figure 1.11). Thereby the lifespan of the mutants is increased at high food concentrations and decreased at lower food concentrations (Clancy *et al.* 2002; Giannakou *et al.* 2008). In contrast, another study has suggested that over-expression of *dFOXO* in the fat body only extends lifespan at low food concentrations (Min *et al.* 2008). Furthermore, studies involving rodents have shown conditional longevity of long-lived mutants on different diets (Bartke *et al.* 2001; Flurkey *et al.* 2001; Bonkowski *et al.* 2006; Taguchi *et al.* 2007; Selman *et al.* 2008). For example, the lifespan of long-lived Ames dwarfs could be further extended by DR (Bartke *et al.* 2001); however, the long-lived Laron dwarf mice do not exhibit a further extension when subjected to DR (Bonkowski *et al.* 2006). In addition, insulin receptor substrate 2 (IRS2) mutants have extended longevity when fed a 9% fat diet (Taguchi *et al.* 2007), but not when fed a 5% fat diet (Selman *et al.* 2008). Overall, the majority of growth hormone, insulin and insulin growth factor mutations extend lifespan on a high food / calorie diet.

In the current study, the response of insulin receptor dominant-negative flies (*dInRDN*) was tested on three different diets; a control DR diet, a methionine-supplemented diet and a DR diet supplemented with all EAAs, which had previously been shown in wild-type flies to cause almost identical fecundity and lifespan phenotypes to the effects observed with a high yeast diet (full feeding). Similarly to previous studies which have shown that mutations in various components of the IIS pathway can extend lifespan (Clancy *et al.* 2001; Tatar *et al.* 2001; Giannakou *et al.* 2004; Hwangbo *et al.* 2004; Broughton *et al.* 2005), *dInRDN* flies also exhibited extended lifespan on all three diets. Interestingly the magnitude of lifespan increase

relative to controls was greatest when *dInRDN* flies were fed a DR diet supplemented with all EAAs. These data suggest that insulin receptor dominant-negative activity can partially block the life-shortening effect of the essential amino acids, although lifespan was not completely rescued since *dInRDN* flies were even longer-lived on DR and methionine-supplemented diets. Furthermore *dInRDN* flies fed all EAA or methionine supplemented diets displayed a negligible or even no increase in fecundity respectively, despite control flies on these diets showing a marked increased compared to control flies fed DR diets. Testing the effect of EAAs on *dInRDN* flies which have been cured or *Wolbachia* infection may reveal more about the capacity of *dInR* dominant-negative activity to block the life-shortening effect of EAAs because removal of *Wolbachia* seemingly causes these flies to exhibit normal lifespan on control diets (T. Ikeya, unpublished data).

The IIS pathway is known to be important in controlling reproduction because mutations in several components of the pathway result in sterility or severely reduced reproductive output (Bohni *et al.* 1999; Clancy *et al.* 2001; Giannakou *et al.* 2004; Broughton *et al.* 2005). However, amino acids sensing occurs via the TOR / S6K pathway and the amino acid transporter *slimfast* as opposed to the insulin receptor which is activated by *Drosophila* insulin-like peptides (DILPs) (Figure 5.16). Therefore it is surprising that the *dInRDN* flies exhibit a robust lifespan extension in response to the EAAs and only a nominal increase in fecundity, whilst the effect of methionine supplementation on fecundity is entirely blocked. One possible explanation for these effects is that the *dInRDN* flies, due to their smaller body size and reduced fecundity, have lower nutritional demands and subsequently reduce their feeding activity. This could be investigated further by comparing feeding rates and food uptake at regular intervals throughout life to determine whether *dInRDN* flies

feed less. Moreover, the reduced body-size of *dInRDN* flies may limit their physiological reproductive capacity, hence the addition of methionine or EAAs would have negligible effects because their egg-laying capabilities are already maximised and restricted by the size of their ovaries for example.

Future studies might investigate the response of other IIS pathway mutants including *chico* heterozygotes which are long-lived, but have normal body size and fecundity (Clancy *et al.* 2001) to determine whether elevated fecundity due to EAAs or methionine in the diet can also be (partially) blocked by these mutants. c*hico* heterozygotes are also thought to have similar feeding behaviour to controls on DR and fully-fed diets (R. Wong, unpublished data), making them a suitable mutant line to use for future experiments. In addition, it would be interesting to establish whether IIS signalling is affected by methionine or EAA supplementation in specific tissues, particularly ovaries. This could be tested by dissecting ovaries of wild-type flies fed EAA or methionine supplemented diets and performing Western blots using phospo-Akt (PKB) as an output of insulin signalling. Furthermore, an ovary specific GAL4 driver, such as c323a or c825 (Manseau *et al.* 1997; Beaucher *et al.* 2007), could be used to express *dInRDN* or *dS6K* dominant-negative activity solely in the ovaries.

5.4.7 Concluding remarks

Previous dietary restriction (DR) studies in multiple organisms have revealed that the beneficial effects of lifespan extension are commonly traded-off with decreased fecundity. Furthermore, the results of chapter 4 suggested that the concentration of essential amino acids in the diet (EAAs) regulate a direct trade-off between high fecundity on one hand and reduced lifespan on the other, mimicking the effects of

full feeding. Fascinatingly, upon further dissection of the role of individual EAAs, it was demonstrated that the long lifespan of DR-fed flies and the high fecundity of fully-fed flies could be simultaneously achieved by supplementing the DR diet with methionine alone. Flies fed methionine-supplemented diets exhibited elevated fecundity, to a similar magnitude achieved with full feeding, whilst maintaining the lifespan extension observed with DR. Moreover, these extraordinary phenotypes could not be explained by increased feeding or *Wolbachia* infection in the wild-type population. The life-shortening effects of full feeding thus appear to be a consequence of an imbalance of the ratios of amino acids, particularly EAAs, in the diet compared with the ratio required to achieve maximized fecundity.

Preliminary work investigating two potential pathways (TOR / S6K and the IIS pathways) mediating the effects of methionine and EAA supplementation suggests that *dS6* kinase may be acting as a negative regulator of high fecundity because *dS6K* dominant-negative flies fed an EAA-supplemented diet exhibited a further increase in fecundity compared to the already high fecundity of control lines fed the same diet. In addition, reduced IIS signalling, through insulin receptor dominant-negative activity, partially blocked the reduced lifespan associated with adding back all EAAs and the high fecundity of flies fed DR diets with either methionine or all EAAs added back. The precise roles of the IIS and the TOR / S6K pathways in regulation of lifespan and fecundity in response to dietary amino acids would need to be investigated further.

Chapter 6

General discussion and future directions

6.1 Discussion

The objective of the work presented in this thesis was to investigate factors affecting the responses of lifespan and fecundity to *Drosophila* dietary restriction (DR). The effects of DR appear to be evolutionary conserved across taxa, with organisms from unicellular yeast through to mammals, possible including primates, exhibiting lifespan extension. Despite a vast amount of literature on the effects of DR on rodents, shorter-lived model organisms including *D. melanogaster*, *C. elegans* and *S. cerevisiae* have emerged as powerful tools to try and establish the potential mechanisms involved in mediating DR. *Drosophila* was chosen as an ideal model organism for these studies primarily due to its short generation time, relatively short lifespan and the ability to culture large numbers of flies for lifespan experiments. Furthermore, previous studies have shown that *Drosophila* exhibit a robust response to DR and the mechanisms that influence ageing appear to be evolutionary conserved in mammals.

The data presented in chapter 3 in conjunction with the recent work of (Bass *et al.* 2007a) highlight the absolute requirement of optimising lifespan and DR conditions before performing experiments. In addition, the results further demonstrate the importance of performing DR experiments over a range of food concentrations. A comparative analysis of the responses of six different wild-type *Drosophila* strains to a range of food concentrations revealed that the peak extension of lifespan for DR is affected by genetic background. This finding has important implications for research with other model organisms and may explain why previously published work with wild mice (Harper *et al.* 2006) and some mouse laboratory strains (Forster *et al.* 2003) failed to show a lifespan response to the DR regimens tested. These results

may also help to explain why no lifespan extension was reported in male flies (chapter 4) using the two concentrations that had been optimised for DR and full feeding in females. Moreover, studies testing the interaction between nutrition and genetic mutations that extend lifespan should also be conducted over a range of food concentrations. Choosing one DR and one fully-fed / *ad libitum* condition may produce misleading results as the lifespan of mutant lines may peak at different concentrations to controls. Additionally, although *Wolbachia* infection was shown not to mediate the responses to DR in *Drosophila*, its presence can potentially affect the food concentration at which lifespan is optimised. Hence, it is important when examining gene / nutrition interactions that both the genetic background and cytoplasm are standardised.

In multiple organisms, reducing food intake (DR) causes an increase in lifespan coupled with a reduction in fertility. This has led to the suggestions that lifespan extension under conditions of DR are caused by an evolved response to food shortages in nature with reallocation of nutrients from reproduction and towards somatic maintenance. In chapters 4 and 5, the role of specific nutrients regulating the lifespan / fecundity trade-off during DR was investigated to determine whether the same nutrients that increase fecundity also reduce lifespan, as predicted by the reallocation hypothesis. Breaking down the nutritional components of dietary yeast, which had previously been shown to be the key determinant of lifespan (Mair *et al.* 2005), revealed that of the four nutrient groups tested only amino acids and particularly essential amino acids regulate the effects of high fecundity and reduced lifespan with full feeding, whilst carbohydrates, vitamins and lipids had negligible effects. This work adds further support for the increasing evidence in both

invertebrate and rodent models that intake of specific nutrients, as opposed to calories, are as or more important in influencing lifespan.

In chapter 5, the role of specific essential amino acids was investigated in more detail to determine whether individual amino acids mediate the lifespan / fecundity trade-off. Adding back all EAAs except methionine was sufficient to rescue the reduced lifespan observed when adding back all EAAs together, whilst having no effect on fecundity compared with DR. However, fascinatingly, supplementing the DR diet with methionine alone could account for the full increase in fecundity with full feeding whilst increasing lifespan to a similar magnitude seen in dietary restricted flies. Lifespan of flies fed methionine-supplemented diets was reduced when the concentration of other EAAs in the diet was increased. The results of these experiments provide evidence that lifespan and fecundity need not trade-off, as has been revealed in some studies using longevity mutants. Consequently, DR in *Drosophila* does not extend lifespan either through reallocation of resources from reproduction to somatic maintenance and repair or due to reduced damage inflicted by lower egg production because these two hypotheses would predict that high fecundity and extended lifespan would be mutually exclusive.

Experiments conducted investigating potential interactions of EAAs with the IIS and the TOR pathways revealed some fascinating results that would form the basis of a more detailed study trying to elucidate the mechanistic responses to EAAs in the diet. Reduced IIS signalling, using insulin receptor dominant-negative flies, appears to partially block both the effects of reduced lifespan and increased fecundity caused by EAAs in the diet. In addition, experiments involving *dS6K* dominant-negative mutants suggest that *dS6K* may be acting as a negative regulator of high fecundity in

Drosophila because these flies exhibited a further increase in fecundity in response to EAAs compared with the already high fecundity of control lines.

6.2 Future work

Having established that the beneficial effects of DR in *Drosophila* in terms of lifespan do not need to be compromised by reduced fecundity, it will be important to determine whether in other organisms, including mammals, the benefits of DR in the absence of DR itself, can be achieved by a suitable balance of nutrients in the diet. The results of this thesis emphasise the importance of using a standard DR protocol between laboratories using the same model organism because small changes in the concentration a single nutrient, e.g. methionine, can have dramatic effects on the interpretation of DR experiments. Potentially, future research on DR in model organisms should set out to use a standardised synthetic medium.

In *Drosophila*, microarray experiments could be performed to determine whether particularly groups of genes are differentially regulated in flies fed a methionine-supplemented diet compared with flies fed a DR diet supplemented with all EAAs or a control diet. This may provide some valuable mechanistic insight into why adding back methionine alone increases fecundity without a reduction in lifespan, in contrast to the effects of full feeding or when adding back all EAAs. Preliminary experiments in this thesis revealed that the reduced lifespan and high fecundity of flies fed the EAA add-back diet could be partially blocked in insulin receptor dominant-negative flies. However, additional experiments would be required to test whether mutations in other components in the IIS pathway also cause a similar response. Previous work in *Drosophila* has pointed to an interaction between genotype and nutrition, with

lifespan of IIS mutants peaking at a higher food concentration than controls, as observed with *chico* homozygotes and flies with *dFOXO* over-expressed in the fat body (Clancy *et al.* 2002; Giannakou *et al.* 2008).

As methionine is the initiation codon for all protein sequences, further work might investigate the link between protein synthesis / protein turnover and lifespan in *Drosophila*, with particular focus on whether methionine supplementation is affecting protein translation in specific tissues. This could be tested by isolating polysomes (multiple ribosomes) over a sucrose gradient, to give an indication of the level of mRNA being translated (Monzo *et al.* 2006).

The identification of essential amino acids as the key component of the diet influencing lifespan provides the foundation to investigate why EAAs specifically might be mediating the reduced lifespan associated with full feeding. As a starting point, the effects of EAAs on cellular and molecular processes including the production of reactive oxygen species (ROS), cell damage, and further work with TOR signalling and protein translation should provide valuable insight into the mechanistic effects of whole-food DR. Future work might also further explore the interesting pilot experiments performed in this thesis which indicated a potential role of *dS6K* as a negative regulator of high fecundity.

The finding described in this thesis that adding back all EAAs with the exception of methionine (methionine restriction) is sufficient to recover the reduced lifespan when all 10 EAAs are added back to DR, suggests that lifespan extension by methionine restriction is evolutionary conserved with rodents. Whilst the mechanism of methionine restriction in *Drosophila* appeared to conform to a simple trade-off

between increased lifespan and reduced fecundity, mirroring the effects of whole-food DR, supplementing the DR diet with methionine alone could increase fecundity without a reduction in lifespan, suggesting other mechanisms are in operation. In rodents, it has been reported that methionine restriction also increases some antioxidant defences and leads to a reduction in ROS production (Richie *et al.* 1994; Lopez-Torres *et al.* 2002; Zimmerman *et al.* 2003; Pamplona and Barja 2006; Ayala *et al.* 2007). Hence, it would be interesting to determine whether similar cellular phenotypes are also observed in flies during methionine restriction. Primary experiments could establish whether methionine-restricted flies are more resistant to paraquat treatment, which induces oxidative stress. However, first and foremost it will be important to develop reliable biomarkers of ageing in *Drosophila*, because similarly to other invertebrate models, there is currently a lack of specific ageing-related markers. These markers will be paramount to understanding more about how ageing affects cellular and molecular damage in flies and to determine the specific effects of amino acids in the diet.

In *C.elegans*, inhibiting translation initiation factors including eIF4E extends lifespan (Hansen *et al.* 2007; Pan *et al.* 2007; Syntichaki *et al.* 2007a), providing evidence that a reduction in protein synthesis may be the mechanism involved in the effects observed. In *Drosophila*, TOR and S6K mutants are reportedly long-lived (Kapahi *et al.* 2004a), whilst experiments on flies carrying a null mutation for the eIF4E inhibitory protein 4E-BP (*Thor*) has revealed that the presence of 4E-BP is essential for starvation and oxidative stress resistance (Tettweiler *et al.* 2005). Future work should determine whether inhibition of eIF4E can extend lifespan in *Drosophila* as reported in *C.elegans*, and in the case that these *Drosophila* mutants are long-lived, whether methionine restriction can further extend their lifespan. If reduced protein

synthesis is the mechanism for lifespan extension by both inhibition of eIF4E and methionine restriction then no further lifespan extension should be detected. These proposed experiments should shed some light on the potential mechanistic actions upon methionine restriction and supplementation in *Drosophila*.

A more detailed study into the role of specific nutrients influencing male *Drosophila* lifespan over a greater range of concentrations would also enhance our understanding of how DR extends lifespan. The data in this thesis suggest that the enhanced response to DR observed in female *Drosophila* relative to males can no longer be explained simply by the nutritional effects on egg production causing a trade-off between high fecundity and reduced lifespan during full feeding. Hence, it is of interest to investigate the different nutritional requirements of males and females further and whether the underlying mechanisms extending lifespan by DR differ between the two sexes.

6.3 Final conclusions

In conclusion, the work presented in this thesis sheds light on the nutrients and mechanisms mediating extension of lifespan by DR in *Drosophila*, by providing three key findings. Firstly, methionine restriction extends lifespan in *Drosophila*, as previously reported in mice and rats. Secondly, the effects of lifespan of DR in *Drosophila* are mediated by predominantly the concentration of essential amino acids in the diet. Finally, the positive effects of lifespan extension by DR in *Drosophila* need not trade-off with a cost in fecundity if the diet contains a suitable balance of nutrients, suggesting that lifespan extension by DR in *Drosophila* cannot be explained by the reallocation of resources hypothesis or by increased damage due

to elevated fecundity with full feeding. An imbalance in the proportions of amino acids present in food, compared with the ratio that is optimal for reproduction, both shortens lifespan during full feeding and limits fecundity during DR, and hence produces the DR responses. Whether this finding also plays a role in the extension of lifespan in mammals, including humans, remains to be determined. However, these findings suggest interesting roles for individual nutrients in the extension of lifespan without reproductive costs, which should form the basis of further studies in other organisms.

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Appendix 1

Bass, T. M., Grandison, R. C., Wong, R., Martinez, P., Partridge, L. and Piper, M. D. (2007). "Optimization of dietary restriction protocols in *Drosophila*." J Gerontol A Biol Sci Med Sci **62**(10): 1071-81.

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Optimization of Dietary Restriction Protocols in *Drosophila*

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Dietary restriction (DR) extends life span in many organisms, through unknown mechanisms that may or may not be evolutionarily conserved. Because different laboratories use different diets and techniques for implementing DR, the outcomes may not be strictly comparable. This complicates intra- and interspecific comparisons of the mechanisms of DR and is therefore central to the use of model organisms to research this topic. *Drosophila melanogaster* is an important model for the study of DR, but the nutritional content of its diet is typically poorly defined. We have compared fly diets composed of different yeasts for their effect on life span and fecundity. We found that only one diet was appropriate for DR experiments, indicating that much of the published work on fly ''DR'' may have included adverse effects of food composition. We propose procedures to ensure that diets are suitable for the study of DR in *Drosophila*.

DIETARY restriction (DR) refers to a moderate reduction of food intake that leads to extension of life span beyond that of normal, healthy individuals. This intervention has principally been studied in rodents, but it also extends the life span of a wide range of organisms including the fruit fly, *Drosophila melanogaster* (1–7). Although extension of life span in response to DR is taxonomically widespread, it is unknown whether evolutionarily conserved mechanisms are at work or, instead, whether this is a case of evolutionary convergence (8). This issue is important, because upon its resolution depends the utility of the powerful invertebrate model organisms for understanding the mechanisms of the response to DR in mammals.

Considerable attention has been paid to the dietary components that are important for extension of life span by DR in rodents, where reduction of whole food intake can increase life span by approximately 40% (3). These studies have shown that altering the ratio of nutritional components, by reducing lipids, minerals, or vitamins in the diet, had no effect on rat life span, although reduction of the protein quantity or quality effected a relatively small increase (9–13). More recent work has shown that specific reduction of tryptophan (14) or methionine (15–17) can extend rodent life span to a similar magnitude as whole-food DR. On the one hand, these interventions with specific nutrients may reveal useful information about the mechanisms of whole-food DR; on the other hand, each intervention could operate through different molecular pathways to extend life span, thus revealing little or nothing about the mechanisms of whole-food DR (18). Similar debate exists over the potentially different mechanisms by which yeast replicative life span is increased when glucose is reduced from 2% to 0.05% (19) or from 2% to 0.5% (20,21). In *Caenorhabditis elegans*, several possible modes of life-span extension by food reduction exist as life span can be extended by dilution of the bacterial food source (22), complete removal of the bacterial food source (23,24), altering the strain of bacterium used in the worms' diet (25,26), or using synthetic axenic media (27,28). To establish the mechanisms at work for any particular method of DR in any model organism, precise specification and, preferably, standardization of DR methods is desirable as a basis for intra- and interspecific comparisons.

DR is usually imposed in *Drosophila* by dilution of an agar-gelled food medium, which is always present in excess (29). In general, as food is diluted from a high concentration, life span increases to a peak at intermediate nutrient levels through DR, and then falls with further food dilution through starvation. It is generally assumed that the increase in life span with DR is a response to reduced nutrients. However, logically, it could just as well be a response to relief from a nonnutritional, toxic effect of the food (30). This is not an easy issue to address empirically, but some evidence can be drawn from parallel effects of diet on reproductive output, which can provide an independent indication of the effect of the diet on the organism's nutritional status. In a manner similar to that for DR in worms and mice (22,31), a decrease in life span in response to increased nutrition should be accompanied by increased daily and lifetime fecundity (5,6). In contrast, increase in the concentration of a toxin would be expected to cause life span to decrease in parallel with a reduction or no increase in fecundity.

DR in *Drosophila* usually involves reduction of the yeast and sugar components of the diet (29), and yeast appears to account for the majority of the DR effect on life span (5,32). However, different laboratories use different sources of yeast and different concentrations of sugar, yeast, and agar for DR (5,6,33,34). Despite these differences, few laboratories have tested their diets to ensure that the effect of DR

Table 1. Recipe Used to Make Food

Media	Components	Supplier	Name
100 g	Yeast*	Baker's (B.T.P. Drewitt, London, U.K.)	SYBaker
		Brewer's (MP Biomedicals, Solon, OH)	SYBrewer
		Torula (Borregaard, Sarpsborg, Norway)	SYTorula
		Bacto Yeast extract (BD Diagnostics, Sparks, MD)	SYExtract & CSYExtract
50 g	Sucrose	(Tate & Lyle Sugars, London, U.K.)	
50 g	Cornmeal†	(B.T.P. Drewitt, London, U.K.)	
10 g	Agar	(Sigma, Dorset, U.K.)	
3 mL	Propionic acid	(Sigma, Dorset, U.K.)	
30 mL	Nipagin M‡	(Clariant UK Ltd, Pontypridd, U.K.)	
1000 mL	Made to final volume with distilled water		

Notes: The values in this table describe the arbitrary reference condition (1.0) used in dietary restriction (DR) experiments and for rearing flies. Where indicated in the text, the yeast, sugar, and agar concentrations were varied.

*For yeast comparison experiments, the yeast concentration alone was varied from 10 g/L (0.1) to 200 g/L (2.0).

†Cornmeal (organic polenta) was used for the CSYExtract medium only.

‡Solution of 100 g/L methyl 4-hydroxybenzoate in 95% ethanol.

on life span in their experiments is a specific response to nutrition as evidenced by reduced reproductive output. To gauge the importance of these differences, and to establish a validated DR diet that should be reproducible between laboratories, we assembled a range of yeast-based diets and directly compared the life span and fecundity of flies in response to DR on each food type. Of the diets that we investigated, only one showed effects on survival and fecundity that is suitable for DR studies in *Drosophila*.

Methods

Fly Stocks, Maintenance, and Handling Procedures

All experiments were performed with the wild type, outbred, laboratory strain Dahomey. The population is maintained in large population cages with overlapping generations on a 12-hour light/dark cycle at 25°C and 65% humidity.

Media

Rearing of flies and experiments were performed on standard sugar/yeast (SY) food (35). The arbitrary standard condition (1.0) is described in Table 1. In all cases, the food was prepared by adding the agar to water and bringing to a boil on a gas hob. At this point, the appropriate amounts of sugar and yeast (and cornmeal where indicated) were added with continuous stirring until the food was completely mixed. The food was then removed from the heat and allowed to cool to 65°C. At this point, preservatives were mixed in, and the food was dispensed. For the sugar range experiments, baker's yeast (Table 1) was used. Media for the comparison of dietary yeasts were based on that in Table 1, with only the yeast component varied. For the water add-back experiment, a 1% agar solution was made (containing preservatives as for the SY media) and poured into individual 200-μL pipette tips. These tips were trimmed to a length that brought the agar solution close to the level of the food surface after being inserted into the food. A pipette tip filled with cotton wool, to prevent flies from crawling into the pipette tip and becoming trapped, was added to the control treatment.

Life Span and Fecundity Assays

For life-span experiments, larvae were reared at standard density in 200-mL glass bottles containing 70 mL of 1.0 SY food (36). Flies emerged over 24 hours, were tipped into fresh bottles, and were allowed 48 hours to mate. Females were then separated from males under light CO_2 anesthesia and randomly allocated to different food treatments at a density of 10 females per vial. Flies were transferred to fresh vials, and deaths were scored at least every 2 days. The yeast comparison experiment was performed in two batches, the first containing SYBaker's, SYBrewer's, and SYTorula, and the second containing SYBaker's, SYBrewer's, SYExtract, and CSYExtract. Due to the similarity between the two trials of SYBaker's and SYBrewer's (Supplementary Figure 1 and Supplementary Table 1), the data were combined. For each condition in each experiment, 100 flies were used.

For fecundity measurements, the same experimental flies as those used for life spans were kept in the same glass vials for between 18 and 24 hours; they were then transferred to fresh food. The eggs in the vacated vials were counted manually under a microscope. For the sugar concentration experiment, egg counts were performed on days 3, 7, 10, 14, and 21 of treatment. For the first yeast comparison experiment (SYBaker's, SYBrewer's, and SYTorula), eggs were counted on days 5, 9, 12, 16, 19, 23, 26, 30; for the second experiment (SYBaker's, SYBrewer's, SYExtract and CSYExtract), eggs were counted on days 4, 8, 11, 15, 18, 22, 25, and 29. Eggs were counted on days 3, 6, 10, 13, 17, 26, 31, and 38 for the water add-back experiment and on days 4, 11, 18, 25, 32, 46, and 60 for the agar concentration range experiment. As an index of lifetime fecundity, the sum of eggs laid during 24 hours on the days of counting by an average female was calculated. These sampling points cover the period of heaviest laying, and are therefore indicative of relative lifetime fecundity (6).

Data Analyses

Comparison of survivorship data was performed using the log-rank test implemented in Excel. Values of p from comparisons of fecundity data refer to the nonparametric Wilcoxon rank sum test performed in R, v2.2.1 (37). For the

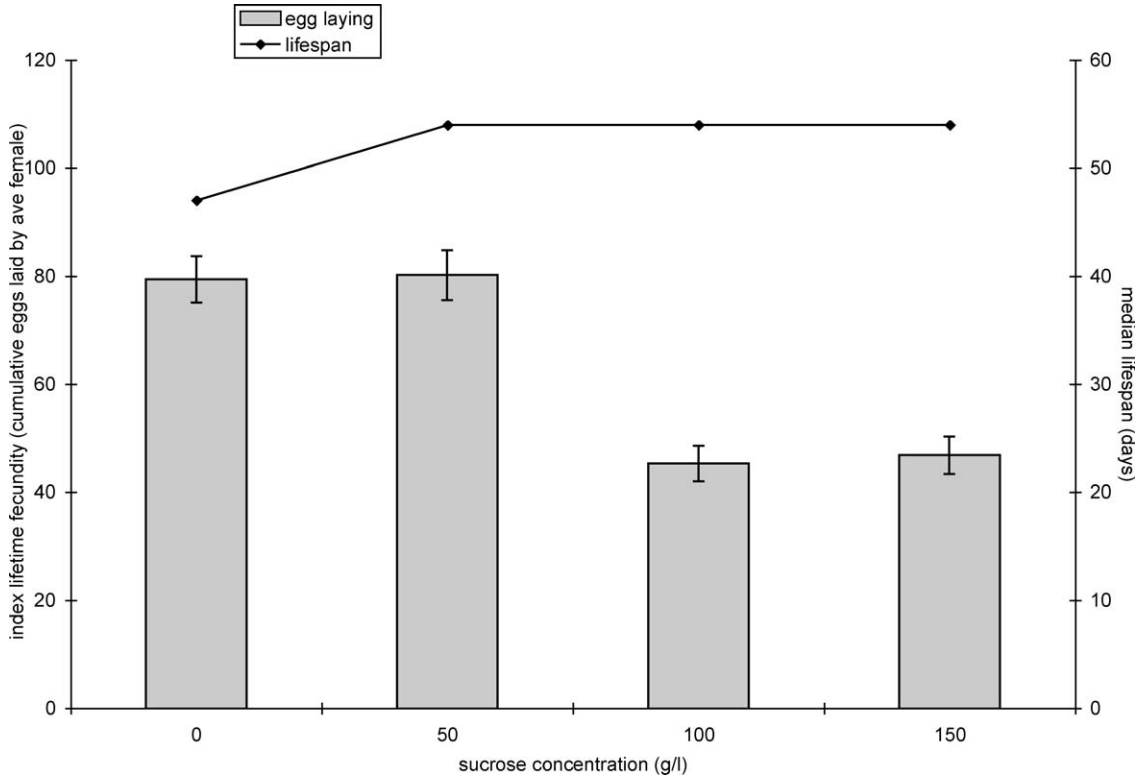

Figure 1. Effect of dietary sucrose concentration on life span and fecundity of mated *Drosophila* females. Increasing concentrations of sucrose were added to a standard food background of 1.5 SYBaker's (Table 1). Over the range of sucrose tested, very little change in life span was observed, whereas a significant decrease in fecundity was observed between 50 g/L and 100 g/L sucrose. *Gray bars:* index of lifetime fecundity (sum of the eggs laid by an average female on the days counted) ± standard error of the mean; *connected black points:* median life span. Representative data from one of two experiments are shown.

more complex comparisons of fecundity data illustrated in Figure 2, the nlme package in R was used (38), specifying a mixed model with yeast type, yeast concentration, and the quadratic function of concentration as fixed terms. Replicate vials were included as a random variable to compensate for multiple females per vial. To deal with the observed increasing variance with increasing fitted values (heteroscedasticity), we modeled the variance as a power function of the fitted values (such weighting of the variance structure improved the fit of the model, although it did not change the results). All factors and interactions were significant. Modeled versus actual data are shown in Supplementary Figure 2.

Nutritional Analysis of Yeast

Chemical analysis of a sample of baker's yeast was performed by Leatherhead Food International (Somerset, U.K.).

Results

High Levels of Dietary Sucrose Adversely Affect Fecundity With Little Effect on Life Span

Although it has been shown that the yeast component of an SY diet is critical for the response to DR in *Drosophila*, sucrose could also produce life-shortening effects similar to those of yeast if raised to sufficiently high concentrations [i.e., higher than those used previously (32)]. To test this, we looked at the effect of varying the sucrose concentration in the diet while keeping all other ingredients at a fixed level.

Interestingly, there was no requirement for dietary sucrose for maximum fecundity and, surprisingly, addition of sucrose at $\geq$100 g/L caused a decrease in female fecundity ($p < .00002$, Wilcoxon rank sum test), indicating that it had a detrimental effect on fly physiology and/or behavior. To ensure that nutrition, and therefore DR, is the key determinant of life span, fecundity should increase for increases in nutrition that cause life span to decrease. These data, therefore, show that sucrose concentrations $>$ 50 g/L are not appropriate for DR studies. For optimum longevity, the flies required the level of dietary sucrose to be at least 50 g/L in an SY diet. This effect of sucrose is shown in Figure 1 as a small, but significant, increase in median life span when sucrose was added to a yeast-only diet (50 g/L vs 0 g/L; $p < .00001$, log-rank test). Raising the sucrose concentration further to 150 g/L caused no decrease in median life span in this experiment, but it has done so in other experiments that we have performed [data not shown and (32)]. As a result, further experiments reported herein used a fixed sucrose concentration of 50 g/L as this was neither detrimental to life span nor inhibitory to egg laying.

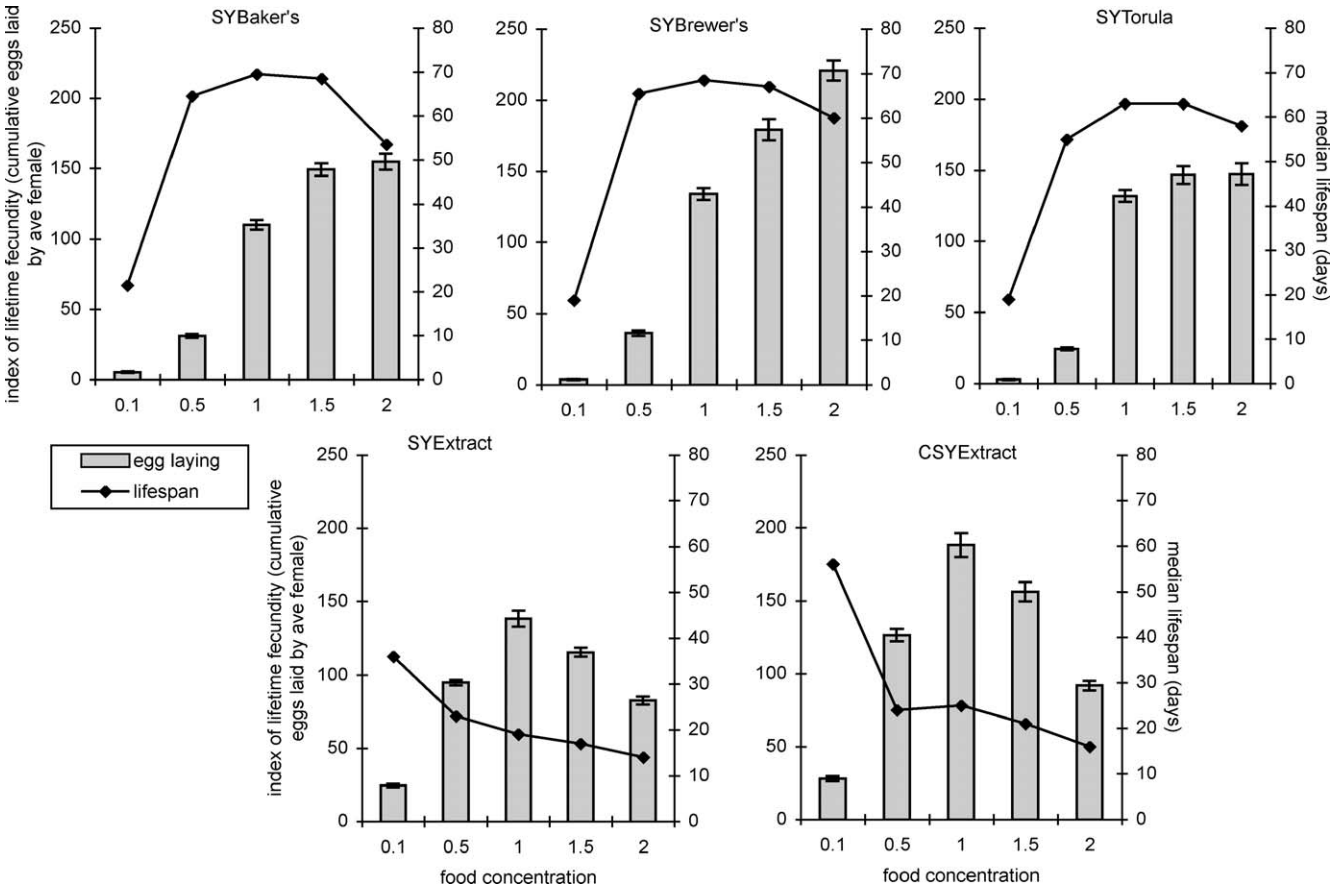

Figure 2. Effect of a range of concentrations of different commercially available yeasts on life span and fecundity. Five different yeast concentrations were prepared for each of five different sugar/yeast (SY) recipes. SYBaker's, SYBrewer's, and SYTorula each refer to food made with different, inactivated whole-yeast preparations, whereas SYExtract and CSYExtract refer to diets based on a water-soluble yeast extract. The nutritional components in each food type were sucrose and yeast or yeast extract and cornmeal (for CSYExtract only). *Bars:* index of lifetime fecundity ± standard error of the mean; *connected black points:* median life-span values. We specified a linear model to describe fecundity (Materials and Methods), which found all factors and interactions to be significant. The predicted values are plotted against observed values in Supplementary Figure 2. Each food concentration range was performed once, except for SYBaker's and SYBrewer's, which were performed twice.

Varying the Quality of the Yeast Supply Produces a Range of Effects on Life Span and Fecundity

The above data and (32) show that DR in *Drosophila* is achieved solely by modulating the yeast component of the diet. We next compared a variety of different yeasts to determine their effects on life span and fecundity. These experiments included four sources of inactivated yeast: a baker's yeast, a brewer's yeast, a torula yeast, and a water-soluble extract of baker's yeast. The first three of these yeasts are whole-cell lysates, whereas the fourth is a purified extract. Each of the yeasts was used over a range of concentrations from 10 g/L (labeled 0.1) to 200 g/L (labeled 2.0) while the other media constituents were held constant (Table 1).

Comparison of the three whole-yeast food types (SYBaker's, SYBrewer's, and SYTorula) showed a similar pattern for median life span, with a peak at 1.0 (100 g/L) and a decline as food concentration was changed above or below this point (top three graphs of Figure 2). SYBaker's and SYBrewer's yielded the longest life spans (69- and 70-day medians, respectively, on 1.0 food), whereas the longest life span on SYTorula (63-day median at 1.0) was significantly shorter ($p < .0001$ in both comparisons, log-rank test). For each of these three yeasts, lifetime fecundity increased with increasing food concentration to 1.5, above which there was no further increase for SYBaker's and SYTorula, but there was for SYBrewer's when the concentration was raised from 1.5 to 2.0 (Supplementary Figure 2). Furthermore, the level of egg laying on 2.0 SYBrewer's was higher than the peak value for any of the other food types tested. Thus, the observed limit to egg-laying on the other food types was not intrinsic to the physiology of the flies, but was restricted by some feature of the foods. In other experiments we have also raised the yeast concentration in SYBrewer's medium to 300 g/L (3.0) and saw a further life-span shortening from 2.0 ($p < .05$, log-rank test). However, this was not accompanied by a further increase in fecundity beyond the level in 2.0 ($p = .53$, Wilcoxon rank sum test; Supplementary Figure 3).

The flies responded differently to the yeast-extract–based media. The most obvious difference was that life span decreased for each addition of yeast extract to the medium. This was similar for CSYExtract and SYExtract (bottom

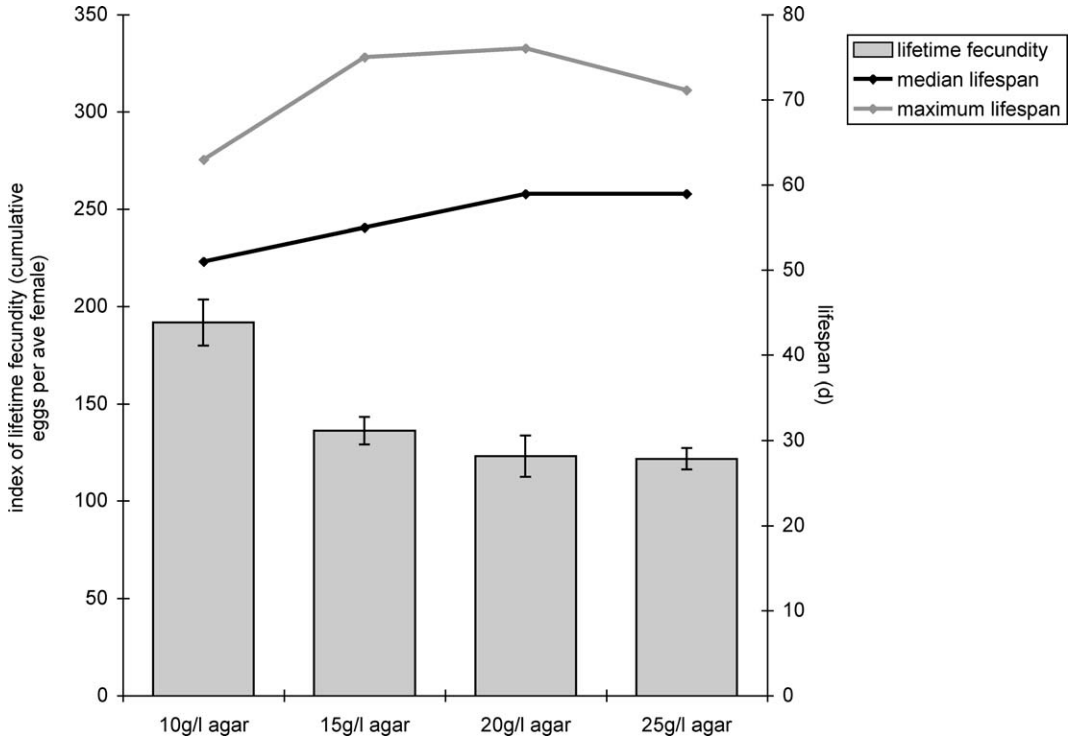

Figure 3. Effect of varying agar concentration on life span and fecundity of females on SYBrewer's medium. The effect of food hardness on life span and fecundity was tested by altering the agar concentration while all other ingredients were held at fixed concentrations (Table 1). This medium contained Brewer's yeast at 200 g/L (2.0 level) (agar concentration ranges were also tested at two other SYBrewer's concentrations; data not shown). *Bars:* index of lifetime fecundity ± standard error of the mean; *connected black points:* median life span; *connected gray points:* maximum life span (median of the last 10% survivorship).

two graphs of Figure 2), except for 0.1, at which level cornmeal addition resulted in a significantly longer life span (36 days on SYExtract vs 56 days on CSYExtract; $p < .0001$, log-rank test). Because the positive effect of cornflour on life span was only seen at the lowest concentration of yeast extract (0.1) and the longest life span on 0.1 SYExtract was low compared with all other treatments, the data are compatible with an argument that yeast extract caused dose-dependent toxicity. The pattern of lifetime fecundity was similar between SYExtract and CSYExtract, increasing with yeast extract addition to a maximum at 1.0, but decreasing at higher concentrations. Cornmeal addition augmented egg laying, which peaked in 1.0 CSYExtract at a level similar to that in 1.5 SYBrewer's and higher than the maxima for the other three food types. In both the presence and absence of cornmeal, yeast extract was apparently more nutritionally dense than whole-yeast powders, because egg laying was greater on CSYExtract (up to 1.0) and SYExtract (up to 0.5) than on the whole-yeast diets at corresponding food concentrations. However, fecundity decreased for additions of yeast extract higher than 100 g/L (1.0). Thus, yeast extract at high concentrations is detrimental to fecundity in addition to negatively affecting life span.

Is DR in Drosophila *a Nutritional Response?*

In order to fulfill the requirements for DR, it is necessary that the longer-lived (restricted) animals are not simply less sick than those with higher nutritional intake. One indication of this comes from increased fecundity with increasing nutrients. However, if the food delivers both nutrients to benefit fecundity as well as a toxic effect that reduces life span, the phenotype would be indistinguishable from a true effect of DR (30). It is therefore important to try and distinguish directly between a toxin-based and a nutrient-based explanation for the life span–shortening effect of the high nutrient concentration.

Increasing the food concentration could mimic a DR effect by increasing the hardness of the food. To test this possibility, we fixed the concentration of all food ingredients (at 2.0 SYBrewer's) and varied the agar concentration on its own (Figure 3). For each increase in agar concentration, there was a trend toward a decrease in lifetime fecundity. However, this trend was only significant for the increase from 10 g/L to 15 g/L ($p < .0005$, Wilcoxon rank sum test). This reduction was accompanied by a significant increase in life span when the agar concentration was raised from 10 g/L to 15 g/L ($p < .01$, log-rank test) and a further, nonsignificant ($p = .09$, log-rank test) increase when agar was raised to 20 g/L. These data are consistent with agar controlling food availability in a nondetrimental way between 10 g/L and 20 g/L agar, and is therefore explicable as a DR effect. When the agar concentration was further increased to 25 g/L there was no change in median life span or lifetime fecundity, but maximum life span decreased from a median of 76 days to 71 days (Figure 3). This result argues that older flies do indeed differentially suffer if the food

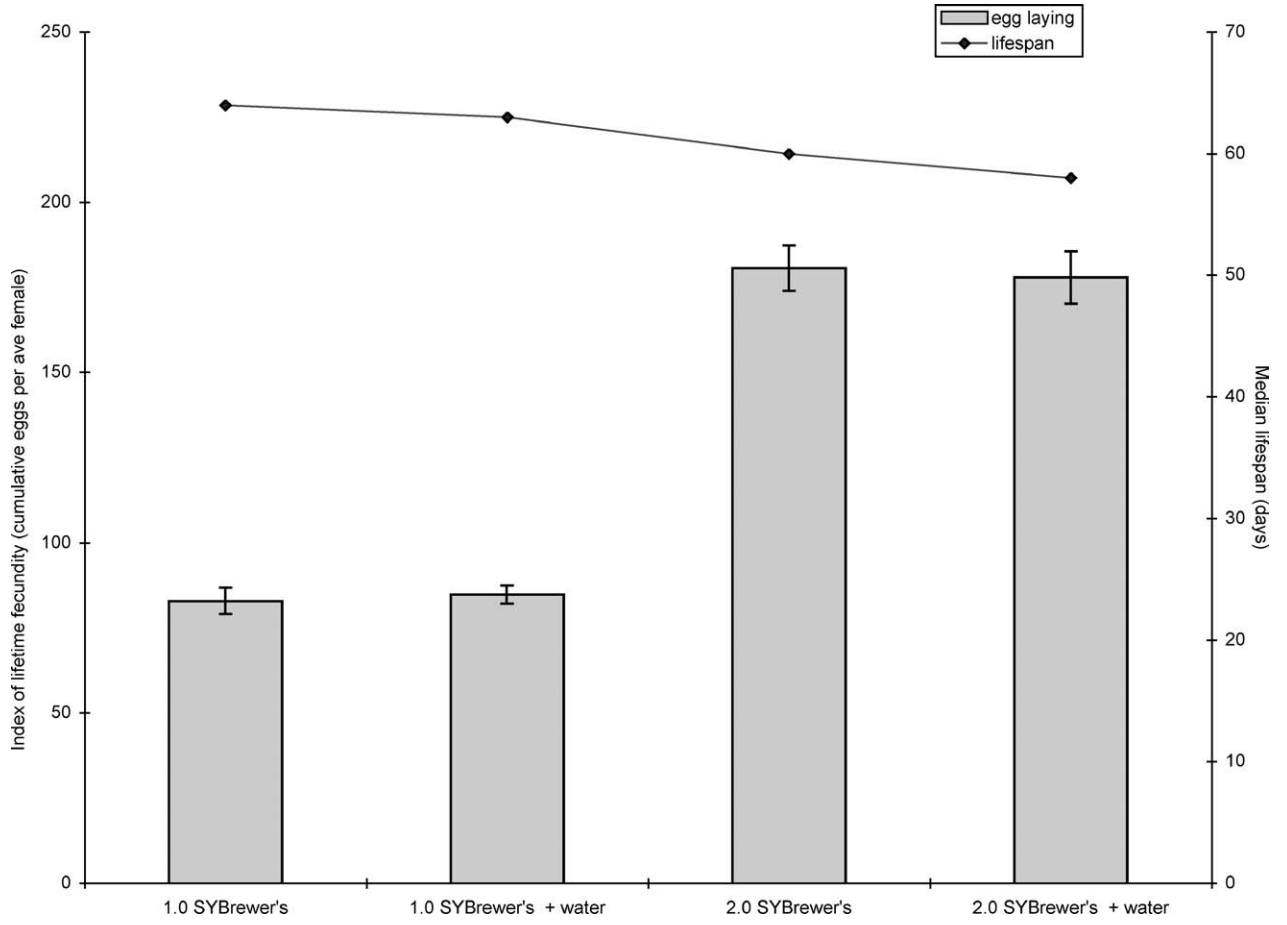

Figure 4. Effect of water addition on the dietary restriction (DR) response of flies on SYBrewer's medium. Free access to water was provided in the form of 1% agar in a pipette tip inserted into the food. *Bars:* index of lifetime fecundity ± standard error of the mean; *connected points:* median life span. Experiment was performed twice.

becomes sufficiently hard, but for agar concentrations < 20 g/L food hardness does not on its own cause the life-shortening (DR) effect seen in Figure 2. We also tested the effect of agar concentration for 1.0 SYBrewer's and 3.0 SYBrewer's (data not shown). Although qualitatively similar, this experiment also revealed an interaction with the yeast concentration, whereby flies were more sensitive to higher agar concentrations at higher yeast concentrations. This result indicates that the yeast content of the food can contribute to overall food hardness and adversely affect life span.

Another possible detrimental effect of high food concentrations concerns water availability, because the food is the only source of water. We therefore tested if water addition could overcome the adverse effects of high nutrition levels on life span. Figure 4 shows that addition of a fresh source of water to 1.0 and 2.0 SYBrewer's could not rescue the life-shortening effect of high nutrient concentrations and had no effect on lifetime fecundity. Therefore, inability to access sufficient free water does not explain the life span–shortening effect accompanying high nutrient concentrations in the food.

Discussion

DR is a well-established intervention for extending fly life span. Indeed, the interaction among diet, life span, and fecundity has formed the basis for both practical and theoretical investigations into the possible trade-offs between these life-history traits (39). Here we have investigated DR more closely and found that, without careful attention to the food composition, studies that claim to be examining extended life span due to DR may simply be studying the rescue of normal life span from the effects of inappropriate food types that prematurely shorten life. It thus follows that any mechanistic conclusions drawn from such studies are likely to be obscured by the detrimental effects of the food and so would be inappropriate to address questions of how DR operates to preserve life span for *Drosophila* or other species.

Drosophila in the wild is thought to coconsume fruit material and microbes from fermenting and/or rotting fruit (40). In the laboratory, *Drosophila* can be maintained on a combination of sugar, yeast, and water (35). We found that addition of sugar > 50 g/L to the culture medium was detrimental for egg laying and that variations from 0 to 150

g/L had little effect on life span (Figure 1). These data indicate that *Drosophila* has a very low requirement for free sugar for maximal life span and fecundity, consistent with the finding that total sugar levels in rotting banana are no more than 20 mM (equivalent to 4.5 g/L sucrose) (41). Other experiments have shown that *Drosophila* modulate their feeding behavior only slightly, or not at all, when sucrose levels rise above 50 g/L (32,42,43). Thus, the dramatically lowered egg laying observed with high sugar is unlikely to be an effect of reduced feeding in response to the altered sucrose concentration, and instead probably reflects an adverse effect on physiology due to the presence of unnaturally high sugar levels. These data show that high sugar should be avoided in *Drosophila* DR experiments.

In contrast, increasing additions of one particular brewer's yeast caused lifetime fecundity to continually increase over a concentration range that also decreased life span and so conformed to the expectations of a DR treatment. When recently changing our yeast supplier, we noted a shift in the concentration at which life span peaked from 65 g/L yeast [0.65 in (32)] to 100 g/L (1.0 shown here). Yeast quality is thus highly variable. Furthermore, high yeast concentrations that reduce life span are not always associated with increasing fecundity. This fact is at odds with the recognized effect of DR on fecundity in worms (22) and rodents (31), and is consistent with an explanation that the life-span decrease on high food concentrations is not an effect of increased nutrition, but due to some detrimental effect of the yeast composition. This could be caused by either a direct effect of a specific toxic element whose increasing concentration reduces life span and perhaps also fecundity or an indirect effect of a nutritionally imbalanced diet that results in ill health.

Under the first explanation, one would expect a pattern of fecundity and life span similar to that seen for the flies fed increasing concentrations of yeast extract. In this situation, both nutrients and the toxin (e.g., a heavy metal) are delivered in the food. This situation results in increasing fecundity as nutrients increase and toxicity remains below a tolerable threshold (e.g., 1.0 in SYExtract and CSYExtract in Figure 2), beyond which fecundity is reduced. For this same concentration range, life span would be ever decreasing. This explanation is consistent with data for *C. elegans* grown on different types of bacteria. It is currently common practice to grow worms on *Escherichia coli*, which can support growth and reproduction and upon dilution elicit an apparent DR response (22). However, when the worms are grown on the soil bacterium *Bacillus Subtilis*, their life span is increased some 50% without changes in development time or reproductive output (26). Thus, any nutrient-dependent life-span shortening when increasing the concentrations of *E. coli* for worms or yeast extract for flies would be combined with the effects of food toxicity.

In contrast, nutritional imbalance would be expected to yield a life-history pattern like that for SYBaker's and SYTorula, where the absence of a nutritional component imposes a limit on egg-laying capacity due to depletion from parental reserves. Previous data on the nutritional requirements of adult *Drosophila* showed that deficiencies for essential amino acids, chloride, phosphorous, or calcium reduced egg laying within 16 days, with little effect on the short-term viability of the adult (44). Thus a trace element shortfall may limit lifetime egg-laying capacity with little effect on immediate risk of death. An example of this phenotype is shown for flies on 1.5 and 2.0 SYBaker's, which have the same level of lifetime fecundity but markedly different life spans (Figure 2). Because they both experience the same limitation to lifetime fecundity, the limitation in itself is not what causes shortened life span on 2.0. Rather, the increasing excess of other dietary components, and so nutrient imbalance, is the most likely explanation for the elevated mortality.

In an attempt to identify any such toxins or nutrient imbalances, we have compared the available nutritional data for each of the yeast types used (Supplementary Table 2). Unfortunately, these analyses have a limited scope because only standard nutritional constituents are measured; therefore, many potentially toxic compounds will be overlooked. It is possible, however, to compare nutrient ratios among yeasts. In this light it is notable that several vitamins are an order of magnitude lower in concentration in SYBaker's than in SYBrewer's. These vitamins include biotin, a deficiency of which is thought to shorten *Drosophila* life span (45). This could be tested by the addition of these vitamins to the food to see if they rescue fecundity and affect life span. As a note, it is possible that similarly subtle effects of food type belie unknown nutrient imbalances in DR experiments that have been performed in other model organisms. For example, rescue from a nutrient imbalance could explain the life-span extension found in rats when the dietary protein source casein was replaced with soy protein (12). Subtle differences in food affecting life span have also been demonstrated by experiments on mice and rats subjected to methionine restriction (15,17,46). Thus, diet optimization is also an important consideration for DR studies in rodents, in which food composition varies depending on the particular commercially available chow that is used.

Despite all these precautions to establish a diet suitable for *Drosophila* DR, it is still possible that the food could have a detrimental effect on life span unrelated to nutrition and with no adverse effect on lifetime fecundity, thus mimicking the DR effect. Because we use a food dilution method for DR, the hardness of the food and water availability are the most likely candidates to produce such an effect. Our experiments showed that neither could account for the life-span shortening seen when varying the yeast concentration. We did note, however, a detrimental effect on maximum life span when agar concentration was raised to an extremely high level (25 g/L, more than twice that used for our other experiments). This effect was exacerbated when the yeast concentration was also raised to 300 g/L, showing that food hardness can reduce *Drosophila* life span. This non-DR–based life-shortening effect of hard food is likely to have contributed heavily to the life-span shortening seen in studies when yeast and sugar are both raised to 300 g/L and agar to 20 g/L (34,47) (Supplementary Figure 3).

Based on the data presented above, we conclude that the brewer's yeast is the most suitable of those that we tested

for DR studies and it now forms the basis for our laboratory recipes. This change has the additional advantage of bringing the nutritional content of our fly diet in line with that of two other laboratories studying fly DR using the same yeast (Helfand and Pletcher laboratories, Scott Pletcher, personal communication, 2005). We are now in the process of extending this study by applying this DR regimen to male flies as well as a variety of commonly used laboratory strains of *Drosophila*. As there is an impact of genotype on the fly response to DR (48), it will be interesting to see if other laboratory strains (both inbred and outbred) exhibit a similar response to these foods. Modulations or even loss of the DR response in these lines may be informative about the mechanisms of DR.

Conclusion

This work highlights the need for validated diets used for DR as a step toward establishing some dietary uniformity in the DR community to allow direct comparison of different experiments with the same species and of different species. For flies, the dramatic variability in quality of yeasts from different suppliers, and presumably between seasons, points to the need for a defined synthetic medium that would avoid the potential problems of unwanted detrimental effects being introduced into *Drosophila* experiments from the yeast or its feedstock.

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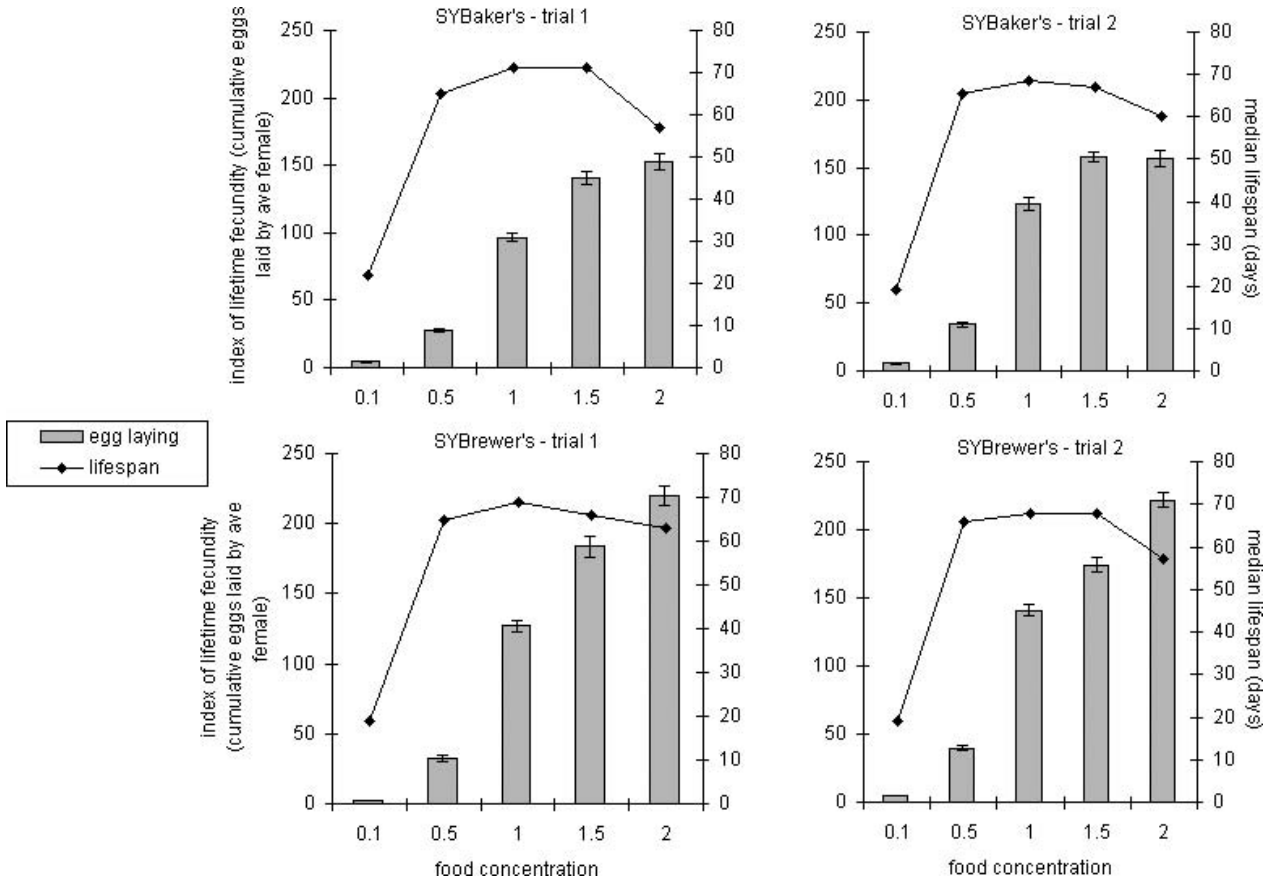
Supplementary Figures and Tables



Supplementary Figure 1. Data for both trials run for SYBaker's and SYBrewer's. These are independently replicated data sets from nonoverlapping generations of flies.

Supplementary Table 1. Life Span and Fecundity Data for Survivorships Referred to in Figures

Condition	Lifetime Fecundity*	(*SEM*)	Median LS	Mean LS	(*SEM*)
Figure 1					
0 g/L sucrose	79.4	(4.3)	47	42.5	(1.4)
50 g/L sucrose	80.2	(4.6)	54	52	(1)
100 g/L sucrose	45.3	(3.3)	54	55	(1.1)
150 g/L sucrose	46.9	(3.5)	54	52	(1.3)
Figure 2 and Supplementary Figure 1					
0.1 SYBaker's – Trial 1	5.9	(0.6)	21	21.9	(0.5)
0.5 SYBaker's – Trial 1	34.2	(1.4)	64	62.7	(1.1)
1.0 SYBaker's – Trial 1	123.3	(4.6)	68	67.0	(0.8)
1.5 SYBaker's – Trial 1	158.2	(3.7)	66	64.7	(0.9)
2.0 SYBaker's – Trial 1	157.0	(5.9)	50	49.4	(1.6)
0.1 SYBaker's – Trial 2	4.4	(0.5)	22	24.8	(1.2)
0.5 SYBaker's – Trial 2	27.9	(1.5)	65	62.8	(1.3)
1.0 SYBaker's – Trial 2	96.7	(3.3)	71	68.8	(1.2)
1.5 SYBaker's – Ttrial 2	140.4	(4.5)	71	68.3	(1.1)
2.0 SYBaker's – Trial 2	152.6	(5.7)	57	54.5	(1.4)
0.1 SYBrewer's – Trial 1	2.4	(0.3)	19	21.0	(0.8)
0.5 SYBrewer's – Trial 1	32.5	(2.0)	65	63.3	(1.2)
1.0 SYBrewer's – Trial 1	126.8	(4.2)	69	67.8	(0.9)
1.5 SYBrewer's – Trial 1	183.9	(7.4)	66	65.2	(1.2)
2.0 SYBrewer's – Trial 1	219.5	(6.9)	63	62.2	(1.1)
0.1 SYBrewer's – Trial 2	4.7	(0.2)	19	19.8	(0.4)
0.5 SYBrewer's – Trial 2	39.8	(1.6)	66	62.8	(1.2)
1.0 SYBrewer's – Trial 2	140.9	(4.0)	68	66.9	(0.5)
1.5 SYBrewer's – Trial 2	174.2	(5.2)	68	67.1	(0.6)
2.0 SYBrewer's – Trial 2	221.9	(5.4)	57	59.0	(0.8)
0.1 SYTorula	2.8	(0.3)	19	21.3	(0.8)
0.5 SYTorula	24.4	(1.1)	55	53.2	(1.2)
1.0 SYTorula	131.9	(4.2)	63	61.4	(1.0)
1.5 SYTorula	146.5	(6.1)	63	63.4	(1.1)
2.0 SYTorula	147.2	(7.6)	58	55.9	(1.4)
0.1 SYExtract	24.5	(1.3)	36	36.4	(1.4)
0.5 SYExtract	94.6	(1.9)	23	23.4	(0.6)
1.0 SYExtract	138.2	(5.4)	19	19.7	(0.3)
1.5 SYExtract	115.4	(3.0)	17	17.1	(0.2)
2.0 SYExtract	82.5	(2.9)	14	14.1	(0.2)
0.1 CSYExtract	27.8	(1.8)	56	54.4	(1.4)
0.5 CSYExtract	126.3	(4.2)	24	25.4	(0.7)
1.0 CSYExtract	188.1	(8.3)	25	25.2	(0.8)
1.5 CSYExtract	156.2	(6.5)	21	21.1	(0.4)
2.0 CSYExtract	91.8	(3.3)	16	16.0	(0.2)
Figure 3					
10 g/L agar	191.6	(11.8)	51	50.8	(1.5)
15 g/L agar	136.3	(7.0)	55	55.3	(1.8)
20 g/L agar	123.2	(10.6)	59	58.8	(1.6)
25 g/L agar	121.8	(5.5)	59	59.8	(1.1)
Figure 4					
1.0 SYBrewer's – water	82.9	(3.9)	64	63	(1.1)
1.0 SYBrewer's + water	84.8	(2.7)	63	61.2	(1.5)
2.0 SYBrewer's – water	180.6	(6.6)	60	59	(1.3)
2.0 SYBrewer's + water	177.8	(7.7)	58	53.9	(1.0)
Supplementary Figure 3					
1.0 SYBrewer's	57.9	(8.0)	67	66.4	(2.7)
2.0 SYBrewer's	136.3	(7.0)	55	55.3	(1.8)
3.0 SYBrewer's	141.6	(6.5)	53	51.5	(1.4)

Notes: *Lifetime fecundity is the sum of eggs laid by an average female on the days of counting.
SEM = standard error of the mean; LS = life span.

Supplementary Table 2. Nutritional Comparison of the Different Yeasts Tested

	Nutrient Composition (g/100 g dry weight)			
Nutrient	Baker's Yeast*	Brewer's Yeast*	Torula Yeast	Bacto Yeast Extract*
Carbohydrates	39	35	28	†
Protein	45.7	45	58	51
Fat	5.8	1	7	
Alanine‡	3.97		7.3	5.6
Arginine	2.01	5.1	5.6	2.6
Asparagine/Aspartate	4.2		10.6	5.3
Cysteine (variable)	0.12	1.2	0.4	Destroyed§
Glutamine/Glutamate	9.1		13.6	9.4
Glycine	1.41	4	4.9	3
Histidine	0.89	2.5	2.2	1.3
Isoleucine	1.68	4.1	5.6	3
Leucine	2.52	5.7	8.4	4.1
Lysine	2.48	6.2	8.8	4.6
Methionine	0.5	1.2	1.7	0.8
Phenylalanine	1.48	3.1	5.1	2.6
Proline	1.41		4.2	2
Serine	1.9		5.7	
Threonine	2.07	4.4	5.8	1.6
Tryptophan	Destroyed	1.1	0.9	Destroyed
Tyrosine	0.99	3.2	4	1.2
Valine	1.89	4.8	6.1	3.5
Inositol		0.47		
Choline		0.47		
Cadmium			0.00004	
Calcium	0.134	0.12	0.4	0.013
Chloride	1			0.38
Cobalt		0.00015		
Copper	0.0002	0.0035	0.0008	
Iron	0.0133	0.02	0.0125	
Lead			0.00002	
Magnesium	0.262		0.1	0.075
Manganese		0.00053		
Phosphate				3.27
Phosphorous	1.603	1.5	0.9	
Potassium	2.447	0.86	1.6	3.195
Sodium	0.041		0.02	1.49
Sulfate				0.09
Sulfite			0.018	
Zinc	0.0062	0.00387	0.01	
Biotin	0.0000236	0.000125		
Ca Pantothenate	0.00211	0.0122		
Folic Acid	0.000871	0.0005		
Nicotinic Acid	0.0182	0.04		
Pyridoxine	0.00046	0.005	0.0425	
Riboflavin	0.00103	0.0045	0.006	
Thiamine-Hcl (aneurin)	0.00067	0.015	0.0002	

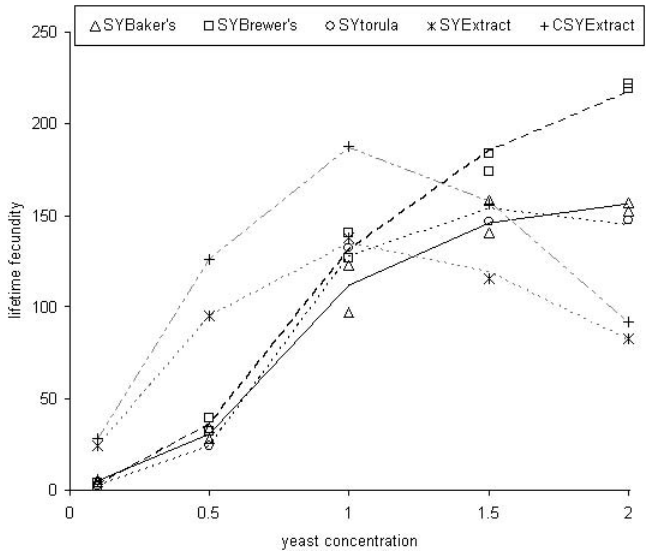
Notes: Each of the analyses above are provided by the manufacturers and describes a typical batch, except for that of baker's yeast, the nutritional breakdown of which was not supplied by the manufacturer. This information was gathered independently using a sample from a bag used in our laboratory for these experiments.

*These yeasts are labeled *Saccharomyces cerevisiae*.

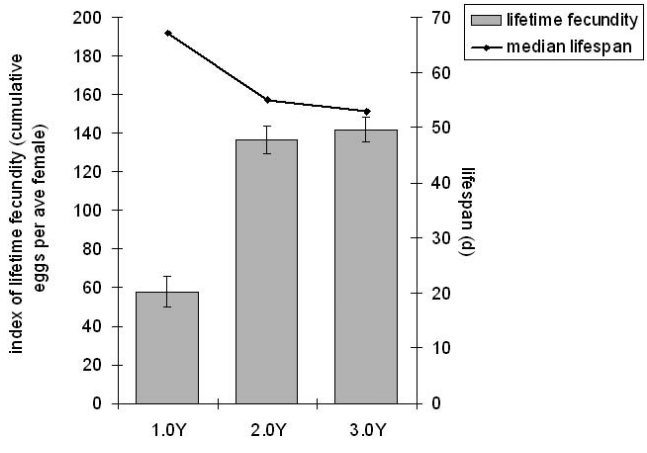
†Missing values are indicative of data not reported, not that the nutrients are absent.

‡Amino acids are reported as total amino acid content. Values for free soluble amino acids are lower.

§Indicates components lost or destroyed by the detection/quantification process.



Supplementary Figure 2. Model predictions versus actual egg-laying data reported in Figure 2. Model predictions are represented by the *lines* and actual data by *symbols*. All fixed terms (yeast type, concentration and the quadratic term for concentration) and interactions were significant.



Supplementary Figure 3. Effect of raising yeast concentration in SYBrewer's above the range used for dietary restriction (DR). Yeast concentration was raised to 300 g/L, and life span and egg laying were monitored. Whereas life span showed a significant decline from that found at 2.0 SYBrewer's, egg laying was not further increased, indicating that the flies did not experience a higher level of nutrition. Agar concentration was 15 g/L.

Appendix 2

Grandison, R. C., Wong, R., Bass, T. M., Partridge, L. and Piper, M. D. (2009). "Effect of a standardised dietary restriction protocol on multiple laboratory strains of Drosophila melanogaster." PLoS ONE **4**(1): e4067.

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Effect of a Standardised Dietary Restriction Protocol on Multiple Laboratory Strains of *Drosophila melanogaster*

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Abstract

Background: Outcomes of lifespan studies in model organisms are particularly susceptible to variations in technical procedures. This is especially true of dietary restriction, which is implemented in many different ways among laboratories.

Principal Findings: In this study, we have examined the effect of laboratory stock maintenance, genotype differences and microbial infection on the ability of dietary restriction (DR) to extend life in the fruit fly *Drosophila melanogaster*. None of these factors block the DR effect.

Conclusions: These data lend support to the idea that nutrient restriction genuinely extends lifespan in flies, and that any mechanistic discoveries made with this model are of potential relevance to the determinants of lifespan in other organisms.

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Introduction

In order to maximise its genetic contribution to posterity, an organism must appropriately direct the use of nutrients to traits such as growth, reproduction and repair. In some circumstances, this will mean maximising one trait at the expense of another. This idea has been used to explain the observation that relatively low food intake can result in longer life, because it comes at the cost of reduced rates of reproduction [1–4]. This particular trade-off phenomenon is widespread and has been termed dietary restriction or DR. Although extensively studied since its first description in 1935 [5], very little is known about the molecular details of exactly what resources are shared in this trade-off and how they are balanced between the traits. Uncovering these mechanisms has now become the holy grail of research into DR, with the aim of harnessing their power for longer and healthier lives.

One of the promising advances towards the goal of uncovering the mechanisms by which DR extends life was the discovery that the effect is evolutionarily conserved [6–10]. However, even with the use of short-lived model organisms for relatively rapid lifespan experiments, the mechanisms remain elusive. This is likely to be largely due to the complexity of physiology involved in determining length of life, but may be also in part due to technical issues in experimental design hampering a clear path of progress [11]. The ease with which complexity can be introduced into these studies can be illustrated by the large effects on fly lifespan caused by very small changes in nutrition. For example, substituting one source of the dietary yeast *Saccharomyces cerevisiae*, with another from a different supplier in an otherwise identical diet can have large effects on fly lifespan [12]. Similarly, lifespan differences have been reported due to the use of different bacterial strains as food for *Caenorhabditis elegans* [13] or by interchanging casein and soy peptone as the source of dietary protein for rodents [14]. In fact, a recent article has proposed that DR itself may have arisen as a by-product of laboratory life as animals are unintentionally subjected to selective breeding in the presence of an artificially rich nutritional environment [15]. Clearly, these issues need to be addressed if we are to uncover the molecular mechanisms of DR.

In our studies on DR in *Drosophila*, we have taken a systematic approach to optimise dietary composition such that fecundity and lifespan are maximised and any non-specific adverse effects of the food are avoided [12]. In this article, we extend this work to examine the effect of different techniques of long-term stock maintenance and microbial infection on the responses of 'wild-type' laboratory-maintained flies to DR. We have undertaken these experiments in order to establish a working protocol that avoids laboratory artefacts and will therefore aid studies seeking the molecular mechanisms of DR. As a result of performing these experiments with flies of different genetic backgrounds, we find interesting differences in the interaction between diet and genotype that form a solid basis for future work to uncover how DR extends the lifespan of flies and other organisms.

Results

An intermittent feeding regime did not affect Drosophila lifespan

We have previously published a description of the optimisation of a sugar/yeast (SY) medium for DR studies in flies [12]. This

study found that yeast dilution in an otherwise unchanged medium effectively limits the flies' nutrient intake, decreases their daily and lifetime fecundity and increases their lifespan.

An alternative DR protocol that extends rodent lifespan is every other day feeding (EOD) [16,17]. In these experiments, the EOD cohort has alternating bouts of 24 h access to unlimited food followed by 24 h starvation, while controls have continuous access to unlimited food. Interestingly, this intervention extends lifespan even though the EOD animals nearly fully compensated for the periods of starvation by eating more. Thus, intermittent periods of starvation could be equally as important as reduced nutrient intake for extending lifespan.

Two *Drosophila* studies have attempted a similar regimen and one reported a generally positive effect on lifespan when flies were subjected to 18 h access to food and 6 h access to water only in every 24 h [18]. In contrast, a more recent study has reported no positive effects of this treatment, or of any other treatments in which the timing of the starvation/feeding periods was altered [19]. However, in this latter study, the treatment was only implemented on 5 out of every 7 days of adult life, making it possible that any beneficial effects of the protocol were masked by the days without treatment. We therefore decided to test this technique using our laboratory strain Dahomey, applying daily bouts of either 3 h or 6 h starvation, during which the flies had access to water only. We found that neither treatment had a positive or negative effect on lifespan (Figure 1). While this could be taken to mean that DR does not work in flies, the lack of any effect on lifespan of the more severe restriction makes it impossible to know to what extent the flies were nutrient restricted or whether the periods of starvation were close to adequate to elicit a protective effect. Without a more extensive set of starvation periods, it is not possible to draw definitive conclusions about the effectiveness of this intervention in *Drosophila*.

Comparison of the DR response between different laboratory strains

In all of our DR optimisation experiments we have used our outbred laboratory strain of *Drosophila*, Dahomey. This strain has been maintained for many years on an SY diet in large population cages with overlapping generations. In contrast, most laboratory wild-type strains are largely inbred and maintained in relatively small numbers in individual containers and may have a varied nutritional history. Some of these housing conditions can easily lead to selection for early reproduction, which is known to cause shortened lifespan [20–22]. We therefore assayed the lifespan of several commonly used wild-type *Drosophila* strains on our standard SY food (1×; Figure 2). In all cases, the lifespans were significantly shorter than that of Dahomey and exhibited median lifespans from 53 days for OregonR to 65 days for Dahomey.

Next we asked what the effect of this variation was on the DR response in these different strains. This was both to assess how our DR protocol is likely to behave when implemented in other laboratories that routinely use fly stocks other than Dahomey, as well as to look for strains with altered DR responses that might provide insights into its mode of action. The operational definition of DR is the range of nutrition that causes lifespan to increase and fecundity to decrease [23]. It should be noted that this definition excludes the dilution from 0.5× down to 0.1×, as this caused the flies to become malnourished and both lifespan and fecundity to decrease (Figure 3). For Dahomey and wDahomey, the DR range was from 2× to 0.5× food, while for w1118 and CantonS it was from 2× to 1×, and for OregonR was from 1.5× to 0.5× (Figure 3). For OregonR only, the highest food concentration caused egg laying to decrease, which indicated that the associated lifespan decrease from 1.5× to 2× was not accompanied by increased intake of biologically valuable nutrition and therefore could be due to a non-specific detrimental effect of high food. It was thus considered outside of the functional DR range for this strain. Finally, for yw, there was a clear DR response from 1× to 0.5× food but, owing to incomplete data, we cannot report any possible broader DR effect. Thus in all cases, a DR response was observed under these conditions although its exact nature was different for different wild-type strains.

In all comparisons from all trials, Dahomey, wDahomey and yw exhibited the longest lifespan (Table 1) with medians from 69 to 73 days over different trials on 0.5× food (Dahomey v wDahomey, $p = 0.69$; Dahomey or wDahomey v highest median lifespan from each other genotype, $p<0.001$, log-rank test). Dahomey and wDahomey also exhibited higher reproductive output than the other wild-types at each food concentrations except 0.1×, as well

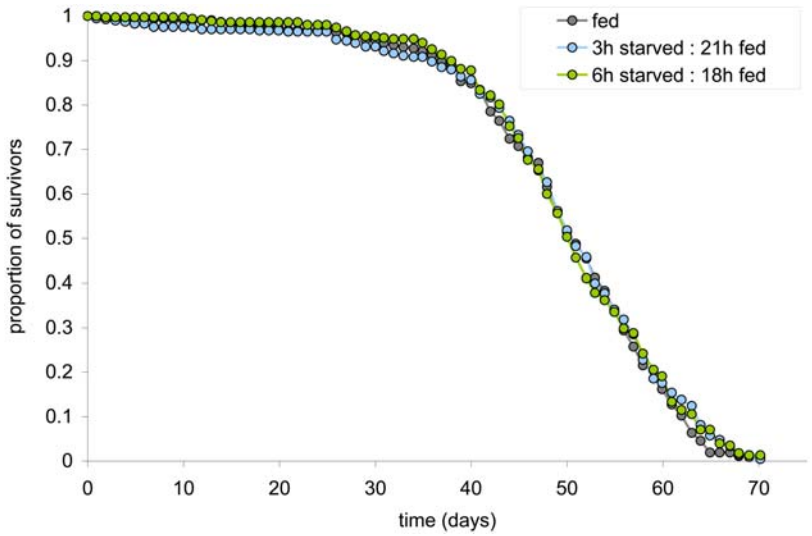

Figure 1. Intermittent exposure of flies to food does not increase their lifespan. Throughout adult life, Dahomey females were exposed to daily cycles of starvation:feeding of either 3 h:21 h or 6 h:18 h. Neither treatment had any effect on lifespan. During the periods of starvation, flies had access to water only.
doi:10.1371/journal.pone.0004067.g001

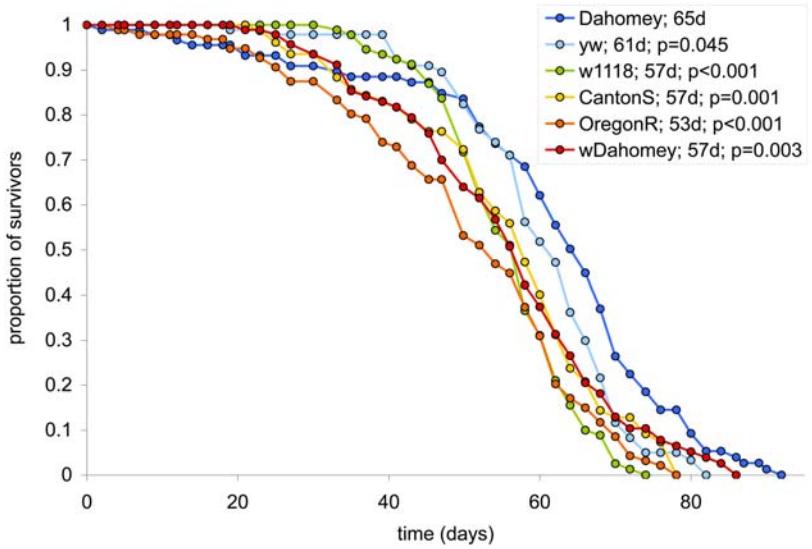

Figure 2. Different laboratory strains of wild-type Drosophila have different lifespans. Each genotype was raised in parallel under the same conditions and assayed on 1× SY for lifespan. All strains that were tested exhibited a shorter lifespan than our outbred laboratory strain Dahomey. The graph legend reports the strain name; median lifespan in days and; p-value from the log-rank test when compared to Dahomey.
doi:10.1371/journal.pone.0004067.g002

as the maximum reproductive output from all conditions (on 2× food) (Dahomey v wDahomey, $p = 0.97$; Dahomey or wDahomey v highest reproductive output for each other genotype, $p<0.003$, Wilcoxon rank-sum test).

Effect of tetracycline treatment on the DR effect

Drosophila are host to a range of microbes, and for many strains, this includes a bacterium of the genus *Wolbachia* that resides in the cytoplasm of reproductive tissues [24]. In some cases, the presence of *Wolbachia* has been shown to alter lifespan [25]. Recently, a vertically inherited factor that was curable by tetracycline treatment was shown to account for at least part of the long lifespan of a long-lived *Drosophila* mutant [26]. We decided it was important to examine the effect of such infections on DR, because if they account for the lifespan difference, it is unlikely DR in *Drosophila* is useful as a model for higher organisms.

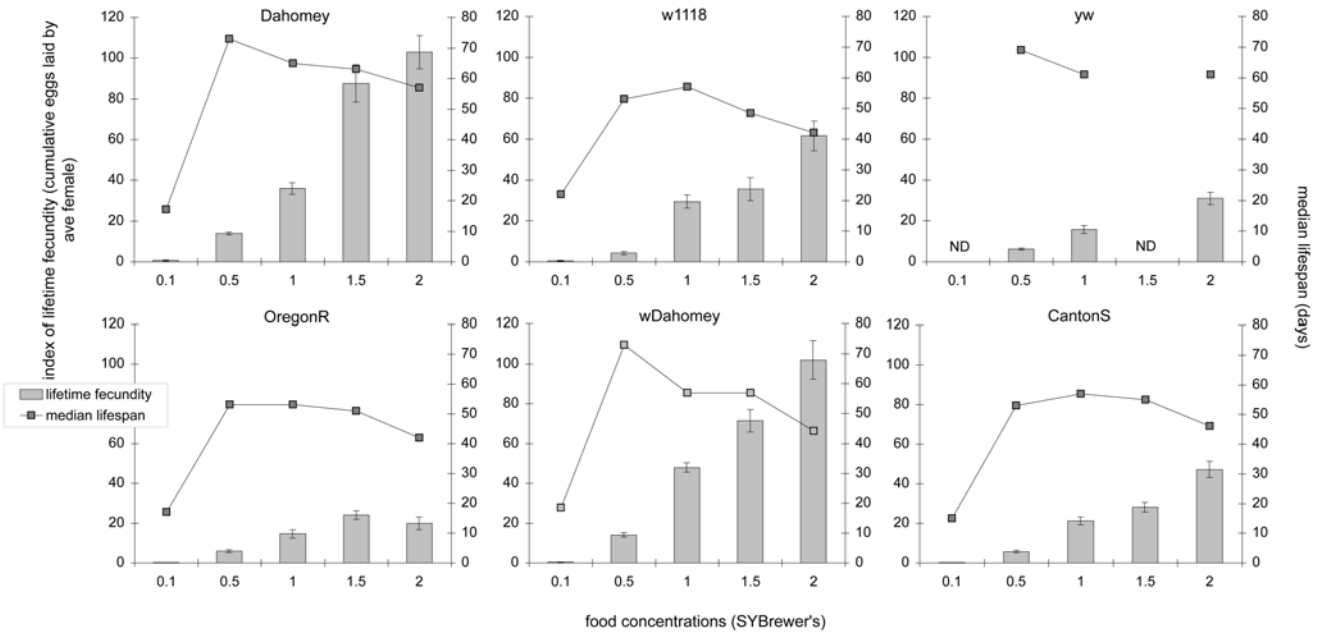

Figure 3. Different laboratory strains subject to DR. When tested in parallel under the same conditions, all wild-type strains tested exhibited a DR response. This is defined as a simultaneous increase in lifespan and decrease in lifetime fecundity when nutrient availability was reduced. Bars: index of lifetime fecundity±standard error of the mean; connected points: median lifespan in days; ND: not determined. Data shown are from a single trial in which all lifespans were run simultaneously. They are representative of triplicate data sets for Dahomey, CantonS and OregonR and duplicates for yw; w118 and wDahomey data are from a single trial.
doi:10.1371/journal.pone.0004067.g003

Table 1.

Wild-type strain	Food Conc (x)	Median lifespans[1]			ave lifespan change due to DR[2]
		Trial 1	Trial 2	Trial 3	
Dahomey	0.1	17.1	18	ND	
	0.5	**73**	**73.5**	**69.1**	
	1	65	66.5	59.5	31%
	1.5	63	64	48	
	2	*57*	*55*	*52.5*	
yw	0.1	ND	ND	ND	
	0.5	**69**	ND	**73.5**	
	1	*61*	ND	*66.5*	12%
	1.5	ND	ND	ND	
	2	61	ND	48	
w1118	0.1	22	ND	ND	
	0.5	53.1	ND	ND	
	1	**57**	ND	ND	36%
	1.5	48.4	ND	ND	
	2	*42*	ND	ND	
CantonS	0.1	15	18	ND	
	0.5	53.1	**57**[3]	**50**	
	1	**57**	**59.5**	48	28%
	1.5	55.1	52.5	*38.5*	
	2	*46.1*	*45.5*	*38.5*	
OregonR	0.1	17	22	ND	
	0.5	**53.1**	45.5	**66.5**	
	1	**53.1**	**52.5**	59.5	15%
	1.5	*50.9*	*48*	*55*	
	2	42	45.5	52.5	
wDahomey	0.1	18.5	ND	ND	
	0.5	**73**	ND	ND	
	1	57	ND	ND	66%
	1.5	57	ND	ND	
	2	*44.1*	ND	ND	

[1]bold numbers denote the greatest median lifespans and italicised numbers the shortest median lifespans, within the DR food range for that strain in that trial.
[2]For all DR ranges for each strain, the longest-lived condition was significantly different from the shortest-lived condition; percentages are derived from the average lifespan difference due to DR.
[3]In cases where there was no significant difference between two food types for the longest or shortest-lived condition, two numbers are in bold or italicised.
doi:10.1371/journal.pone.0004067.t001

Upon testing our wild-type strains for *Wolbachia* we found all except w1118 and OregonR were infected (Figure 4a). Therefore, because all strains exhibited a DR response, *Wolbachia* infection *per se* can not account for the full effect of nutrition on lifespan. To test if tetracycline-treatment could eliminate the DR response by other means, we selected three lines for treatment (Dahomey, CantonS and OregonR). After two generations on tetracycline-containing food, flies were subsequently maintained on normal food to recover for at least five generations. PCR testing revealed that the treatment was effective as both Dahomey and CantonS were cleared of *Wolbachia* (Figure 4b). When subjected to different food concentrations, all three tetracycline-treated lines retained their DR response (Figure 4c). In the trial shown, the lifespan peak for all three strains was at 1× food and fecundity increased to 2× food. While this was qualitatively different from that seen in the previous trials with non-tetracycline-treated flies, a further trial with these lines after an additional five generations on normal food, revealed more similar data to that shown in Figure 3 (data not shown). Thus, tetracycline-treatment may produce a transitory alteration in the way flies respond to food, but its effects can not account for the DR response.

Discussion

Intermittent feeding did not extend fly lifespan but does not rule out DR in flies

There are several different ways to restrict the access of animals to nutrition and thus extend lifespan by DR [27]. For flies, dilution of the concentration of yeast in a diet that is provided in excess, has proven to be practical and effective [12,28]. However, food dilution methods are unique to the invertebrates and in mammalian studies, periodic access to food is used. One such

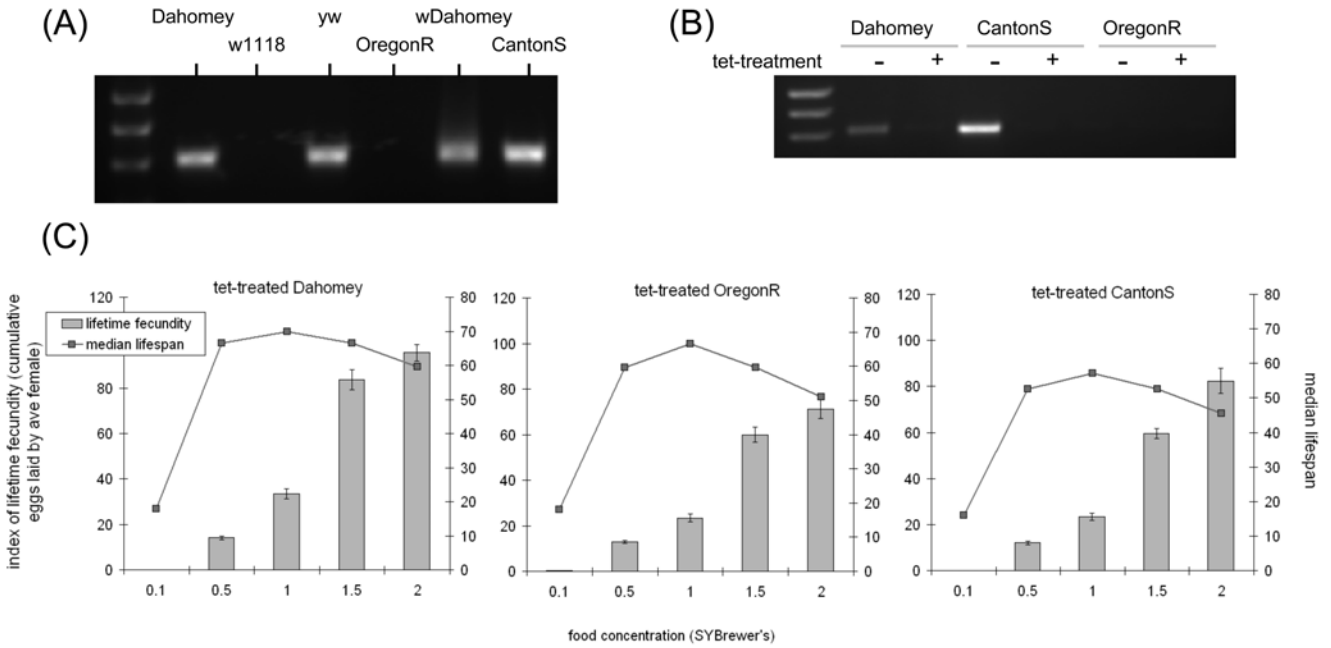

Figure 4. Tetracycline treatment does not eliminate the DR response. (A) Gel showing diagnostic PCR for the presence of the intracellular bacterium *Wolbachia*; (B) three strains were selected from the set of wild types for treatment with tetracycline, which was sufficient to clear *Wolbachia* if present. (C) Each of the three strains was then allowed at least five generations to recover on non-tetracycline-containing food before being assayed for lifespan and fecundity on different concentrations of food. Each of the three strains still exhibited a DR response after tetracycline treatment. Bars: index of lifetime fecundity±standard error of the mean; connected points: median lifespan in days. Data shown are from one of two trials in which all lifespans were run simultaneously.
doi:10.1371/journal.pone.0004067.g004

protocol provides animals with a measured amount of food that is completely consumed before the next meal. While effective for extending rodent lifespan [7], it has been unsuccessful when used on flies [29,30]. An alternative technique is EOD feeding, which extends rodent lifespan by alternating periods of access to excess food with periods of starvation. Importantly, the mice subjected to this regime increased their feeding behaviour such that they consumed nearly the same quantity of nutrients as controls. Thus, regular periods without food maybe just as important as reducing nutrient intake for extending rodent lifespan [16]. In contrast, this protocol has had little or no success when adapted for flies [18,19]. In this study, we also found no extension of life using a similar protocol on *Drosophila* (Figure 1). Thus, our results support the previous invertebrate data and could be used to argue that periods of starvation cannot extend the lifespan of flies [30,31] or that the mechanism by which DR extends lifespan is different between flies and mammals. While both of these explanations are possible, the fact that lifespan was not shortened by the more severe of our restriction treatments means we are unable to determine how much nutrient intake may have been reduced, or exactly what other periods of starvation could be protective for lifespan in our flies. While a more extensive range of starvation periods would be revealing, other factors such as the time of day at which food is removed may also be important since feeding behaviour is controlled by the circadian rhythm [32]. Thus, it is easy to implement an inappropriate methodology when attempting to DR flies in this way and the absence of a positive result does not rule out the possibility of observing a positive effect if protocols were optimised.

If nutrient restriction is the critical factor in these DR experiments then intermittent feeding protocols that use different dietary compositions would also be expected to vary lifespan outcomes in different ways. Figure 5 illustrates how this is possible. When given increasing doses of a relatively concentrated diet (orange line), lifespan would increase as malnutrition lessens to a peak at an intermediate level of food availability. As food availability is increased beyond this point, lifespan decreases via the DR response. At some point, no additional increase in food availability will further shorten lifespan as the organism will reach its limit to ingest more food ('point of satiety' and beyond). If, however, the concentration of the food being provided is low enough (represented by the 'dilute' and 'very dilute' diets in Figure 5), lifespan will increase to a plateau whose onset occurs at the point that the organism's food intake limit is reached. If these dilute food types are used in an intermittent feeding protocol, it would be impossible to find an intermediate level of food exposure which increases lifespan, falsely giving the impression that DR does not exist. It is possible that this can explain why some studies have been published that did not find a DR response (eg [33–35]). As mentioned above, food dilution has proven to be the most successful intervention to implement DR in flies [11]. The connection between this intervention, where the food remains in excess, and intermittent feeding can be found by taking the lifespan values at any one level of food availability above the point of satiety in the left panel of Figure 5. A cross-section of these values is shown in the right panel of Figure 5; this represents the standard DR effect in flies (eg Figure 2). It should be noted that in reality, this illustration is somewhat simplistic in that the lifespan-sensitive nutrients represented on the x-axis are unlikely to be accurately represented by the term 'food availability'. Furthermore, nutrient composition variations are likely to alter the point of onset of satiety, which in turn changes the onset of the lifespan plateau. Thus, although further work on diet composition, feeding intervals and measured food availability may uncover an

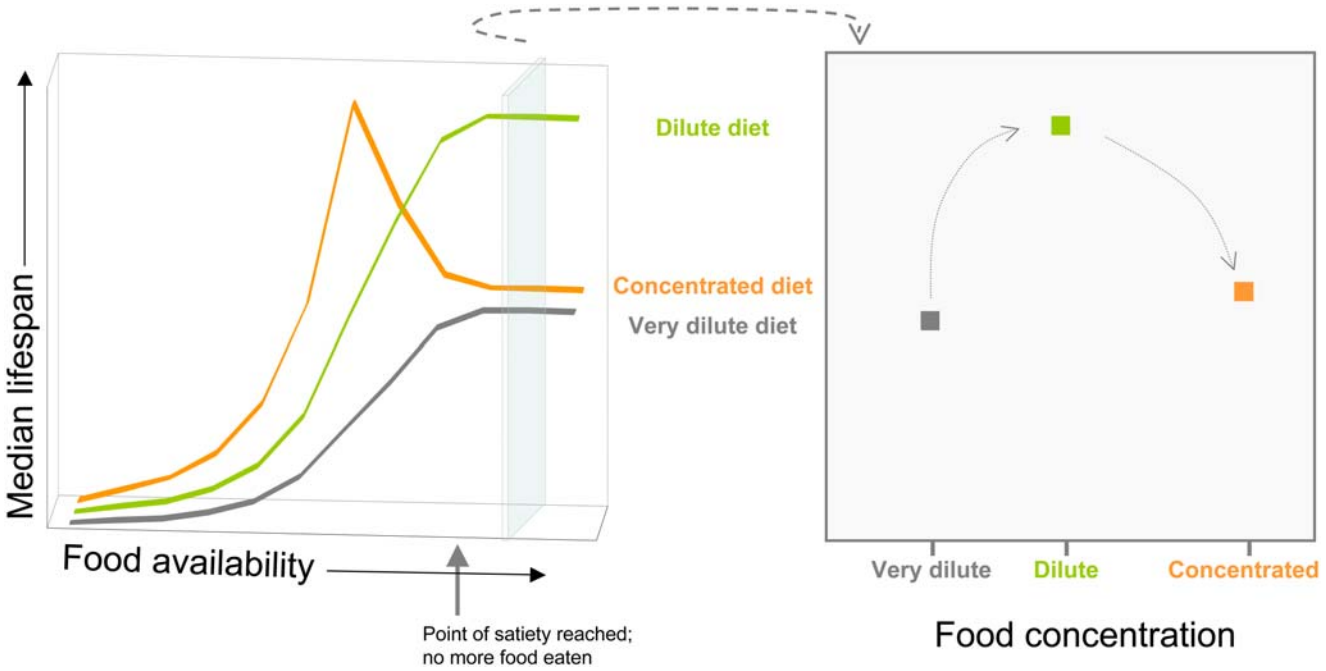

Figure 5. Model of the relationship between lifespan and DR protocols that reduce access to food either by intermittent exposure (left panel) or nutrient dilution (right panel). These demonstrate how the composition of food used for intermittent feeding protocols could lead to the false conclusion that DR does not exist for an organism. Three different diets are shown that vary in a given nutrient concentration from 'very dilute' to 'concentrated'. In this example, increasing access to the concentrated diet causes lifespan to rise to a peak (DR) beyond which lifespan decreases. At some point (marked here as the 'point of satiety') the animal will no longer be able to eat any more food, meaning the nutrition level it experiences is capped and no further increase in availability will further decrease lifespan. For the dilute and very dilute diets, the point of satiety is reached before the level of nutrients ingested has a chance to cause lifespan to reduce. Thus, there is no lifespan increase for any intermediate level of food restriction, making it look like the organism does not exhibit a DR response. For flies, these problems can be avoided by assaying lifespan in the presence of excess food that is diluted to differing extents. The relationship of this situation to DR by intermittent feeding is represented by taking a cross-section through the graph on the left. The plot on the right shows the type of data presented herein and for other invertebrate studies.
doi:10.1371/journal.pone.0004067.g005

alternative intermittent feeding regime suitable for flies, it is likely to be a labour intensive process that may not provide any more information about DR than dietary dilution.

DR in Drosophila does not appear to be a laboratory artefact

For ease of handling and to extend generation times, fly stocks in the laboratory are often kept in small numbers, under relatively poor nutrient conditions and at low temperatures. Over time, these factors are likely to exert selective pressures that could influence lifespan. Importantly, when transferring stocks to fresh food for maintenance, it is relatively easy to select for early age of reproduction, which is known to reduce adult lifespan [36]. That this happens in the laboratory has been demonstrated by comparing the lifespans of flies maintained for years in the laboratory under normal stock-handling conditions with others selected for early or late reproduction as well as others freshly caught from the wild [37]. This study showed that the laboratory stocks were as short lived as those selected for early reproduction, while the wild-caught lines had a much longer lifespan, similar to flies selected for late reproduction and were much longer lived. In our laboratory, we have maintained a wild-type outbred stock (Dahomey) since 1970 in large population cages with overlapping generations. When compared with other laboratory wild-type strains that we have maintained using routine stock handling techniques, we found that Dahomey demonstrated the capacity for both the longest lifespan and the greatest lifetime egg laying output (Figure 6). Thus, maintenance of flies using large population cages with overlapping generations appears to preserve the life history characteristics of wild-flies for long periods of time. This is in agreement with previous work that demonstrated this fact for flies maintained in the laboratory during a three year period [38].

Importantly, despite the differences between strains in their selection histories, all exhibited a DR response (Figure 3). Recently, it has been proposed from work with mice that lifespan extension by DR could simply be an artefact of laboratory domestication because a wild-caught strain was reported whose longevity was not increased in response to a typical DR regime [15]. In contrast, a recent study of several wild-derived strains of *C. elegans* showed that all exhibited a DR response [39]. Although we have not directly tested DR using flies recently caught from the wild, our study indicates that they would exhibit a DR response because of the strong effect seen with Dahomey (Figure 3 and Figure 4). It should be noted that the invertebrate studies were conducted using a DR technique that deprived worms of bacteria, while the rodent study used a food restriction protocol with only one level of limitation. Thus, as explained above, diet design and an incomplete range of food concentrations could be important factors in explaining why the DR effect was apparently absent from wild mice [15].

DR in Drosophila is not sensitive to tetracycline treatment, but varies with diet quality and genotype

We show here that DR is not sensitive to infection with the bacterium *Wolbachia*, or indeed any other tetracycline-sensitive infection that may be present in flies (Figure 4). Interestingly, the levels of fecundity at a given food concentration differed after

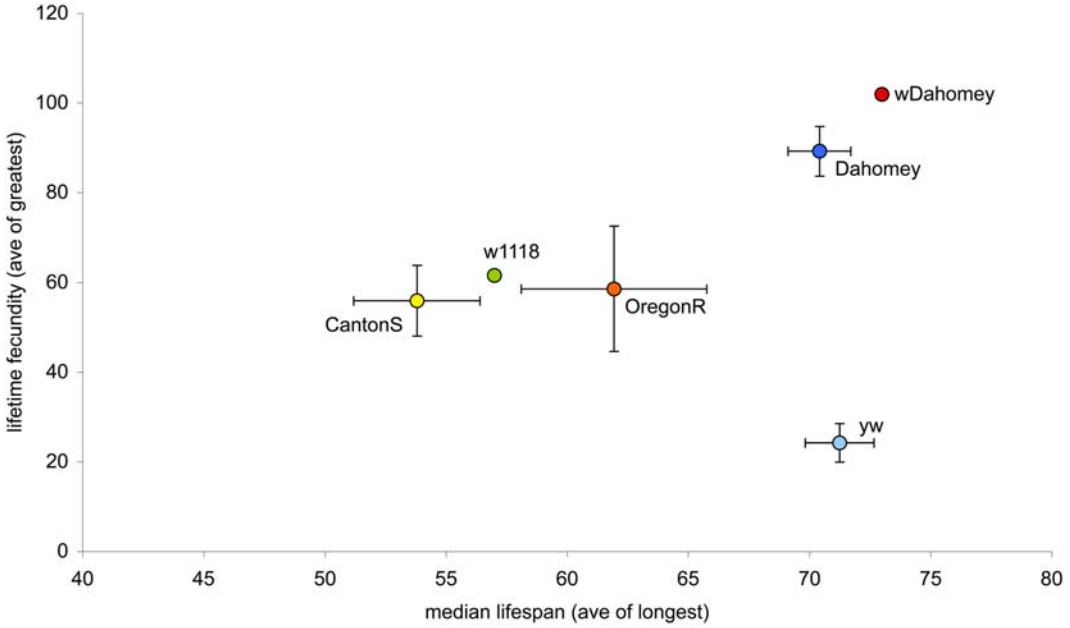

Figure 6. The Dahomey genetic background is capable of the longest lifespan and greatest reproductive output of the wild-type strains tested. For median lifespan, the data are the averages from the longest lived conditions for each strain. For lifetime fecundity they are the average of the condition producing the greatest lifetime reproduction. It should be noted that the conditions under which these occur is different for the two traits, as predicted by the expectations of DR, and that they may be different for each different strain. Data from n independent repeats, where n = 5 for Dahomey, CantonS and OregonR; n = 2 for yw, and; n = 1 for w1118 and wDahomey.
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tetracycline treatment (compare Figure 3 with Figure 4c). This indicates that some tetracycline-sensitive microbes carried by some flies might be involved in the control of fecundity. However, further data would be required to validate this observation since a subsequent trial with the tetracycline-treated flies saw fecundity levels return to those previously observed (as for Figure 3; data not shown). Together, these data extend previous work we have performed to optimise a DR protocol to avoid lifespan variations from non-nutrient dependent effects [12]. From this work, we have sought to generate a standardised DR protocol to aid studies into the mechanisms of DR. However, we here report that the food concentration to yield the longest lifespan in Dahomey was at 0.5×, which is less than the 1× reported in [12]. This demonstrates an inherent problem with using a natural ingredient like yeast whose nutritional content varies seasonally due to production methodology and the quality of its feedstock. In doing so, it also highlights the need for a standardised synthetic defined medium to replace yeast-based diets to study the details of how lifespan varies with food composition. Interestingly, not all strains exhibited a lifespan peak at the same food concentration as Dahomey (Figure 3). It is already known that genotype can affect the interaction between lifespan and food [27,40–42] and could indicate the breadth of the DR effect on fly health. One interesting possibility from these data is that if flies of different genotypes die from different pathologies, DR has the ability to delay the onset of each of these causes of death, which agrees with data from rodent studies [7,43]. Future work on the exact molecular mechanisms of DR via interactions with different genotypes on precise dietary manipulations will be key to exploring this further.

Materials and Methods

Fly stocks and maintenance

Dahomey: This strain has been in the laboratory since 1970, having been collected in West Africa in what is now the Republic of Benin. Four population cages (dimensions: 20 cm H×21 cm W×30 cm D) have been maintained in parallel at 25°C on a 12-hour light/dark cycle. At all times, 12 bottles of food are in each cage, being replaced gradually. Each week, three half-pint bottles containing 70 ml of food (1× SY) are supplied to each cage and the three oldest bottles removed.

wDahomey was generated by backcrossing the white gene from w1118 into the Dahomey genetic background. It has since been maintained in one large population cage with a feeding regime as described above for Dahomey.

w1118, yw, OregonR and CantonS have been maintained in the lab for many years under a variety of conditions. Generally, this involves transferring each new generation to a fresh set of several half-pint bottles or vials of food. These are usually kept at 18°C to extend each generation's lifecycle and are fed either 1× SY food or a cornmeal-based diet (see below).

Media

The SY food reported here is the same as SYBrewer's in [12]. Standard (1×) contains per litre: 100 g autolysed Brewer's Yeast (MP Biomedicals, Solon, OH), 100 g sucrose (Tate & Lyle sugars, London, UK), 15 g agar (Sigma, Dorset, UK), 3 ml propionic acid (Sigma, Dorset, UK), 30 ml Nipagin M solution (100 g/l methyl 4-hydroxybenzoate in 95% ethanol) (Clariant UK Ltd, Pontypridd, UK), distilled water to 1 l. Cornmeal-based diet used in stock keeping contains: 60 g cornmeal (organic polenta; B.T.P. Drewitt, London, UK), 20 g autolysed Brewer's yeast, 85 g sucrose, 10 g agar, 25 ml Nipagin M and 1 l distilled water.

Tetracycline treatment was carried out by the addition of 25 μg/ml tetracycline to 1× SY food for two generations.

For stock maintenance, food was cooked in a 60 l Joni Multimix food preparation kettle (Joni Foodline, Munkebo, Denmark), while experimental food was prepared on a gas hob as described in [12].

Lifespan and fecundity assays

Flies were reared at a standard density for at least two generations before being used for lifespan experiments as previously described [12,44]. All experiments were performed with female flies that were allowed 48 h to mate after emerging as adults. On the second day of adult life, flies were lightly anaesthetized with CO_2, sorted and counted at 10 per vial. The minimum number of flies per condition was 100. For the intermittent feeding experiment, five replicate 1 l cages, each containing 100 flies was used for each condition. These cages have two side-arm inlets that can each accommodate a food vial. The periods of starvation were initiated at 10:00 (lights on), whereupon the food vial (2× SYBrewer's) was replaced with an empty vial. In all cages at all times, flies had constant access to a vial containing water that was plugged with wet cotton wool. This was housed in the side-arm not containing the food vial. In all cases, flies were transferred to fresh food at least three times a week, at which point deaths were scored.

For fecundity measurements, eggs were counted after the flies had been in the vials for between 18 and 24 h. Generally, these counts were performed once a week for the first six to seven weeks of adult life. Importantly, the first egg count was only conducted after at least four days exposure to the new food in order to allow time to adjust to the new nutritional conditions.

PCR detection of Wolbachia infection

PCR for detection of Wolbachia was performed using primers wsp81F and wsp691R (kind gift from G. D. D. Hurst) as described in [45]. In each case, a sample of flies form the experimental generation was used for PCR testing.

Data analyses

Lifespans were recorded and analysed using spreadsheets created in-house in Excel. Comparisons using the Wilcoxon rank sum test were performed in R, v2.5.1 [46].

Author Contributions

Conceived and designed the experiments: RCG RW TMB LP MDWP. Performed the experiments: RCG RW TMB MDWP. Analyzed the data: MDWP. Contributed reagents/materials/analysis tools: RCG RW TMB MDWP. Wrote the paper: LP MDWP.

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