Understanding the mechanisms of dietary restriction to extend healthy lifespan in *Drosophila melanogaster*

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Abstract

Dietary restriction (DR), defined as a moderate reduction in food intake short of malnutrition, has been shown to extend healthy lifespan in a diverse range of organisms, from yeast to primates. In this work we aim to uncover the mechanism by which DR extends lifespan. The prevailing theory of somatic maintenance by resource reallocation proposes that the balance of nutrient allocation is weighted either towards reproduction, when environmental nutrients are abundant, or towards maintenance of the soma when food is limited, thereby aiding organismal survival during food shortages. This theory has found support in reports that dietary restricted *Drosophila melanogaster* (fruit fly) benefit from increased lifespan but have compromised reproduction, and that the inverse is true of fully fed flies. It has recently been found that addition of the ten essential amino acids (EAA) to a DR diet is sufficient to decrease lifespan and increase fecundity to the same degree as full feeding, implicating EAAs as the dietary mediators of the responses of lifespan and fecundity to DR. In this thesis I characterise the physiological and metabolic parameters that define DR flies, fully fed flies and EAA-supplemented DR flies, with the aim of identifying candidate factors that consistently correlate with lifespan for the three treatments in order to identify the causes of longer life in response to DR. We also use genetic tools to explore the role of nutrient signalling pathways in mediating the relationship between nutrition and ageing, with special focus on the amino acid sensitive target of rapamycin (TOR) pathway, the insulin/insulin-like growth factor signalling (IIS) pathway, and the general amino acid control (GAAC) pathway. These studies find a role for TOR signalling in mediating the effects of DR on lifespan and this effect appears to be different from those caused by altered IIS and GAAC pathways. These data also implicate accumulation of fat as consistently correlated with, and so possibly causal for, longer life. Finally, I investigated the potential roles that these nutrient sensing/signalling pathways might play in modifying feeding behaviour in response to changes in dietary nutrient quality. Here, the GAAC pathway proved to play an important and specific role in the way single amino acid deficient foods are detected to alter feeding behavior. These data are somewhat consistent with mammalian studies on nutrient-specific feeding alterations and establish the groundwork for detailed studies into the molecular processes involved. As a combined body of work, this thesis outlines important data on the mechanisms of DR to extend life as well as new information about the nutrients and molecular signals involved in shaping feeding choices.

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 thesis.

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Sahar Emran

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Contents

Figures and Tables

Abbreviations

DR – Dietary restriction

FF – Full feeding

AA – Amino acid

EAA – Essential amino acid

NEAA – Non-essential amino acid

NSP – Nutrient sensing/signalling pathway

TOR – Target of rapamycin

IIS – Insulin/ insulin growth factor signalling

GAAC – General amino acid control

Rag – Ras-related GTP-binding

Dilp – *Drosophila* insulin like

GCN2 – General control non-derepressible 2

tRNA – Transfer ribonucleic acid

UAS – Upstream activating sequence

GS – Gene switch

SY – Sugar/yeast food medium

S – Sugar

Y – Yeast

Rapa – Rapamycin

Arg – Arginine

Trp –Tryptophan

Leu – Leucine

Met – Methionine

Chapter 1 - General introduction

1.1. Ageing

The broad definition of ageing encompasses chronological, biological, psychological and social factors. Biogerontology is an active field of science researching the biological causes, effects, and mechanisms of ageing. In the biological sense, ageing is defined as a functional deterioration over time that increases mortality (Finch, 1990), also known as senescence. Senescence can arise at any level of biological organisation, from macromolecules, to cells, to tissues and organs, and contributes to dysfunction at the whole organism level, thereby increasing the incidence of disease and the probability of death. Ageing has been defined by four major features: it is universal, intrinsic, progressive and deleterious (Strehler, 1977; Viña et al., 2007).

Ageing affects most multicellular organisms and its universality suggests that the basic mechanism may be conserved across species. Whether the cumulative effect of physical and chemical changes that give rise to senescence is caused by extrinsic factors or whether it is initiated by some intrinsic clock is still a point of uncertainty. It is likely that both environmental and genetic factors contribute to senescence. The degree to which these two factors influence senescence and whether there is an interaction between them is the focus of research in the ageing field. The maximum potential lifespan of an organismal population is probably largely determined by genetics, whereas the mean lifespan is subject to change in response to environmental influences (Finch, 1990), and ageing research is largely based on this assumption. Efforts to lengthen the lifespans of model organisms through both genetic and non-genetic interventions have done so to a limited extent - as yet, no single intervention has lengthened the lifespan of an organism indefinitely. Thus, if there is a single master regulator of ageing, either it has not yet been identified or its effects on aging are independent of the influence of external factors. It is possible that the interventions that have been found to extend lifespan have done so by targeting one of potentially many downstream effectors of such a master regulator, which may explain the relatively modest effects on lifespan of interventions so far reported. An interesting feature of the ageing process is that within a given species the characteristic

phenotypes of ageing manifest at around the same age – there appears to be a degree of synchrony with the onset of various age-associated diseases, such as cancer, cardiovascular disease, cerebrovascular disease and diabetes (Holliday, 1996), suggesting the workings of some intrinsic determinant of ageing onset. Additionally, there is evidence for lifespan-extending interventions having ameliorative effects for other aspects of ageing pathology. For example, downregulation of the target of rapamycin (TOR) pathway in mice, a lifespan-extending intervention, also extends healthspan, protecting against age-related pathologies such as immunosenescence, motor dysfunction and insulin resistance (Selman et al., 2009). Thus, it may be possible to ameliorate more than one aspect of the ageing phenotype with a single environmental or genetic intervention.

1.1.1. Evolutionary theories of ageing

Most living organisms exhibit biological senescence, culminating in their eventual death. However, some organisms, such as *Hydra vulgaris*, appear not to undergo biological senescence (Martínez, 1998), and the jellyfish *Turritopsis dohrnii* is able to reverse the ageing process and escape death (Piraino et al., 1996). Thus, ageing appears not to be inevitable - so why do most organisms age? In light of Darwin's theory of Evolution, which postulates that randomly-arising heritable traits that bestow some fitness advantage to an individual will accumulate in a population, where fitness is defined as reproductive success (Darwin, 1859), could there be some fitness advantage of ageing? Senescence does not have any apparent benefit for reproductive fitness – in fact, most organisms experience a decline in fecundity with age, that is, the reproductive rate as measured by the number of offspring, gametes or asexual propagules (Aigaki and Ohba, 1984; Partridge and Barton, 1993; Rauser et al., 2003). We might predict that the lack of senescence would increase reproductive fitness, presuming that reproduction is limited by declining somatic integrity. So how is such a deleterious and deadly process so widespread? Is ageing actively conserved, or has it managed to escape elimination by natural selection? The apparent paradox of ageing has led to a number of evolutionary theories to explain it.

The underlying basis for the evolutionary theories of ageing is that aging is a matter of evolutionary neglect - traits with deleterious effects manifesting later in life fail to be selected against, since the force of natural selection declines after reproduction (reviewed

in Partridge and Gems, 2002). Most animals in nature fail to reach an age where these deleterious effects would manifest, since their environment is such that they are prone to death by disease, starvation, predation, or by other environmental hazards (Haldane, 1941). Therefore, there is little reason for the body to maintain fitness for the long-term, so there is little selection pressure for traits that would maintain survival beyond the time when environmental hazards would claim the lives of most animals anyway. As such, late-acting deleterious mutations/alleles in the population evade the action of natural selection and are thus allowed to persist in the subsequent generation, under a "selection shadow" (Fabian and Flatt, 2011; Medawar, 1952; Figure 1.1). The result is the accumulation of maladaptive mutations manifesting later in life, and culminating in the ageing phenotype. This is known as the Mutation Accumulation theory of ageing.

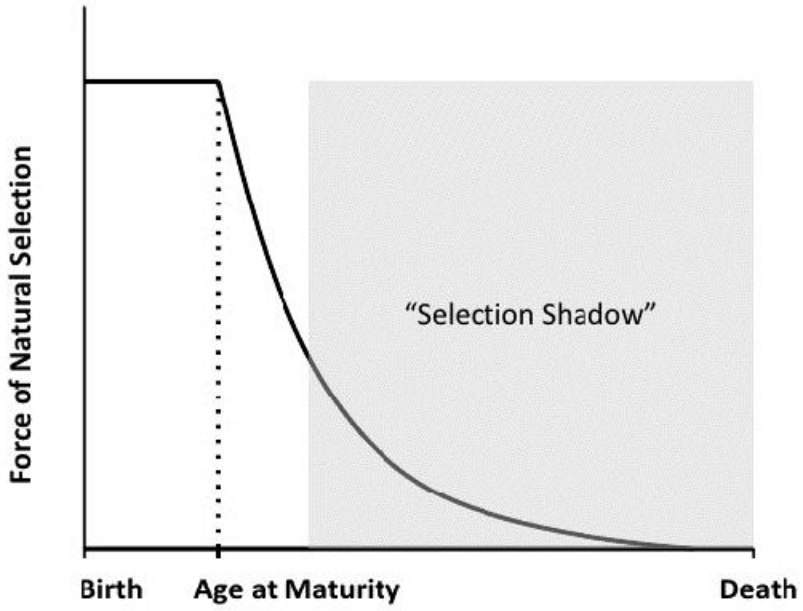


Figure 1.1. The force of selection as a function of age. Figure from Fabian and Flatt (2011).

The Antagonistic Pleiotropy theory of ageing was developed from the Mutation Accumulation theory and hypothesises that there exist alleles with multiple distinct phenotypes that occur at different life history stages, where at least one is beneficial and manifests early in life, and another is detrimental and manifests later in life (Williams, 1957). Since evolution aims to maximise offspring production, strong selection for enhanced fertility early in life would favour mutations with beneficial effects on reproduction and survival before the reproductive period, even if these mutations have deleterious effects later in life. The result is that the deleterious effects of such alleles

manifest later in life as age-related pathology. In this sense, ageing can be viewed as a by-product of enhanced fertility, or as the result of a trade-off between reproduction and longevity. Although Antagonistic Pleiotropy has become a prevailing theory of aging, it has yet to be sufficiently experimentally verified. Some of the reported ageing genes seem to have an associated beneficial effect on fertility, but not all.

The related Evolutionary Life History theory predicts that an animal's lifespan is determined by the allocation of finite resources (e.g. nutrients, time, energy) between traits that contribute to fitness, and that variations of life history characteristics such as ageing, reproductive lifespan, and number and size of offspring between different species reflect differential allocation of resources. The allocation of these resources is shaped by natural selection and is influenced by environmental selection pressures (Hamilton, 1966; Kirkwood and Shanley, 2005). Theoretically, an animal with an infinite supply of resources would not be subject to such trade-offs, and would therefore be able to simultaneously maximise all aspects of fitness, resulting in an unlimited lifespan. This hypothetical organism is referred to as the 'Darwinian Demon' and is used in thought experiments to explore the life history strategies among different organisms (Law, 1979). Intuitively, natural selection would favour the maximisation of both fecundity and longevity since both facilitate genetic contribution to the next generation. In reality, however, these two traits commonly display an inverse relationship with each other, such as in mammals and birds (Holmes et al., 2001; Read and Harvey, 1989). Moreover, experimental manipulations often have antagonistic effects on lifespan and fecundity (Reznick et al., 2000). Experiments have shown that fruit flies that were selected for later-life reproduction were long-lived but their early-life fecundity was compromised, suggesting that ageing is under pleiotropic genetic control. Moreover, unstable environments select for flies with shorter lifespans and higher fecundity, presumably because in such an environment when survival is threatened it is better to invest resources into producing offspring than to potentially waste resources on survival (Luckinbill et al., 1984; Rose, 1984; Rose and Charlesworth, 1980).

The Disposable Soma theory attempts to explain the trade-off between reproduction and longevity from a metabolic viewpoint, where the limiting resource is nutrients (Kirkwood, 1977). The theory proposes that an organism invests its limited nutritional resources in either reproduction or somatic maintenance, depending on the amount of this resource

available in its environment, where reproduction is favoured under conditions of nutrient abundance and somatic maintenance is favoured during nutrient scarcity. The Disposable Soma theory is based on the idea that longevity is a function of an organism's ability to maintain its body to optimal levels, and argues that this degree of maintenance lasts only for as long as the typical reproductive period, after which the body is disposable and deterioration is not prevented. The optimal level of resource allocation to somatic maintenance evolves to be lower than that required for infinite survival - a greater level of investment is not likely to pay off as the organism will most likely die at the hands of extrinsic mortality before any benefits of such an investment would be realised. Thus, according to the Disposable Soma theory, intrinsic longevity is defined by the degree of resource allocation to somatic maintenance, and conversely it is the compromised allocation of resources to somatic maintenance that causes the body to deteriorate with age. Thus, a trade-off exists as alleles that use energy to increase reproductive fitness are favoured at the expense of energy investment in the soma for long-term survival, which fits the Antagonistic Pleiotropy model. The molecular mechanism of this phenomenon remains to be elucidated, but it seems to be one that is evolved and conserved, and necessitated by a resource-limited environment. An alternative hypothesis to that proposed by the Disposable Soma theory is that the process of reproduction directly inflicts somatic damage, thereby increasing the rate of ageing (Barnes and Partridge, 2003; Williams, 1966). However, this seems unlikely since sterile female flies exhibit extension of lifespan under DR, suggesting that there is no obligatory trade-off (Mair et al., 2004).

It has been suggested that ageing is not entirely neglected by natural selection and that senescence is indeed actively selected against (Williams, 1957). The competitive environment of nature is such that even slight degrees of senescence can be fatal to an animal if, for example, it causes the animal to be slower or if it reduces the efficiency of its immune system. Demographic studies have demonstrated the influence of senescence on the rate of death in nature.

There has also been some opposition to the idea of aging being maladaptive, and this has given rise to the theories of "Programmed Death" (Goldsmith, 2013) which support the idea that aging evolved as an adaptation and is actively selected for. Implicit in these theories is the notion that limiting lifespan has some evolutionary benefit, in effect causing the retention of adverse traits that give rise to ageing. Theories of Programmed Ageing

generally propose non-individual (population-wide) benefits of limited lifespans, where ageing evolved to the advantage of the species rather than to the individual, which is also known as group selection (Wynne-Edwards, 1986). One such theory hypothesises that ageing is necessary to remove older individuals in a population to free up resources and make room for the younger, more prolific generation, thereby sustaining the genetic turnover required for evolution (Weismann, 1889; Yang, 2013). However, while ageing may indeed have this advantage for a population in the long-term, this theory has not been substantiated by an accompanying mechanistic theory – how would individuals acquire 'ageing genes', and how would these individuals be more successful than other individuals without such genes? It seems counter-intuitive since ageing reduces individual fitness, and this theory has largely been discounted. However, another theory that supports the idea that longevity is actively selected for is the Grandmother Hypothesis (Hawkes, 2003; Hill and Hurtado, 1991), which is rooted in the Kin Selection strategy theory of Evolution (Bourke, 2007). The Grandmother Hypothesis attributes the reproductive success of an individual to the post-reproductive lifespans of their mother and grandmother. The reasoning is that post-reproductive mothers contribute to the reproductive success of their offspring through altruistic behavior - helping to care for the young, thereby enabling their offspring to breed earlier and more often (Hamilton, 1963). Demographic analysis of pre-modern Finnish and Canadian human populations revealed that women with a greater post-reproductive lifespan have more grandchildren, indicating greater fitness (Lahdenperä et al., 2004). In the context of the Grandmother Hypothesis, it is possible that natural selection may have selected for menopause in females to preserve a post-reproductive lifespan, thereby permitting greater investment in existing offspring and increasing the reproductive success of those offspring in turn. An alternative theory for the origin and preservation of menopause is that it is the result of a physiological trade-off (or antagonistic pleiotropy) where the genes that determine menopause onset may have primarily been favoured for their contribution to reproductive success early in life (Peccei, 2001).

1.1.2. Theories of the downstream mechanisms of ageing

There are several theories that attempt to explain the molecular and cellular mechanics of ageing, owing to the fact that ageing is a complex process with many and varied biological manifestations. The Damage Accumulation theory postulates that the build-up of various forms of molecular and cellular damage over the course of life results in the failure of maintenance processes that counter deterioration, such as wound healing, cell replacement, cell growth, homeostasis, detoxification and immunity, in turn giving rise to age-associated pathology and disease (Holliday, 1997). This theory encompasses a number of sub-theories, which are not necessarily mutually exclusive. One form of damage accumulation that has been widely studied in the context of ageing is that inflicted upon the genetic material. Known as the DNA Damage theory, it is proposed that DNA alterations contribute to aging by interfering with the processes of transcription and translation, thereby causing aberrant protein synthesis resulting in inhibition of protein formation or impaired protein function, and in turn cellular dysfunction (Gensler and Bernstein, 1981). Insults to DNA mostly come in the form of hydrolysis, oxidation, and alkylation and can be caused by reactive oxygen species (ROS), viruses, ionising radiation, and environmental chemical pollutants (Ames and Gold, 1991; Best, 2009). Although specialised proof-reading mechanisms exist within cells to recognise and repair DNA damage, the DNA repair processes are not entirely proficient and the resultant accumulation of unrepaired DNA damage is what is thought to bring about some of the pathologies of ageing (Best, 2009; Freitas and de Magalhães, 2011). Moreover, genes involved in DNA repair can themselves become damaged, rendering them incapable of recognising DNA damage, resulting in the replication of this mutated DNA. In this way, cells can accumulate DNA damage and mutation, themselves consequently becoming increasingly dysfunctional. Studies have drawn correlations between DNA repair capacity and lifespan (Hart and Setlow, 1974).

Damage to DNA by ROS is encompassed in the Free-Radical theory of ageing, which also implicates ROS in the damage to cellular structures, which givies rise to senescence (Harman, 1956). Free radicals, a type of ROS, are molecules with an unpaired electron, which makes them extremely and non-specifically reactive, with damaging effects. They are by-products of respiration within mitochondria (Harman, 1972), but external factors such as X-rays and UV-light can also cause free-radical production. Free radicals readily

attach to and react with almost any cellular structure, including DNA and cell membranes, disrupting their structure and compromising the integrity of their function (Barja, 2004; Fridovich, 1978). Several protective mechanisms are employed by organisms to defend against free radicals, including enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase, as well as various antioxidants including vitamins C, E, selenium, and glutathione (Cutler, 1991). It is thought that the efficiency of these defenses to clear free-radicals decreases over time and becomes less sufficient to prevent the constant accumulation of persistent low-level damage.

Another mechanism of damage accumulation within cells is through the harmful effects of waste build-up. The Waste Accumulation theory (Terman, 2001) states that over time cells accumulate waste products from metabolic processes and that this can have a toxic effect to interfere with normal cell function. The cell is well-equipped to remove metabolic waste products such as urea, electrolytes and carbon dioxide through urine, faeces, sweat and breathing. Larger waste molecules such as proteins and nucleic acids must first be broken down by specialised digestive enzymes for removal. Cross-linked proteins and other cross-linked cellular structures are particularly difficult to remove and are thus prone to accumulation into large poorly-soluble aggregates. Although proteases exist to break down such protein aggregates, at some point the rate of their production exceeds their break-down, resulting in aggregation and accumulation. One example is the long-term build-up of lipofuscin, which is a brown lipid pigment, in the lysosomes of cells (Mann and Yates, 1974; Reichel et al., 1968). In humans, lipofuscin exists in most tissue types, particularly the heart, brain, nerves and muscle. Lipofuscin and other age-pigments do not have any toxic effect, rather they are thought to exert deleterious effects on cells by taking up space, thereby interfering with the normal traffic of molecules, nutrients and structural units around, into and out of the cells and slowing the removal of wastes. Significant correlations have been noted between the amount of lipofuscin in neurons and heart muscle cells and the severity of dementia or heart failure, respectively (Dowson, 1982; Allaire et al., 2002).

Underlying these collective damage accumulation theories of ageing is the idea that the body's ability to repair damage becomes exceeded by the rate of damage accumulation. Not only is the amount of damage insufficiently cleared by the repair processes, but the repair processes themselves become a victim of the damage. The Misrepair Theory of

Ageing argues that faulty repair molecules contribute to further damage accumulation (Wang et al., 2009).

An alternative theory to the damage accumulation theories of ageing is that ageing is the detrimental result of the failure of developmental processes to be turned off, which may lead to hypertrophy and/or atrophy (Blagosklonny, 2006a). For example, unregulated biosynthesis, driven by growth-promoting pathways such as target of rapamycin (TOR) and insulin/IGF-like signalling (IIS), can lead to detrimental hypertrophy. This theory can be reconciled with antagonistic pleiotropy, since growth promotes early life fitness, yet its continuation into later life is harmful. The theory argues that ageing is 'quasi-programmed' in that such early-life fitness-promoting programmes fail to be regulated in later life, due to the decreased force of natural selection post-reproduction. This theory is supported by the observation that decreased TOR and IIS activity extends lifespan in laboratory organisms (reviewed in chapter 1.3).

1.1.3 Ageing research

With advances in medical research and improvements in living standards, life expectancy in developed countries has been steadily increasing over the past 160 years (Murray, 2015; Wilmoth, 2000). This increase in lifespan, however, is not owed to research into ageing – instead it is the result of improved hygiene, immunisation and advancements in treatments for disease. In fact, one consequence of these improvements to public health is an increase in age-associated diseases, since more people are living long enough to suffer such late-onset diseases. As global life expectancy continues to increase, it becomes more important to improve healthspan as well as lifespan, because elongation of lifespan is unappealing and increases suffering without enhanced behavioural vitality and improved functional capacity. Thus, there is now a greater need to improve health during old age.

A principle goal of ageing research is to understand the mechanisms that give rise to loss of function at different levels of biological organisation in an organism over time. At some point, irreversibility of this functional decline occurs. Thus if we are to intervene to slow or prevent the ageing process, treatments must be targeted to cellular and tissue changes before the point of irreversibility (Partridge et al., 2005). It is therefore necessary, in

consideration of possible interventions, to identify the root cause of the biological changes that amass to physical senescence. In this research we explore the role of diet in modulating the physiological changes that give rise to ageing, and how dietary status is signalled to the body to maintain homeostasis. We use *Drosophila melanogaster* as a biological system to study ageing through genetic and dietary interventions.

1.1.4. Drosophila melanogaster as a model for ageing

Research into ageing takes advantage of the genetic conservation of metabolic and developmental pathways, and other genetic material, across different species. *Drosophila* is a good model for ageing in humans because they share many genes and interventions that alter lifespan between species. For example, certain proposed biological determinants of lifespan, such as the TOR nutrient signalling pathway and the IIS pathway, are present in both humans and in *Drosophila* (to be discussed later). Approximately 75% of known disease genes in humans have homologs in the *Drosophila* genome (Reiter et al., 2001). *Drosophila* have an advantage over other commonly-used model organisms, such as the nematode worm *Caenorhabditis elegans*, in that they have greater genetic similarity to humans, making them comparably more relevant for studying human ageing. Many basic physiological properties are conserved between *Drosophila* and mammals, and *Drosophila* also displays measurable changes in morphology and function with age.

The are many practical advantages of using *Drosophila* as a model organism in research; they have a relatively short lifespan of 3 months, and a development time of 10 days from egg to adult (at 25^{o}C, 65% humidity), meaning that rapid progress can be made, especially in the context of ageing research, compared to the use of mice, which typically live up to 3 years old.

Mated female *Drosophila* can lay ~400 fertilised eggs during their lifetime. 21-24 hours after egg-laying, these embryos hatch into larvae, which feed and grow for ~4 days, molting twice – once at 24 hours after hatching and once at 48 hours. The larvae then wander from their food and enter a pupal phase for ~4 days, during which they undergo metamorphosis into adult flies. Flies eclose from their pupae 9-10 days from the egg stage (Figure 1.2). *Drosophila* have 4 pairs of chromosomes – an X/Y and 3 autosomes.

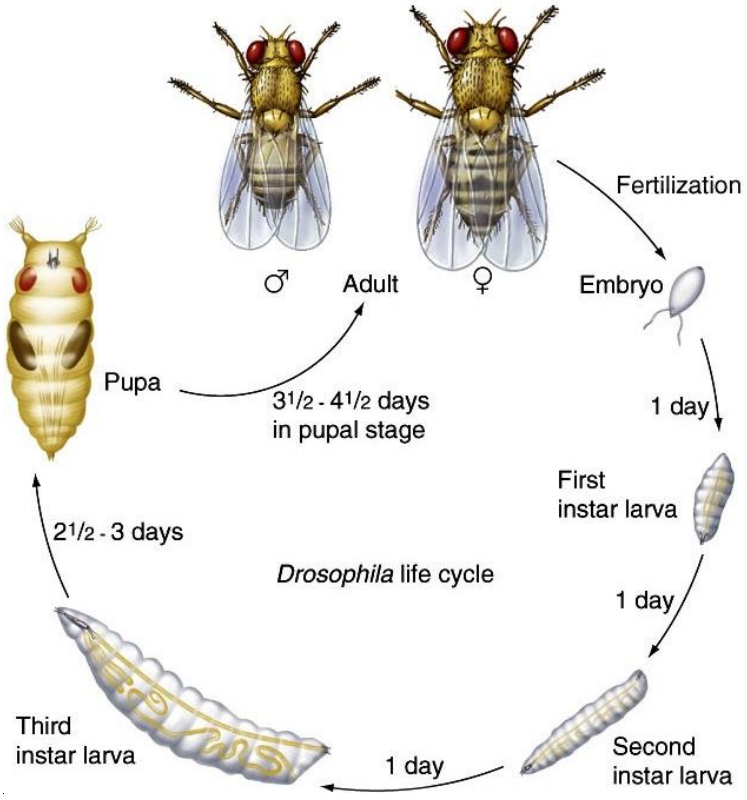


Figure 1.2. Life cycle of *Drosophila melanogaster* from egg to adult.
Image taken from (Hartwell et al., 2011).

Other practical advantages of using *Drosophila* for research include their ease of culture to large numbers, their ease of handling and the relatively low costs required for their maintenance. Moreover, sequencing of the *Drosophila* genome has made it possible to genetically manipulate them for gene function studies.

The use of *Drosophila* in biological research allows the use of genetic tools that permit the spatial and temporal control of gene expression in the fly, and this has proved to be very useful for analysing gene function. Tissue-specific expression of a gene of interest is enabled by the UAS (upstream activating sequence)/GAL4 system (Brand and Perrimon, 1993; Figure 1.3). Two distinct transgenic fly lines are required; in the first, a yeast transcriptional activator gene, *GAL4*, is placed under the control of the promoter (or enhancer) of a tissue-specific driver from *Drosophila*. In the second transgenic fly line, the upstream activation sequence (UAS), containing GAL4-binding sites, is cloned upstream of the gene of interest. GAL4 induces the transcription of this GAL4-responsive

(UAS) target gene. Only upon crossing of the GAL4 line to the UAS-target gene line is the target gene activated (in the progeny).

A further level of control can be introduced by the gene switch (GS) system. Here, temporal activation of the UAS/GAL4 system can be achieved with the addition of the inducing drug RU486 (Osterwalder et al., 2001). This is desirable in cases where the misexpression or overexpression of the target gene is lethal at the developmental stage, thereby preventing analysis of gene function in adults using drivers that also express during development. In this case, the driver lines express a modified version of the GAL4 transcriptional activator that is RU486-dependent. In the absence of the RU486 activator, the GAL4 protein is expressed in the tissues of interest but remains transcriptionally silent, and the transgene remains unexpressed. Administration of RU486 (e.g. orally via food) activates the GAL4 protein, thereby permitting the expression of the gene of interest in only the tissues containing the GAL4 protein. Transgene activation is only possible in the progeny of the cross between the two transgenic fly lines.

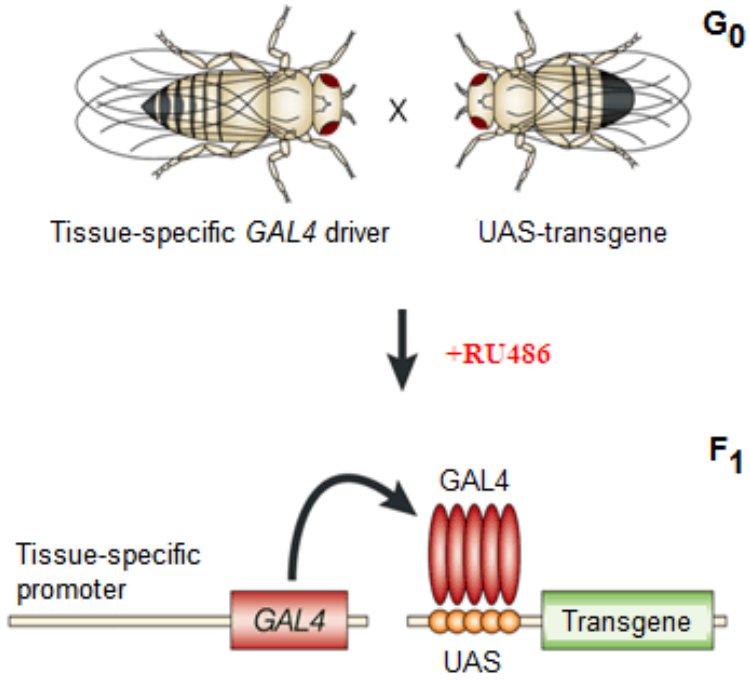


Figure 1.3. The Gene Switch/UAS expression system in *Drosophila.*

Mutagenised fly lines expressing the tissue-specific GAL4 transcriptional activator are crossed to UAS-reporter fly lines with the target gene fused to GAL4 binding sites. In the F_1 progeny, expression of the *GAL4* gene is activated by a tissue-specific promoter. In the presence of the RU486 activator, the resulting GAL4 protein in the target tissues becomes transcriptionally active and binds to and activates the transcription of the UAS (upstream activating sequence), which in turn activates expression of the transgene within the target tissues.

Although *Drosophila* has many strengths as a model system for ageing research, the ultimate aim of this research is to enhance human healthspan, and so the focus of such research must be on those aspects of ageing that are relevant to humans. It is in consideration of genetic resemblance to humans that *Drosophila* stands at a disadvantage in comparison to mammalian models. Thus, any experimental result that comes from *Drosophila* must be verified in multiple different animal models to establish evolutionary conservation of function, thereby increasing the relevance to humans. Indeed *Drosophila* has been instrumental in establishing evidence of conservation for an effect of the insulin/IGF pathway and dietary restriction on aging (Piper et al. 2005; Giannakou and Partridge 2007).

1.2. Dietary restriction

Dietary restriction (DR) is an intervention that extends lifespan in a diverse range of organisms. DR is defined as a moderate reduction in food intake that is just short of malnutrition. Originally, DR was defined as a 20-40% reduction from *ad libitum* feeding. However, it now refers to a broader spectrum of dietary interventions, including more specific limitations of one or more macronutrients to varying degrees. DR is considered one of the most robust environmental interventions that extends lifespan in diverse animal models, including yeast (Jiang et al., 2000), worms (Klass, 1977), fruit flies (Chapman and Partridge, 1996), rats (McCay, 1935) and even dogs (Lawler et al., 2008). Almost 100 years after it was first demonstrated that the lifespan of an animal could be extended by restricting its diet (Osborne et al., 1917; McCay, 1935), and despite subsequent research efforts, the biological mechanism underlying this phenomenon remains to be elucidated.

In most organisms, DR results in reduced fecundity, however, it has been shown to have a wide range of health benefits. DR rodents have a delayed onset or a lesser severity of age-related diseases such as cancer, type 2 diabetes, cardiomyopathy, nephropathy, chronic lung diseases, autoimmune diseases, and motor dysfunction and neurodegenerative diseases (Blackwell et al., 1995; Fontana and Klein, 2007; Ingram et al., 1987; Martin et al., 2006; Masoro, 2005; Weindruch et al., 1979). DR rodents are also protected against sarcopenia, left ventricular diastolic dysfunction, decline in heart rate variability, and age-related hearing loss (Martin et al., 2006; Marzetti et al., 2009; Meyer et al., 2006; Someya et al., 2010). Moreover, data from postmortem pathological studies have shown that 30% of the DR rats versus 6% of fully-fed rats die without any sign of morphologic lesions (Shimokawa et al., 2003). Thus DR appears to maintain physiological functions in more youthful states, rather than just extending the moribund period of life.

DR studies in rhesus monkeys suggest that the beneficial effects of DR on health are conserved in primates. The percentage of rhesus monkeys free of age-related diseases has been reported to be higher in DR monkeys compared to control groups. For example, monkeys maintained on a DR diet long-term have been shown to have a reduced incidence of type 2 diabetes, glucose intolerance, cancer, cardiovascular disease and brain atrophy, and DR improves age-related survival in some, but not all, cases (Bodkin et al., 2003;

Colman et al., 2009, 2014; Kemnitz, 2011; Mattison et al., 2012). Short-term DR has also been correlated with beneficial effects on health in humans; it was reported that DR reduced risk factors for type 2 diabetes and atherosclerosis, including reduced obesity, increased insulin sensitivity, reduced cholesterol, reduced blood pressure, and improved left ventricular diastolic function (Fontana et al., 2004; Holloszy and Fontana, 2007; Fontana and Klein, 2007). However, the optimal degree of DR implementation for humans remains unknown. This is dependent on what is considered as the optimal body weight, level of adiposity, and metabolic profile.

Longevity is difficult to study in humans, thus the effects of long-term DR on human lifespan are as yet unknown. However, 'natural experiments', such as the conditions producing the high centenarian population of Okinawa, Japan, have offered insights into the effect of DR on lifespan in humans. Okinawa has the highest proportion of centenarians in the world (Chan et al., 1997). The people of Okinawa not only have the longest life expectancy in the world, but are also comparatively healthier – they have the lowest frequency of coronary heart disease, stroke, and cancer, and they spend an average of 97% of their lives disease and disability-free (Bernstein et al., 2004; Willcox et al., 2008b). The Okinawa Centenarian Study, which began in 1975, aims to determine the genetic and lifestyle factors owing to the success of this particular aging population. It is believed that the Okinawans owe their exceptional health and longevity to their low calorie, but nutrient-rich, diet. They are reported to consume only 83% of the average caloric intake for Japan (Kagawa, 1978; Willcox et al., 2007, 2014). The Okinawan diet is necessitated by a rural lifestyle, however the quality of their diet is sufficient to protect them against malnutrition and infectious diseases. The case of the Okinawan centenarians is often used as evidence that DR can decelerate ageing in humans and protect against age-related diseases.

The broad spectrum of health benefits associated with DR suggest that the effect of DR to extend life is not achieved by averting some single cause of death, but rather by slowing the ageing process itself. Alternatively, DR may alter the rate of aging by averting multiple causes of death that would otherwise manifest as senescence and increase age-related mortality. In *Drosophila*, DR increases both mean and maximum lifespan. DR delays the onset of age-specific mortality, but subsequently advances at the same rate as control flies (Pletcher et al., 2002). Age-specific mortality trajectories generated for DR and control

female flies, as well as for DR flies switched to control food (and vice versa) at 14 and 22 days of treatment, revealed that control flies that were switched to DR food rapidly adopted the mortality profiles of chronically treated DR flies (Mair et al., 2003). This study suggested that DR removes the short-term risk of death, rather than slowing age-related damage, since the rapid (48hr) drop in mortality seen for the control-to-DR switched flies implies that any pre-existing damage before the switch had no effect on mortality at the ages tested.

1.2.1. Caloric restriction Vs. dietary restriction

Historically, the lifespan and health benefits of DR have been attributed to a reduction of dietary calorie intake; as such, DR is often referred to as ‘caloric restriction' or 'CR'. Some studies have shown support for this theory - restriction of calories in rats without a reduction in protein resulted in lifespan extension (Masoro et al., 1989). Another study showed that rats fed an isocaloric diet with reduced fat and mineral levels did not have increased lifespan (Iwasaki et al., 1988a). One problem with these kinds of studies is that when a particular dietary component is omitted from the diet, it is necessarily replaced by something else to compensate for the physical reduction in volume. Consequently, not only is there a reduction of calories, but the balance of all the other dietary components is altered.

The idea that the caloric content of diet is responsible for the DR effects on lifespan is under scrutiny and other work in the field suggests that it is the balance of particular dietary components that mediates the effects of DR on lifespan. One study showed that the restriction of protein had lifespan-extending effects in rats, where calorie intake was kept the same (Yu et al., 1985). In another study, changing the protein source from casein to soy, again while keeping the caloric content the same, conferred an increase in the lifespan of rats (Iwasaki et al., 1988b). Moreover, rodent studies that apply DR by intermittent-day fasting regimens, which do not affect the overall calorie consumption compared to control animals, have reported increases in lifespan (Anson et al., 2003; Goodrick et al., 1990). The discrepancy between findings from different laboratories, animal strains, and methods of DR application only highlights the complexity of the mechanism of DR to enhance longevity, and it strengthens the notion that the lifespan and health benefits of DR cannot

always be attained simply by reducing ingested calories. The level of mechanistic complexity implied from these studies suggests that the lifespan response to DR is governed by specific nutritional components of the food and their relative abundance.

In *Drosophila*, it was demonstrated that reducing the levels of dietary yeast or sugar increased lifespan, but to different degrees and by amounts unrelated to calorie content (Mair et al., 2005). Moreover, flies that were switched between DR and control yeast concentrations quickly adopted the mortality profiles of flies that had been chronically maintained on the food to which flies were switched. In contrast, switching between sugar concentrations had no effect on the mortality trajectory. Furthermore, the greater effect of altering dietary yeast, compared to altering sugar, on survival suggests that the nutritional mix found in yeast, which consists mainly of protein, is a more important determinant of lifespan than carbohydrate.

1.2.2. Role of amino acids in dietary restriction

Evidence suggests that the dietary determinants of the lifespan effect of DR may be narrowed down further from protein to amino acids. The absence of the non-essential amino acid cysteine, and low amounts of the essential amino acid methionine, resulted in lifespan extension in rats (Orentreich et al., 1993; Richie et al., 1994; Zimmerman et al., 2003). Methionine restriction has also been shown to increase lifespan in mice (Miller et al., 2005). Similarly, tryptophan restriction extended lifespan in both mice (De Marte and Enesco, 1986) and rats (Ooka et al., 1988; Segall and Timiras, 1976). Methionine-restricted animals, in addition to extended lifespan and reduced growth, also exhibit physiological changes that are similar to those seen in calorie-restricted animals, as well as some that are dissimilar, suggesting that methionine-restricted animals are not calorie-restricted. It also suggests that the mechanistic pathways that govern the effect of methionine restriction and calorie restriction on physiology are separate, but perhaps overlapping to some degree.

Recently in the Partridge lab, it was shown that it is the balance of amino acids that mediates the lifespan differences between DR and full feeding (FF) (Grandison et al., 2009). This study used *Drosophila* as a model system for ageing. DR was implemented by

a two-fold dilution of yeast in the standard sugar / yeast diet. Supplementing the DR diet with the ten essential amino acids (EAA) produced the same relatively short lifespan as that in the FF condition. In the same study, EAAs were also shown to be responsible for the fecundity differences between DR and FF, where DR flies supplemented with EAAs had increased egg-laying to the same degree as the FF condition. Moreover, specific EAAs were implicated as being responsible for the increase in fecundity with EAA addition; supplementing a DR diet with methionine alone was sufficient to increase fecundity to the same level as FF. Omitting methionine from the artificial FF condition (DR+EAA) reduced egg-laying, indicating that methionine is necessary and sufficient to increase fecundity to the same level as FF. Methionine addition did not shorten lifespan, as would be expected under a model involving an obligate nutrient trade-off between traits. The omission of methionine from the DR+EAA condition increased lifespan, and this effect was not due to reversal of a detrimental effect of high levels of methionine since methionine addition to the DR condition increased egg-laying. Not only did this study demonstrate that it is the balance of EAAs that is responsible for the lifespan effects of DR, but also that a different balance of EAAs is responsible for the fecundity response of FF, such that the effect of DR on lifespan and fecundity in *Drosophila* cannot be attributed to reallocation of nutrients from somatic maintenance to reproduction, as the prevailing theory for the mechanism of DR would predict.

DR extends lifespan in most species tested to date, and it is probable that the lifespan response to DR evolved very early as a mechanism to increase survival during times of environmental food shortage. The comparatively more modest effects of DR on primates, compared to simpler organisms, may be due to the fact that simpler organisms have shorter lifespans to begin with, and exposure to a period of famine represents a greater fraction of their lives, necessitating a more dramatic adaptation. It is not yet clear whether the mechanism is evolutionarily conserved or whether it evolved independently in these species.

Many of the genetic interventions that have extended lifespan downregulate the activity of nutrient sensing pathways which regulate growth and metabolism in response to nutritional cues, making them attractive candidate mediators of DR-induced longevity. As such, research is focussed on uncovering the nutrient effector(s) of DR, how they are sensed, integrated and relayed as a signal, and how this in turn promotes longevity assurance

mechanisms to extend lifespan and healthspan. Moreover, studies into the workings of these pathways may uncover genes and proteins as targets for drug interventions that mimic DR, with the aim of achieving longer and healthier lives.

1.3. Nutrient sensing/signalling pathways

In order to survive in a dynamic and ever-changing environment, animals must be able to sense and respond to change. Indeed, animals have evolved distinct signalling response pathways for different environmental stressors. The activity of such signal transduction pathways is induced by an extra-cellular signal, which then triggers a cascade of molecular events that culminates in a physiological and/or behavioural change that allows the organism to adjust to the environmental conditions in order to maintain metabolic homeostasis. Several biological pathways have been proposed to mediate the adaptive changes to environmental nutrient supply, and are collectively termed nutrient sensing/signalling pathways (NSP). The response to nutritional stress via these pathways often involves changes in gene expression that promotes the conservation of resources, for example, by downregulating global protein synthesis (Spriggs et al., 2010), thereby allowing the organism to endure the stress period.

The three most widely studied NSPs are the target of rapamycin (TOR) pathway, the insulin/IGF (insulin-like growth factor) signalling (IIS) pathway, and more recently the general amino acid control (GAAC) pathway. These pathways are distinct, but they also share a considerable degree of mechanistic overlap with crosstalk between them. All three pathways regulate protein synthesis, and in each case have been shown to influence ageing in animal models. As such, these pathways are being studied as mediators of the lifespan-extending effects of dietary restriction (DR). It is hoped that understanding the workings of these pathways will uncover genes and proteins that can serve as targets for drug interventions that mimic DR.

1.3.1. The target of rapamycin (TOR) pathway

The TOR pathway integrates a range of environmental inputs, such as nutrient availability, energy availability, stress and growth factors, to regulate protein synthesis, thereby ensuring that an organism's growth rate matches its resources (Martin and Hall, 2005) (Figure 1.4). The TOR pathway has recently been referred to as the mechanistic target of rapamycin, abbreviated mTOR. However, this abbreviation is also used for mammalian target of rapamycin. To avoid confusion, we will refer to the pathway here simply as TOR. We will use mTOR when referring to mammalian TOR, dTOR when referring to *Drosophila* TOR, yTOR for yeast TOR and ceTOR for *C. elegans* TOR. We will omit the leading letter if we are referring to the TOR pathway in a general sense. We also distinguish between organism-specific names of the pathway components, summarised in Table 1.1.

Table 1.1. The main genes of the target of rapamycin (TOR) pathway in mammals, and their orthologous genes in *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*, along with the biochemical functions of the gene products in mammals.

Mammals	***D. melanogaster***	***C. elegans***	***S. cerevisiae***	**Biochemical function in mammals**
mTOR	*dTOR*	*let-363*	*TOR1/2*	4E-BP1 and S6K1 phosphorylation
Raptor	*dRaptor*	*daf-15*	*KOG1*	mTOR binding protein
Rheb	*dRheb*	*CeRheb*	*ScRheb*	GAP activator of mTOR
Tsc1/2	*dTsc1/2*	No orthologue	*Tsc1/2*	GAP activator of Rheb
RagA/RagB	*dRagA*	*raga-1*	Gtr2	GAP regulator of mTORC1 localisation
RagC/RagD	*dRagC*	*ragc-1*	Gtr2	GAP regulator of mTORC1 localisation
S6K1	*dS6K*	*rsks-1*	*SCH9*	Ribosomal protein S6 phosphorylation
4E-BP1	*d4E-BP*	*spn-2*	*PHAS-1*	Repressor of eIF4E

TOR pathway mechanisms

The TOR pathway has been best described in mammals, and genetic orthologues of the components of the TOR pathway have been identified in yeast, in *Drosophila* and in *C. elegans*. In mammals, the TOR pathway exists in two multiprotein complexes; mTORC1 and mTORC2, which localise to different subcellular compartments (Betz and Hall, 2013) and differ in some of their constituent proteins and functions. Both complexes share a common catalytic subunit - mTOR - a serine-threonine kinase that relays extracellular signals to downstream effectors. mTORC1 functions to regulate cell size and proliferation (Miron et al., 2001; Montagne et al., 1999; Oldham et al., 2000; Zhang et al., 2000), and its associated proteins, in mammals, include Raptor (regulatory associated protein of TOR), mLst8 (mammalian lethal with SEC13 protein 8), and PRAS40. mTORC2 has a role in the regulation of the actin cytoskeleton (Jacinto et al., 2004), and its associated proteins include Rictor (rapamycin insensitive companion of mTOR), Sin1, Proctor/PRR5L, mLst8, and Deptor (Guertin and Sabatini, 2005, 2009; Wullschleger et al., 2006). The two TOR complexes have distinct effector pathways; mTORC1 signals downstream to ribosomal S6 kinase (S6K1) and eIF4E binding protein-1 (4E-BP1), which are translational regulators that are activated when TORC1 is active, resulting in increased growth. mTORC2 signals to downstream AKT, a protein kinase with roles in glucose metabolism, apoptosis inhibition, and the promotion of cell survival.

The tuberous sclerosis proteins 1 and 2, also known as TSC1 (hamartin) and TSC2 (tuberin) form a heterodimeric protein complex and serves to integrate signals that indicate low energy availability and stress (e.g. DNA damage, hypoxia) into mTORC1 signalling (Figure 1.4). These stress signals activate the TSC1/2 complex, which is a negative regulator of mTOR signalling, resulting in a downregulation of protein synthesis. Growth factor signals are also integrated through the TSC1/2 complex, but they inhibit the complex, and therefore derepress mTOR signalling and promote protein synthesis. The GTPase activity of TSC2 in turn stimulates the GTPase activity of the mTOR activator Rheb (Ras homolog enriched in brain) (Inoki et al., 2003).

Activated mTOR phosphorylates the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). 4E-BP1 is a translational repressor that binds the eukaryotic translation initiation factor 4E (eIF4E), preventing its association with the translation

initiation complex, which is required for protein synthesis. Phosphorylation by mTOR causes the dissociation of 4E-BP1 from eIF4E, thereby permitting assembly of the initiation complex and enabling the translation of mRNA. Another major downstream target of mTOR phosphorylation is ribosomal protein S6 kinase 1 (S6K1). S6K1 is a serine/threonine kinase that phosphorylates the S6 ribosomal protein, which is a component of the 40S ribosomal subunit involved in mRNA translation, resulting in increased protein synthesis.

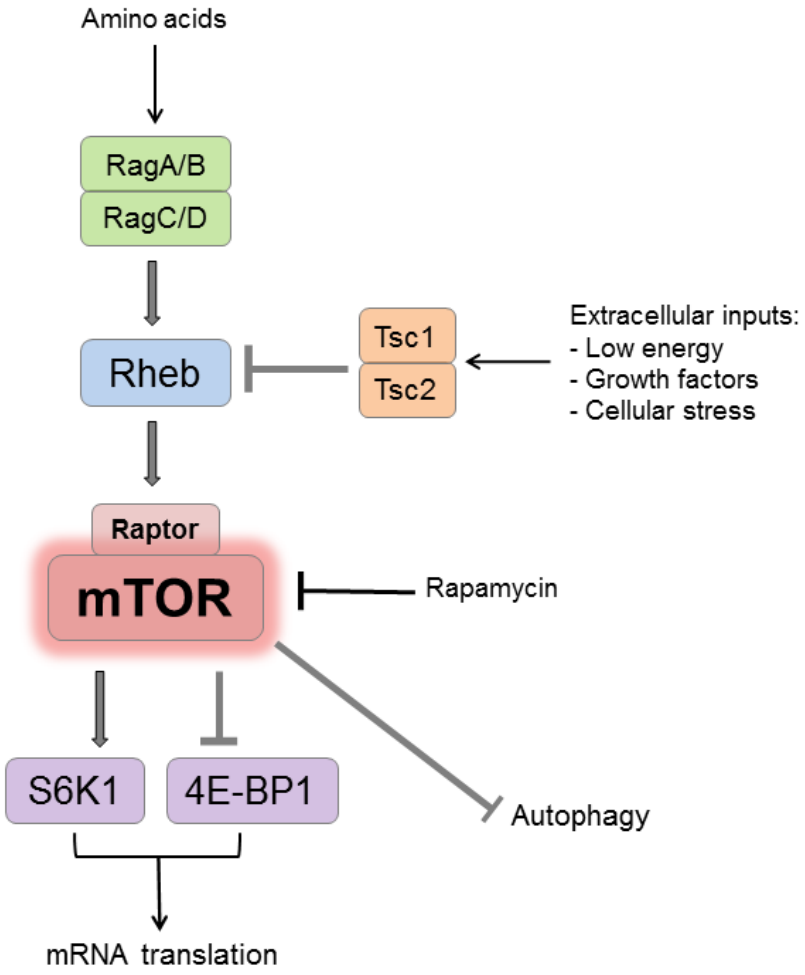


Figure 1.4. The mammalian target of rapamycin complex 1 (mTORC1) signalling network.

The mTORC1 pathway integrates extracellular amino acid signals via the Rag proteins and other extracellular inputs, such as energy, stress, and growth factors via TSC1/2 to regulate cell growth. These signals are transmitted to downstream effectors via Rheb, Raptor and mTOR. S6K1 phosphorylation and 4E-BP1 inhibition result in an upregulation of mRNA translation and increased protein synthesis. Activation of mTORC1 also inhibits autophagy. mTOR is inhibited by the drug rapamycin.

Amino acid sensing and the Rag proteins

The specific nutrients that modulate TOR pathway activity have been shown to be amino acids. Withdrawal of amino acids from cell culture medium was shown to result in the deactivation of the downstream mTORC1 effectors S6K1 and 4E-BP1. Even in the presence of growth factors, mTORC1 remained inactive in the absence of amino acids, implicating amino acids as a main activator of mTORC1 pathway activity. This was reversed upon supplementation with amino acids (Hara et al., 1998). Deficiencies of most amino acids singly have been shown to lessen mTORC1 signalling to different degrees, however, deficiencies in leucine and arginine in particular reduces TORC1 signalling in cell culture, as measured by dephosphorylation of S6K1 and 4E-BP1, as effectively as the withdrawal of all amino acids (Hara et al., 1998), suggesting a particularly important role for these amino acids in the mediation of mTORC1 activity. In another study, leucine-mediated activation of mTORC1 was shown to be dependent on the preloading of cells with glutamine, which itself has no effect on mTORC1, but rather acts as an efflux solute enabling the intracellularisation of leucine through an amino acid transporter (Nicklin et al., 2009).

Exactly how the presence, quality and quantity of nutrients is sensed within the cell cytoplasm and how these nutrient signals are relayed to downstream translation machinery remains to be fully elucidated. However, the recently discovered heterodimeric Rags (Ras-related GTP-binding) GTPases, RagA/RagC and RagB/RagD, have been found to have a significant role in transmitting the amino acid signal to mTORC1 (Kim et al., 2008; Sancak et al., 2008). In mammalian cells it was found that in the presence of amino acids, RagD dimerises with GTP-bound RagB and associates with the Raptor subunit of mTORC1, and in doing so promotes the translocation of mTORC1 to endosomal membranes within the cell, where its activator, Rheb, is thought to reside (Sancak et al., 2008; Figure 1.5). The specific endosome that hosts these mTORC1 interactions has been identified as the lysosomal membrane, and in particular the Lamp2 (lysosome-associated membrane protein 2) and Rab7 positive regions, which are markers of lysosomes and late endosomes. Within this membrane region a heterodimeric protein complex, dubbed Ragulator, was discovered as being the intermediate link between mTORC1 and the lysosomal membrane (Sancak et al., 2010). The three proteins that make up the mammalian Ragulator complex are MP1, p14 and p18, encoded by the genes *MAPKSP1*, *ROBLD3* and *c11orf59* respectively. The interaction of the Ragulator with the Rag proteins

is not dependant on amino acids, however the integrity of the Ragulator has been shown to be critical for mTORC1 activation by amino acids. Amino acids, through a mechanism unknown, encourage the loading of RagB with GTP, inducing the binding of the Rag heterodimer to the Raptor subunit of the mTORC1 protein complex. mTORC1 then translocates to a Ragulator region bound area of the lysosomal membrane where it interacts with the Ragulator via Rag, forming a Rag-Ragulator-mTORC1 super-complex which in turn promotes the interaction of mTORC1 with its lysosome-localised activator, Rheb.

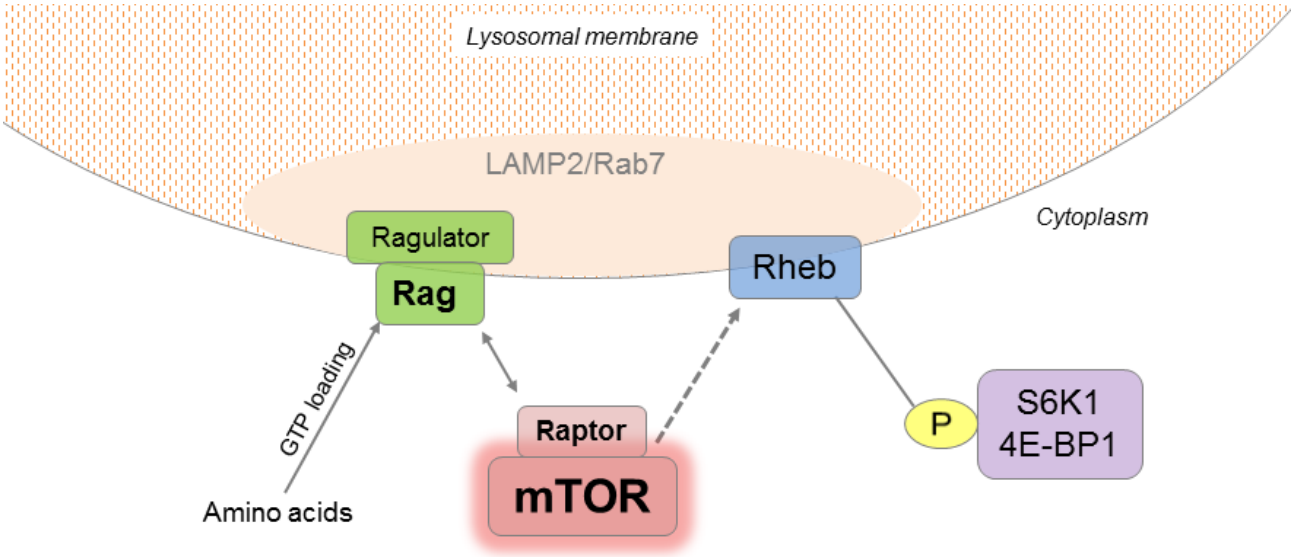


Figure 1.5. A model for Rag-mediated amino acid-induced activation of mTORC1 in mammals.

In amino acid depleted cells, mTOR cannot associate with the lysosomal membrane on which its activator, Rheb, resides. The Rag GTPases are held to the lysosomal surface by the Ragulator. Amino acids promote the loading of RagB with GTP, thereby enabling the association of the Raptor subunit of mTORC1 with the Rag-Ragulator complex. This conformation brings mTORC1 to a close enough proximity to interact with Rheb, which stimulates its kinase activity.

Rapamycin as an inhibitor of the TOR pathway

As the name suggests, TOR is sensitive to suppression by the drug rapamycin (also known as sirolimus), which is an antifungal macrolide produced by the bacterium *Streptomyces hygroscopicus*. Rapamycin was originally discovered in a soil sample from Easter Island, or Rapa Nui in the native language (Sehgal et al., 1975; Vezina et al., 1975), and was soon found to be a potent inhibitor of the mammalian cell cycle, arresting activity at the G1 phase (Houchens et al., 1983). Further genetic studies that screened for rapamycin-resistant mutants of the baker's yeast, *Saccharomyces cerevisiae*, revealed three genes whose protein products were targeted by rapamycin; *yTOR1* and *yTOR2* (Heitman et al., 1991) and *FPR1*. Several years later, the mammalian TOR pathway was identified as an orthologue of the yTOR1/2 proteins and confirmed as being the rapamycin target in mammals (Brown et al., 1994; Sabatini et al., 1994). The single mammalian TOR protein has a 42% amino acid sequence similarity to the yeast TOR1/2 proteins (Hay and Sonenberg, 2004). Subsequently, the mammalian homologue of yeast *FPR1* was identified – called *FKBP12*.

Further work showed that rapamycin binds to FKBP12, and this rapamycin-FKBP12 complex in turn attaches to a binding domain on mTORC1. This binding domain lies adjacent to the mTOR kinase domain, which becomes disrupted upon FKBP1 binding, compromising the interaction between mTOR and its activator Raptor. In reference to the fact that rapamycin can only inhibit mTOR as a complex with FKBP12, mTOR has also been called FRAP (FKBP-rapamycin-associated protein) and RAFT (rapamycin and FKBP target). Rapamycin has been thought to bind to the mTOR in complex 1 exclusively, indicating that only mTORC1 is subject to inhibition by rapamycin (Loewith et al., 2002; Sarbassov et al., 2004). However, recent evidence suggests that prolonged treatment with rapamycin can also inhibit complex 2, shown both *in vitro* (Sarbassov et al., 2006) and *in vivo* (Guertin et al., 2009; Lamming et al., 2012). Rapamycin's anti-proliferative properties have meant that it has a clinical use in treating cancer patients - rapamycin inhibits tumour cell proliferation by inducing apoptosis of the tumour cell and suppressing angiogenesis in the tumour. Additionally, the ability of rapamycin to inhibit T and B cell proliferation makes it a useful immunosuppressant, and is often administered to organ transplant patients to prevent organ rejection.

The role of TOR in growth control

Loss of function experiments support a critical role for TOR in organismal growth control; deficiency of CeTOR in *C. elegans* resulted in developmental arrest at larval stage L3, as well as gonadal degeneration and intestinal cell atrophy. Similar phenotypes were also observed for *C. elegans* with loss of function of *daf-15*, an orthologue of mammalian *Raptor* (Jia et al., 2004). These phenotypes are attributed to an inhibition of mRNA translation (Long et al., 2002). In *Drosophila*, *dTOR* (*Drosophila* orthologue of mammalian *TOR*) null flies exhibited phenotypes including developmental arrest and decreased size of endoreplicating tissue (Oldham et al., 2000). Studies in mice have shown that disruption of the kinase domain of *TOR* results in early embryonic lethality due to defective cell proliferation (Hentges et al., 2001; Murakami et al., 2004), and the absence of *Raptor* only from mouse skeletal muscles induces muscular dystrophy due to impaired protein synthesis (Bentzinger et al., 2008). Altogether these results confirm a conserved role for the TOR pathway in the control of cell growth.

The role of TOR in lifespan regulation

The TOR pathway was first found to be involved in the regulation of ageing when deletion of the yeast homolog of *S6K1* - *SCH9* - doubled the chronological lifespan of the yeast (Fabrizio et al., 2001). Soon after, reduced TOR activity was shown to extend lifespan in worms: l*et-363* (*C. elegans* orthologue of mammalian *TOR*) deficiency doubled the lifespan of worms (Vellai et al., 2003), RNAi knockdown of *daf-15* (*C. elegans* orthologue of mammalian *raptor*) also increased lifespan (Jia et al., 2004), as did mutations in *rsks-1* (*C. elegans* orthologue of mammalian *S6K1*) (Pan et al., 2007). Lifespan extension by inhibition of TORC1 pathway genes was subsequently demonstrated in flies (Kapahi et al., 2004), further in yeast (Kaeberlein et al., 2005), and in mice (Selman et al., 2009). It was also demonstrated that pharmacological downregulation of TORC1 by rapamycin extends lifespan in yeast (Powers et al., 2006), worms (Robida-Stubbs et al., 2012), flies (Bjedov et al., 2010) and mice (Harrison et al., 2009). Crucially, TOR pathway downregulation has been shown to confer broad-spectrum health benefits in mice (Selman et al., 2009). Deregulation of the TOR pathway has also been implicated in age-related diseases in humans, such as cancer, diabetes, obesity, atherosclerosis, osteoporosis, arthritis, psoriasis, Alzheimer's disease, and Parkinson's disease (Tee and Blenis, 2005; Tsang et al., 2007), due to over-proliferation.

TORC1 regulates several processes that could be involved in the lifespan response to DR. One major effect of TORC1 downregulation is reduced global mRNA translation. There are several hypothesises for how reduced protein synthesis may be favourable for longevity, and one such theory proposes that the resultant alleviation of stress on protein folding produces fewer misfolded proteins that could otherwise aggregate in a harmful way. Accordingly, mouse models of Huntington Disease showed a decrease in the formation of toxic huntingtin aggregates upon rapamycin treatment (Ravikumar et al., 2004). Another theory postulates that aberrant growth and differentiation with high TORC1 activity can exhaust the stem cell pool. In fact, the abundance of stem cells and their ability to differentiate has been shown to decline with time and this causes cellular senescence (Janzen et al., 2006; Molofsky et al., 2006).

As well as alleviating the effects of damage, inhibition of TORC1 may also trigger repair mechanisms to counter any damage. One such repair mechanism brought about by TORC1 inhibition is autophagy, which is a catabolic mechanism that employs the action of lysosomes to degrade and recycle excess or dysfunctional cellular components, thereby limiting their potential to cause deterioration of normal cell function. During this process, targeted cellular constituents are isolated within an autophagosome, which then fuses with a lysosome and unloads its contents for degradation. Autophagy also promotes survival by degrading unnecessary proteins, thereby freeing amino acids and bioenergic components to be synthesised into proteins that are essential for survival. As such, autophagy may be regarded as an adaptive response to stress. Autophagy is induced during starvation, via TORC1, enabling short-term viability through the starvation period (Lum et al., 2005). Work from several model organisms implicates autophagy in the ageing process; in worms and flies activation of autophagy has been shown to extend lifespan (Bjedov et al., 2010; Hansen et al., 2008; Hars et al., 2007; Jia et al., 2007; Meléndez et al., 2003; Tóth et al., 2008), and in mice overexpression of Atg5, a protein involved in the formation of autophagosomes, extends median lifespan by 17.2%. *Atg5* transgenic mice were also leaner, and had increased insulin sensitivity and improved motor function, and embryonic fibroblasts cultured from these mice were more resistant to oxidative stress (Pyo et al., 2013).

Suppression of growth programs like those activated by TORC1, or reduced food intake, result in the activation of stress-response mechanisms, like autophagy, that preserve the functionality of cells for extended periods of time (Fontana et al., 2010). In yeast, Gis-1, a transcription factor lying downstream of yTOR, regulates stress response programs. In flies, 4E-BP1 activation by DR resulted in the increased translation of components of the mitochondrial electron transport chain, leading to more efficient mitochondrial respiration (Zhang and Cuervo, 2008). In worms, TOR downregulation enhances thermotolerance (Hansen et al., 2007).

Thus, downregulation of TOR may extend lifespan either via reduced protein synthesis and/or via upregulation of stress-response mechanisms, improving the animal's ability to protect against damage by preventing it or repairing it (Syntichaki and Tavernarakis, 2006). According to the Disposable Soma Theory of Ageing, reduced nutrient availability shifts cells from a state of nutrient usage for protein synthesis and growth to one favouring cell maintenance and survival. It is possible that TOR mediates such a shift in resource allocation by inhibiting translation, and therefore growth, to allow for mechanisms involved in maintenance and repair, thereby enhancing survival (Hansen et al., 2007).

1.3.2. The insulin/IGF signalling (IIS) pathway

IIS is a highly conserved biochemical pathway with a primary role in regulating growth, differentiation and metabolism in response to nutrient availability (Figure 1.6). In mammals, insulin is produced by the beta-cells of the pancreas in response to increased blood glucose after food consumption, primarily in response to digestible carbohydrates, and to a lesser degree, protein. Insulin binds to the α-subunit of its receptor, which is embedded in the cell membrane, forming a receptor-ligand complex. Upon extracellular ligand binding, the β-subunit of the receptor, a tyrosine kinase, phosphorylates, and consequently binds, the insulin receptor substrate (IRS) within the cytoplasm. IRS is a signalling adaptor protein that plays a key role in transmitting signals from the receptor, to downstream effector proteins. IRS phosphorylates and activates two signalling pathways: the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-3-kinase (PI3K) pathway. The MAPK pathway (Zhang and Liu, 2002) regulates mitogenic processes such as cell growth and gene expression, and the PI3K pathway regulates metabolic processes, as well as acting as a negative regulator of the forkhead box protein O (FOXO) transcription factor. After glucose enters the cell, PI3K binds the glucose transporter, GLUT4, and sends it back to the cell membrane (Chang et al., 2004). The isolated glucose is then sent to the mitochondria where it is either used to make ATP, or is stored as glycogen if in excess. PI3K also regulates protein and lipid synthesis. Downstream of PI3K lies the serine/threonine-specific protein kinase AKT (also known as protein kinase B, PKB), which plays a key role in glucose uptake and protein, lipid and glycogen synthesis (Manning and Cantley, 2007).

AKT phosphorylates FOXO, thereby preventing it from entering the nucleus and inducing FOXO-dependent transcription of genes involved in apoptosis, cell cycle arrest, cell differentiation and protection against reactive oxygen species (ROS). It is instead sequestered in the cytoplasm, where it is ubiquitinated and degraded (Greer and Brunet, 2008). AKT is also a major link between the IIS and the TOR pathway (McCormick et al., 2011). It suppresses the inhibitory action of the TSC1/TSC2 complex on the TOR activator Rheb, which in turn activates the TOR pathway.

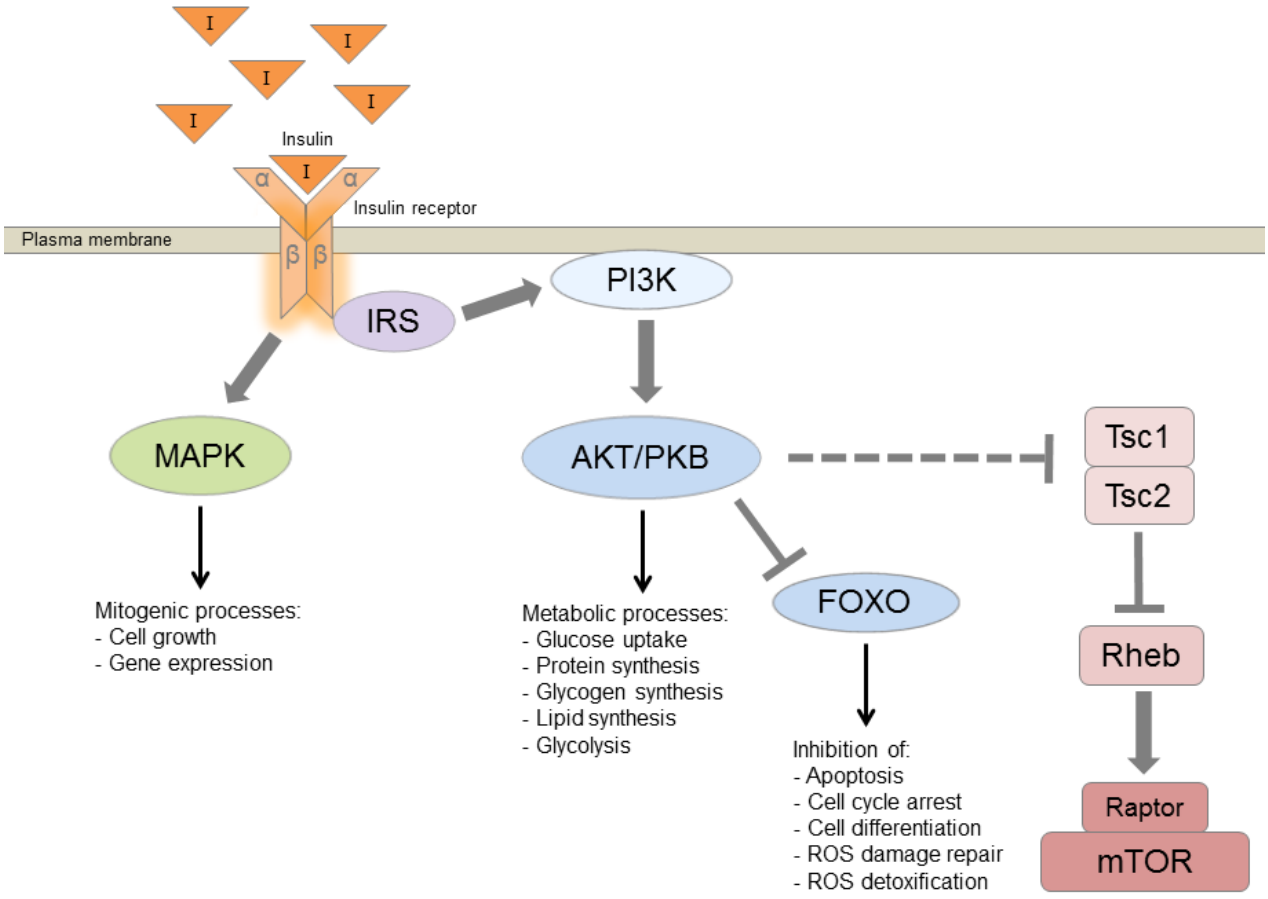


Figure 1.6. The mammalian insulin/IGF signalling (IIS) signalling pathway.
Insulin binds to the insulin receptor, which in turn phosphorylates and binds, the insulin receptor substrate (IRS). IRS phosphorylates and activates the mitogen-activated protein kinase (MAPK) pathway, which regulates cell growth and gene expression. IRS also phosphorylates the phosphatidylinositol-3-kinase (PI3K) pathway, which involves activation of AKT/PKB to regulate metabolic processes such as protein, lipid and glycogen synthesis, and inhibition of the forkhead box protein O (FOXO) transcription factor, which suppresses processes such as apoptosis, cell cycle progression, stress resistance and DNA repair. AKT also links IIS to the target of rapamycin (TOR) pathway, suppressing the inhibitory action of the TSC1/TSC2 complex on the TOR activator Rheb, thereby activating the TOR pathway.

The role of IIS in lifespan regulation

IIS was the first pathway to be shown to influence ageing in a model organism; in *C. elegans*; mutation of *age-1*, a gene encoding the *C. elegans* homologue of PI3K, was found to extend lifespan (Friedman and Johnson, 1988; Morris et al., 1996). Additional components of the IIS pathway have also been shown to play a role in the regulation of ageing in *C. elegans*; a loss-of-function mutation in *daf-2*, encoding an insulin/IGF

receptor homologue, resulted in more than doubling of lifespan (Kenyon et al., 1993). Lifespan extension with both *daf-2* and *age-1* downregulation requires *daf-16*, the *C. elegans* homologue of FOXO (Kenyon et al., 1993). In accord with this finding, upregulating *dfoxo* in *Drosophila* adipose tissue extends lifespan (Giannakou et al., 2004; Hwangbo et al., 2004; Tatar et al., 2003), and flies with overexpression of a dominant negative form of the insulin receptor also have increased lifespans - as with *C. elegans*, this lifespan extension is dependent on dFOXO (Slack et al., 2011). The requirement of FOXO for lifespan extension upon reduction of IIS suggests that FOXO plays an important role in mediating the physiological changes that give rise to extended lifespan. Indeed, the multitude of roles attributed to FOXO fall in line with a change in physiology conducive to a lower rate of cellular senescence (Eijkelenboom and Burgering, 2013). For example, FOXO upregulation results in increased resistance to oxidative stress, induction of apoptosis, cell cycle inhibition and a metabolic shift towards catabolism.

Mouse models suggest a conserved effect of IIS downregulation to extend life; fat-specific insulin receptor knockout mice (FIRKO) (Bluher et al., 2001), mice with heterozygous knockout of the insulin growth factor-1 receptor (IGF-1R) (Holzenberger et al., 2003; Kappeler et al., 2008), mice lacking the insulin receptor substrate 1 (IRS-1) (Selman et al., 2008, 2011) and mice with brain-specific IRS-2 knockdown (Taguchi et al., 2007) all had enhanced longevity. Moreover, hepatic deletion of *Foxo1* in mice rescued the adverse effects of IIS disruption by Akt1 and Akt2 deletion in the liver, such as glucose intolerance and insulin resistance (Lu et al., 2012). In humans, variants of the FoxO3A locus are correlated with longevity (Willcox et al., 2008a).

Animal models of IIS pathway component mutants have been used to study the role of IIS in mediating the lifespan-extending effects of DR. In *Drosophila*, loss of *chico*, deletion of *dilps 2*, *3* and *5*, overexpression of a dominant negative form of the insulin receptor, and fat-body-specific *dfoxo* overexpression – all of which downregulate IIS – resulted in a shift of the lifespan peak to a higher food concentration than that for wildtype flies (Clancy et al., 2002; Giannakou et al., 2008; Grandison et al., 2009; Grönke et al., 2010). These observations suggest that reduced IIS induces a DR-like state at higher food-intake levels, implicating IIS as having a role in regulating, at least partially, the effects of dietary restriction on lifespan. Moreover, flies that have defects in an odorant receptor have extended lifespans, and exposure of flies to odorants partially reverses the lifespan-

extending effects of DR (Libert et al., 2007). In humans, the sight or smell of food alone can induce increased insulin secretion, known as cephalic phase insulin secretion (Johnson and Wildman, 1983; Sjostrom et al., 1980).

Reduced IIS is proposed to extend lifespan by the upregulation of processes that shift physiology towards somatic maintenance, which originally evolved as an adaptive survival response to harsh environmental conditions, but also has the potential to counter somatic assaults that cause cellular senescence and accelerates ageing. When starved, *C. elegans* L1-L2 larvae have the ability to enter a form of diapause called dauer, during which development is arrested and various physiological changes ensue; their old cuticle is replaced with a more impermeable cuticle and they cease to feed as a result of pharyngeal muscle inactivity. Interestingly, they retain a normal level of motility for the first 2-3 weeks, and develop heightened resistance to harsh chemicals. The dauer state can last up to 70 days, and upon refeeding larvae exit the dauer stage and resume their moulting cycle to adulthood (Klass, 1977). Extraordinarily, there is no effect of the duration of the dauer state on post-dauer lifespan or fecundity. Because the dauer stage is induced by nutrient starvation, it has been hypothesised that the molecular mechanisms that give rise to the increased longevity of dauer worms may also underlie lifespan extension under DR. Several genes control dauer formation in *C. elegans*, collectively called *daf* (*da*uer *f*ormation) genes, and some of these encode constituents of the IIS pathway; namely, *daf-2* and *daf-16* (Kenyon et al., 1993; Kimura et al., 1997). It is therefore possible that IIS may be responsible, to some degree, for the lifespan difference between dauer and non-dauer *C. elegans*. Adult *Drosophila* also exhibit a form of diapause, affecting reproductive development; in response to low temperatures coupled with a short-day photoperiod, female flies have reduced vitellogenesis (Saunders et al., 1989). Ovarian diapause in flies is subject to control by the juvenile hormone (JH). There is evidence for cross-talk between JH signalling and IIS in *Drosophila* (Jindra et al., 2013); long-lived insulin receptor mutants have reduced JH levels. Lifespan was shortened and vitellogenesis was restored upon administration of methoprene, a JH analogue (Tatar et al., 2001). It was subsequently discovered that the insulin receptor is required for JH biosynthesis (Belgacem and Martin, 2007). *Drosophila* and *C. elegans* diapause seem to have some parallels at the molecular level, which could prove to be interesting for ageing studies given that the involvement of IIS is implicated in both processes, as well as in the regulation of ageing - there may be some overlap between these pathways.

1.3.3. The general amino acid control (GAAC) pathway

The GAAC pathway primarily responds to environmental nutrient starvation, and functions to decrease protein synthesis and to enhance cellular amino acid uptake and biosynthesis (Dever and Hinnebusch, 2005). The activity of the GAAC pathway changes in response to direct nutritional cues, and affects pathways involved in metabolism and biosynthesis. It is thus considered to be a nutrient sensing/signalling pathway. The GAAC pathway was originally described in the yeast *Saccharomyces cerevisiae* (Roussou et al., 1988; Wek et al., 1989; Hao et al., 2005) and has since been found to be conserved in mammals (Sood et al., 2000). The central component of the GAAC pathway is GCN2, a serine/threonine-protein kinase. Upon stimulation by uncharged transfer ribonucleic acids (tRNA), which accumulate during amino acid starvation, GCN2 phosphorylates and inactivates the eukaryotic initiation factor 2α (eIF2α), resulting in a decrease in global protein synthesis, whilst the translation of selected mRNAs coding for proteins involved in amino acid biosynthesis is allowed. In this way, resources are efficiently conserved, and cell division is limited under inadequate growth conditions (Figure 1.7).

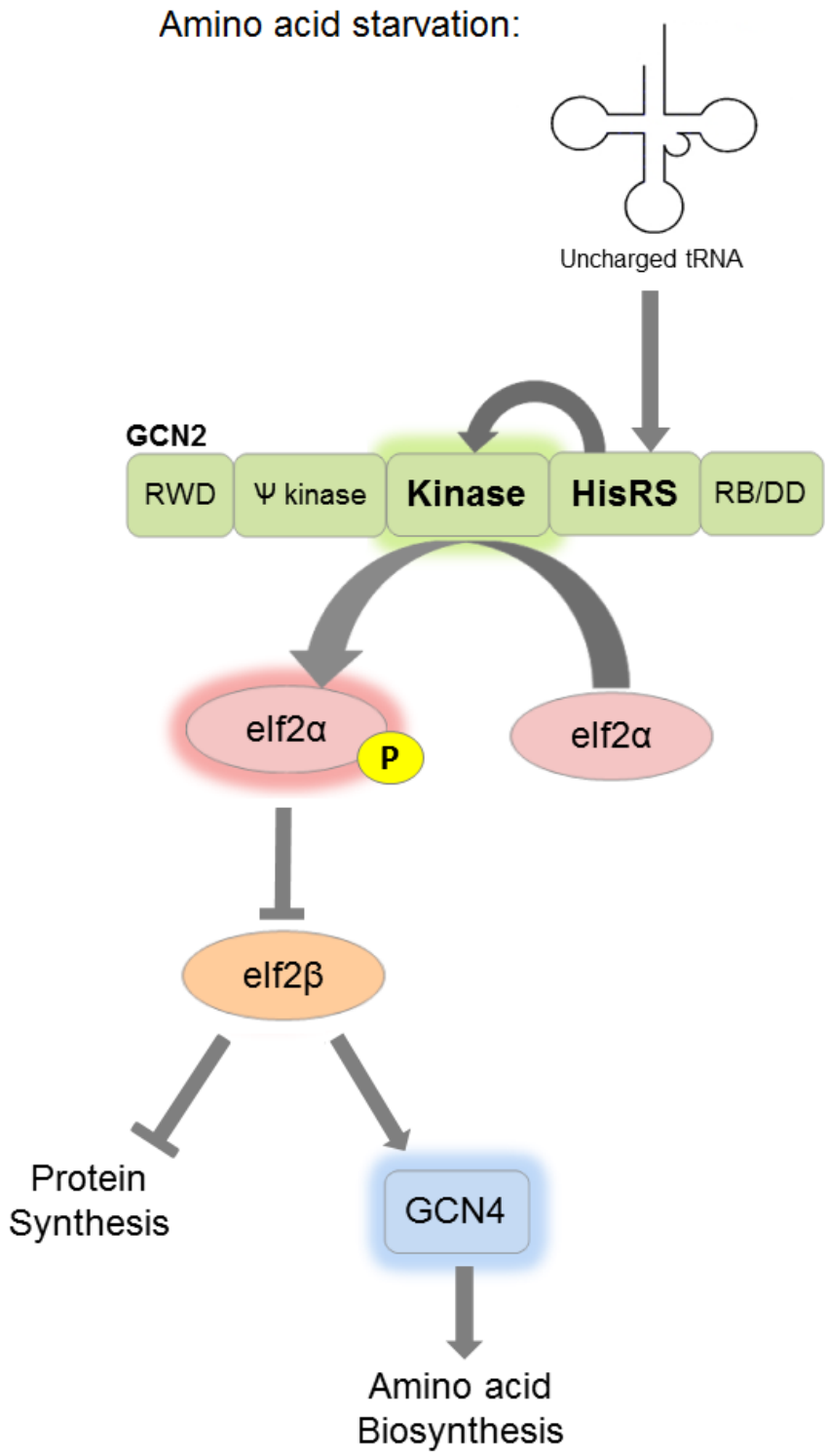


Figure 1.7. The GAAC pathway in yeast.

Amino acid starvation results in the accumulation of uncharged tRNAs, which bind to the HisRS-related domain of GCN2, resulting in the activation of the adjacent kinase domain. The kinase in turn phosphorylates the α-subunit of eIF2 on serine 51, thereby inhibiting eIF2β and blocking protein synthesis. The inhibition of eIF2β leads to the translation of *GCN4* mRNA, which encodes a transcription factor that activates transcription of genes encoding enzymes involved in amino acid biosynthesis.

GAAC mechanism

GCN2 is a multi-domain protein; at its carboxy-terminus, juxtaposed to a kinase catalytic moiety, is a region that is homologous to the entire sequence of histidyl-tRNA synthetase (HisRS) (Wek et al., 1989). Based on the fact that aminoacyl-tRNA synthetases bind uncharged tRNAs, it was proposed that the HisRS-like region of GCN2 also binds uncharged tRNAs. In yeast, mutations in the HisRS-related domain of *GCN2* prevented the binding of uncharged tRNAs and impaired eIF2α phosphorylation (Wek et al., 1995). GCN2-phosphorylation of the α subunit of eIF2 *in vivo* was stimulated by histidine starvation (Dever et al., 1992), and mutating the gene encoding histidyl tRNA synthetase was found to induce GCN2 phosphorylation of eIF2α, even in the presence of histidine (Wek et al., 1995). In fact, GCN2 binds several de-aminoacylated tRNAs with similar affinities and limitation of several amino acids can activate the GAAC (Dong et al., 2000; Wek et al., 1995), making it a sensor of general amino acid limitation. The binding of uncharged tRNAs to the HisRS-like region of GCN2 induces a conformational change of the entire GCN2 protein that disrupts its autoinhibitive conformation (Dey et al., 2007). As a result, the kinase domain is activated, permitting the inhibitive phosphorylation of eIF2α at Serine 51 (Dever et al., 1992). Other domains of the GCN2 protein include a ribosome binding and dimerisation domain at the C-terminal domain (Qiu et al., 1998), a RWD domain at the N-terminus proposed to have a function in protein interaction (Kubota et al., 2000; Sattlegger et al., 2004) and a pseudo kinase domain (ψK) with unknown function.

During translation initiation, an eIF2-GTP-$tRNA^{met}$ ternary complex is formed which facilitates the binding of the initiator $tRNA^{met}$ to the 40S ribosomal subunit to form the 43S preinitiation complex. $tRNA^{met}$ is transferred to the ribosome in a GTP-dependant manner and binds to the mRNA at the capped 5'end, forming the 48S complex (Hershey, 1991). This complex scans the mRNA until it encounters an AUG start codon. Upon completion of initiation, The GTP is hydrolysed to GDP and the GDP-bound eIF2 is released from the ribosome. eIF2 must exchange its GDP for GTP, via its β-subunit which acts as a guanine nucleotide exchange factor, before it can engage in another round of translation initiation. Phosphorylation of eIF2α impairs this rate of GDP-GTP exchange, thereby inhibiting protein synthesis. In mammals, eIF2α is a target for four kinases – GCN2, PERK, PKR and HRI – all of which are regulated by different stimuli; amino acid deprivation, ER stress, double stranded RNA and heme deficiency, respectively (Figure 1.8).

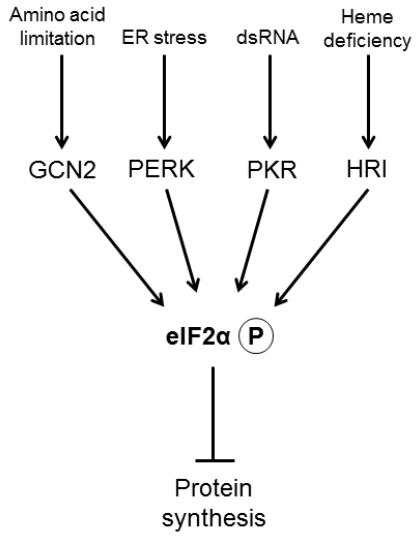


Figure 1.8. Regulation of eIF2α phosphorylation in mammals.

Phosphorylation of eIF2α is regulated by four known protein kinases that are activated by different cellular stresses. Phosphorylation of eIF2α inhibits mRNA translation and in turn protein synthesis. Figure adapted from Kimball and Jefferson, 2004.

Another downstream effect of GCN2 phosphorylation of eIF2α in yeast is the activation of the transcription factor GCN4. Paradoxical to the effect of eIF2α phosphorylation to inhibit general protein synthesis, translation of *GCN4* activates more than 30 genes involved in amino acid biosynthesis and also genes encoding aminoacyl-tRNA synthetases (Dever, 2002). *GCN4* translation is controlled by four short upstream open reading frames (uORFs) in the leader strand of *GCN4* mRNA, which is dependent on both GCN2 and eIF2α phosphorylation. When amino acids are in abundance, the uORFs restrict the migration of ribosomes downstream towards the start codon of *GCN4*, thereby hindering the initiation of translation of *GCN4* mRNA. When amino acid supply is limited and the levels of ternary complex are low, the inhibitory effect of the uORFs is overcome and the scanning ribosome is able to bypass the start codons of the uORFs in the *GCN4* mRNA and initiate translation at the authentic start codon of *GCN4* (Abastado and Miller, 1991; Dever et al., 1992). *ATF4* encodes a transcription factor and is the mammalian counterpart of yeast *GCN4*. It, too, is subject to control by uORFs in its mRNA leader, and is activated by eIF2α phosphorylation. ATF4 upregulates amino acid biosynthesis and activates genes encoding amino acid transporters, such as SLC7A5, so as to enhance cellular amino acid uptake (Chen et al., 2014; Harding et al., 2000, 2003; Malmberg and Adams, 2008; Vattem and Wek, 2004). As well as inducing *GCN4*/*ATF4* translation, amino acid starvation also increases the levels of their mRNA.

The role of GAAC in amino acid sensing

The GAAC regulatory response allows cells to conserve their amino acid stores and channel resources into amino acid biosynthesis during environmental nutrient limitation. Not only is this regulation in effect at the cellular level, but there is evidence that this signal transduction pathway culminates in some behavioural manifestation that induces an aversive response to an imbalanced food source, thereby encouraging the organism to seek an alternative nutritional environment that can better support reproduction and survival. Yeast, which have the ability to synthesise all 20 amino acids, regulate metabolism through the GAAC, producing an adaptive response to nutrient availability (Niederberger et al., 1981). In contrast, mammals can only produce a subset of amino acids, and must obtain the others from the environment. It has been known that animals have an innate ability to detect dietary imbalances; rats, for example, are able to sense and decisively reject diets deficient in a single amino acid, without the influence of peripheral sensations such as taste (Markison et al., 1999) and smell (Leung et al., 1972), implicating some internal mechanism that senses a dietary imbalance and communicates it to neural regulators of feeding behaviour. Because GCN2 activity depends on the level of uncharged tRNAs, which is a direct indicator of amino acid supply, it was hypothesised that GCN2 may play a role in coupling environmental nutrient availability to foraging behaviour. Indeed, increased eIF2α phosphorylation on serine 51 was reported in the brains of rats fed a diet lacking threonine (Gietzen et al., 2004), and *GCN2* null mice are unable to avoid imbalanced diets when starved of essential amino acids. It is possible that ATF4 links eIF2α phosphorylation to downstream effectors that control feeding behaviour (further discussed in chapter 5).

Interaction between GAAC and other nutrient sensing pathways

There is some indication of coordination between the GAAC pathway and other NSPs, such as TOR and IIS. The TOR inhibitor, rapamycin, has been shown to increase eIF2α phosphorylation in a GCN2 dependent manner; a phosphorylation site at Serine 577, located between the kinase and the pseudo kinase domains of GCN2, is the target of an unknown kinase (Garcia-Barrio et al., 2002). Rapamycin inhibits phosphorylation at this Ser-577 site (Cherkasova and Hinnebusch, 2003), thereby activating GCN2 and increasing its affinity for uncharged tRNAs *in vitro* (Kubota et al., 2003). Mutation of this site resulted in the constitutive activation of GCN2, even in low levels of uncharged tRNAs, but also impaired the effect of rapamycin to increase eIF2α phosphorylation. Thus,

downregulation of the TOR pathway, which primarily reduces protein synthesis through decreased S6K1 phosphorylation and increased 4E-BP1 phosphorylation, also decreases protein synthesis through activation of GCN2. Moreover, TOR-mediated upregulation of autophagy during starvation was found to be dependent on ATF4 (B'chir et al., 2013; Rouschop et al., 2010; Rzymski et al., 2010); knockdown of ATF4 inhibited TOR activation upon nutrient stimulation and resulted in high levels of autophagy (Chen et al., 2014).

The GAAC pathway also interacts with IIS; in mice, leucine deprivation increased insulin sensitivity by activating hepatic *GCN2*, as well as decreasing TOR pathway activity (Xiao et al., 2011). In fact, this study placed GCN2 as an upstream negative regulator of TOR, since *GCN2* null mice did not have reduced TOR signalling in the liver under leucine- or valine-deprived conditions. Moreover, insulin increases the levels of *ATF4* mRNA and its translation, implicating a role for ATF4 in regulating the anabolic effects of insulin (Malmberg and Adams, 2008). Accordingly, *ATF4* null mice exhibit growth defects (Hettmann et al., 2000; Masuoka and Townes, 2002; Tanaka et al., 1998). Interestingly, insulin-mediated regulation of ATF4 is not dependent on GCN2, and in fact the effects of the GAAC and the IIS pathways are additive, suggesting that these pathways work in parallel to regulate amino acid synthesis. It has been suggested that signalling pathways that offer an additional level of hormonal control over metabolism, like IIS, evolved after the ancient cell-autonomous regulatory systems, like GAAC, of unicellular organisms (Malmberg and Adams, 2008).

There is abundant evidence implicating TOR and IIS as having vital roles in modulating lifespan in a wide range of organisms, and the interaction of GAAC with these pathways makes it an additional interesting molecular target for studies into longevity. Coupled with the fact that it has an important role as an amino acid sensor, it is likely that the GAAC pathway is an important member of the nutrient sensing web that coordinates the regulation of metabolic processes that influence lifespan.

1.4. Aims and Objectives

As previously discussed, the effect of DR to extend lifespan has been robustly observed for a range of taxonomically diverse animal species. While there is a broad literature describing the contributions of the NSPs to regulating the DR effect on longevity, these data can sometimes be contradictory or inconclusive. Moreover, although it is clear that the NSPs interact with each other within a complex feedback web, the exact nature of the links between the NSPs have yet to be defined, highlighting the complexity of the biology underlying the relationship between nutrition and lifespan. Little is also known about the specific effector nutrients that govern the DR effect on longevity, although recent work has begun to highlight the importance of dietary amino acid balance, i.e. the relative abundance of individual amino acids to each other, as well as collectively in relation to other dietary components.

The main aim of this PhD project was to elucidate the molecular mechanisms by which DR confers benefits to lifespan and health. Because the nature of nutrient sensing is so broad and complex, we sought an approach that could narrow the focus of this task. Previously, DR has been experimentally studied as a dilution of the entire diet, and only relatively recently is the importance of more specific dietary effects being realised. We reasoned that identifying the specific dietary components responsible for the DR effect on lifespan could concentrate our investigations on more targeted experimental questions. We were particularly interested in the influence of the amino acids, since they have been previously shown to be an important dietary regulator of the lifespan response to DR (Grandison et al., 2009), therefore we have limited our dietary manipulations mainly to the protein/amino acid component of the diet. We have focused on the evolutionarily conserved TOR, IIS and GAAC nutrient signalling pathways, since these are most likely to bear relevance to human health and have previously been implicated in regulating health and longevity in laboratory animals. The hypothesis that I have been investigating is that nutrient signalling pathways mediate the lifespan-extending effects of DR in flies. Here, we define DR by the balance of amino acids in the diet relative to other dietary components. This specific definition of DR has not yet been experimentally explored in flies.

To address this hypothesis, I used *Drosophila* as an established biogerontological model to study ageing in a living system. We used genetic interventions to alter the activity of nutrient sensing pathways in order to assess the effect on fly physiology and to correlate this to any roles that these nutrient sensing pathways might have in mediating the effect of DR on longevity. We also took advantage of a recently-developed holidic fly food medium (Piper et al., 2014), where the amount of each dietary component is defined and can be manipulated at the single nutrient level, to assess the effect of specific nutrients on fly physiology and lifespan. Combining these two interventions (genetic and dietary) enabled us to identify interaction effects between diet and nutrient sensing, and to characterise the nature of this interaction.

In parallel to lifespan measurements, we characterised various aspects of fly physiology in response to our interventions in order to draw correlations between lifespan and physiology. This allowed us to build a physiological profile characteristic of long-lived versus short-lived flies, and ask whether these profiles were shared by all long-lived models. Any differences could provide clues as to the unique causation factors by which a particular intervention conferred changes to fly lifespan. Using this unique approach, we identified fly triacylglycerides (TAG) levels as being correlated with TOR-mediated DR dependent lifespan change, where elevated levels of TAG was associated with a longer lifespan under DR.

We also investigated the hypothesis that animals regulate their food intake in order to achieve a diet optimal for various aspects of physiology, including reproduction and somatic maintenance. This hypothesis was based on published work from rodent models that show that mice and rats readily reject a nutrient-deficient diet, particularly amino acid deficiency, with evidence that this is at least partly regulated by the GAAC pathway. We further characterised this phenomenon in flies, establishing support for a role of the GAAC pathway in regulating the feeding response to single amino acid deficiency, where methionine deficiency was highlighted as being of particular interest in this context. We also characterised the effect of genetically downregulating the GAAC pathway in flies in the context of lifespan and physiology, of which little was known, and which revealed further support for a role for GAAC in mediating dietary methionine signals.

Chapter 2 - Materials and methods

2.1. *Drosophila melanogaster* stocks

2.1.1. Wildtype flies

Our wildtype stock, Dahomey, was originally collected in 1970 from Dahomey (now known as the Republic of Benin, Africa) and since maintained outbred in large population cages (measuring 45x25x25cm) in the Partridge laboratory at 25^{o}C in a non-humidified controlled-temperature room, on a 12h light:12h dark cycle. Each population cage contains 8 bottles of 1x SY food (see chapter 2.2), 3 of the oldest of which were replaced with 3 bottles of fresh food routinely every 7 days. These conditions allow for inter-generational breeding and the life expectancy of flies remain similar to that of newly caught wild flies (Sgro et al., 2001).

The w^{Dah} stock, used as the controls in this project, were generated by backcrossing the mutated *white* gene (*w*-) from stock w^{1118} into a wildtype Dahomey background (Broughton et al., 2010), thereby producing a stock of Dahomey with white eyes. This white-eyed Dahomey background made it possible to follow transgenes marked with a *w+* (wildtype) eye colour. The w^{Dah} stocks were maintained in the same way as described for Dahomey above.

2.1.2. Transgenic flies

In *Drosophila* research, genetic markers are commonly used as a means of detecting the presence of a transgene in a fly stock and its inheritance through subsequent generations. The gene of interest is closely linked to a marker gene, whose expression is identifiable in transformed flies. The transgenes used in this project were linked with a *mini-white* marker gene (a truncated version of the *white* gene). One copy of the transgene partially rescues the *white* mutation, producing yellow/orange eyes. A more complete rescue with two copies produces darker, red eyes in a white mutant background. This allows homozygous flies to be distinguished from heterozygous flies for a particular insertion (Klemenz et al., 1987).

dilp2-3,5 null flies:

Insulin/IGF signalling mutant flies lacking three of the *Drosophila* insulin-like peptides (DILPs), *dilp*2, *dilp*3 and *dilp*5 were generated as described in Grönke et al. (2010).

UAS-*dRagA* transgenic flies:

Mutant flies with alterations of the upstream dTOR signalling component, dRagA, were generated as described in Kim et al. (2008), and supplied by T. P. Neufeld. The three different *dRagA* transgenic flies used in this work are described in Table 2.1.

Table 2.1 Description of the molecular effects of the UAS-*dRagA* transgenes used: UAS-*dRagA*^{Q61L}, UAS-*dRagA*^{T16N}, UAS-*dRagA*^{WT} (Kim et al., 2008)**.**

Transgene name	Activity	Description
UAS-*dRagA*^{Q61L}(CA)	Constitutively active	*dRagA* gene contains a mutation on the catalytic residue Q61, which is responsible for stabilising the transition state for GTP hydrolysis. The result is that GTP hydrolysis is prevented and the gene is locked in an active state.
UAS-*dRagA*^{T16N}(DN)	Dominant negative	*dRagA* gene product is altered so that it binds less GTP, rendering it inactive. It decreases normal dRag function by competing with the corresponding wildtype protein for dimerization with wildtype dRagC.
UAS-*dRagA*^{WT}	Wildtype	Overexpression of the wildtype *dRagA* gene.

dGCN2 null flies:

In collaboration with Sebastian Grönke (Max Planck Institute for the Biology of Ageing, Cologne), we generated a *Drosophila* mutant with a knockout of the entire open reading frame (ORF) of the *dGCN2* gene by homologous recombination (unpublished; Figure 2.1a). The absence of the *dGCN2* gene in the mutants was confirmed by PCR analysis targeting distinct regions of the ORF (Figure 2.1b), and western blot analysis probing for reduced phosphorylation of the *dGCN2* target, eIf2α (Figure 2.1c).

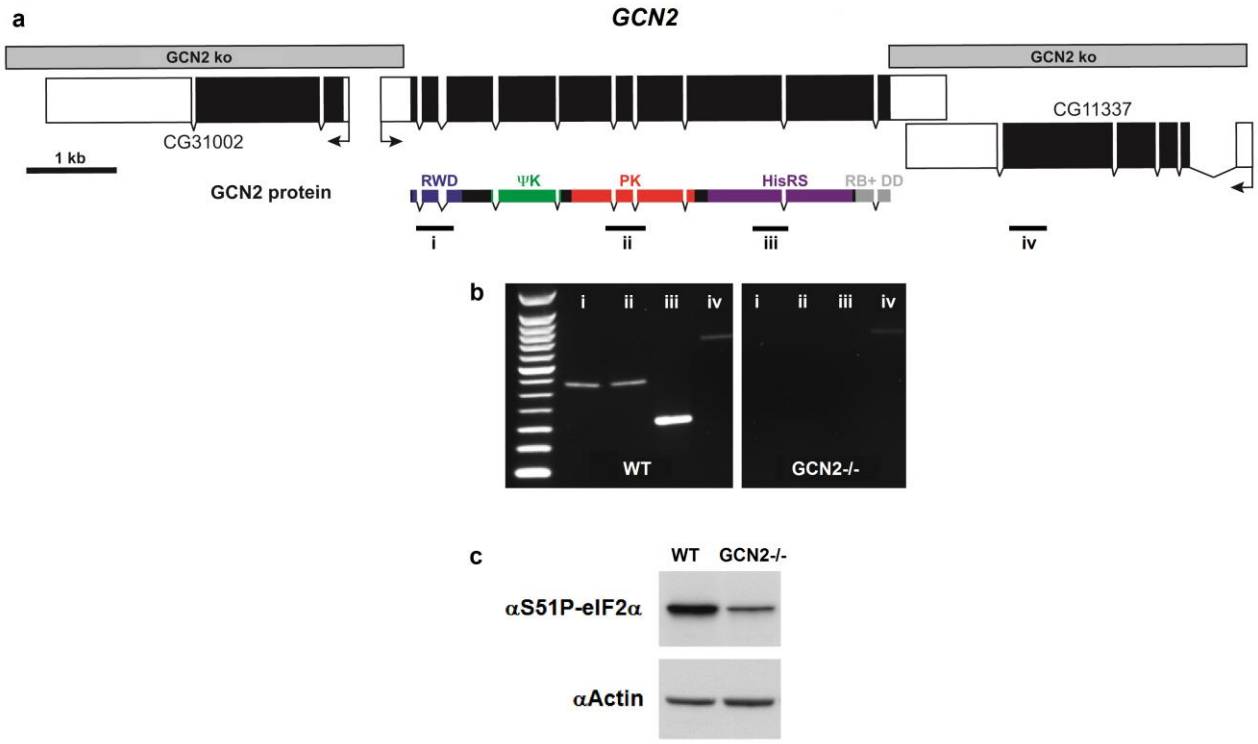


Figure 2.1. Generation of *dGCN2* null flies.

(a) The *dGCN2* transgene used in our experiments is a knockout of the entire open reading frame (ORF) of the *dGCN2* gene, including the protein interaction domain (RWD), the pseudo kinase (ψK) domain, the protein kinase (PK) domain, the Histidyl tRNA synthetase (HisRS) tRNA binding domain, and the ribosome binding (RB)/dimerization domain (DD) (functions of these gene regions are reviewed in chapter 1.3.3). Flies were generated by homologous recombination (unpublished, supplied by S. Grönke). **(b)** PCR analysis of genomic DNA, confirming the absence of the *dGCN2* ORF in *dGCN2* null flies; lanes i-iv represent amplification target regions of dGCN2, representing the RWD, the PK, the HisRS, and a region outside of the ORF, respectively. **(c)** eIF2α phosphorylation is reduced in *dGCN2* null flies compared to wildtype flies, as measured by western blot analysis. α-Actin levels were measured as a loading control. Samples were prepared from fly head extracts.

2.2. *Drosophila* food media

2.2.1. Standard laboratory food

Our standard laboratory media is composed mainly of sugar, yeast and agar and henceforth referred to as 1x SY. This medium was used for the development and maintenance of all flies used, unless otherwise stated. 1 litre of 1x SY was prepared according to the recipe and method in Bass *et al*. (2007): 15g agar (Sigma-Aldrich, Dorset, UK) in 700ml distilled water was heated to boiling while stirring, at which point 100g of autolysed yeast powder (1x; MP Biomedicals, Solon, OH, USA) and 50g of sugar (Tate & Lyle, London, UK) was added. This was thoroughly mixed using a whisk until returning to the boil, at which point the heat was turned off and an additional 170ml water was added. This mixture was left to cool to below 60^{o}C before adding 30ml of nipagin (100g/l in 95% ethanol (EtOH) (nipagin supplied by Chemlink Specialities, Manchester, UK; EtOH supplied by Sigma-Aldrich, Dorset, UK) and 3ml propionic acid (Sigma-Aldrich, Dorset, UK), which act as anti-microbial agents. The mixture was then dispensed into glass vials or bottles, in volumes of ~4ml or 60ml respectively, and allowed to set at room temperature overnight before being stored at 4^{o}C. Details of any variations of this basic recipe will be given within the relevant chapters.

2.2.2. Specialised SY food

Essential amino acid supplementation (chapter 3):

In order to test the effects of supplementing a DR diet with only the 10 essential amino acids, we used the standard 1x SY food (representing the DR diet) and added to it a solution containing the 10 essential amino acids. The essential amino acids were dissolved in MiliQ water to the concentrations shown in Table 2.2. Per litre of 1x SY food, 50ml of this stock solution was added upon cooling of the SY food to below 60^{o}C. As a control measure, MiliQ water was added to the food conditions within the same experiment that did not contain essential amino acids.

Table 2.2. Stock solution of essential amino acids for yeast food media.

Essential amino acids are dissolved in MiliQ water, pH adjusted to 4.5 with hydrochloric acid, and sterilised by passing through a 45µm filter.

Essential amino acid	Stock concentration (mol/L)
L-arginine	0.041
L-histidine	0.027
L-isoleucine	0.052
L-leucine	0.073
L-lysine	0.057
L-methionine	0.013
L-phenylalanine	0.031
L-threonine	0.062
L-tryptophan	0.009
L-valine	0.068

Rapamycin (chapters 3 & 4):

We used rapamycin as a pharmacological inhibitor of the target of rapamycin (TOR) pathway to test its effects on essential amino acid-supplemented flies. Rapamycin (LC Laboratories, MA, USA) was dissolved in ethanol and added to essential amino acid-supplemented 1x SY food (see above), to a final concentration of 200µM. As a control measure, ethanol alone was added to the food conditions within the same experiment that did not contain rapamycin.

RU486 (chapter 3):

The drug Mifepristone (RU486; Sigma-Aldrich, Dorset, UK), required for *dRagA* transgene expression, was dissolved in ethanol and added to 1x SY food at a final concentration of 200µM. As a control measure, ethanol alone was added to the food conditions within the same experiment that did not contain RU486.

Yeast:sugar manipulations (chapter 4):

We altered the amounts of yeast against sugar in the SY medium to test the relative effects of altering the protein and carbohydrate supply to the flies. Table 2.3 shows how the amounts of yeast, sugar and water in the standard SY medium (1x yeast : 1x sugar) were

altered to produce food conditions varying in yeast and sugar concentration combinations. The 'final volume' value refers to the volume of water added at the final stage of the SY preparation.

Table 2.3. Amounts of yeast, sugar and water required for SY food conditions varying in yeast:sugar concentration combinations.

		Yeast concentration		
		0.5x	**1x**	**2x**
Sugar concentration	**1x**	Yeast: 50g/l Sugar: 50g/l Final water: 196ml/l	Yeast: 100g/l Sugar: 50g/l Final water: 170ml/l	Yeast: 200g/l Sugar: 50g/l Final water: 118ml/l
	2x	Yeast: 50g/l Sugar: 100g/l Final water: 170ml/l	Yeast: 100g/l Sugar: 100g/l Final water: 144ml/l	Yeast: 200g/l Sugar: 100g/l Final water: 92ml/l
	4x	Yeast: 50g/l Sugar: 200g/l Final water: 118ml/l	Yeast: 100g/l Sugar: 200g/l Final water: 92ml/l	Yeast: 200g/l Sugar: 200g/l Final water: 40ml/l

2.2.3. Defined medium

In order to test physiological and behavioural responses of flies to foods lacking specific components of the diet, we used a chemically defined and tractable medium (Piper et al., 2014). This holidic medium is agar-based and contains all the necessary dietary components required to support *Drosophila* development, growth and fecundity. The medium allowed us to readily omit specific dietary components of interest. Below we describe the preparation of 1 litre of the standard nutritionally-complete medium; details of any variations of this basic recipe will be given within the relevant chapters.

In the first step of the preparation of the medium, the nutritional components shown in Table 2.4 were combined and made up to 799.4ml with MiliQ water. All components were supplied by Sigma-Aldrich (Dorset, UK), except for acetic acid, which was supplied by Fisher Scientific (Surrey, UK). Stock solutions were sterilised by passing through a 45µm filter.

Table 2.4. Components of defined medium combined and made up to 799.4ml MiliQ water before the autoclaving step of the preparation (to make 1L medium).

Nutritional component	Preparation of stock	Quantity in 799.4ml MiliQ water	Order of addition
Sucrose		17.12g	1
Acetate buffer	Acetic acid (30g/L), KH_2PO_4 (30g/L) and $NaHCO_3$ (10g/L) in MiliQ water	100ml	1
Tyrosine		0.84g	1
$CaCl_2$	250g/Lin MiliQ water	1ml	2
$MgSO_4$	250g/Lin MiliQ water	1ml	2
$CuSO_4$	2.5g/Lin MiliQ water	1ml	2
$FeSO_4$	25g/L in MiliQ water	1ml	2
$MnCl_2$	1g/L in MiliQ water	1ml	2
$ZnSO_4$	25g/L in MiliQ water	1ml	2
Agar		20g	3
Cholesterol	0.02g/ml in EtOH	15ml	3

This solution was autoclaved for 15 minutes at 121^{o}C, and then allowed to cool to below 60^{o}C. Table 2.5, Table 2.6 and Table 2.7 show the nutritional components added to the defined medium after autoclaving, and after the solution was allowed to cool to 60 ^{o}C. The solution was continuously mixed using a magnetic stirrer. All components were supplied by Sigma-Aldrich (Dorset, UK), except for nipagin which was supplied by Chemlink Specialities (Manchester, UK).

Table 2.5. Components of defined medium added after the autoclaving step of the preparation, once the solution cooled to below 60OC (to make 1L medium).

Nutritional component	Preparation of stock	Quantity of stock in 1L defined medium	Order of addition
Nucleic acid/lipid solution	choline chloride (6.25 g/L), myo-inositol (0.63g/L), inosine (8.13g/L), and uridine (7.5g/L)	8ml	1
Vitamin solution	thiamine (0.1g/L), riboflavin (0.05g/L), nicotinic acid (0.6g/L), Ca pantothenate (0.775g/L), pyridoxine (0.125g/L) and biotin (0.01g/L)	14ml	1
Folic acid	0.5g/L in MiliQ water	1ml	1
Non-essential amino acids	(See Table 2.6)	60.51ml	1
L-glutamate	0.1g/ml in MiliQ	18.21ml	1
L-cysteine	0.05g/ml in MiliQ	5.28ml	1
Essential amino acids	(See Table 2.7)	90.77ml	1
Nipagin	100g/L in 95% EtOH	15ml	2
Propionic acid		6ml	2

Table 2.6. Stock solution of non-essential amino acids for defined medium.

Essential amino acids are dissolved in MiliQ water, pH adjusted to 4.5 with hydrochloric acid, and sterilised by passing through a 45µm filter.

Non-essential amino acid	Stock concentration (mol/L)
L-alanine	0.295
L-aspartic acid	0.080
L-glycine	0.238
L-asparagine	0.105
L-proline	0.081
L-glutamine	0.206
L-serine	0.119

Table 2.7. Stock solution of essential amino acids for defined medium.

Essential amino acids are dissolved in MiliQ water, pH adjusted to 4.5 with hydrochloric acid, and sterilised by passing through a 45µm filter.

Essential amino acid	Stock concentration (mol/L)
L-phenylalanine	0.061
L-histidine	0.048
L-isoleucine	0.097
L-lysine	0.105
L-leucine	0.138
L-methionine	0.025
L-arginine	0.074
L-threonine	0.120
L-valine	0.126
L-tryptophan	0.024

The solution was thoroughly mixed and then dispensed into sterilised (autoclaved) glass vials at a volume of 3ml per vial. After being left to set at room temperature for ~2 hours, the vials were inverted into plastic trays lined with paper towels, covered with plastic film and stored at 4^{o}C.

2.2.4. Specialised defined media

Amino acid dilution (chapter 5):

In order to test the effect on feeding of differing amounts of dietary amino acids, we used the defined medium which allowed us to specifically alter the concentration of amino acids in the diet, while keeping the amounts of other nutrients constant. Food conditions as outlined above except that the volumes of the amino acid stock solutions were diluted in MiliQ as shown in Table 2.8. In the case of food containing 1.5x amino acids, the stated amount of water (75.65ml per 1L food) was subtracted from the volume of water in the defined medium mixture before autoclaving.

Table 2.8. Volumes of essential and non-essential amino acid solutions required for defined media food conditions with varied concentrations of amino acids.

Target concentration of amino acids	Quantity of essential amino acid stock solution (ml)	Quantity of non-essential amino acid stock solution (ml)	MiliQ water (ml)
0	0	0	+151.28
0.25x	22.69	15.13	+113.46
0.5x	45.39	30.26	+75.63
1x	90.77	60.51	0
1.5x	136.16	90.77	-75.65

Amino acid group omissions (chapter 5):

We wanted to distinguish the effects on feeding of the essential amino acids from the non-essential amino acids from the total amino acid effect. Where a food condition required the absence of all the essential or all of the non-essential amino acids, or both, the stock solutions corresponding to those groups (i.e. Table 2.7 and Table 2.6, respectively) were omitted from the medium and replaced with the equivalent volume of MiliQ water to control for the reduced overall volume of the medium.

Single essential amino acid omissions (chapters 4 & 5):

The defined medium allowed us to investigate the effect on feeding, lifespan and fecundity of removing a single amino acid from the food source. Where a food condition required the absence of a particular amino acid, that amino acid was omitted from the stock solution in Table 2.7, and the defined medium recipe outlined above was followed through as normal.

2.2.5. Grape medium

An agar-based grape juice medium was used as a surface for egg collection, required for the 'egg squirt' protocol (see chapter 2.3.3). Grape plates were made by melting 25g agar in 500ml distilled water and heating to boiling, at which point 300ml red grape juice (Young's, West Midlands, UK) was added, thoroughly mixed, and brought to the boil again. The medium was removed from the heat and combined with a further 50ml of distilled water and, once left to cool to below 60^{o}C, 21ml nipagin (100g/l in 95% EtOH) was added and stirred in. The mixture was poured into plastic petri dishes, filling to half-way, and left to set at room temperature before being stored at 4^{o}C.

2.3. General methods and animal husbandry

2.3.1. Backcrossing

The backcrossing of transgenic lines is necessary to ensure that any differences seen between mutant and control flies are due to the expression of the transgene alone, and not some non-specific effect of hybrid genetic backgrounds (Partridge and Gems, 2002). For this reason, before the transgenic stocks could be used for experimentation, they were backcrossed into the w^{Dah} background. In the first cross, transgenic males were mated with w^{Dah} females to ensure that the maternally-inherited cytoplasmic and mitochondrial components in the offspring originates from w^{Dah}. From this first generation, transgenic (identified as having red or orange eye pigmentation) virgin females were collected and crossed to w^{Dah} males. From the resultant offspring of this cross, transgenic virgin females were collected and again crossed to w^{Dah} males. This type of cross was repeated a further 5 times with each generation of transgenic offspring, totalling 6 backcrossing rounds overall. This is expected to result in 98.44% of the genetic material being of w^{Dah} origin.

2.3.2. Virgin collection

It was necessary to collect unmated female flies in order to control crosses to desired male flies for setting up backcrosses and for generating experimental flies. Female *Drosophila* do not respond to copulating males during the first 12 hours after eclosion at 25^{o}C (Manning, 1967). Therefore, any emerged female flies collected within 4 hours after clearing the bottles of adults were virgins. Female virgins, which can be identified by their pale complexion, shrivelled wings, and meconium (a ventrally-visible dark spot on their abdomens, a remnant of their last larval meal), were collected under light CO_2 anaesthesia using a fine paint brush. Collected female virgins were maintained in glass vials containing 1x SY, with 20 females per vial, for at least 2 days as a check to ensure that no larvae appeared, thereby confirming that the females were unmated. Only flies from larvae-free vials were used for subsequent crosses. In the case of larvae being detected, all the females in that vial were discarded.

2.3.3. Lifespan set up and maintenance

When studying traits relating to fitness, such as lifespan and fecundity, it is important that all variables are controlled. A number of measures were taken to ensure that flies used for experimentation could be comparable between experimental groups.

To control for the effects of parental age on longevity (Priest et al., 2002), it was necessary to ensure that the parents of the experimental flies were all the same age at egg laying and that they were reared under the same conditions as each other.

To standardise the effect of larval density on longevity (Priest et al., 2002; Zwaan et al., 1991), an 'egg squirt' protocol (Clancy and Kennington, 2001) was undertaken. Flies were allowed to lay eggs for ~18 hours on grape medium plates, with a small amount of live yeast paste to increase egg laying and encourage remating. The eggs were collected from the plate by washing with phosphate buffered saline (PBS; Sigma-Aldrich, Dorset, UK) solution (in MiliQ water) and the wash collected into a screw cap 'Falcon' tube. The eggs were left to settle to the bottom of the tube and the excess supernatant was discarded. Using a 100µl Gilson pipette and a wide-bore tip, ~18µl of the PBS egg suspension was taken up and dispensed into 200ml glass bottles containing 70ml 1x SY food. This equates to a standard density of ~300 eggs per bottle (Bass et al., 2007).

After 9.5 days of development at 25^{o}C, the flies emerged from their pupae and were transferred from each of the egg-squirted bottles into bottles containing fresh 1x SY medium. Experimental adults were collected within a 12 hour period after eclosion. In order to standardise their mating status, flies were left to mate for 48 hours after eclosion. The experimental female flies were then separated from males under CO_2 anaesthesia using a fine paintbrush. These females were randomly allocated to the experimental food treatments and housed in glass vials containing the food (or disposable plastic vials when the food contained a drug, e.g. RU486 or rapamycin), at a density of 15 flies per vial. For each genotype or experimental food condition 10 vials were set up, totalling 150 flies per genotype per condition as standard. In order to minimise the effects of any variation between experimental flies emerging from different bottles, flies were sorted between vials such that those from the same bottle were distributed between all the experimental food treatment conditions.

All experiments were conducted at 25^{o}C on a 12h light:12h dark cycle, at a constant humidity of 65%. Flies were transferred to fresh vials of food three times a week throughout life. If left unchanged, the fly larvae hatching from the eggs laid by the experimental flies would burrow into the food, making it moist and posing a risk to the adult flies of becoming stuck in it. Regularly changing the food ensured that any case of death could not be attributed to reduced food quality. The number of dead flies found during each transfer was recorded, beginning from the first transfer. Accidental deaths and escapees were distinguished from deaths and were censored from the experiment.

From this data, a survivorship graph could be generated, making it possible to compare survival curves over time between different genotypes and/or food conditions. Survivorship is a measure of the probability of an individual surviving until a given age, and these data were expressed as proportion survival for each experimental group. Statistical analysis of the differences between the survivorship of experimental groups was done using the log-rank test, which uses a cumulative chi-squared statistic to assign a P-value to assess null hypotheses. Differences were regarded as significant if the P-value was less than 0.05. Cox Proportional Hazards (CoxPH) regression analysis was used to assess any interaction between the effects of one or more variables on survival.

2.3.4. Fecundity assay

Fecundity was measured as the mean number of eggs laid per female fly over a 24-hour period. ~18 hours after transferring experimental flies to fresh vials, the number of eggs laid in these new vials was counted. The counting was done by eye under a light microscope. Differences in egg-laying between conditions were analysed using a non-parametric Wilcoxon rank-sum test and a significance threshold of $P<0.05$ was used.

2.3.5. Feeding behaviour

Feeding was measured by observing fly proboscis extension onto a food source, where the probability of proboscis extension is used as a quantitative measure. Proboscis extension has been shown to be correlated with electrophysiological responses of the gustatory receptor neurons (Dahanukar et al., 2007) and is also correlated with calcium responses to

tastants (Marella et al., 2006), and is therefore a reliable measure of feeding. Experimental flies were generated in the same way as described for the lifespan assay set-up. Flies were allowed to mate for 24 hours after eclosion and were then transferred to the treatment food types for 4 days before the assay. Flies were sorted as 5 per vial under CO_2 anaesthesia at least 12 hours prior to the assay, to ensure sufficient recovery from the anaesthesia. Also during this time, flies were assembled onto observation racks and left to acclimatise overnight (25^{o}C, non-humidified). To avoid observer bias, assay vials with different food types and/or genotypes were anonymised to the experimenter and were assembled in random order. Observations were started one hour after lights on, at 11am, and this time was kept consistent for all feeding experiments in order to account for any effects of circadian rhythm. During the assay, the number of flies feeding in each vial was recorded in turn. Flies were scored as feeding if their proboscis was extended onto the surface of the food, and each vial was observed for no longer than 2 seconds. Each vial was scored in succession and the entire set counted repeatedly every ~5 minutes over a 90-minute period. The identity of each vial was decoded at the end of the observation period.

2.4. Stress Experiments

We measured flies' ability to resist a range of different stresses. The responses to various stressors can inform about the physiological status of the flies under a particular intervention, and in turn provide clues as to the underlying molecular changes.

Experimental flies were reared and housed as described for the lifespan experiment. Mated female flies were kept on the experimental food types for 7 days before being transferred to the stress conditions.

2.4.1. Heat shock

A minimum of 12 hours before the start of the assay, flies under CO_2 anaesthesia were individually assigned to a vial containing the experimental food that they had previously been maintained on. This was done to ensure sufficient recovery from the anaesthesia before the start of the assay. At the time of the assay the flies were transferred to dry empty 2ml glass vials, which were then plugged with cotton wool. The vials were placed in a rack and arranged so that each different pre-treatment condition was represented evenly. The rack was then placed inside a glass water bath in which the water temperature was maintained at 39^{o}C. The racks were positioned such that the vials were submerged in the water up until the point before the rim, so that the cotton wool plug was kept dry. The assay vials were observed by eye from the outside, and the time taken for each fly to fall onto their backs and stop twitching (knockout) was recorded.

2.4.2. Starvation

Measuring the resistance of flies to starvation can provide an insight into the status and availability of internal energy stores. Flies were transferred to vials containing plain agar medium (1.5%). Flies were assayed at a density of 10 flies per vial, and deaths were scored 3 times per day.

2.4.3. DDT stress

DDT (dichlorodiphenyltrichloroethane) induces the unfolded protein response by blocking the formation of disulfide bonds necessary for the folding of many endoplasmic reticulum (ER) proteins, and is therefore used in the laboratory to induce ER stress. Flies were transferred to vials with 1x SY medium food containing 0.03% DDT, made by adding a 2% stock solution of DDT (Supelco Sigma-Aldrich, Dorset, UK) dissolved in EtOH. Flies were assayed at a density of 10 flies per vial, and deaths were scored 3 times per day.

2.4.4. Paraquat stress

Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) induces oxidative stress in flies – it is an electron acceptor in redox and radical reactions, thereby producing destructive reactive oxygen species. Flies were transferred to vials with 1x SY medium food containing 20mM paraquat, made by addition of a 1M stock of methyl viologen dichloride hydrate (paraquat; Sigma-Aldrich, Dorset, UK) dissolved in MiliQ water. Flies were assayed at a density of 10 flies per vial, and deaths were scored 3 times per day.

2.4.5. H_2O_2 stress

Hydrogen peroxide (H_2O_2) induces an acute oxidative stress in flies. Flies were transferred to vials with 1.5% agar medium food containing 5% H_2O_2 made from a 30% stock solution of H_2O_2 (Sigma-Aldrich, Dorset, UK) and 50g/l sucrose. Flies were assayed at a density of 10 flies per vial, and deaths were scored 3 times per day.

2.4.6. Immune stress

Adult septic injury

Erwinia carotovora (strain Ecc15) were grown overnight in Luria Bertani (LB) at 29°C and adjusted to optical density (O.D.) 200. Adult flies were anesthetised with CO_2 and were pricked in the lateral thoracic epithelium, just under the wing attachment, with a thin metal needle (0.5 mm diameter) dipped in the bacterial culture. Control flies were pricked with a sterilised needle dipped in LB to ensure that the pricking itself had no effect. The flies were separated from the needle with a brush and transferred to a clean vial containing 1x SY food medium at density of 10 flies per vial. Deaths were scored daily.

Larval oral infection

Erwinia carotovora (strain Ecc15) were grown overnight in Luria Bertani (LB) at 29°C and adjusted to O.D. 200. 200µl of a bacterial pellet was then combined with 400µl of crushed banana in a 2ml microfuge tube. 20 third-instar larvae were transferred into this tube at the same time and shaken to mix thoroughly. The tube was then plugged with a small amount of cotton wool to allow an air supply and to prevent larvae from wandering out. After 30 minutes at room temperature the entire contents of the tube was emptied into a clean vial containing 1x SY food and maintained at 25°C through pupation. Eclosing flies were emptied from the vial to avoid eggs being laid by the emerging adults. After 10 days the rate of eclosion of adults was scored by observing the pupae within the vials under a light microscope. Uneclosed pupae were distinguished from empty pupal cases as having a darker colour, due to the dark-coloured fly still inside the semi-transparent pupal case.

2.5. Metabolic measurements

Experimental flies were reared and housed as described for the lifespan experiment. Mated female flies were kept on the experimental food types for 7 days before being frozen in liquid nitrogen and stored at -80 until use.

2.5.1. Triacylglycerides

To quantify triacylglyceride levels, a colorimetric assay kit (Thermo Fisher Scientific, Surrey, UK) was used; 6 replicas of 5 female flies were homogenised in 0.05% Tween 20 (Sigma-Aldrich, Dorset, UK) in MiliQ water and incubated for 5 minutes at 70ºC. The samples were then centrifuged for 5 minutes at 7000 rpm. The supernatant was transferred to fresh Eppendorfs and centrifuged for 10 minutes at maximum speed. For each sample, 175µl was transferred to a fresh Eppendorf. 10µl of each sample was dispensed into a well on a 96-well plate, with each sample in triplicate. To each well, 200µl of Thermo Infinity Triglycerides solution was added and the plate was left to incubate at 37ºC for 10 minutes, after which time absorbance in each well was measured at 574nm. The lipid standards, made from a stock solution of triglycerides (20mg/ml; Fisher Scientific, Surrey, UK), were treated in the same way as the samples. The samples were prepared as 7 serial dilutions in 0.05% Tween 20, and these were 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 µg/µl triglyceride.

2.5.2. Glycogen

10 replicas of 2 female flies were homogenised in 200µl saturated Na_2SO_4 (Fisher Scientific, Surrey, UK) solution and centrifuged for 1 minute at 10,000 rpm. 80µl of each sample was transferred to new Eppendorf tubes and 800µl chloroform:methanol (1:1) solution was added (chloroform supplied by Sigma-Aldrich, Dorset, UK; methanol supplied by VWR International, West Sussex, UK). Samples were then centrifuged for 5 minutes at 10,000 rpm. The supernatant (containing lipids) was removed and the remaining pellets, containing precipitated glycogen, were resuspended in 1ml anthrone solution (anthrone in 50ml 70% H_2SO_4; anthrone supplied by Sigma-Aldrich, Dorset, UK; H_2SO_4 supplied by Fisher Scientific, Surrey, UK). These were then incubated at 90^{o}C for 20 minutes, mixing several times to ensure dissolving of the pellet, and a colour developed during this time. The glycogen standards were treated in the same way as the samples and were prepared as 5 serial dilutions in water from a stock (2.5µg/µl MiliQ; glycogen

supplied by Sigma-Aldrich, Dorset, UK). These were 1.25µg/µl, 0.63µg/µl, 0.31µg/µl, 1.16µg/µl, 0.08µg/µl and 0µg/µl. 200µl of each sample and standard was dispensed into a 96 well plate, and the absorbance was measured at 620nm.

2.5.3. Trehalose

10 replicas of 2 female flies were homogenised in 50µl 0.2M Na_2CO_3 (Sigma-Aldrich, Dorset, UK) and incubated for 2 hours at 95ºC. The pH was adjusted to 5.2 by addition of 30µl 1M acetic acid (Sigma-Aldrich, Dorset, UK) and 120µl 0.2M Na-acetate pH 5.2 (Sigma-Aldrich, Dorset, UK). 0.05 U of trehalase (Sigma-Aldrich, Dorset, UK) was then added to each sample and left to incubate overnight at 37^{o}C. A 0-10mM dilution series of D-(+)-Trehalose (Sigma-Aldrich, Dorset, UK) in 0.2M Na_2CO_3 was made, and these standards were treated in the same way as the samples. Liberated glucose was measured using the Glucose Affinity kit (Thermo Fisher Scientific, Surrey, UK); 1µl of each sample and standard was dispensed into a 96-well plate, and to each well 150µl of Affinity reagent was added. The plate was left to incubate for 5 minutes at 37ºC, and the absorbance was measured at 340nm.

2.6. Western blot analysis

Protein extracts for western blot analysis were made from whole flies, sampled after 7 days of food treatment, using a TCA-based extraction protocol. 10µl of each sample was loaded into a 12% SDS-PAGE gel and blots were probed with anti-phospho-Thr398-S6K antibody (#9209, Cell Signaling Technologies, MA, USA), and total-S6K (re-made using a peptide sequence previously used to generate the total S6K antibody in Stewart et al., 1996 [54]). Both antibodies were used at a dilution of 1:12000 and normalised by probing with an anti-actin antibody at a dilution of 1:5000. Secondary antibodies conjugated to HRP (AbCam, Cambridge, UK) were used at a dilution of 1:5000, and the signals were detected by chemiluminescence.

Chapter 3 - The role of nutrient sensing/signalling pathways in mediating lifespan extension with dietary restriction by essential amino acid alteration

The contents of this chapter have been published in part in Emran et al., (2014) - *see appendix*.

3.1. Introduction

Dietary restriction (DR) to extend lifespan has been shown to be effective in a wide range of evolutionarily diverse organisms, and is considered one of the most robust environmental interventions to extend lifespan (Mair and Dillin, 2008). The molecular mechanisms underlying the physiological changes elicited by DR have yet to be elucidated. However, experimental data point towards nutrient sensing/signalling pathways (NSP) as playing an important role. Two such NSPs are the target of rapamycin (TOR) pathway (Hansen et al., 2007; Kaeberlein et al., 2005; Kapahi et al., 2004), and the insulin/insulin-like growth factor signalling (IIS) pathway (Apfeld and Kenyon, 1999; Clancy et al., 2002). There is evidence for significant roles played by both of these pathways in determining organismal lifespan in response to DR.

DR-mediated lifespan extension has often been correlated with a reduction in TOR complex 1 (TORC1) signalling, and genetically downregulating TORC1 has been shown to modify lifespan in a similar way to DR, suggesting that the effect of DR on lifespan may be mechanistically regulated by the TORC1 pathway. In the roundworm, *Caenorhabditis elegans*, *eat-2* mutants with pharyngeal pumping defects, which presumably eat less food, have been used as a DR model (Lakowski and Hekimi, 1998). Indeed, these mutants have been shown to have increased lifespans relative to control worms, and TOR pathway inhibition in these worms did not further extend lifespan (Chen et al., 2009), suggesting overlapping mechanisms for the action of DR and decreased TORC1 activity to increase lifespan. In the yeast *Saccharomyces cerevisiae*, glucose restriction, as a means of implementing DR, extended replicative lifespan, but did not further extend the lifespan of long-lived *tor1Δ* mutants (Kaeberlein et al., 2005). In the fruit fly *Drosophila melanogaster*, inhibition of the TORC1 pathway by overexpression of

dTsc1/2 (orthologue of mammalian *Tsc1/2)*, which are negative upstream regulators of TORC1, significantly increased lifespan (Kapahi et al., 2004). These mutant flies were not further long-lived under DR, as implemented by dietary yeast extract dilution. Also in *Drosophila*, rapamycin treatment, as a means of inhibiting dTORC1 activity, extended lifespan beyond that which could be achieved by DR, and lessened the reduction in lifespan with full feeding, suggesting that the mechanism by which DR extends lifespan and that by which decreased TORC1 pathway activity extends lifespan is overlapping (Bjedov et al., 2010). However, studies using *Drosophila* null mutants of *d4E-BP*, encoding a translational repressor downstream of dTORC1, have yielded conflicting results; one study showed a diminished extension of lifespan for these mutant flies upon DR (Zid et al., 2009), such that it suggests an overlapping mechanism between TORC1 downregulation and DR, whereas another study reported a robust DR effect on lifespan for these flies (Partridge et al., 2011), suggesting that dTORC1, or at least the d4E-BP effector branch of dTORC1, is not required for DR-dependent lifespan extension. Moreover, a recent study using mice suggests that DR- and rapamycin-induced lifespan extension may result from distinct mechanisms, given the observed differences in endocrine and metabolic profiles from mice subjected to either of these two interventions (Miller et al., 2013).

The IIS network is commonly associated with biological ageing. Mutations in components of the IIS pathway have extended lifespan in a host of model organisms (reviewed in chapter 1.3.2). Because the IIS pathway signals nutrients, and IIS is reduced by fasting (Ikeya et al., 2002), considerable effort has been made to assess the role of IIS in modulating the longevity responses to DR. In *C. elegans, eat-2* mutants that were also null for *daf-2*, the gene encoding the worm orthologue of the insulin-like growth factor 1 (IGF-1) receptor, not only retained their longevity, but were even longer lived than *eat-2* single mutants (Lakowski and Hekimi, 1998), suggesting that the lifespan benefits of the mutants were additive and independent. Likewise, an increase in lifespan with DR was achieved by both wildtype worms and those with a null mutation in *daf-16*, the gene encoding the worm orthologue of the FOXO family of transcription factors (Houthoofd et al., 2003). In *Drosophila*, *dfoxo* null flies exhibit the same lifespan response to DR as control flies (Min et al., 2008), as do *chico* heterozygote flies (Clancy et al., 2002). Moreover, flies with individual knockouts of the insulin-like ligands *dilp2*, *dilp3* or *dilp5*, which are normally expressed in brain median neurosecretory cells of the adult fly brain (Broughton et al.,

2005), exhibit a normal response to DR (Grönke et al., 2010). However, when mutations for *dilps 2, 3* and *5* were combined in the same fly the DR response was attenuated, suggesting that these three dilps are required for the lifespan response to DR, and that in the case of the single mutants there is compensation from the remaining functional dilps. While IIS does not seem to be solely accountable for DR, the published data suggest a partially common mechanism for IIS- and DR-mediated lifespan extension.

In this chapter, we explore the potential roles of the TOR and IIS pathways in mediating the *Drosophila* lifespan response to DR. We use genetic fly models and a pharmacological approach to characterise the nature of the association between NSPs and DR, specifically asking whether it is the *signalling* of nutrients through the particular nutrient sensing pathway or the end fate of those nutrients that affects lifespan.

Work from the Partridge lab has shown that adjustments to the dietary amino acid balance can mimic the benefits to lifespan by DR in *Drosophila* (Grandison et al., 2009). Supplementing a DR diet with the 10 essential amino acids (EAAs) phenocopies the lifespan and fecundity effects of a diet corresponding to high yeast levels, referred to herein as full feeding (FF), indicating that the beneficial effects of DR are a consequence of improved amino acid balance. Experimentally, the addition of the EAAs to DR (DR+EAA) offers a sharper instrument with which to dissect the potential causes of lifespan change in response to nutritional balance than the FF condition, which is achieved by increasing the concentration of dietary yeast.

Long-lived animal models often have an enhanced ability to resist environmental stresses, assumed to reflect a general increase in their health, which may also reveal something about the underlying cause of lifespan extension through increased somatic maintenance. Long-lived IIS mutant flies have been shown to be resistant to acute toxic doses of DDT, paraquat and hydrogen peroxide (H_2O_2) (Broughton et al., 2005; Grönke et al., 2010; Slack et al., 2010). Long-lived DR *C. elegans* have increased resistance to heat stress (Chen et al., 2009; Kaeberlein et al., 2006). We therefore characterised the physiological and metabolic parameters that define DR and EAA-supplemented flies, with the aim of identifying physiological changes that explain the cause of the lifespan response to DR.

3.2. Results

3.2.1. The effect of EAAs on lifespan and egg-laying

Wildtype w^{Dah} flies maintained under DR are longer-lived and lay fewer eggs than FF flies exposed to a high proportion of yeast in their diet (DR vs FF: lifespan, P<0.001, log-rank test; egg-laying, P<0.001, Wilcoxon rank-sum test; Figure 3.1a-c). Moreover, we found that the effect of full feeding to shorten lifespan and increase egg-laying can be mimicked by the addition of the 10 EAAs to DR food (FF vs DR+EAA: lifespan, P=0.94, log-rank test; egg-laying, P=0.19, Wilcoxon rank-sum test; Figure 3.1a-c), supporting the notion that the decrease in lifespan with FF can be accounted for by EAAs alone (Grandison et al., 2009).

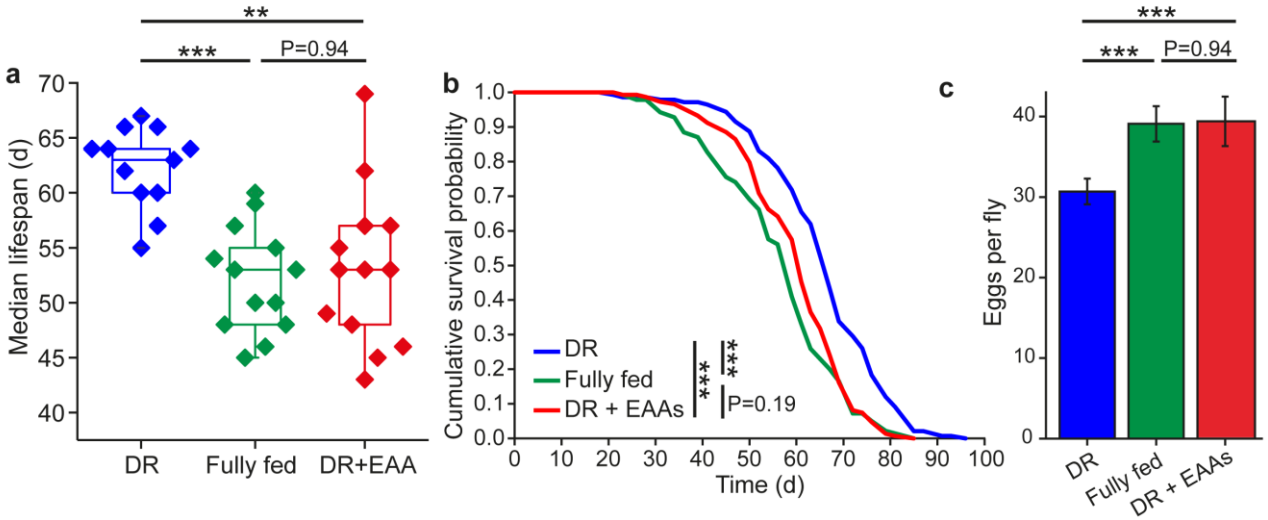


Figure 3.1. The effect of EAAs to mediate lifespan and fecundity changes under DR.
(a) Summary of *Drosophila* median lifespans under dietary restriction (DR), full feeding (FF) and essential amino acid supplementation of DR (DR+EAA) (DR vs FF, P<0.001; FF vs DR+EAA, P=0.94; DR vs DR+EAA, P<0.01; n=13 biological replicates; Wilcoxon rank-sum test) **(b)** A representative lifespan experiment: adding EAAs to DR food shortened lifespan (P<0.001) to that of FF flies (P=0.194); n=150 per treatment, compared using the log-rank test. **(c)** Adding EAAs to DR food increased egg-laying (P<0.001) to that of FF flies (P<0.936); n=15; compared using the Wilcoxon rank-sum test. Egg counts were made at 8 days of food treatment.

3.2.2. The role of NSPs in mediating the effect of EAAs on lifespan

We used genetically modified fly models for the IIS and TORC1 signalling pathways to test the effect of EAA supplementation on lifespan upon down-regulation of these longevity-associated NSPs. Dietary amino acids have been shown to stimulate the IIS pathway in *Drosophila* (Britton et al., 2002; Zhang et al., 2000). *dilp2-3,5* null flies, which are long lived due to deletion of three of the eight *Drosophila* insulin-like peptide genes, *dilp*2, *dilp*3 and *dilp*5 (Grönke et al., 2010), showed no difference from wildtype flies in their responses to the addition of EAA to DR food, indicating that although these mutations to IIS are sufficient to extend life, they are not required for the lifespan extension by DR (Figure 3.2; work done by Jelle Zandveld).

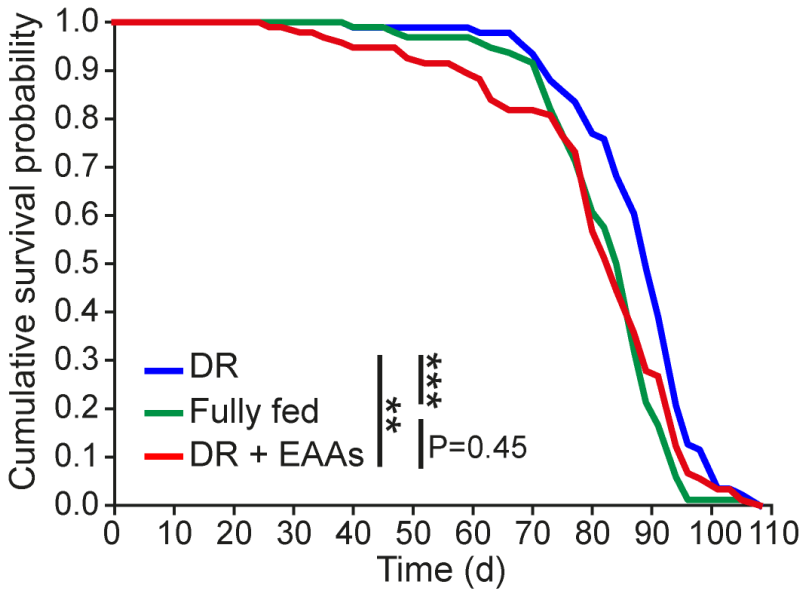


Figure 3.2. The lifespan response of *dilp2-3,5* null flies to DR.

dilp2-3,5 mutants were longer-lived under dietary restriction (DR) than when maintained on a fully fed (FF) diet or a DR diet supplemented with the essential amino acids (EAA) (DR vs FF, P<0.001; DR vs DR+EAA, P=0.007; n=100 per treatment; log-rank test). Median lifespans (days): DR=88, FF=85.5, DR+EAA=83. Data supplied by Jelle Zandveld.

To test whether the dTOR pathway might have a role in mediating the DR response to lifespan, we used flies with mutations of *dRagA*, an upstream amino acid sensing component of the dTORC1 pathway (Figure 1.4, Figure 1.5). We tested the effect of both the upregulation and downregulation of *dRagA* using three transgenic fly lines overexpressing a different modified version of *dRagA*: constitutively active (CA), UAS-$dRagA^{Q61L}$; dominant negative (DN), UAS-$dRagA^{T16N}$, and; wildtype, UAS-$dRagA^{WT}$ (Kim et al., 2008).

One of the main outputs of TORC1 activity is growth (Loewith and Hall, 2004). Using the apterous-*GAL4* (ap-*GAL4*) driver, which drives the expression of a UAS transgene to the dorsal cell layer of the fly wing (Calleja et al., 1996), we were able to assess dTOR activity by observing the growth effects of our transgenic UAS-*dRagA* fly lines, such that increased cell growth should result in a downward curvature of the wing, or upward wing curvature in the case of decreased cell growth. When driven with ap-*GAL4*, flies expressing UAS-$dRagA^{Q61L}$(CA) had downward curvature of the wings (Figure 3.3a) indicating increased growth of the dorsal layer of cells of the wing compared with those of the ventral layer. This is compatible with overgrowth caused by UAS-$dRagA^{Q61L}$(CA) increasing dTORC1 activity. Flies expressing UAS-$dRagA^{T16N}$(DN) had an upward curvature of the wings (Figure 3.3b), suggesting reduced growth of the dorsal layer of cells of the wing, consistent with downregulation of TORC1 activity. Flies expressing UAS-$dRagA^{WT}$ showed no curvature of the wing (Figure 3.3c), indicating no effect of this transgene on growth. This could be because although *dRagA* may still be overexpressed, an activator (amino acids) is required for the growth phenotype to manifest. In this case, perhaps the level of amino acids in the DR food medium was insufficient to cause increased cell growth.

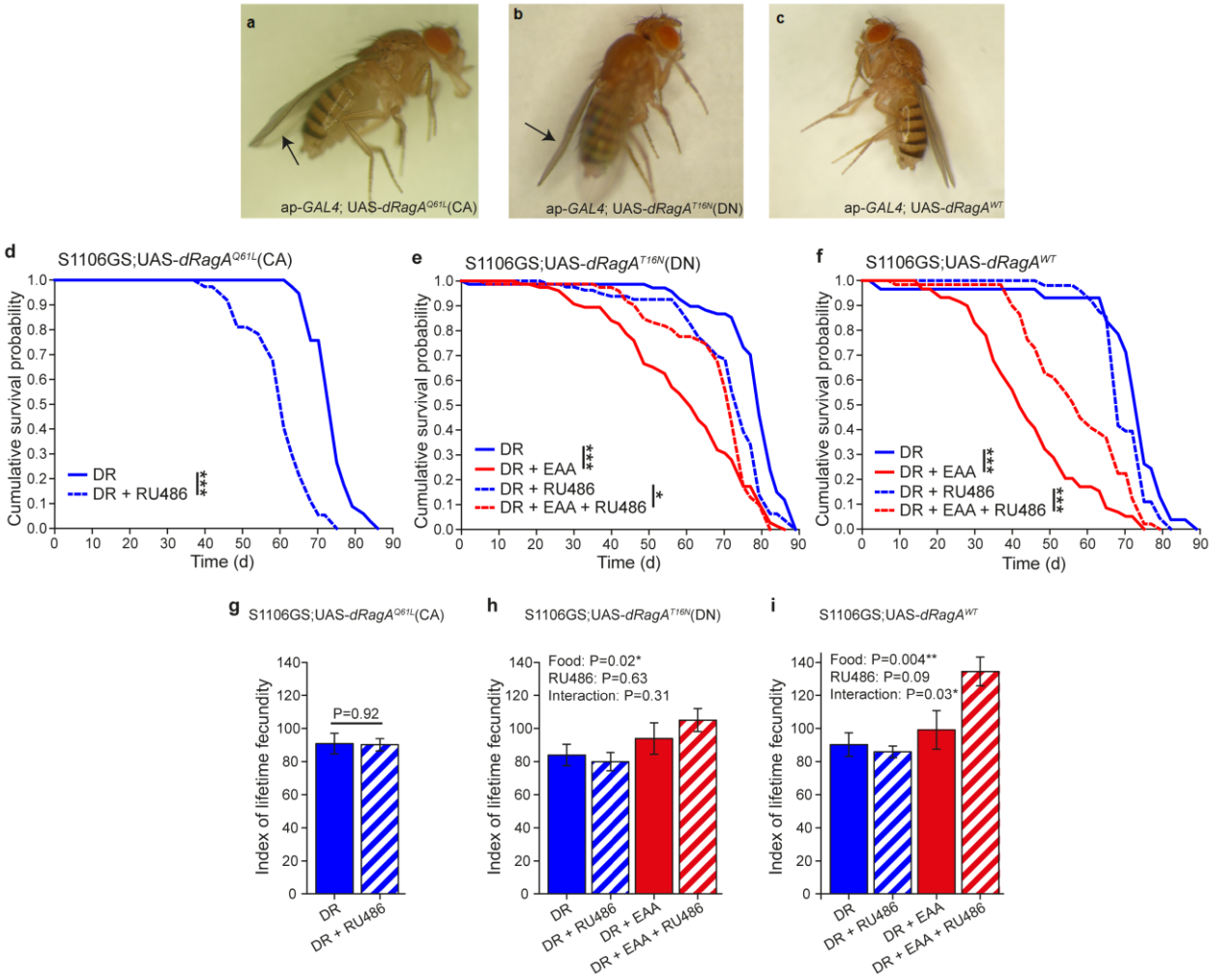


Figure 3.3. The effect on lifespan and fecundity in response to DR of genetically altered dRagA activity.

Wing curvature phenotypes of the offspring of the ap-*GAL4* wing-specific driver fly line crossed with **(a)** UAS-*dRagA*^{Q61L}(CA), showing wing tips curved down due to overgrowth of dorsal cells, **(b)** UAS-*dRagA*^{T16N}(DN), showing wing tips curved up due to reduced growth of dorsal cells, and **(c)** UAS-*dRagA*^{WT}, showing no curvature of wing tips, indicating no effect of this transgene on growth. These data are consistent with the anticipated effect of each transgene to alter dTOR activity. **(d)** The effect on lifespan of UAS-*dRagA*^{Q61L}(CA) transgene expression under dietary restricted (DR). Flies expressing the transgene upon administration of RU486 showed a significant decrease in lifespan (median lifespan 59.5 days), compared to controls (median lifespan 73.5 days, P<0.001, log-rank test, n=40 per condition). **(e)** The effect on lifespan of UAS-*dRagA*^{T16N}(DN) transgene expression under DR and with EAA supplementation. Both control flies and transgene-expressing flies showed a DR effect on lifespan, where the lifespan of DR flies was significantly greater than DR+EAA flies (DR vs DR+EAA, P<0.001; DR+RU486 vs DR+EAA+RU486, P=0.02; log-rank test; n=60 per condition). The magnitude of the difference in the DR effect was greater in the control flies (24%) than in the transgene-induced flies (3%). Median lifespans (days): DR=78.1, DR+RU486=73.5, DR+EAA=59.5, DR+EAA+RU486=71.1. **(f)** The effect on lifespan of

UAS-*dRagA*^{WT} transgene expression under DR and with EAA supplementation. Both control flies and transgene-expressing flies showed a DR effect on lifespan, where DR flies were significantly longer lived than DR+EAA flies (DR vs DR+EAA, P<0.001; DR+RU486 vs DR+EAA+RU486, P<0.001; log-rank test, n=80 per condition). The magnitude of the difference in the DR effect was greater in the control flies (44%) than in the transgene-induced flies (14%). Median lifespans (days): DR=73.5, DR+RU486=66.6, DR+EAA=40.9, DR+EAA+RU486 =57. **(g)** Index of cumulative lifetime egg-laying for control and transgene-induced UAS-*dRagA*^{Q61L}(CA) flies maintained under DR conditions. There was no significant difference in fecundity between the two groups (P=0.92, Wilcoxon rank-sum test, n=40 per condition). **(h)** Effect on egg-laying of UAS-*dRagA*^{T16N}(DN) transgene expression under DR and with EAA supplementation. There was an effect on egg-laying of food, no effect on egg-laying of RU486, and no interaction effect between food and RU486 on egg-laying (food, P=0.02; RU486, P=0.63; interaction, P=0.31; linear model; n=60 per condition). **(i)** Effect on egg-laying of UAS-*dRagA*^{WT} transgene expression under DR and with EAA supplementation. There was an effect on egg-laying of food, no effect on egg-laying of RU486, and an interaction effect between food and RU486 on egg-laying (food, P=0.004; RU486, P=0.08; interaction, P=0.03; linear model; n=80 per condition). For figures g-i, error bars represent the s.e.m.

3.2.3. The effect of RagA transgene expression on lifespan

We tested the effect of increased dRagA activity on lifespan by driving the UAS-*dRagA*^{Q61L}(CA) transgene in the gut and fat-body using the RU486-inducible S1106 gene-switch UAS/GAL4 driver (Roman et al., 2001) (see Figure 1.3 for an overview of the gene switch system). These tissues are particularly interesting since the gut is where nutrients are initially broken down and absorbed into the body, and the fat-body functions as a site of nutrient storage and has been shown to have a humoral role in sensing nutrient availability and controlling the growth of peripheral tissues accordingly (Arrese and Soulages, 2011; Colombani et al., 2003). Moreover, it was shown that targeted downregulation of dTOR in the fat-body was sufficient to extend lifespan (Kapahi et al., 2004). We refer to the flies that were not given the RU486 inducer as the controls.

Expression of UAS-*dRagA*^{Q61L}(CA) resulted in a significant reduction in lifespan compared to controls (P<0.001; log-rank test; Figure 3.3d), consistent with dRagA increasing dTORC1 activity, which in turn is expected to decrease lifespan.

To test if amino acid signal transduction through RagA is responsible for the effect of EAAs to shorten lifespan, we measured the effect on survival of each of the UAS-*dRagA* transgenes when they were exposed to a DR diet supplemented with the 10 EAAs. For both UAS-$dRagA^{T16N}$(DN) and UAS-$dRagA^{WT}$ control flies (i.e. not expressing the transgene) DR flies were longer-lived than flies fed DR+EAA (UAS-$dRagA^{T16N}$(DN), P<0.001; UAS-$dRagA^{WT}$, P<0.001; log-rank test; Figure 3.3e-f).

Having verified a DR effect for each set of control flies, we tested whether inducing expression of each of the UAS-*dRagA* transgenes could modify this response. As with the controls, both induced UAS-$dRagA^{T16N}$(DN) and induced UAS-$dRagA^{WT}$ transgenic flies were still longer-lived under DR than when supplemented with EAAs (UAS-$dRagA^{T16N}$(DN), P=0.02; UAS-$dRagA^{WT}$, P<0.001; log-rank test; Figure 3.3e-f). However, expression of both UAS-$dRagA^{T16N}$(DN) and UAS-$dRagA^{WT}$ significantly changed the way the flies responded to EAA supplementation (P-value for interaction between diet and transgene expression P<0.001; Cox Proportional Hazards); the magnitude of the decrease in lifespan with EAA addition was reduced upon transgene expression for both UAS-$dRagA^{T16N}$(DN) (24% decrease to 3% decrease in median lifespan upon transgene expression) and UAS-$dRagA^{WT}$ flies (44% decrease to 14% decrease in median lifespan upon transgene expression). In summary, modification of *dRagA* did not change the direction of the lifespan response of flies to EAA-supplementation relative to DR (i.e. EAA-supplemented flies were still shorter lived than DR flies), but the magnitude of this difference was reduced upon transgene induction, and more drastically so for UAS-$dRagA^{T16N}$(DN) flies than for UAS-$dRagA^{WT}$ flies.

We could not conduct these comparisons for the UAS-$dRagA^{Q61L}$(CA) transgenic flies because we did not have enough parental flies to generate a sufficient number of progeny for experimentation.

3.2.4. The effect of RagA transgene expression on egg-laying

To further characterise the effects of EAA signalling through dTORC1 we measured the changes in fecundity upon altering nutrient availability and nutrient signalling from dRagA to dTORC1. We compared the egg-laying of control and UAS-*dRagA* transgene-expressing flies under DR. For all three UAS-*dRagA* transgenic lines tested, no significant difference was detected between transgene induced and control flies (UAS-$dRagA^{Q61L}$(CA), P=0.92; UAS-$dRagA^{T16N}$(DN), P=0.65; UAS-$dRagA^{WT}$, P=0.62; Wilcoxon rank-sum test; Figure 3.3g-i).

For UAS-$dRagA^{T16N}$(DN) flies we found that there was an effect of EAA supplementation on egg-laying, such that flies maintained on DR+EAA food laid more eggs than those maintained on DR food (P=0.02; linear model; Figure 3.3h). There was no effect on egg-laying upon administration of RU486 (i.e. transgene induction) for these flies (P=0.63; P=0.09; linear model). We found no effect of an interaction between food and RU486 on egg-laying (P=0.31; linear model), indicating that the induction of UAS-$dRagA^{T16N}$(DN) transgene does not affect the egg-laying response to food.

UAS-$dRagA^{WT}$ flies had higher egg-laying when supplemented with EAAs (P<0.01; linear model; Figure 3.3i). There was no effect on egg-laying upon transgene induction for these flies (UAS-$dRagA^{WT}$, P=0.09; linear model). When we tested for an interaction effect between transgene expression and food treatment on egg-laying we found that induction of the UAS-$dRagA^{WT}$ transgene resulted in a significantly more pronounced increase in egg-laying upon EAA supplementation compared to control flies (P=0.03; linear model), suggesting that the modification of egg-laying in response to EAAs depends on the induction of the UAS-$dRagA^{WT}$ transgene.

Again, egg-laying data for these conditions could not be obtained for UAS-$dRagA^{Q61L}$(CA) flies due to inadequate numbers.

In summary, genetically altering dTOR activity resulted in a reduction of the magnitude of lifespan difference between long-lived DR and shorter-lived EAA-supplemented flies and increased the difference in egg-laying between these food conditions.

3.2.5. Rapamycin in mediating the effect of EAAs on lifespan and egg-laying

We used the TOR inhibitor drug rapamycin as an alternative method for genetically downregulating dTOR to test whether it could rescue the effect of EAAs on lifespan. Consistent with the effect of *dRagA* downregulation in $dRagA^{T16N}$(DN) flies, addition of rapamycin extended the lifespan of wildtype flies maintained on EAA-supplemented DR food ($P<0.001$, log-rank test) such that their lifespan was not shorter than those subjected to DR (Figure 3.4a). Rapamycin treatment also prevented the increase in egg-laying seen for EAA addition to DR food. In fact egg-laying was effectively blocked by rapamycin treatment ($P<0.001$, Wilcoxon rank-sum test; Figure 3.4b). We confirmed that phosphorylation of the TORC1 target S6K was reduced by the addition of rapamycin as anticipated (Figure 3.4c). Together with data from the UAS-$dRagA^{T16N}$(DN) flies, these data are consistent with dTOR signalling playing a role in mediating the change in lifespan upon DR.

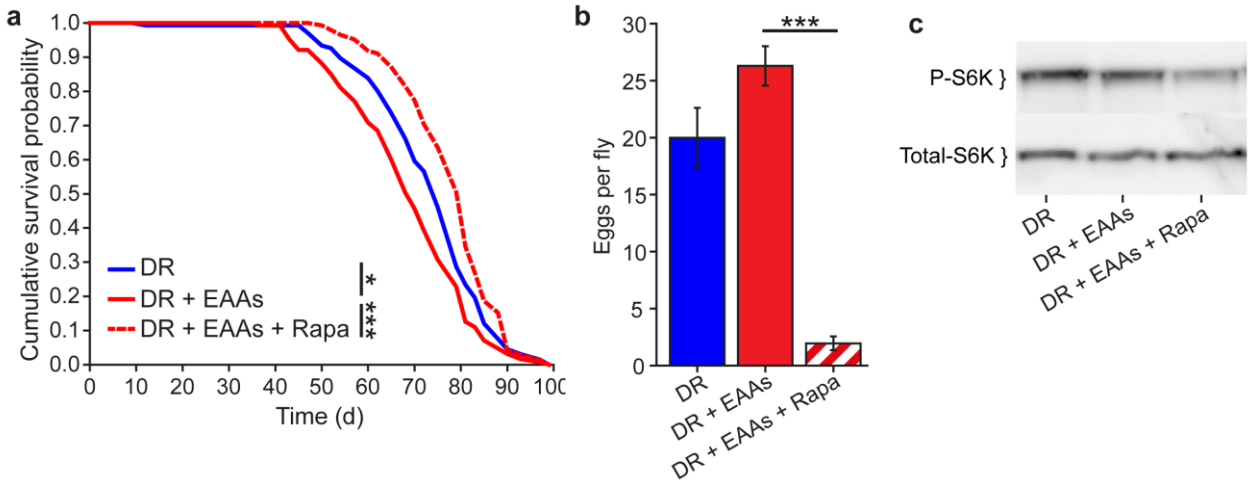


Figure 3.4. The effect of rapamycin treatment on EAA-supplemented flies.

(a) Rapamycin treatment extended the lifespan of DR+EAA flies beyond that of DR (DR+EAA vs DR+EAA+Rapamycin, $P<0.001$; DR vs DR+EAA, $P=0.01$; $n=150$ per treatment; log-rank test). **(b)** Rapamycin treatment decreased the fecundity of DR+EAA flies ($P<0.001$, $n=10$ flies per condition, error bars represent the s.e.m., Wilcoxon rank-sum test). Egg counts were made at 8 days of food treatment. **(c)** Levels of phospho-T398-S6K were measured from whole-fly protein extracts. Treatment with rapamycin for 7 days decreased phospho-T398-S6K levels in DR+EAA+Rapamycin flies relative to DR+EAA flies.

3.2.6. Identifying phenotypic correlates of lifespan change under DR

Because long-lived animal models often have an associated increase in the ability to resist environmental stresses, we tested whether long-lived DR flies are protected from the harmful effects of a number of stress-inducing compounds. These phenotypic correlates of lifespan change under our dietary conditions may help us to understand the causal mechanisms of increased lifespan under DR. Phenotypes that are consistently correlated with longevity may indicate something about the mechanisms that regulate longevity, just as those phenotypes that differ between long-lived fly models can be said to be uncoupled from longevity. These data can help us to address the overarching aim to understand the mechanism of lifespan modulation in response to DR.

Toxic stressors

DR flies were significantly more resistant than DR+EAA flies to a toxic dose of H_2O_2 ($P<0.01$, long-rank test, Figure 3.5a). In contrast, DR flies were no better able to survive under paraquat stress than DR+EAA flies ($P=0.52$, long-rank test, Figure 3.5b). DR flies were more sensitive to a toxic dose of DDT than DR+EAA flies ($P=0.04$, long-rank test, Figure 3.5c), indicating that, at least for DDT resistance, DR does not protect against this toxin in the same way that lowered insulin signalling has been shown to (Grönke et al., 2010).

Environmental stressors

We also tested the response of DR flies to environmental stressors. Under conditions of a 39^{o}C heat stress we found that DR flies were significantly less resistant than DR+EAA flies ($P<0.001$, long-rank test, Figure 3.5d), indicating that in flies, longevity associated with EAA reduction comes at a cost to heat stress resistance. We found that DR flies showed greater resistance to starvation than DR+EAA flies ($P<0.001$, long-rank test, Figure 3.5e), suggesting a possible mechanistic coupling between longevity and starvation resistance.

Metabolic measurements

Resistance to starvation stress could depend on the availability of enhanced energy stores within the fly. While we found no difference between dietary treatment groups in the levels of the storage carbohydrates glycogen ($P=0.66$, t-test, Figure 3.5f) or trehalose

(P=0.63, t-test, Figure 3.5g) we did find that DR flies had significantly higher levels of stored fat as triacylglycerides (TAG) than DR+EAA flies (P<0.001, t-test, Figure 3.5h). It is possible that this difference in TAG levels is causative of the longevity differences between DR and DR+EAA flies such that increased TAG confers some benefit to survival.

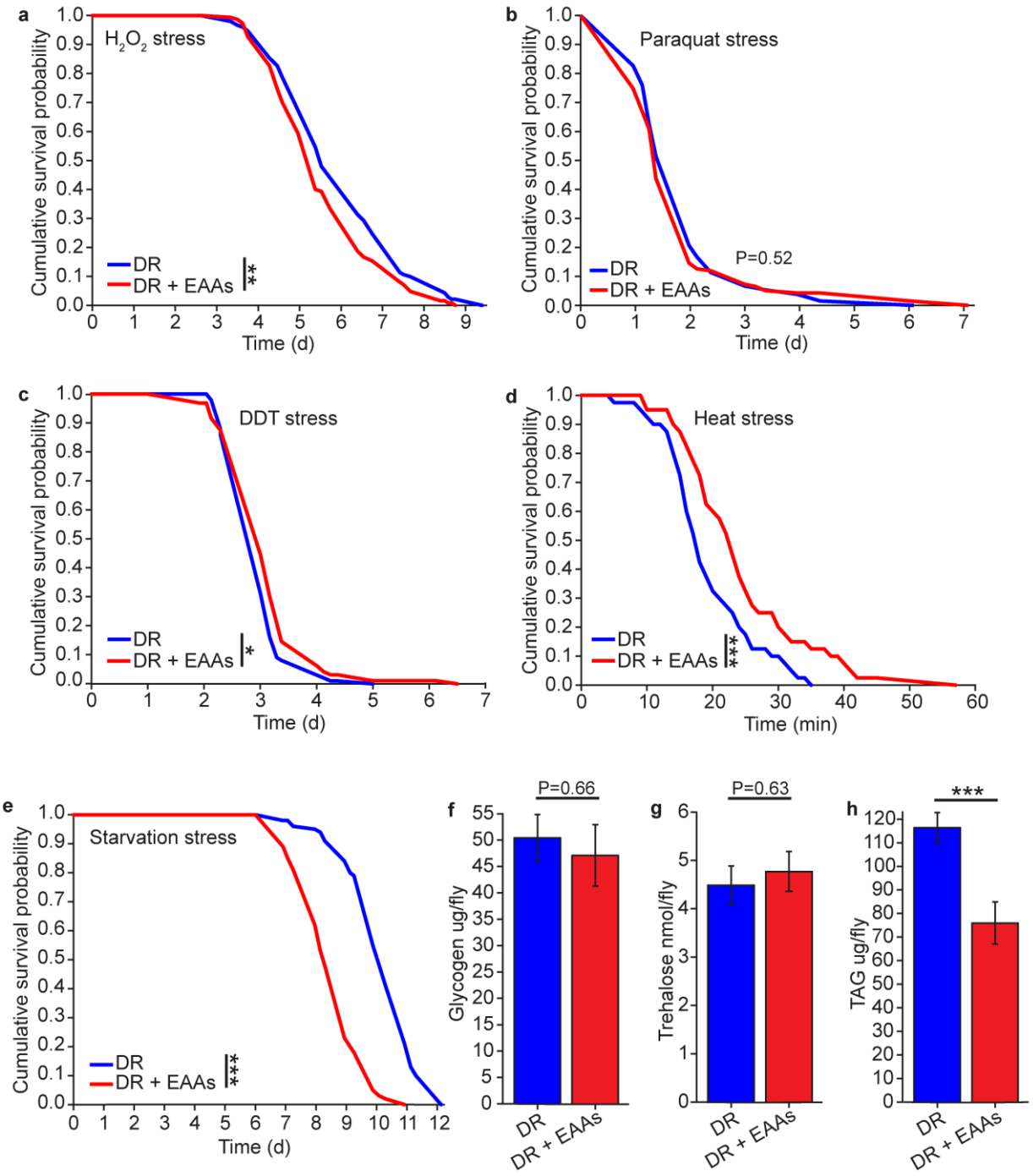


Figure 3.5. Phenotype comparisons between DR and EAA-supplemented flies.

(a) DR+EAA flies showed a decreased resistance to hydrogen peroxide toxicity compared to DR flies (P=0.01; n=150 flies per condition). **(b)** There was no difference between DR and DR+EAA flies in their sensitivity to paraquat stress (P=0.52; n=150 flies per condition).

(c) DR+EAA flies showed a significantly improved tolerance to DDT compared to that of DR flies (P=0.04, n=100 flies per condition). **(d)** DR+EAA flies were significantly more resistant to a 39ºC heat stress compared to DR flies (P<0.001; n=40 flies per condition). **(e)** DR+EAA flies were significantly more sensitive to starvation than DR flies (P<0.001; n=100 flies per condition). **(f)** After 7 days of treatment there was no difference in the amounts of glycogen measured for DR+EAA flies compared to DR flies (P=0.66; n= 6). **(g)** There was no difference in the levels of trehalose measured for DR+EAA flies compared to DR flies (P=0.63; n=6). **(h)** DR+EAA flies had significantly reduced levels of TAG compared to DR flies (P<0.001; n=6). For figures a-e, P values were calculated using the log-rank test. For figures f-h, P values were calculated using the t-test, and error bars represent the s.e.m.

3.2.7. The effect of rapamycin to alter DR phenotypes

If the above phenotypes induced by DR are causally linked to longevity through reduced TOR signalling, it should be possible to reproduce the same physiological outcomes by treating flies with rapamycin. We therefore tested the effect of rapamycin on DR+EAA flies for H_2O_2 stress resistance, starvation sensitivity, heat shock stress resistance and TAG levels. Of these, heat stress resistance and TAG levels are particularly interesting because the changes observed upon rapamycin treatment of DR+EAA flies meant they more closely resembled DR flies. There was no effect of rapamycin on the response of EAA-treated flies to H_2O_2 stress (P=0.96, log-rank test, Figure 3.6a) or to starvation stress (P=0.07; Figure 3.6b). Like DR, rapamycin treatment increased the sensitivity of EAA-treated DR flies to a 39ºC heat stress (P<0.001, Figure 3.6c), and increased their TAG content (P=0.01, t-test, Figure 3.6d). It is therefore possible that dTOR-mediated lifespan extension under DR is a result of the physiological processes that result in higher TAG levels, or an alteration in the mechanisms required to respond to a heat stress, or both.

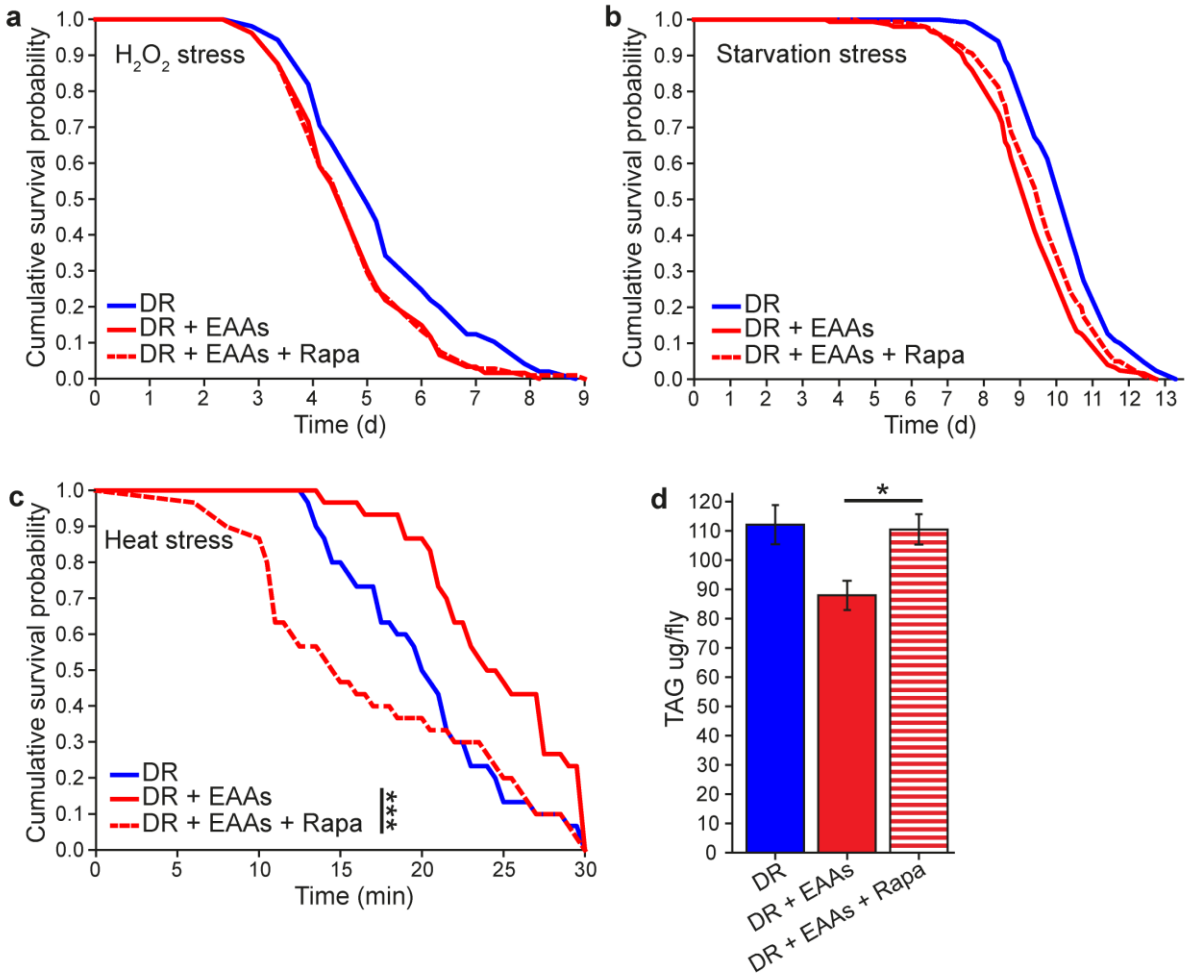


Figure 3.6. The effect of rapamycin to alter phenotypic differences between DR and EAA-supplemented flies.

(a) Rapamycin had no effect on the sensitivity of DR+EAA flies to H_2O_2 stress (P=0.96; n=105 flies per condition). **(b)** Rapamycin had no effect on the sensitivity of DR+EAA flies to starvation stress (P=0.07; n=150 flies per condition). **(c)** Rapamycin, like DR, increased the sensitivity of DR+EAA flies to a 39ºC heat stress (P<0.001; n=30 flies per condition). For figures a-c, P values were calculated using the log-rank test. **(d)** Rapamycin treatment increased the triacyglyceride (TAG) levels of DR+EAA flies to the level of DR (P=0.01, t-test, n=6, error bars represent the s.e.m).

3.3. Discussion

In this chapter, we set out to understand the mechanisms that govern DR-induced longevity. We took advantage of a more nutritionally defined intervention than classically used in DR studies; supplementing a DR medium with EAAs to mimic the effect on lifespan of full feeding by increased yeast. As an initial step, we used fly models for genetically downregulated IIS and TOR signalling to pinpoint the nutrient signalling pathway(s) that might play a role in mediating the lifespan response to DR. We thus identified TOR signalling, an evolutionarily conserved amino acid sensor, as required for longevity upon amino acid reduction. We then looked for phenotypic correlates of longevity by DR to ask whether the TOR signalling pathway could also alter those phenotypes and thus point to mechanisms by which it modifies lifespan.

The lifespan of IIS-mutant flies responded in a similar way to DR as wildtype flies, indicating that although IIS downregulation increases lifespan, it is not required for the lifespan-extending effects of DR. In support of this finding, other studies in both flies and worms have reported that the effect of DR to extend lifespan is independent of IIS (Houthoofd et al., 2003; Lakowski and Hekimi, 1998; Min et al., 2008). However, such additive interactions do not necessarily prove that DR and IIS act independently, since each intervention singly could induce the same longevity mechanism at submaximal levels (Gems et al., 2002), suggesting perhaps that DR and IIS work in parallel to increase lifespan via a common longevity-assurance mechanism. Our findings appear to contrast several other studies that have reported that lifespan modification in response to yeast dilution is abolished in some IIS mutant flies (Broughton et al., 2010; Grandison et al., 2009; Grönke et al., 2010). These differences could be due to the fact that in the current study we modulated lifespan by adjusting EAAs alone, rather than by diluting the yeast concentration in the diet. In doing so, we report a markedly different sampling of nutritional space than for yeast dilution, since we change the ratio of EAAs to all other dietary components, such as lipids, carbohydrates, non-essential amino acids, vitamins and trace elements. Moreover, these other studies use flies with different genetic manipulations of IIS, which could modify the outcomes for interactions with food. Interestingly, our experiments also showed that long-lived DR flies had increased sensitivity to DDT, which is the opposite phenotype seen for IIS mutant flies, in which longevity is accompanied by dFOXO-dependent DDT resistance (Grönke et al., 2010; Slack et al., 2011). Together,

these data indicate that if the correlated stress phenotypes seen for IIS mutants are causative of longevity, then the mechanism of DR to extend lifespan do not require intact insulin signalling. These data suggest that the beneficial effects on lifespan of DR can be achieved independently of IIS.

It is known that decreased dTORC1 activity extends lifespan in flies (Bjedov et al., 2010; Kapahi et al., 2004). In this study we have shown, through both genetic and pharmacological downregulation of dTOR, that the TOR pathway is required for lifespan modification in response to EAA availability. Using either method we found that flies were as long lived as those under DR in the presence or absence of added EAAs.

We found that increased dRagA activity was associated with decreased lifespan when either the UAS-*dRagA*^{WT} or UAS-*dRagA*^{Q61L}(CA) transgenes were overexpressed under DR conditions, consistent with the prediction that high TOR activity shortens lifespan (Figure 3.7a-b). Induction of the UAS-*dRagA*^{WT} transgene did not shorten lifespan as much as induction of the UAS-*dRagA*^{Q61L}(CA) transgene, which is consistent with it being a less potent activator of signalling through dTOR. However, when UAS-*dRagA*^{WT} flies were supplied with additional dietary EAAs, lifespan was further shortened, consistent with further activation of dTOR activity.

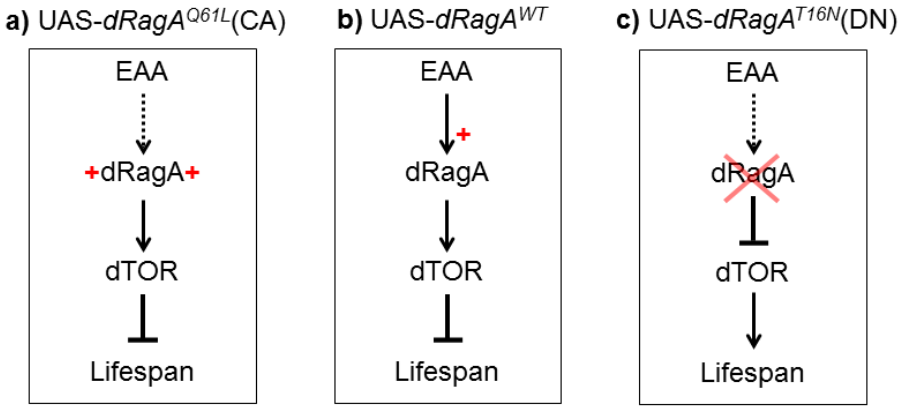


Figure 3.7. Predicted effects of *dRagA* transgenes on lifespan.
In the constitutively active UAS-*dRagA*^{Q61L}(CA) transgene model **(a)** the dRagA protein does not require EAAs for activation, while in the UAS-*dRagA*^{WT} model **(b)** dRagA requires activation by EAAs. In both models, dRagA activation increases dTOR activity which is predicted to lower lifespan. In the dominant negative UAS-*dRagA*^{T16N}(DN) model **(c)**, downregulated dRagA activity inhibits dTOR, which is in turn predicted to increase lifespan.

We tested whether a dominant negative form of *dRagA* could rescue the negative effects of EAAs on lifespan, as predicted by our model that decreased dRagA activity would dampen EAA signalling through TOR, and in turn alleviate the detriment to lifespan caused by supplementation of a DR diet with EAAs (Figure 3.7c). EAA supplementation decreased the lifespan of control flies by 24%, but only 3% for transgene-expressing UAS-*dRagA*^{T16N}(DN) flies. This observation is in line with our model in which reduced dRagA signalling blocks the effect of EAAs to increase dTOR activity and so prevents their lifespan shortening effect. This result is also supported by data from *C. elegans*, where dominant negative *raga-1* (the *C. elegans* homologue of *dRagA*) lengthened lifespan (Robida-Stubbs et al., 2012; Schreiber et al., 2010). This data supports our prediction that it is primarily the *signalling* of EAAs to/through dTOR, rather than the end fate of the EAAs themselves, that dictates lifespan. It is likely that dTOR signalling decides the fate of the ingested EAAs based on a 'sample' signal that informs about the environmental abundance.

The effect of EAAs to reduce lifespan was almost completely abolished in our UAS-*dRagA*^{T16N}(DN) flies. However, there was still a small, statistically significant reduction in lifespan upon EAA addition, suggesting that EAAs may have still been activating the functional native dRagA gene product which is still expressed in these flies. Alternatively, it may be the case that dTOR is not the only effector of altered lifespan in response to EAA addition. Interestingly, under DR conditions overexpression of the UAS-*dRagA*^{T16N}(DN) transgene resulted in a significant decrease in lifespan compared with controls, which indicates a limit for the beneficial effects of reducing dRagA, and in turn, dTOR activity (Figure 3.8a). Thus downregulating dTOR activity appears to be detrimental to flies under DR conditions, during which dTOR activity is expected to be optimal for lifespan, possibly because it may push flies into a state of perceived starvation. This can be harmful, for example, if energy stores start to get broken down in order to provide energy for short-term survival of the perceived starvation. It therefore may be informative to measure fat levels in our UAS-*dRagA*^{T16N}(DN) flies upon DR. There are several examples in the literature that demonstrate the detrimental effects of signalling being mismatched to the actual abundance of environmental nutrients; one such example comes from work using flies lacking the fat-body amino acid transporter, *slimfast* (*slif*), as a genetic tool to mimic amino acid deprivation (Colombani et al., 2003). These flies were shown to exhibit phenotypes similar to that of starved flies, such as low mass and small

wing size in the adults, and storage vesicle aggregation in the larvae. Moreover, *slif* null flies have reduced S6K phosphorylation, suggesting that it may lie upstream of the TOR pathway. Alternatively, dRagA may also be involved in some other signalling pathway that, when downregulated, has negative effects on physiology that decreases lifespan.

Induction of the UAS-$dRagA^{WT}$ transgene under DR conditions shortened lifespan by 9% - a small but significant reduction, contrary to our prediction that lifespan would be unaffected in this line due to the requirement for additional dietary amino acids to act as coactivators of the transgene to enhance dTOR signalling.

Together, our data are compatible with the lifespan response to modified TOR activity, as represented by a bell-shaped curve (Figure 3.8a). The position of an organism's lifespan on this curve is subject to changes in TOR activity, which is plotted along the x-axis, and lifespan peaks at a narrow window of TOR activity. To the left of the lifespan peak, survival declines as TOR activity is reduced in response to poor nutrient supply reflecting a state of relative malnutrition. To the right of the peak, progressively increased TOR activity becomes harmful for survival, due to unknown mechanisms, discussed further below. Interventions that reduce TOR activity, such as genetically downregulating *dRagA,* or rapamycin treatment, would shift an organism's position on the curve to the left. Conversely, because EAAs increase TOR activity, an increased EAA supply or genetically increasing TOR activity would shift an organism's position on the curve further to the right. Thus, whether an intervention to modify TOR activity lengthens or shortens lifespan depends on the starting position of the organism on this curve (Figure 3.8b).

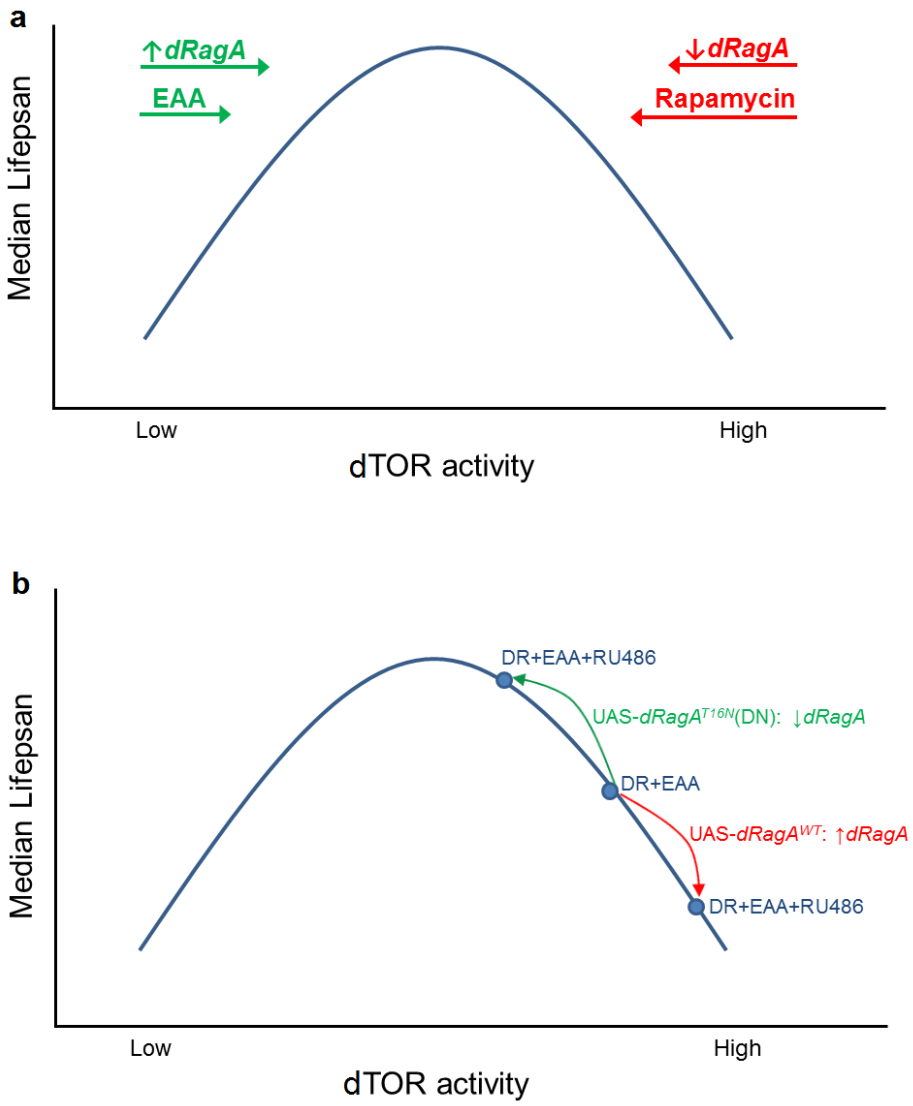


Figure 3.8. A schematic showing the relationship between lifespan and dTOR activity and the effect of dTOR modifiers to alter lifespan.

(a) Modifiers that increase dTOR activity, such as upregulation of dRagA activity and dietary essential amino acid (EAA) supplementation, are expected to push lifespan in the left-to-right direction, where too much dTOR activity becomes detrimental after an optimal level of activity. Modifiers that decrease dTOR activity, such as downregulation of dRagA activity and rapamycin treatment, are expected to push lifespan in the right-to-left direction, where lowered dTOR activity favours lifespan extension to a peak level, beyond which too little dTOR activity becomes detrimental. **(b)** The predicted effects on lifespan of upregulated and downregulated dRagA activity on short-lived EAA-supplemented flies. We would expect that upon transgene induction with RU486, lowered dRagA activity, and in turn lowered dTOR signalling, in UAS- UAS-*dRagA*^{T16N}(DN) flies would extend the lifespan of EAA-treated flies. Upon transgene induction, increased dRagA and dTOR activity in UAS-*dRagA*^{WT} flies would be expected to cause a further reduction in lifespan of EAA-supplemented flies.

Interestingly, in our experiments the magnitude of the lifespan response to DR was greatly reduced with overexpression of the UAS-*dRagA*^{WT} transgene when compared to control flies (44% difference in median lifespan between DR and DR+EAA, to 14% difference upon transgene induction). This amelioration of lifespan shortening with additional amino acids is somewhat at odds with our initial hypothesis that activation of overexpressed UAS-*dRagA*^{WT} together with EAA supplementation would increase dTOR activity and so cause a greater decrease in lifespan than when compared to a case where EAAs are added to a normal-functioning dTOR environment (i.e. uninduced transgene) (Figure 3.8b). This unexpected result may be explained if we consider that the starting position of the control flies on the curve may have been at a level of dTOR activity lower than predicted, and increasing dTOR activity with transgene expression may have rescued lifespan so that the position of transgene-expressing flies lies closer to the peak of the curve. Alternatively, gut-confined expression of the transgene may have somehow disrupted the lifespan shortening effects of EAA addition. Also, potential feeding differences, for which we did not test, may have affected the amount of nutrients taken in by the flies, and in turn the degree to which dTOR signalling was active.

We wanted to determine the effects on egg-laying of altering nutrient dependent signalling from dRagA to dTOR. For UAS-*dRagA*^{WT} flies, transgene induction exaggerated the increase in egg-laying with EAA addition, which is in line with what we would expect when dTOR signalling, and in turn protein synthesis, is upregulated. The induction of the UAS-*dRagA*^{T16N}(DN) transgene did not affect the egg-laying response to food, and any difference that we saw in egg-laying arises only from the food type. While we may have expected a decrease in egg-laying with dTOR dowregulation, as we saw with rapamycin treatment, it is possible that egg production may be controlled by other/additional protein synthetic pathways involved in directing nutrient signals to egg-producing processes, perhaps in a compensatory manner in response to dTOR activity. One such alternative is IIS, which has been implicated in *Drosophila* oogenesis. IIS within the germline has a direct role in regulating germline cyst development, vitellogenesis, germline stem cell division (Hsu and Drummond-Barbosa, 2009; Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005), and indirectly controls follicle cell proliferation (LaFever and Drummond-Barbosa, 2005).

Together, our data indicate that expression of the UAS-*dRagA* transgenes significantly affected the response of lifespan to EAA addition in a manner that is partially consistent with a role for dTOR in mediating these effects. However, the extent to which dRagA is involved in nutrient signalling, the exact mechanisms through which it functions, and any interaction it may have with other NSPs has yet to be elucidated. Some of the seemingly anomalous results we obtained could be explained by deleterious effects of the transgene expression itself. For example, the dRagA protein forms a heterodimeric complex with dRagC in vivo (Sekiguchi et al., 2001) so perhaps overexpression of any *dRagA* transgene might disrupt heterodimeric dRagA function in an unanticipated way, creating an unanticipated dominant negative effect. This can be tested via western blot analysis of dS6K phosphorylation, a downstream effector of dTOR. Decreased dS6K phosphorylation should indicate decreased dTOR activity, and presumably decreased dRagA activity.

In further work it would be interesting to test whether ubiquitous alteration of *dRagA* affects lifespan and egg-laying in the same way as tissue-specific expression. It may also be interesting to disentangle the effects of combined gut and fat-body *dRagA* alteration by using separate drivers that target these tissues singly. Mammalian TORC1 has also been shown to be required for skeletal muscle protein synthesis (Dickinson et al., 2011), therefore muscle-specific *dRagA* expression may reveal a specific role for dRagA in muscle growth and senescence and how this may in turn impact whole organismal ageing. Further, a comparison of these findings with altered dRagC (with which dRagA forms a heterodimer) activity could inform us of the dynamics of the Rag heterodimer subunits and whether or not their roles are distinct. It would also be useful to investigate whether the observed phenotypes are a specific effect of decreased signalling via the amino acid sensitive dRagA input to TOR, or whether they are the result of generally decreased TOR activity in response to any upstream modifier. This can be tested using flies with alterations in pathway components that modify TOR activity, such as dTSC1/2 and dRaptor, in combination with flies with altered dRagA activity. These experiments would shed light on the question of whether the effects we have observed are specific to amino acid stimulation of TOR activity, since other environemtnal factors, such as energy and growth factors, also affect TOR activity. Ongoing work in the laboratory is using whole transcriptome sequencing to analyse the gene expression profiles of DR, EAA-supplemented and rapamycin-treated flies to further investigate the molecular signatures associated with lifespan regulation via EAA signalling to dTOR.

One of the strategies taken to understand the mechanisms by which amino acid abundance could modify lifespan downstream of TOR is to seek out correlated physiological changes that may provide markers into the treatment's mode of action. A common mechanistic explanation for longevity requires enhancing systems that protect against internal and environmental injury, such as the damaging side-effects of aerobic metabolism caused by oxidative stress or endogenous lipophilic toxins (McElwee et al., 2004, 2007), and several lines of evidence show that the capacity for DNA repair decreases with age (Goukassian et al., 2000; Hasty et al., 2003; Lombard et al., 2005), and that this may be due in part to reduced energy availability (McCarroll et al., 2004; Meyer et al., 2007). In the case of DR, it has been theorised that the limited amount of nutrients available in a DR diet is invested into somatic maintenance, at the expense of reproduction, in an effort to increase the likelihood of organismal survival to the event of the return of nutrient abundance (Holliday, 1989; Kirkwood, 1977). Thus, resources are invested into maintenance programmes such as DNA repair and DNA proof-reading mechanisms, which are costly resource-consuming reactions. The expected result of this investment is a general increase in functional efficiency of biological processes and this would be indicated by greater efficiency of stress defense pathways. We measured the survival responses of DR flies to a range of stressors. We found no evidence for broad-spectrum enhanced protection against stressors under DR, suggesting that the mechanism for increased longevity under DR may not involve a general enhanced resistance to stress. If organisms can live long with an apparently unaffected capacity for somatic maintenance, what causes ageing?

The Free Radical Theory of Ageing proposes that an accumulation of free radicals, harmful by-products of metabolism, cause oxidative damage within cells and that this leads to ageing (Balaban et al., 2005; Harman, 1956). DR has been proposed to decrease mitochondrial utilisation of oxygen, resulting in the decreased production of free radicals and in turn the attenuation of damage, leading to lifespan extension (López-Lluch et al., 2006; Lopez-Torres et al., 2002; Sohal et al., 1990). However, several conflicting reports show that DR animals in fact have increased mitochondrial respiration (Cerqueira et al., 2012; Hempenstall et al., 2012; Nisoli et al., 2005). We found that DR had no or negative effects on resistance to the oxidative stressors paraquat and hydrogen peroxide stress. The evidence for the Free Radical Theory of Ageing in the literature is inconsistent (Masoro, 2000, 2005; Merry, 2004; Sinclair, 2005), and our data does not provide support for it.

Studies in *C. elegans* showing that lifespan could not be extended by alleviation of oxidative stress (Doonan et al., 2008; Gems and Doonan, 2009) has led to the hypothesis that age-related functional decline, and ultimately death, is caused by 'hypertrophy' brought about by inappropriate continuation of early-life growth programmes into later life. This mismatch of growth to physiological demand beyond the developmental period is physically overwhelming to the organism and manifests as accelerated senescence, causing ageing and ageing-related diseases (Blagosklonny, 2006a, 2006b, 2008; Gems and de la Guardia, 2013). High levels of TOR signalling driving growth sits at the heart of this theory. Our data is not incompatible with this theory, although readouts of growth would be needed to substantiate this idea.

The finding that DR flies survive longer under starvation compared to fully fed control flies has been observed before (Burger et al., 2007; Chippindale et al., 1993). The higher lipid levels of DR flies has been proposed to account for their increased resistance to starvation (Rauser et al., 2004), with the reasoning that increased metabolic stores provide the flies with a more sustained source of energy. The starvation stress and fat phenotypes were not uncoupled from longevity in our experiments, whether lifespan was modified by diet manipulation or rapamycin addition. If the enhanced longevity of organisms in response to DR evolved as a mechanism to protect animals from food scarcity, it would make sense that the post-ingestive storage of limited resources would be central to such a mechanism. The question remains - is the storage of nutrients actively increased as a direct response to food limitation, or is nutrient storage a secondary effect of decreased fecundity due to insufficient resources required to fulfil egg production? In other words - which comes first, decreased egg-laying or nutrient storage? Or do both responses occur independently?

DR by yeast restriction in *Drosophila* has previously been shown to increase lipid content (Bradley and Simmons, 1997; Chippindale et al., 1993; Skorupa et al., 2008), and several rodent studies also show that higher fat levels correlate with increased lifespan (Berryman et al., 2004; Coschigano et al., 2000; Flurkey et al., 2001; Harrison et al., 1984; Liao et al., 2011; List et al., 2009; Liu et al., 2004; Miller et al., 2005; Olsson et al., 2005). The counterintuitive nature of these findings suggests that the effect of TAG on lifespan is likely to be a complicated relationship and may be accounted for by the fact that TAGs exist in various types with different structural and functional properties. TAG fatty acids

vary in their chain length and saturation, occupying specific stereopositions on the glycerol molecule. The factors that determine the assembly of TAGs are not clear, but this stereopositioning determines their specificity as substrates for different lipases and how they are metabolised, having distinct effects on cellular processes and physiological functions (Karupaiah and Sundram, 2007). The molecular nature of these TAGs and the expression levels of enzymes that act on them may be influenced by environmental cues and/or internal signals, and collectively these factors may account for the reported complexity of the relationship between fat and lifespan.

A recent study from *C. elegans* proposes that DR promotes expression of lipases that hydrolyse lipids, resulting in increased levels of ω-6 polyunsaturated fatty acids, that in turn induce autophagy, thereby promoting longevity (Rourke et al., 2013). The study hypothesises that these lipid species serve as signals of nutrient scarcity. The findings complement our model of TOR-mediated lifespan extension under DR, where long-lived flies with downregulated TOR activity have elevated TAG levels, like DR flies. Further identifying the specific TAG species that are elevated in these flies and measuring any accompanying changes in the levels of autophagy would be informative in determining whether the relationship between DR, fat and autophagy is also conserved in flies.

During fasting 20%–50% of fatty acids undergo the energy-costly process of re-esterification (Weber and Reidy, 2012), which is inconsistent with the role of TAG as an energy source, especially during fasting when conservation of energy would be essential for survival. It has been suggested that this remodeling of TAGs may have some, as yet unknown, important biological function (Kniazeva and Han, 2013). Thus, fat seems to have an important physiological role beyond energy storage that promotes the activation of protective mechanisms. Indeed, the adipose tissue is being realised as an important endocrine organ, and not just a TAG storage organ (Lubbers et al., 2013). The adipose tissue produces and secretes adiponectin, a cell-signalling protein, which has been positively correlated with longevity (Arai et al., 2011; McKee Alderman et al., 2010). Genetically altered mice that express increased levels of human adiponectin have increased lifespans, and accordingly some long-lived mice have been shown to have increased levels of adiponectin (Lubbers et al., 2013). Moreover, as like mammalian insulin, *Drosophila* Dilps regulate energy metabolism, and it was shown that inhibition of the adiponectin receptor in the insulin producing cells (IPCs) in the fly brain resulted in elevated levels of

TAG in the whole body, and that this was correlated with accumulation of Dilp2 protein in IPCs, decreased levels of circulating Dilp2, and decreased insulin signaling in the fat body (Kwak et al., 2013). This study indicates that insulin signalling controls lipid metabolism, providing a direct mechanistic link between NSPs and lipid metabolism. It would be interesting to investigate whether the TOR pathway plays a role in this system, and whether we may be able to reconcile this with our own observed associations between TOR, fat and lifespan.

Katewa et al. (2012) have shown that DR flies have increased TAG levels, and demonstrated an increased requirement for muscle-specific fatty-acid synthesis and breakdown in extending lifespan under DR. Moreover, some long-lived TOR and IIS pathway mutants have increased fat levels (Bjedov et al., 2010; Böhni et al., 1999; Teleman et al., 2005; Zhang et al., 2000). Given that not all fat mutants are long-lived (Grönke et al., 2005), it is likely that if fat storage is causally involved in extending life, the type of fat accumulated is important. It would be interesting in future work to determine how lipid profiles change under different dietary conditions, to identify the specific types of lipids that are altered, and whether experimental manipulation can enhance lifespan.

One of the main limitations of our study has been that the correlations that we have drawn between lifespan and phenotype are based on the phenotypes of flies at a single time point early in life (7 days old). Thus, our data are unlikely to be wholly indicative of the impact of our treatments on ageing per se. However, they may indicate the impact of early life physiology on lifespan, if indeed these early-life traits are decisive of the course of longer-term ageing. Ideally we would sample flies at multiple ages throughout their lifespan to deduce if and how the phenotypes change through their lifetime. Some studies have demonstrated how traits may change over the lifetime of an organism. Burger et al. (2007) report that at 4 days old, DR-treated flies are significantly more resistant to starvation than control flies, but by day 20 this is reversed and DR flies are more sensitive than control flies to starvation stress, and remain so when sampled at 33 days and 47 days. This study also found age-dependent changes in DR and control flies in response to oxidative stress, infection and cold stress. Furthermore, it has been shown that mice treated with rapamycin exhibit different effects on metabolism in response to different lengths of rapamycin treatment (Fang et al., 2013). For example, mice treated with rapamycin for 2 weeks

showed no difference in adiposity compared to control mice, whereas 20-week old rapamycin-treated mice had significantly lower fat compared to control mice. Detailed longitudinal studies on the molecular bases of these changes will be important to understand how the phenotypic changes in the flies relate to their longevity.

Our method of DR implementation is different from other studies in that we phenotype DR flies as relative to flies supplemented with additional EAAs. This may explain why the phenotypes of our short-lived flies are somewhat different from those of other organisms subjected to FF. It is thus possible that the associated physiological correlates of lifespan, causal or not, that we have observed may be specific to our particular dietary intervention, i.e. that the phenotyping profile that we have characterised is specific to DR relative to FF as implemented by EAA supplementation, as opposed to FF by increased yeast. These two FF controls are very different, since in the latter not only is the level of EAAs being elevated, but also the relative levels of all other nutritional components in the yeast. Of course, inter-species differences may also be accounted for by the very fact that they are different species with distinct physiologies.

Together, our findings implicate a role for the TOR pathway in mediating the lifespan effects of DR, as implemented by EAA alteration. In an attempt to understand the mechanisms by which longevity is achieved, we have described the physiological and metabolic features that define long-lived DR flies. Our data indicate that dietary amino acids modify dTOR signalling, which in turn alters lifespan outcomes. We found that both dietary EAA manipulation and dTOR modification in flies alter TAG levels, such that increased TAG correlated with longer life in our flies subjected to DR or rapamycin treatment. Despite finding a requirement for functional TOR in mediating the DR response, our data also indicate there are likely to be other mechanisms important for longevity. This may include other nutrient sensing pathways. In the next chapter we explore the role of the, as yet uncharacterised, general amino acid control (GAAC) pathway in DR-mediated lifespan extension.

Chapter 4 - Phenotypic characterisation of dGCN2 deficiency in *Drosophila* and its effects on dietary restriction-mediated longevity

4.1. Introduction

Organisms require an adequate intake of amino acids, where a deficiency in one or more amino acids in the diet compromises growth, development and survival. Thus, sensors of amino acid availability are crucial for homeostatic control. Amino acids not only serve as the building blocks of protein, but also have important signalling roles to regulate processes such as metabolism, development, growth, gene expression, and immunity, and are precursors for important bioactive molecules including neurotransmitters and hormones (Wu, 2010). It would therefore be expected that mechanisms that ensure adequate amino acid intake would be tightly regulated. Indeed, one such proposed control mechanism is the general amino acid control (GAAC) pathway, which is a major regulator of protein synthesis and is activated by amino acid starvation.

In the GAAC pathway, amino acid limitation is signalled directly via the binding of uncharged tRNAs to the GCN2 kinase, which in turn phosphorylates the eukaryotic initiation factor-2 (eIF2), resulting in the inhibition of mRNA translation and a decrease in global protein synthesis (reviewed in chapter 1.3.3) (Wek et al., 1995; Yang et al., 2000). Because GCN2 can bind different species of uncharged tRNAs with similar affinities, the GAAC can in theory detect deficiencies in any amino acid. Knockout of GCN2 function has been described for rodents. Phenotypically, *GCN2* null rodents resemble their wildtype counterparts under adequate nutritional conditions. However, phenotypic differences tend to reveal themselves under conditions of nutritional stress (Zhang et al., 2002). For example, when fed a diet devoid of leucine, *GCN2* null mice produced fewer pups than wildtype mice, suggesting a role for GCN2 in the control of nutritional stress during embryogenesis. Postnatally, *GCN2* null mice are more sensitive to leucine deprivation and do not survive well compared to control mice, further implicating a role for GCN2 in regulating growth under nutritional stress (Anthony et al., 2004). One study investigating the role of tryptophan restriction in the resistance to surgical stress following renal

ischemia reperfusion showed that *GCN2* null mice had increased protection against surgical stress, compared to wildtype mice, when fed a complete diet, but did not benefit from a tryptophan deficient diet, whereas wildtype mice did (Peng et al., 2012). Moreover, some markers of inflammation, such as the level of circulating neutrophils and the levels of growth hormone receptor mRNA and Igf1 mRNA, were reduced for wildtype mice, but not for *GCN2* null mice, upon tryptophan deficiency. It has also been shown that mice treated with asparaginase, which depletes levels of the amino acids asparagine and glutamine, incurred hepatic triglyceride accumulation and had increased DNA damage from oxidative stress and inflammation (Wilson et al., 2013). These observations are analogous to those in yeast, where *GCN2* mutants have a reduced growth rate relative to control yeast when grown on amino acid-deficient media or when treated with inhibitors of amino acid biosynthesis (Dever et al., 1992; Kilberg et al., 1994; Zaborske et al., 2009).

In accord with the findings above, Rousakis et al. (2013) show that GCN2 aids the survival of the nematode worm *Caenorhabditis elegans* under nutrient stress. *GCN2* null worms under normal nutritional conditions were phenotypically similar to wildtype worms, with no difference in lifespan, growth, development, movement or fecundity. However, upon concomitant knockdown of genes encoding either lysil- or leucyl-tRNA synthetase (as a means of inducing amino acid limitation), *GCN2* null worms incurred a greater shortening of lifespan compared to wildtype worms, which is consistent with previous work showing that animals with loss of *GCN2* are more sensitive to conditions of nutrient stress. This study also proposes that GCN2 regulates lifespan extension under dietary restriction (DR), based on the finding that loss of *GCN2* suppressed the longevity of *eat-2* mutant worms, which are often used as a model of DR due to their pharyngeal pumping defects. In addition, the study makes a connection between the GAAC pathway and the target of rapamycin (TOR) and insulin/insulin-like growth factor signalling (IIS) pathways in worms. They present evidence to suggest that GCN2 mediates lifespan extension conferred by inhibition of the TOR pathway and that these two pathways converge on the PHA-4/FoxA transcription factor, lying downstream of the IIS pathway.

In this work we set out to characterise the role of the GAAC pathway in regulating the physiological responses of *Drosophila melanogaster* to nutritional stress. We also investigate the role of GCN2 in the lifespan response to DR, as well as any connection it may have with the TOR pathway.

4.2. Results

The kinase activity of the GAAC pathway is dependent on amino acid availability. This led us to ask whether, like other nutrient sensing/signalling pathways (NSP), the GAAC pathway has a role in mediating lifespan modifications in response to dietary change. Using flies deficient in dGCN2, the *Drosophila* homologue of mammalian GCN2, we tested whether the *Drosophila* GAAC pathway may be involved in mediating lifespan extension under DR. Although commercially available *Drosophila* RNAi lines targeting *dGCN2* exist, they have been found to have off-target effects (Carlos Ribeiro, personal communication). Therefore, we generated a *Drosophila* line with a knockout of the entire *dGCN2* gene (see chapter 2.1.2, figure 2.1), in collaboration with Sebastian Grönke (Max Planck Institute for the Biology of Ageing, Cologne).

4.2.1. Physiological characterisation of dGCN2 null flies

Since the function of dGCN2 in flies is only inferred from similarity to yeast GCN2, we carried out a number of phenotyping assays to characterise flies lacking *dGCN2*. We tested several phenotypes under varying concentrations of yeast: 0.2x Y, 1x Y, and 2x Y (see chapter 2.2.2, table 2.3).

We determined the development time of flies reared on different concentrations of dietary yeast, as measured by time to pupal eclosion. None of the diets tested compromised the proportion of larvae eclosing as adults. There was no difference in development time between *dGCN2* null and wildtype flies on 2x yeast ($P=0.10$, Wilcoxon rank-sum test, Figure 4.1a), nor when reared on 0.2x yeast ($P=0.37$, Wilcoxon rank-sum test). However *dGCN2* null flies developed slightly quicker than wildtype flies on 1x Y (3.8% improvement of median development time, $P<0.001$, Wilcoxon rank-sum test), suggesting that there may be some interaction between dietary protein and dGCN2 to regulate development. Interestingly, only development on 0.2x yeast resulted in a significant reduction in adult body weight for *dGCN2* null compared to wildtype flies (0.2x Y, $P<0.01$; 1x Y, $P=0.68$; 2x Y, $P=0.13$; t-test; Figure 4.1b).

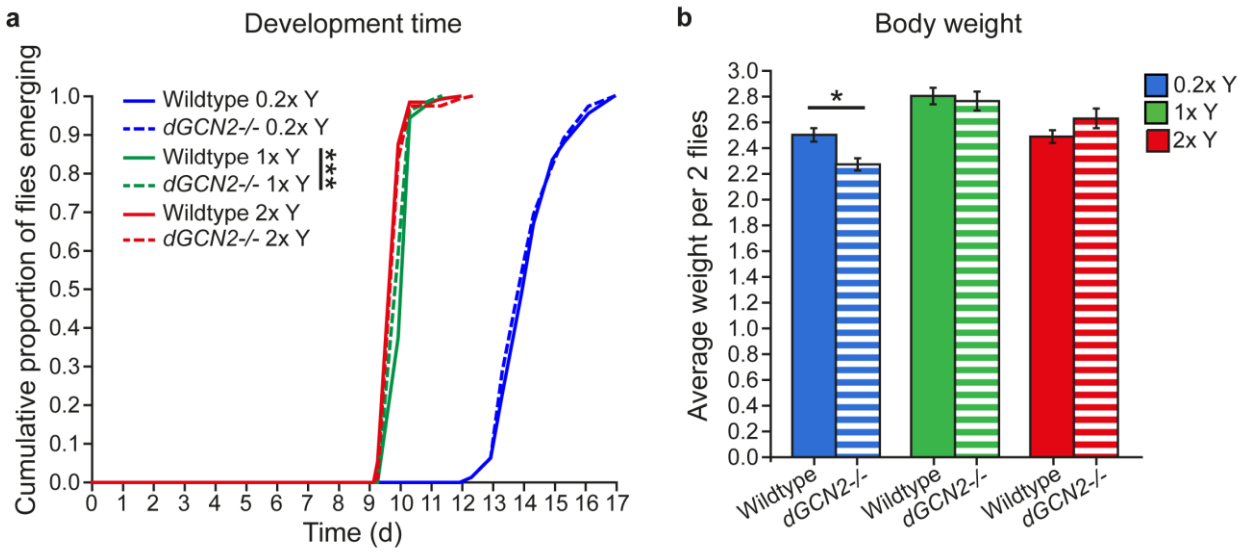


Figure 4.1. The effect on development time and body weight of *dGCN2* deletion for flies reared on varying concentrations of yeast (Y).

(a) There was no difference in development time between female wildtype and *dGCN2-/-* flies on 0.2x Y (P=0.37; Wilcoxon rank-sum test) or 2x Y (P=0.10). On 1x Y, *dGCN2-/-* flies developed significantly faster than wildtype flies (P<0.001). Median development times (days from 1st instar larvae): Wildtype: 0.2x Y=14.3, 1x Y=10.3, 2x Y=9.9; *dGCN2-/-*: 0.2x Y=13.9, 1x Y=9.9, 2x Y=9.9. n=30 flies per condition per genotype. **(b)** Body weights of 3-day old female flies after eclosion. Measurements were made per 2 virgin female flies, and values are expressed as an average. There was no difference in body weight between wildtype and *dGCN2-/-* flies reared on 1x Y (P=0.68; t-test) and 2x Y (P=0.13), but on 0.2x Y wildtype flies weighed significantly more than *dGCN2-/-* flies (P<0.01). n=30 flies per condition per genotype.

Several studies have suggested a role for translation in regulating the immune response (Lemaitre and Girardin, 2013). Since GCN2 is a key regulator of protein synthesis in yeast, we tested the ability of *dGCN2* null flies to withstand an immune challenge. We tested their resistance to infection with the pathogenic bacterium *E. carotovora carotovora* (*Ecc*). Adult *dGCN2* null flies were significantly more resistant to *Ecc* infection than wildtype flies, as determined by survival analysis following infection by septic injury (P<0.001, log-rank test, Figure 4.2a; assay done with assistance from Jenny Regan). Unlike adults, *dGCN2* null larvae were more sensitive to *Ecc* infection compared to wildtype larvae, as measured by pupal eclosion rate following infection by ingestion (P=0.04, t-test, Figure 4.2b; assay done with assistance from Jenny Regan).

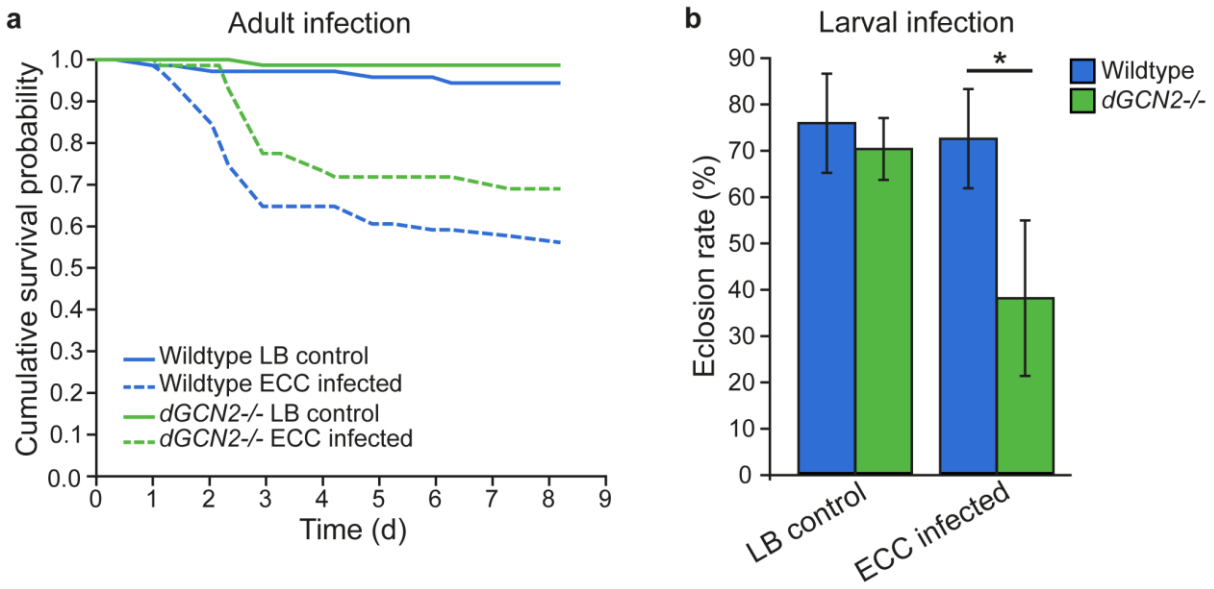


Figure 4.2. The effect on survival of an immune challenge in wildtype and *dGCN2* null adult flies and larvae.

(a) *dGCN2-/-* adult flies were more resistant than wildtype flies to infection by septic injury with *E. carotovora* (O.D. 200) ($P<0.001$, log-rank test, n=75 flies per condition per genotype). **(b)** *dGCN2-/-* larvae were more sensitive to oral infection with *E. carotovora* (O.D. 200) compared to wildtype flies ($P=0.04$, t-test, n=30 larvae per condition per genotype).

4.2.2. The role of the GAAC pathway in mediating lifespan under DR

The GAAC pathway is considered to be a major nutrient signalling pathway, and so we tested how dGCN2 activity might alter lifespan in response to dietary change. We manipulated the yeast:sugar ratios of our standard SY food medium to produce our test conditions, totaling nine combinations of three yeast concentrations (0.5x, 1x, and 2x) and three sugar concentrations (1x, 2x and 4x). From this data we were able to generate a heat map, which graphically displays the median lifespan responses of flies to each treatment within the yeast and sugar nutrient space that we tested. It should be noted that these surfaces are interpolated from the nine data points available, whose values are indicated on the plots. The lifespan heat maps for wildtype and *dGCN2* null flies seem to have a very similar distribution of lifespan effects within the nutritional frame (Figure 4.3), with maximal lifespan at a yeast:sugar ratio of 1x:1x for both genotypes, and at its lowest at 0.5x yeast : 4x sugar. In fact, for both genotypes, excess sugar relative to yeast seems to be more detrimental than the reverse imbalance. Most notably, absence of dGCN2 increased

median lifespan on almost all yeast:sugar concentration combinations tested. Analysing this data by Cox Proportional Hazards (CoxPH) confirmed a significant effect of genotype on lifespan ($P<0.001$). CoxPH analysis also revealed both a significant effect of yeast and sugar, independently, on the lifespan outcomes ($P<0.001$ for both variables), as well as a combined effect of yeast and sugar ($P<0.001$). We found that genotype affected the lifespan response to sugar ($P=0.01$), but we did not find a significant interaction between genotype and yeast ($P=0.12$). However, genotype altered the interaction between yeast and sugar to affect lifespan ($P=0.02$). In essence, the absence of dGCN2 altered the lifespan responses of flies to manipulations in the yeast:sugar ratio.

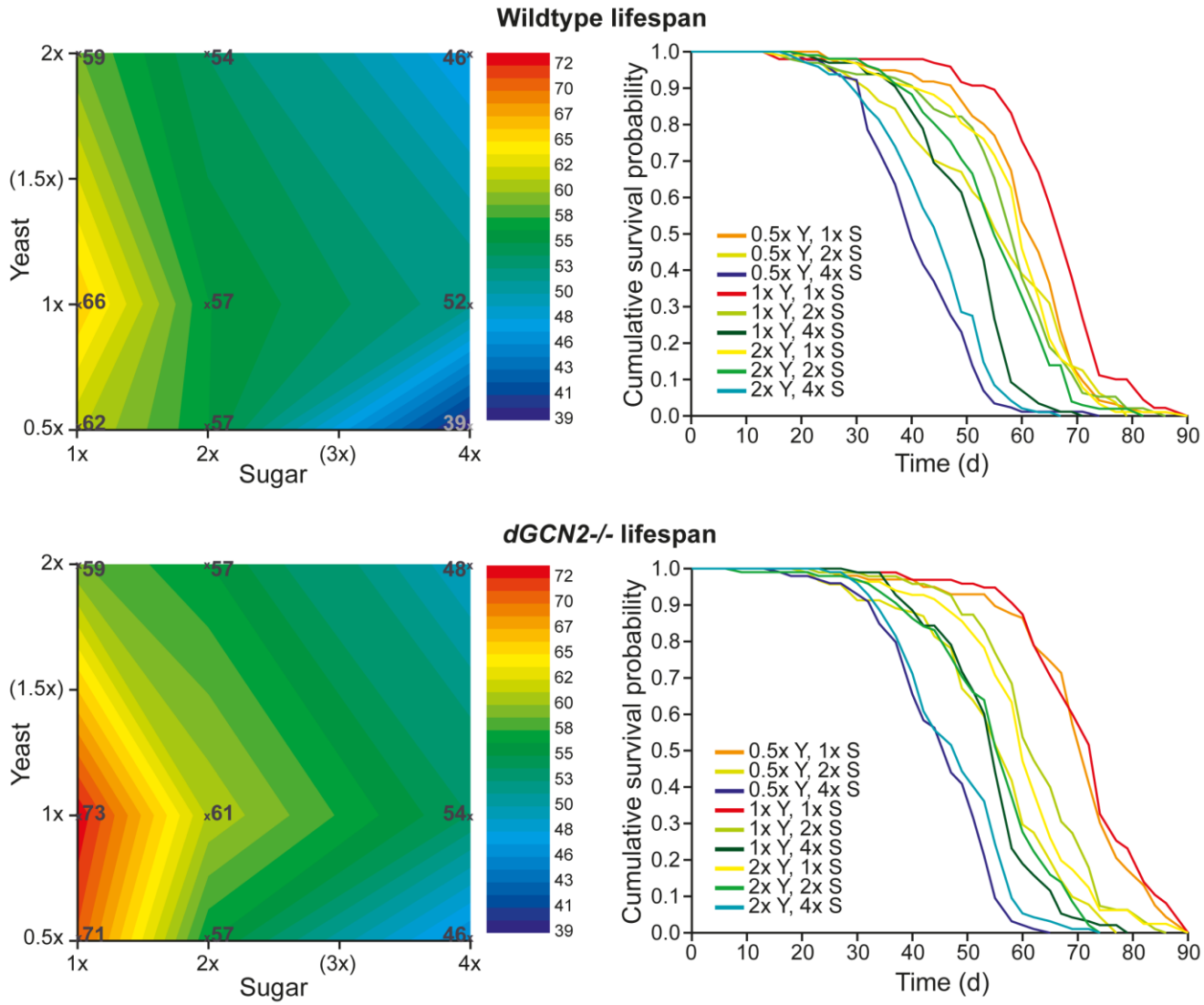


Figure 4.3. The effect of *dGCN2* deletion on the lifespan response to dietary yeast and sugar manipulation.

Lifespan was measured in wildtype and *dGCN2-/-* female flies maintained on 9 different diet regimes, varying in their yeast and sugar compositions. The median lifespans for each food condition were used to estimate diet response surfaces, with the values indicated on the plots. The corresponding survivorship graphs for each response surface plot are also shown. Cox Proportional Hazards analysis was carried out to determine the significance of the effects on lifespan of genotype, of diet, and of the interaction between them: genotype, $P<0.001$; yeast, $P<0.001$; sugar, $P<0.001$; yeast-sugar interaction, $P<0.001$; genotype-yeast interaction, $P=0.12$; genotype-sugar interaction, $P=0.01$; genotype-yeast-sugar interaction, $P<0.001$. n=150 flies per genotype per condition.

NSPs are often implicated as candidate mediators of the effect of DR to extend lifespan and reduce fecundity. Thus, we characterised the lifespans and egg-laying responses of *dGCN2* null, as well as *dGCN2* heterozygous, flies under DR conditions in order to establish a role, if any, for the GAAC pathway in mediating the lifespan and fecundity responses to DR. Other work has shown that DR is defined only by the yeast axis (Mair et al., 2005), and so we imposed DR by altering dietary yeast concentration, while keeping the concentration of sugar consistent at lx. The lifespan response of wildtype *Drosophila* has been shown to be typically tent-shaped (Chapman and Partridge, 1996), where lifespan peaks at an intermediate food concentration, beyond which higher concentrations are associated with shorter lifespan, and in the opposite direction lower concentrations fall into starvation. The egg-laying peak usually occurs at a concentration of food higher than that which maximises lifespan, but short of concentrations so high that neither trait improves (reflecting a general detrimental effect of overfeeding). In order to define the DR response range for *dGCN2* mutants, we sampled five different concentrations of yeast in our standard SY food media; 0.2x, 0.5x, 1x, 1.5x and 2x yeast, and we measured the effects on lifespan and egg-laying.

All three genotypes exhibited a tent-like response of lifespan to altered yeast concentration, with a decline in lifespan at the lowest and highest yeast concentrations, peaking at an intermediate yeast concentration (Figure 4.4). CoxPH analysis revealed independent effects of both food and genotype on lifespan ($P<0.001$ for both variables), as well as a significant interaction between genotype and food ($P=0.03$). The lifespan of wildtype flies was highest at 1x and 1.5x yeast, equally, while the lifespan of *dGCN2* null flies peaked at 1.5x yeast. *dGCN2* heterozygous flies were equally long-lived at 0.5x, 1x and 1.5x yeast. Noteworthy is that lifespan was extended by *dGCN2* deletion on both 1x and 2x yeast, representing DR and full-feeding (FF) treatments respectively. In fact, *dGCN2* null flies were longer-lived than wildtype flies on all concentrations of yeast tested. The lifespan response of *dGCN2* heterozygous flies were intermediate to wildtype and *dGCN2* null flies.

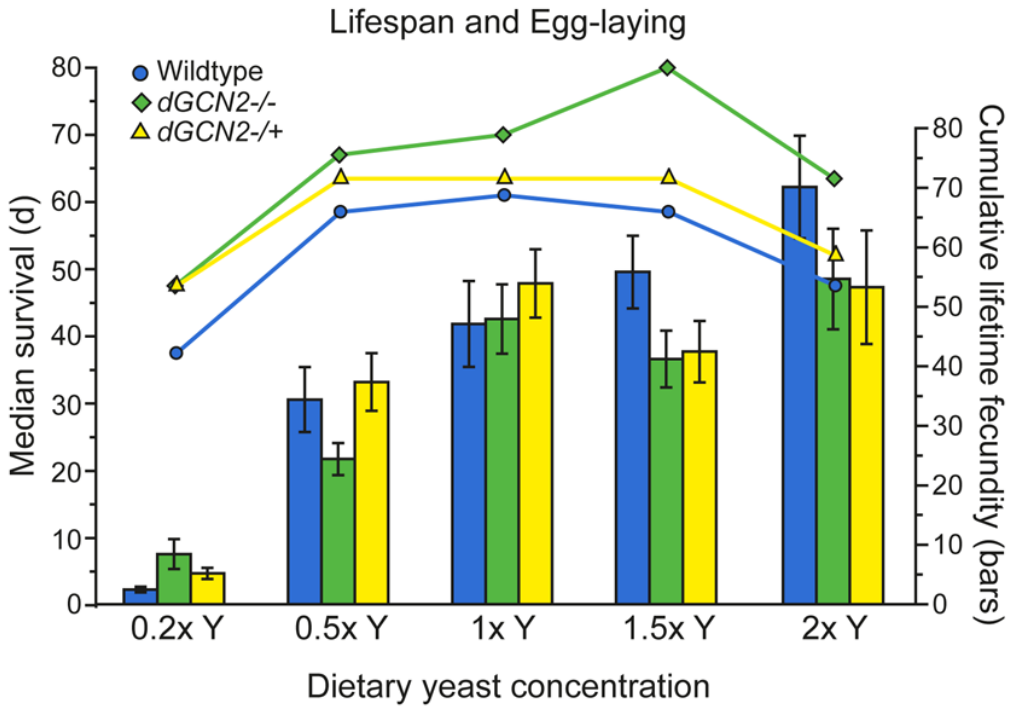


Figure 4.4. Dietary restriction (DR) lifespan tents and corresponding egg laying for wildtype, *dGCN2* null and *dGCN2* heterozygous flies.

Points (joined by lines) represent the median lifespans, and bars represent the average number of eggs laid per female per day with errors representing the s.e.m. All strains exhibited a tent-shaped response to DR with the lowest median lifespan values at 0.2x and 2x yeast concentrations, and peak values at 1x and 1.5x yeast for wildtype flies, at 0.5x, 1x and 1.5x yeast for *dGCN2-/+* flies, and at 1.5x yeast for *dGCN2-/-* flies. For all strains, female fecundity showed an incremental increase with yeast concentration. Cox Proportional Hazards analysis for lifespan, and a linear model for egg-laying, were used to determine the significance of the effects on lifespan of genotype, of yeast concentration, and of the interaction between them: both lifespan and fecundity were significantly affected by yeast ($P<0.001$ for both traits). Genotype affected lifespan ($P<0.001$), but not egg-laying ($P=0.20$). There was a significant interaction effect between genotype and yeast for lifespan ($P<0.001$), but not for egg-laying ($P=0.29$). n=150 flies per condition per genotype. n=150 flies per genotype per condition.

Egg-laying increased with yeast concentration ($P<0.001$, linear model, Figure 4.4), and despite apparent differences between genotypes, there was no significant difference ($P=0.20$, linear model). There was no interaction effect of genotype and food on egg-laying ($P=0.29$, linear model). Together, these data indicate that altered *dGCN2* activity modifies the lifespan response to DR, but not the egg-laying response.

4.2.3. The role of the GAAC pathway in mediating lifespan responses to single EAA deficiency

In yeast, GCN2 activity is stimulated by the presence of uncharged tRNAs (Wek et al., 1995). It is possible that GCN2 binds different tRNAs with different affinities, and in turn this may affect downstream effectors differentially, and potentially resulting in different physiologies. Comparably, the TOR pathway has been shown to be activated more strongly by some amino acids than others. Namely, leucine has been shown to be an especially potent activator of the TOR pathway (Proud, 2002). This prompted us to investigate the effect of more specific nutritional manipulations on lifespan. We selected four essential amino acids (EAA) - one from each functional group; namely arginine, tryptophan, methionine and leucine, representing cationic, aromatic, sulphur-containing, and branch-chained groups, respectively. We omitted each of these EAAs singly from defined media food and measured the lifespans of *dGCN2* null flies upon treatment on each of these deficient conditions. All four EAA omission conditions were detrimental to both wildtype and *dGCN2* null flies compared to a complete food medium containing all EAAs ($P<0.001$ for all comparisons, long-rank test, Figure 4.5a). *dGCN2* null flies were generally more sensitive to EAA deficiency compared to wildtype flies, even though they were longer-lived on a complete food medium ($P=0.05$, log-rank test). The degree of detriment caused by the single EAA deficiencies was different for each EAA, and also different for each genotype; *dGCN2* null flies were more sensitive to arginine, leucine and tryptophan omission compared to wildtype flies ($P<0.001$ for all comparisons, log-rank test). Interestingly, however, *dGCN2* null flies were more resistant than wildtype flies to methionine deficiency ($P<0.001$, log-rank test). Together, our data indicate a role for dGCN2 in regulating survival under conditions of EAA deficiency, particularly methionine deficiency.

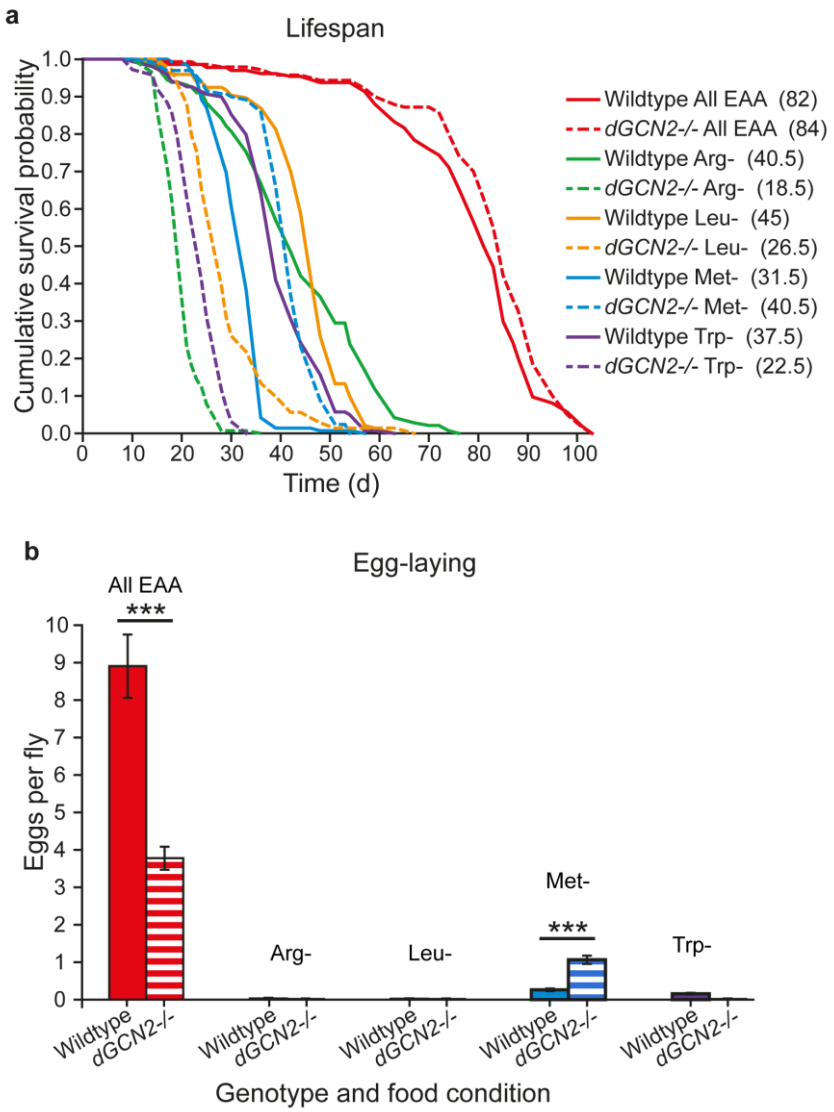


Figure 4.5. The effect *dGCN2* deletion on lifespan and egg-laying under conditions of single essential amino acid (EAA) deficiency.

(a) Both wildtype and *dGCN2-/-* female flies had significantly reduced lifespans when either arginine (arg), leucine (leu), methionine (met) or tryptophan (trp) are omitted from the diet ($P<0.001$ for all comparisons, long-rank test). *dGCN2-/-* flies were more sensitive than wildtype flies to single EAA deficiency for all EAAs tested ($P<0.001$) except in the case of met deficiency when they were more resistant than wildtype flies ($P<0.001$). Median lifespans (days) are quoted in brackets on the graph. n=150 per genotype per condition. **(b)** *dGCN2-/-* flies laid fewer eggs than wildtype flies when maintained on a complete food medium ($P<0.001$, Wilcoxon rank-sum test). Both wildtype and *dGCN2-/-* flies had significantly reduced egg-laying when either arg, leu, met or trp were omitted from the diet ($P<0.001$ for all comparisons). Wildtype and *dGCN2-/-* flies both laid equally few eggs on all single EAA omission conditions, except in the case of met deficiency when *dGCN2-/-* flies laid significantly more eggs than wildtype flies ($P<0.001$). Egg counts were made at 8 days of food treatment. n=100 per genotype per condition.

Food type and genotype also had a significant effect on egg-laying independently (P<0.001 for both, linear model, Figure 4.5b), as well as interactively (P<0.001, linear model). Under all four single EAA-deficient conditions, both wildtype and *dGCN2* null flies had dramatically lower egg-laying compared to that on the complete medium. However, *dGCN2* null flies laid more egg-laying when deprived of methionine compared to the other EAA deficiencies tested (P<0.001 for all comparisons, Wilcoxon rank-sum test), and this was significantly higher than methionine-deprived wildtype flies (P<0.001, Wilcoxon rank-sum test), which is particularly noteworthy given that wildtype flies laid more than double the number of eggs on a complete medium compared to *dGCN2* null flies (P<0.001, Wilcoxon rank-sum test). Together, these data suggest that dGCN2 deficiency confers some protection against the detrimental effects on lifespan and egg-laying of methionine deficiency specifically.

4.2.4. Lifespan correlated phenotypes of dGCN2 null flies

In chapter 3 we identified *Drosophila* phenotypes that correlated with DR-induced lifespan extension. We found that increased triacylglyceride (TAG) fat levels, and increased resistance to starvation were characteristics of long-lived DR flies. We assayed *dGCN2* null flies, which are longer lived than wildtpye flies under both DR and full feeding, for these DR-correlated phenotypes. We measured TAG levels of flies following a 7-day treatment period on the nine combinations of yeast and sugar concentration ratios used for lifespan. We generated a heat map to illustrate these findings (Figure 4.6a). *dGCN2* null flies exhibited the highest levels of TAG when the concentration of sugar was higher relative to that of yeast (0.5x yeast: 4x sugar), and the lowest TAG levels were evident in the reverse scenario where the concentration of yeast was higher relative to sugar (2x yeast: 1x sugar). Wildtype flies, however, showed a very different TAG profile across this nutritional space, displaying a peak at 0.5x yeast: 1x sugar, with no apparent increase or maintenance of this high TAG across increasing sugar concentrations at this level of yeast. Analysing this data using a linear model revealed a significant effect on TAG of both food and genotype independently (P>0.001 for both variables), as well as an interaction between the two (P<0.001). For the conditions that represent DR (1x yeast: 1x sugar) and FF (2x yeast: 1x sugar) there was no difference in the levels of TAG between wildtype and *dGCN2* null flies (DR, P=0.30; FF, P=0.80; t-test).

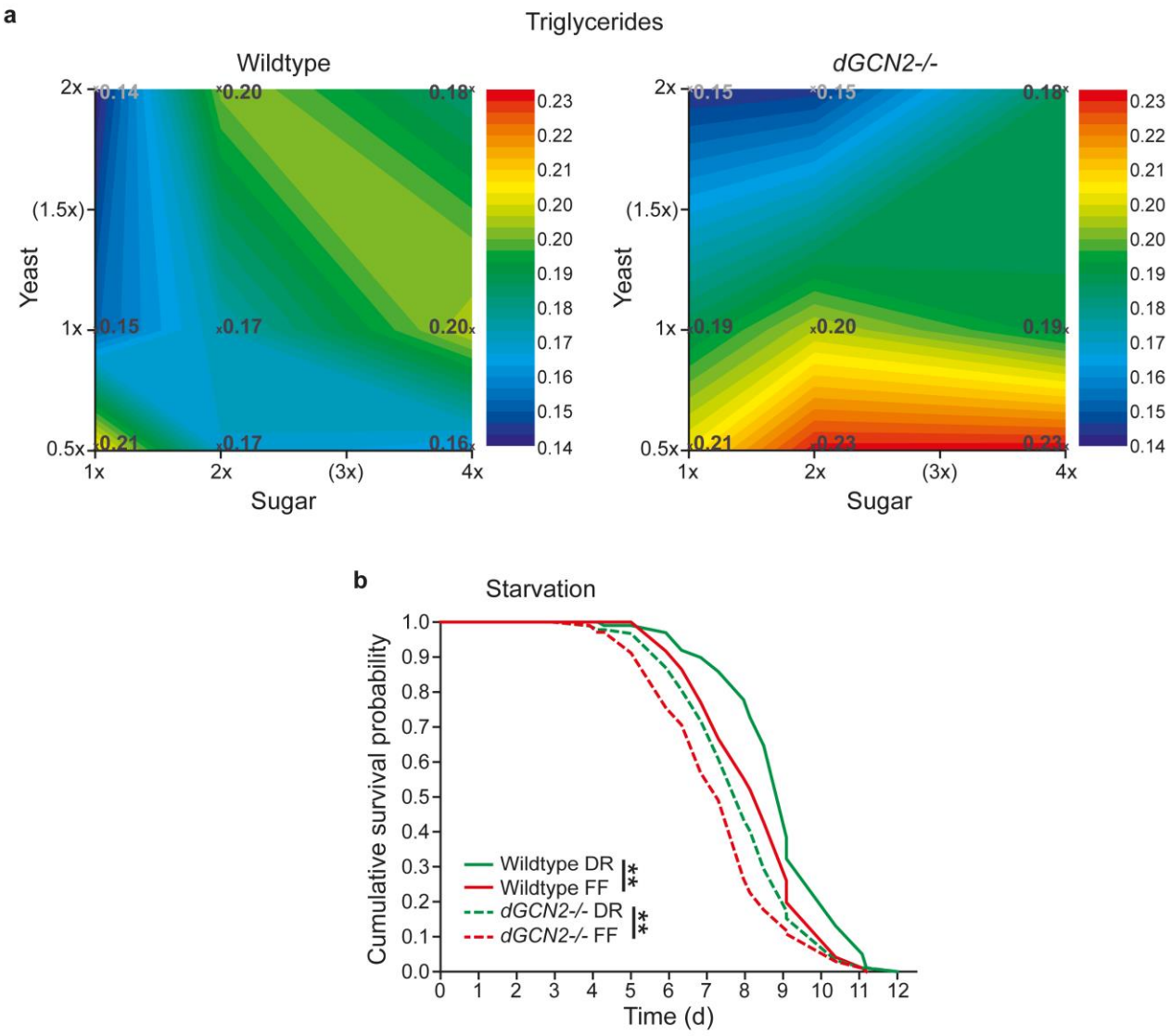


Figure 4.6. Diet response surfaces for triacylglyceride (TAG) levels and the effect of *dGCN2* deletion on starvation stress resistance.

(a) Levels of TAG were determined in wildtype and *dGCN2-/-* female flies after 7 days of treatment on 9 different diet regimes, varying in their yeast and sugar compositions. The average TAG content (µg per fly) for each food condition was used to estimate a diet response surface. These values are indicated on the plots. Statistical analysis using a linear model suggests a significant effect of genotype and food type, both independently and interactively, on TAG ($P<0.001$ for all comparisons). **(b)** There was no difference in the response of dietary restricted (DR) and fully fed (FF) wildtype and *dGCN2-/-* flies to starvation – for both genotypes, DR-treated flies were more resistant to starvation than FF flies ($P=0.001$ for both genotypes, log-rank test). Median survival (days): wildtype DR=8.8, wildtype FF=8.3, *dGCN2-/-* DR=7.6, *dGCN2-/-* FF=7.1). n=105 per genotype per condition.

We tested the ability of *dGCN2* null flies to resist starvation stress following pre-treatment on either DR or FF food. Like wildtype flies, *dGCN2* null flies pre-treated on DR food were more resistant to starvation than those pre-treated on FF food (P=0.001 for both genotypes, log-rank test, Figure 4.6b). Both DR and FF *dGCN2* null flies were less resistant than wildtype flies to starvation stress (P<0.001 for comparisions of both food conditions, log-rank test).

Our data suggests that the effects of *dGCN2* deletion on lifespan under DR conditions cannot be attributed to TAG levels, or the ability to resist starvation stress.

4.2.5. Testing for interaction between the dTOR and GAAC pathways

The TOR pathway interacts with other NSPs, such as IIS, through a complex and extensive web of interactions (for review, see Grewal, 2009). To test whether there is any cross-talk between the dTOR pathway and the GAAC pathway to alter lifespan, we treated *dGCN2* flies with rapamycin, a pharmacological inhibitor of dTOR, and assessed their survival under DR conditions. We previously showed that rapamycin rescues the detrimental effects of EAAs on by dTOR downregulation (chapter 3), and we have used this same paradigm to test whether *dGCN2* null flies are also sensitive to EAA supplementation and whether this can be rescued with rapamycin treatment. Indeed, EAA supplementation significantly decreased the lifespan of *dGCN2* null flies from the level of DR, and this was rescued by the addition of rapamycin, as for wildtype flies (Figure 4.7a). CoxPH analysis of the lifespan data confirms that there is no interaction between genotype and food (P=0.42), despite significant effects on lifespan of genotype and food independently (P<0.001 for both variables).

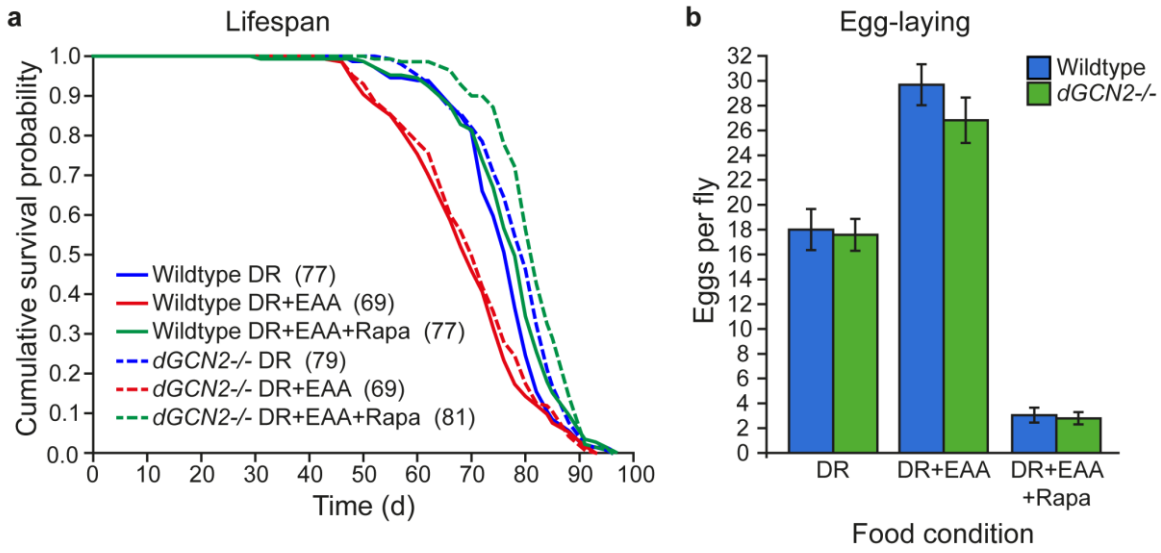


Figure 4.7. The lifespan and fecundity effects of rapamycin treatment on *dGCN2* null flies.

(a) Both wildtype and *dGCN2-/-* female flies maintained on dietary restricted food supplemented with the essential amino acids (DR+EAA) had significantly shorter lifespans compared to flies maintained under DR, and for both genotypes rapamycin addition to the DR+EAA condition (DR+EAA+Rapa) rescued lifespan to the level of DR. Cox Proportional Hazards analysis, used to determine the significance of the effects of genotype and food treatment on lifespan, confirmed a significant effect of food on lifespan ($P<0.001$), and also an effect of genotype ($P<0.001$), but no interaction effect of genotype and food ($P=0.42$). Median lifespans (days) are quoted in brackets on the graph. n=150 per genotype per condition.

(b) There was no difference in egg-laying between *dGCN2-/-* and wildtype flies on any of the food conditions. For both genotypes, EAA-supplemented flies laid more eggs than DR flies and rapamycin addition significantly reduced egg-laying. Statistical analysis using a linear model revealed a significant effect on egg-laying of food condition ($P<0.001$), but no effect of genotype ($P=0.29$), nor was there an interaction effect between food treatment and genotype ($P=0.57$). Egg counts were made at 8 days of food treatment. n=100 per genotype per condition.

Also in a similar manner to wildtype flies, EAA supplementation of DR food boosted the egg-laying of *dGCN2* null flies, and this was dramatically reduced upon additional rapamycin treatment ($P<0.001$ for the effect of food condition on egg-laying; $P=0.29$ for the effect of genotype on egg-laying; linear model; Figure 4.7b). This response of egg-laying to food condition was not significantly altered by *dGCN2* deletion ($P=0.57$, linear model). Together, these data suggest that the lifespan and egg-laying effects seen for *dGCN2* null flies are not achieved through alteration of dTOR signalling.

4.3. Discussion

In this work we set out to establish a role for the GAAC nutrient signalling pathway in mediating the lifespan extending effects of DR. We tested the responses of *dGCN2* null flies to a range of nutritional manipulations. Our results implicate the GAAC pathway in mediating, at least partially, lifespan alteration under DR.

Flies lacking *dGCN2*, although still being long-lived under DR compared to when fully fed, exhibit a lifespan response 'tent' to a range of dietary yeast concentrations that differs from wildtype flies. While the lifespan response to DR was not completely abolished for *dGCN2* null flies, the absence of *dGCN2* conferred some effect on the way that lifespan changed with yeast dilution; *dGCN2* deletion shifted the response tent such that lifespan peaked at a higher yeast concentration compared to wildtype flies, effectively changing the range of foods qualifying as DR. Our data supports a role for *dGCN2* in playing a partial role in mediating the lifespan effects of DR. It would be interesting to learn whether GCN2 deletion could change the DR response for worms and rodents.

In order to understand the interaction between dGCN2-mediated lifespan regulation and DR-mediated lifespan regulation, we selected phenotypes that we had previously predicted to be coupled to lifespan extension under DR (chapter 3), namely, increased TAG levels and improved resistance to starvation. We characterised *dGCN2* null flies for these phenotypes. We found no evidence that *dGCN2* modified these phenotypic responses to DR, suggesting that *dGCN2* downregulation may not extend lifespan via the same mechanisms that DR might, if the phenotypic correlates of DR-induced longevity that we identified in chapter 3 are indeed causal. Alternatively, increased TAG and starvation resistance are not causal for longer life. *dGCN2* null flies still exhibit the same response to DR as wildtype flies, indicating that if fat is important for both lifespan and starvation resistance under DR, then it is likely that they are affected by different types of fat, and modification of *dGCN2* affects the type that governs the response to starvation. *dGCN2* null flies are equally as fat as wildtype flies when maintained on either a DR or a FF diet, yet they are significantly more sensitive to starvation than wildtype flies for both of these conditions. This may suggest that they have a defect in utilising stored fat for survival under starvation. Indeed, previous studies have implicated a role for GCN2 in the regulation of lipid metabolism; *GCN2* null mice, upon dietary leucine deprivation, are

unable to repress the expression of lipogenic genes and the activity of fatty acid synthase, and are unable to mobilise adipose lipid stores. As a result, they develop liver steatosis, an accumulation of fat in the liver (Guo and Cavener, 2007).

GCN2 has been shown to be involved in the protective effect of EAA restriction against ischemia reperfusion injury, a type of surgical stress, in a mouse model (Peng et al., 2012). It is therefore possible that similar protective responses occur in flies upon starvation, and this may account for the observation that dGCN2 null flies are sensitive to starvation stress. However, we would not expect that GCN2 offers globally heightened stress protection, since we found that *dGCN2*-deficient flies were more resistant to an immunological challenge compared to wildtype flies.

The *Drosophila* lifespan response to DR is often accompanied by decreased egg-laying with dilution of dietary yeast. This pattern was observed for both our wildtype and *dGCN2* null flies, indicating no effect of *dGCN2* deletion on egg-laying. If dGCN2 does indeed regulate the lifespan response to DR, then it is not achieved via altered egg-laying, which in turn implies that egg-laying is not intimately linked to lifespan under DR. In other words, that increased lifespan and decreased egg-laying are correlated effects of DR, but not necessarily functionally coupled to each other. Alternatively, the potential role of *dGCN2* to regulate lifespan is so subtle that any accompanying egg-laying effect does not manifest as a significant change. Further replication would resolve this issue.

The effect on lifespan of *dGCN2* heterozygous flies across the yeast dilution concentrations that we tested were largely intermediate to that for *dGCN2* null flies, as we might expect since *dGCN2* heterozygous flies have one functional copy of *dGCN2*, as opposed to two functional copies in wildtype flies and no copies in *dGCN2* null flies, perhaps suggesting a dose dependent effect for dGCN2 activity. This could be tested by measuring the phosphorylation of eIF2α as a quantitative readout of dGCN2 activity.

Loss-of-function mutations in yeast and mouse *GCN2* have no apparent phenotypic effect under standard dietary conditions. However, negative effects have been shown to manifest when the mutants encounter dietary imbalanced conditions. We found that *dGCN2* null flies had very similar development times to wildtype flies on the various dietary yeast concentrations that we tested, perhaps suggesting that dGCN2 is not involved in

developmental processes. We did, however, see a decrease in body weight for GCN2 null flies maintained on 0.2x Y, and this condition may be considered imbalanced since the yeast:sugar ratio is changed from that which is optimal for lifespan, egg-laying or most aspects of physiology. Given that the phenotypic manifestation of *GCN2* deficiency has been shown to arise under a dietary imbalance, it is possible that this is the case for the body weight phenotype that we have observed here.

Our data indicate a role for *dGCN2* in the regulation of lifespan responses to diet in *Drosophila*, satisfying the expectations of the GAAC pathway as a nutrient sensing/signalling pathway. Previous studies have shown that *GCN2* null animals are more sensitive, compared to wildtypes, to nutrient stress (Anthony et al., 2004; Marion et al., 2011; Rousakis et al., 2013). Interestingly, and counter to this, *dGCN2* null flies are longer lived than wildtype flies on the lowest yeast concentration that we tested (0.5x Y), which either means that this concentration of yeast was not low enough to qualify as a condition of nutrient stress, or that in the context of dGCN2 signalling a nutrient stress is defined as an imbalance of the constituent nutrients relative to each other, which we do not consider to be the case in our 0.5x Y condition since it is just a diluted form of the complete medium. This suggests that dGCN2 is activated by the relative deficiency of one or more specific nutritional components – most likely an amino acid(s) since it is known that GCN2 is activated by uncharged tRNAs. Indeed, we find support for this hypothesis in our observation that *dGCN2* null flies have heightened sensitivity to omission of a single EAA from the diet, compared to wildtype flies, even though *dGCN2* null flies are longer-lived than wildtype flies on a complete food medium. Our data also indicate that this response is specific to a subset of amino acids, since we did not observe the same relative sensitivity of *dGCN2* null flies to methionine deficiency.

Unlike for the other EAAs tested, *dGCN2* deletion appears to attenuate the detriment to lifespan conferred by methionine deficiency, which implies that in a wildtype fly dGCN2 functions to sensitise the fly to methionine deficiency. *dGCN2*-deficient flies also lay more eggs than wildtype flies upon methionine deficiency. The evolutionary selection of a mechanism that decreases lifespan and egg-laying in the face of methionine deficiency seems counter intuitive. However, it may have evolved as a protective measure, perhaps serving to alert the animal to the dietary insufficiency in its environment, thereby prompting the pursuit of a more sustainable food source. In chapter 5 we present data

supporting the hypothesis that flies show aversion to deficient food sources, presumably as a mechanism to increase the likelihood of subsequent foraging for an alternative food source in the environment.

The TOR and IIS nutrient signalling pathways, although have distinct constituent proteins, have overlaps within their extended networks, and we hypothesised that the GAAC pathway may also lie within this web of interaction. However, we found no evidence to suggest an interaction between the GAAC pathway and the dTOR pathway. Despite our findings, studies in yeast have shown that rapamycin treatment leads to GCN2 activation (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003), and a recent study in *C. elegans* suggests that the GAAC pathway interacts with the TOR pathway to regulate survival under nutrient stress (Rousakis et al., 2013). Further work to characterise the relationship between the GAAC and TOR pathways in *Drosophila* would benefit from molecular analysis of downstream readouts of both dGCN2 and dTOR, such as eIF2α phosphorylation and S6K phosphorylation, respectively. Additionally, generating double mutant fly lines that have deficient *dGCN2* and *dTOR* activity could be useful to study the reciprocal effects of these pathways.

Chapter 5 - Molecular regulation of feeding behaviour

5.1. Introduction

An essential physiological objective for all organisms is to monitor and regulate their nutritional intake to support requirements for processes such as growth, reproduction, metabolism and general physiological maintenance. This regulation seeks not only to optimise energy balance, but also to optimise the balance of the macronutrients relative to each other. In addition, dietary components such as vitamins, minerals, cholesterol and fibre, which do not have a significant energetic value, may still play important roles in regulating metabolic and other physiological processes, and their intake must be regulated.

Animals need to be able to sense and adapt to encounters of sub-optimal nutritional conditions to avoid malnutrition, and they also need to be able to recognise when they are fully nourished to avoid the harmful effects of overeating. How an organism's internal nutritional state is communicated to influence its feeding choices is a question of great interest, and several studies have sought to elucidate the mechanisms that dictate the behavioural adaptations through which animals attain and maintain a nutritional optimum. One of the major drivers of brain evolution is thought to be foraging, and correlations have been noted between an animal's ecological condition and their brain size. For example, fruit-eating spider monkeys are considered to be more intelligent than the leaf-eating howler monkeys, and this has been attributed to the fact that spider monkeys have to forage more extensively to find fruits, which are less abundant than leaves (Milton, 1993).

The physiological state of an organism can change in response to many factors. For example, with the dietary environment, in response to biological processes such as development, reproduction and ageing, and in the face of environmental stressors such as starvation, infection and toxicity. It is likely that the physiological status of an animal will influence how it proceeds to feed in order to optimise survival for that physiological state.

An abundant literature suggests that animals carefully regulate their dietary protein intake and this has been demonstrated experimentally with dietary manipulations both in the

context of changes in protein quantity and protein quality. Changes in absolute protein quantity have been shown to affect feeding in rats, such that diets high in protein suppress feeding, diets moderately low in protein increase food intake, and diets with insufficient amounts of protein suppress feeding (Bensaïd et al., 2003; Du et al., 2000; Peters and Harper, 1985). When given the choice, rats readily abandon high protein food sources for the control diet (Peters and Harper, 1984). The hyperphagic response of rats fed a moderately low protein diet is thought to be due to an effort to increase protein intake (White et al., 1994). However, for food sources with insufficiently low amounts of protein, where hyperphagia is not able to overcome the protein deficit, rats were found to abandon the food and reduce their intake (Du et al., 2000). Moreover, the hyperphagic effect on the moderately low protein diet, was more apparent in young rats compared to older rats, likely due to an increased demand for protein for growth in young rats (White et al., 2000). Similarly, growing pigs were shown to be able to differentiate between foods differing in their protein content and selected between these diets according to their changing demands for protein (Bradford and Gous, 1991). Birds bred for high muscle mass choose diets with a higher percent protein in comparison to control birds (Forbes and Shariatmadari, 1994). Similarly, rats subjected to chronic injections of growth hormone show a higher preference for protein (Roberts et al., 1995). In locusts, hunger for protein has been found to be the reason for swarming, during which they eat everything in their path, including other non-swarming locusts, with the aim of attaining a *protein intake target* (Simpson et al., 2006), which is the level of nutrient intake that satisfies an animal's optimal requirements and maximises its performance, where performance can be fecundity, survival, stress resistance, etc. The target intake is plastic, as protein demands change with changes in physiology. Locusts sated with protein stop swarming (Simpson et al., 2006), and injecting amino acids into the haemolymph of protein-deprived locusts reduces their feeding rate (Abisgold and Simpson, 1987).

There is also data to suggest that humans subconsciously regulate, to some extent, their ingested nutrient balance. However, food choice decisions for humans are influenced by a complex social context and by conscious preferences for certain tastes that could potentially override any nutritional requirements. Nevertheless, it has been shown that people regulate their food intake, and that they regulate protein intake more strongly than carbohydrate and fat (Martinez-Cordero et al., 2012; Simpson and Raubenheimer, 2005; Simpson et al., 2003). The protein leverage hypothesis proposes that people have a

dominant appetite for protein, and when a diet does not allow both the protein and carbohydrate intake targets to be achieved simultaneously, protein intake is prioritised and people will eat more of a food source with a low protein to fat/carbohydrate ratio in an effort to meet their protein intake target. It has been theorised that this is one of the main drivers of obesity, since the consequence of attaining a protein intake target in this way is an excess intake of fat and carbohydrate. A test of protein leverage in humans revealed that protein intake was maintained at a constant level across a range of diets containing different proportions of protein (Gosby et al., 2011). In this short-term study, subjects had ad libitum access to the fixed menus, which were designed to be of similar palatability and sensory quality in order to minimise any variability in food-choice attributed to taste. At the population level, there has been a progressive reduction of protein intake over the past few decades, and this has been associated with an increase in energy intake and obesity (Austin et al., 2011; Simpson and Raubenheimer, 2005), perhaps owing to economic pressures that encourage greater consumption of cheap sugar and fatty foods (Brooks et al., 2010), as well as the fact that these foods are generally considered to be more appetising (Austin et al., 2011; Simpson et al., 2003). Protein leverage has also been demonstrated in several other species, such as non-human primates (Felton et al., 2009), pigs (Kyriazakis et al., 1991), mice (Sørensen et al., 2008), fish (Raubenheimer et al., 2005) and insects (Raubenheimer and Simpson, 1997). In one experiment, rats that were given eight different imbalanced but complementary food pairings ingested a similar intake of both protein and carbohydrate (Simpson and Raubenheimer, 1997; Theall et al., 1984).

Metabolically, protein represents nitrogen, amino acids, and digested peptides, so which of these dietary components is being selected for by animals that regulate protein intake? Evidence indicates that rather than just aiming towards a bulk nitrogen target, animals specifically select amino acids. Animals display aversive behaviour towards amino acid -deficient or -imbalanced food sources and actively select more balanced foods when given the choice, even if the diets contain the same level of nitrogen. Rats appear to be able to detect imbalanced diets and avoid them, and will preferentially select a diet containing an adequate amount of a particular essential amino acid (EAA) over a diet that is deficient in that EAA. Similarly, they will select a diet with a balanced amount of all of the EAAs over a diet with excessively high amounts of one or more EAAs (Harper and Peters, 1989). Behavioural responses to manipulations of particular EAAs have been demonstrated for threonine (Gietzen et al., 1992; Leung et al., 1968), tryptophan (Mori et al., 1991),

isoleucine (Naito-Hoopes et al., 1993), methionine (Peng et al., 1975), histidine (Rogers and Harper, 1970; Sanahuja and Harper, 1962), lysine (Markison et al., 1999; Torii et al., 1987) and valine (Murphy and King, 1989). Rats stop eating an imbalanced food before satiation, usually within 20 minutes (Koehnle et al., 2003). This timeframe is too long to be attributed to taste, but is also too rapid to be explained by a transcriptional response. Lysine-deficient rats identified and readily ingested a bitter solution of a lysine and hydrochloric acid (HCl), which they preferentially selected within a choice of 15 other solutions, to replete their lysine stores (Mori et al., 1991; Tabuchi et al., 1991; Torii et al., 1996). After being fed a diet containing adequate lysine, the rats no longer opted for the lysine HCl solution (Mori et al., 1991). The same response has also been shown for histidine-deficient rats offered a histidine HCl solution (Rogers and Leung, 1977).

There seems to be some physiological mechanism that evaluates the balance of internal protein with feedback to regulate dietary protein/amino acid selection. However, it is not known whether animals feed for a specific limiting amino acid, whether they seek a balance of all amino acids, or whether they just avoid dietary imbalances. Moreover, it is not known whether this selection is based on an ability to directly detect these dietary components, or whether it is a secondary learned response that couples sensory cues with postingestive consequences (Morrison et al., 2012). Internal nutrient sensors may act by enabling effector neurons to directly sense the levels of circulating nutrients, or alternatively by employing hormones or other such nutrient-sensitive signals to relay information about nutrient status to the nervous system to elicit a behavioural response. The post-oral gastrointestinal (GI) tract expresses sweet, bitter, and umami taste receptors that can ‘taste’ specific nutrients (Raybould, 2008; Tsurugizawa and Torii, 2010), and given that the GI system co-ordinates the release of gut hormones, it is possible that dietary signals can influence the brain in this way. However, the evidence rules out the GI system as the chemosensor for dietary amino acid imbalance, and also rules out the involvement of smell and taste in this mechanism (Gietzen, 1993; Gietzen and Rogers, 2006; Gietzen et al., 1998; Harper et al., 1970; Rogers and Leung, 1973, 1977). Organs such as the liver, which is the site of protein metabolism, and skeletal muscle, which is the primary storage site for amino acids, may play significant roles in conveying information about amino acid metabolism to the brain, just as leptin from adipose tissue informs about internal energy stores.

How feeding behaviour is neurologically regulated is the subject of current investigation in the field. The first suggestion that the brain is involved in sensing dietary amino acid imbalance came from the observation in rats that following an amino acid-imbalanced meal, the levels of the limiting amino acid decreased in brain tissue as quickly as it did in the plasma (Peng et al., 1972). Moreover, amino acid-deficient rats infused with the limiting amino acid through the carotid artery showed a reduction in their feeding aversion to the imbalanced diet, whereas infusion through the jugular vein had little effect (Leung and Rogers, 1969). The anterior piriform cortex (APC) brain region in particular has been shown to play an important role in the feeding response to dietary amino acid imbalance. In rats, using brain-lesioning techniques to destroy brain areas associated with food selection and feeding, the chemosensor for EAA imbalance was identified to be located in the APC (Leung and Rogers, 1971). Rats with lesions in the APC no longer showed aversion to an amino acid imbalanced. This was also demonstrated in birds (Firman and Kuenzel, 1988). Moreover, amino acid-deficient diets deplete the APC of the limiting amino acid within 30 min (Koehnle et al., 2004). The accumulation of uncharged tRNAs, indicating low amino acid levels, in this brain region is thought to be responsible for the feeding response to EAA imbalanced diets, since microinjection of tRNA synthetase inhibitors into the APC of rats decreased food intake just 20 minutes after injection (Hao et al., 2005). Reciprocally, other studies have shown that microinjection of a limiting amino acid into the APC re-established a level of feeding to that on an EAA balanced diet (Beverly et al., 1990; Monda et al., 1997; Russell et al., 2003). Evidence for the involvement of the APC in nutrient sensing seems to be limited to the sensing of protein quality, rather than protein quantity – lesions of the APC which have been shown to prevent aversive behaviour towards EAA-deficient diets do not appear to prevent the aversion of rats to high protein food sources (Leung and Rogers, 1971). In one study in humans, stimulation of the dorsolateral prefrontal cortex, a brain area involved in 'executive functions', increased cravings for high-calorie diets (Lowe et al., 2014). Several neurophysiological pathways have also been implicated in subconscious food choice (reviewed in Cohen, 2008).

The specific search for a molecular mechanism of behavioural aversion to amino acid imbalanced foods has led to interest in the potential roles of nutrient sensing pathways (NSP), the three best characterised of which are the general amino acid control (GAAC) signalling pathway, the target of rapamycin (TOR) signalling pathway, and the

insulin/insulin-like growth factor signalling (IIS) pathway (see chapter 1.3). The GAAC pathway has attracted much interest as a potential control mechanism of dietary intake since mammalian GCN2 directly binds and is activated by uncharged tRNAs (Hao et al., 2005). Many reports indicate that GCN2 is involved in the detection of protein quality, such that *GCN2* null animals are insensitive to EAA imbalance in comparison to their wildtype controls, and unlike wildtypes they do not display an aversion to these imbalanced foods, likely due to the fact that they cannot detect the inadequacy in the food source. A food source deficient in threonine has been shown not only to be rejected by wildtype rodents, but also results in the increased phosphorylation of eIF2α, which is a downstream effector of GCN2 activity, in the APC. *GCN2* null mice do not reject a threonine-devoid diet, and also do not show the same increase in eIF2α phosphorylation (Gietzen et al., 2004; Hao et al., 2005; Maurin et al., 2005). The GAAC in the APC is activated in the requisite 20-minute period for the recognition of amino acid imbalance, and brain-specific deletion of GCN2 in mice prevented the aversion to amino acid-imbalanced diets (Maurin et al., 2005). IMPACT, an actin-binding protein, primarily expressed in the rat hypothalamic brain region, was shown to inhibit the activation of GCN2, and accordingly decreased phosphorylation of eIF2α (Pereira et al., 2005). The lack of IMPACT in the APC brain region is consistent with a role for GCN2 in the APC in sensing amino acid imbalances (Gietzen et al., 2007).

Work from *Drosophila* has shown that larvae exhibit an aversion phenotype on amino acid-deficient food, and normal feeding can be restored upon addition of the limiting amino acids to the food (Bjordal et al., 2014). This study also pinpoints the neurological basis of these observations to the dopaminergic neurons, and proposes that the dopaminergic circuitry negatively regulates feeding. Both reduction of dopaminergic neuronal signalling and dopaminergic-targeted *GCN2* downregulation attenuated the aversion phenotype on the amino acid imbalanced food, as did the knockdown of *ATF-4*, which lies downstream of GCN2 in the GAAC pathway. Conversely, targeted overexpression of GCN2 in the dopaminergic neurons resulted in the larvae exhibiting aversion to both control and amino-acid imbalanced food, and this aversion phenotype was also observed in adult flies with overexpression of GCN2 in the dopaminergic neurons.

The TOR pathway has also been implicated in the modulation of feeding behaviour (Ribeiro and Dickson, 2010; Vargas et al., 2010; Wu et al., 2005a), as has the IIS pathway (Britton et al., 2002; Wu et al., 2005b; Zhao and Campos, 2012). The TOR nutrient-sensing pathway regulates feeding via the sex peptide receptor (SPR) (Ribeiro and Dickson, 2010). Male and female *Drosophila* deprived of protein preferentially choose a yeast-containing food over a non-yeast food source, and in females this behaviour has been found to be dependent on mating status. This feeding decision in females relies on the binding of a sex peptide ligand, present in the seminal fluid of males, to the SPR in the neurons of the female reproductive tract. This process is regulated by the dTOR pathway, possibly functioning to signal the nutritional status of the fly, thereby matching the fly's feeding choices to its nutritional needs. TOR signalling has also been implicated in mediating the increased preference of female *Drosophila* for yeast following mating (Vargas et al., 2010). Virgin female flies overexpressing constitutively active *dS6K*, encoding a downstream effector of dTOR, had increased serotonin production in the neuronal tissue and exhibited an increased preference for yeast, which was not seen for mated females, suggesting a role for dS6K in mediating the post-mating dietary switch in flies. It has also been shown that overexpression of the *Drosophila* insulin-like peptides (DILPs) in the neurons of fasted larvae attenuates the hyperphagic feeding upon exposure to a sub-optimal food source, and that neuronal dS6K is involved in the regulation of this hunger-driven response (Wu et al., 2005a).

In rats, mTOR signalling in the hypothalamus brain region responds to nutrient availability to regulate food intake - leucine increases hypothalamic mTOR signalling resulting in an inhibition of food intake by 4 hours (Cota et al., 2006). Moreover, the satiety hormone leptin was shown to increase hypothalamic mTOR signalling, and inhibition of mTOR attenuates the ability of leptin to suppress appetite, suggesting a role for mTOR as a fuel sensor in the regulation of energy intake. However, despite its well described role in amino acid sensing, in particular leucine, there is no evidence to suggest that mTOR signalling plays a role in the detection of amino acid deficiency (Gietzen et al., 2007). Moreover, injection of the TOR inhibitor rapamycin into the APC of rats did not affect food intake of either an amino acid-deficient diet or a control diet (Hao et al., 2010).

Adult *Drosophila melanogaster* are composed mainly of post-mitotic tissue, and have little cell proliferative activity. Therefore, their nutritional requirements are mostly for egg-laying, physical activity and general somatic maintenance. Reproduction responds rapidly to nutrition, such that *Drosophila* exhibit an increase in egg-laying as well as an increase in re-mating frequency (Chapman and Partridge, 1996; Chippindale et al., 1993). In particular, egg-laying and lifespan are limited by dietary protein (chapter 3), (Chippindale et al., 1993; Mair et al., 2005; Piper et al., 2005). In this chapter we aim to characterise the feeding responses of *Drosophila* to different nutritional manipulations, starting at changes in the amount of total protein, to more specific alterations at the level of single EAAs. Using genetic fly models, we investigate the role of nutrient sensing pathways, such as the GAAC and IIS pathways, in mediating these behavioural responses to dietary change.

5.2. Results

5.2.1. The feeding responses of flies to alterations of dietary protein

To assess the potential role of protein in modifying fly feeding behaviour, we altered the concentration of the yeast component in our standard laboratory diet (SY food), which is the flies' only source of protein, to 0.2x, 1x and 2x. The highest level of feeding was seen on 1x yeast, with slightly lower feeding on 2x yeast, and very low feeding on 0.2x yeast ($P<0.001$, linear model; Figure 5.1a). The 0.2x yeast condition is severely yeast-compromised, as demonstrated by delayed larval development and low egg-laying (Figure 5.1b-c).

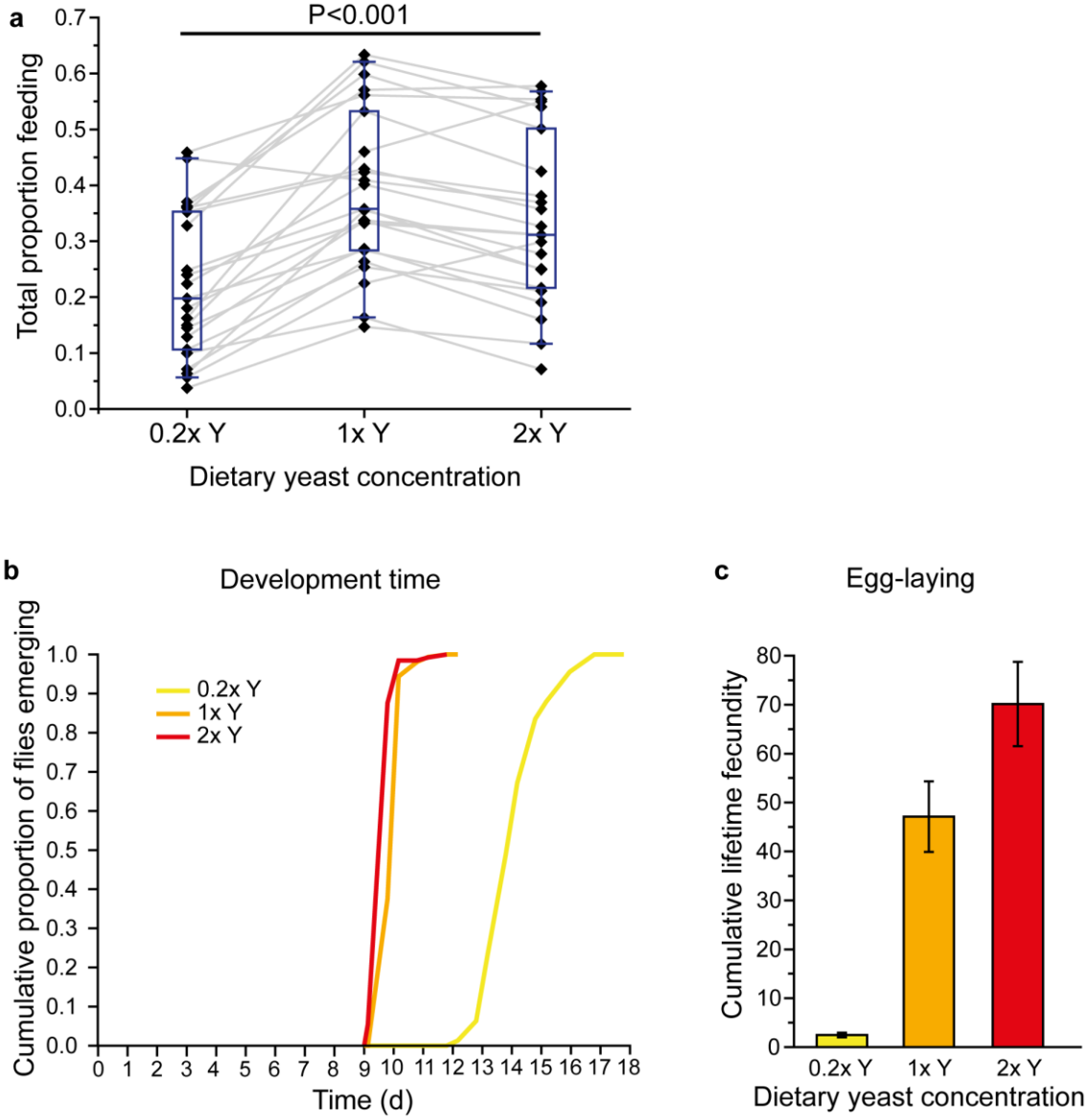


Figure 5.1. Feeding, development and egg-laying phenotypes of wildtype female flies on varying yeast concentrations.

(a) Flies fed at different rates across diets with different yeast concentrations: 0.2x, 1x and 2x yeast (of the standard SY diet) ($P<0.001$, linear model). The lowest level of feeding was observed on 0.2x yeast, with peak feeding on 1x yeast. Results from 23 independent trials are displayed. Scatter points represent the mean proportion feeding, with grey lines connecting cohorts in the same experiment. Box plots indicate the variation between trials. **(b)** The development time of flies increased with each dilution of yeast concentration of the food on which they were reared. Flies reared on the 0.2x yeast condition had dramatically delayed development times compared to those reared on food containing 1x and 2x yeast ($P<0.001$; Wilcoxon rank-sum test; days from 1st instar larvae). **(c)** Egg-laying decreased with lowered concentrations of dietary yeast ($P<0.001$ for each comparison; Wilcoxon rank-sum test). Errors represent the s.e.m.

Since yeast contains essential nutrients other than protein that could be responsible for the feeding phenotype we observed above, we further examined the effect of protein dilution using a holidic medium, a fully defined diet in which individual nutrients can be independently manipulated (Piper et al., 2014; see chapter 2.2.3). In this diet, protein is substituted for by a mixture of the 20 standard protein-encoding amino acids. We compared feeding in flies maintained on a complete food source containing all 20 amino acids to those maintained on food lacking all amino acids but whose composition was otherwise unchanged. We found that flies fed, on average, 66% less on food without amino acids ($P<0.001$, linear model; Figure 5.2a).

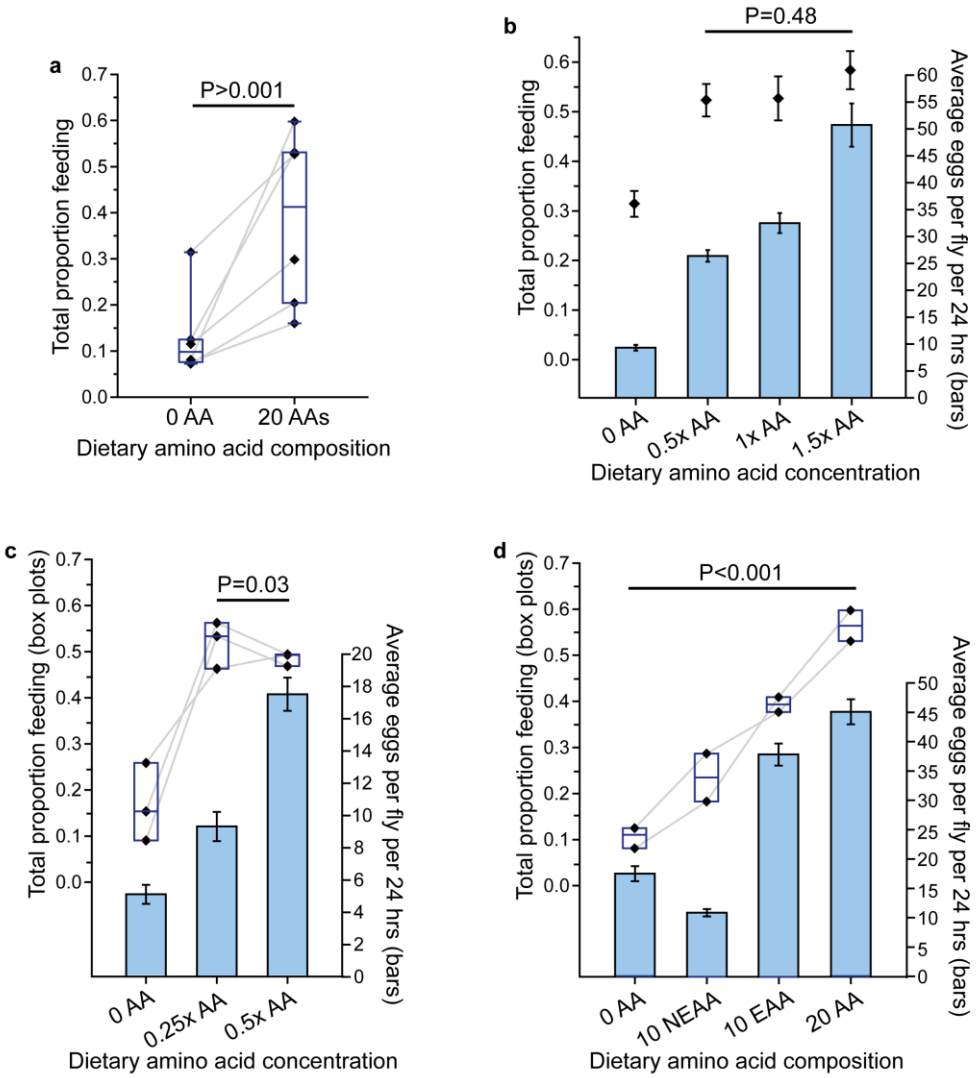


Figure 5.2. Feeding rates and egg-laying of wildtype female flies treated for 5 days on food media with manipulations of amino acid content.

(a) Flies fed at a higher rate on food containing all 20 amino acids (AA), compared to food containing no amino acids. Results from 6 independent trials are displayed. **(b)** Flies fed at a higher rate with addition of the 20 AAs in the food media, but there was no observable difference in feeding between diets containing 0.5x, 1x and 1.5x AAs (P=0.48, linear model, n=1 trial). Egg-laying was higher with each increase in AA concentration (P<0.001, linear model). **(c)** Flies fed at a higher rate on food containing 0.25x AA than on food containing 0.5x AA (P=0.03, n=3 independent trials), with an accompanying increase in egg-laying from 0.25x AA to 0.5x AA (P<0.001, linear model, n=1 trial). **(d)** Flies displayed a higher rate of feeding (n=2 independent trials) and egg-laying (n=1 trial) on food containing only the 10 essential AAs (EAA) than on food containing only the 10 non-essential AAs (NEAA) (P<0.001 for both traits; linear model). Scatter points represent the mean proportion feeding, with grey lines connecting cohorts in the same experiment. Box plots indicate the variation between trials. Bars represent mean egg-laying ±s.e.m from one representative trial. P-values for proportion feeding are displayed on the graphs (linear model).

We tested whether flies change their feeding upon exposure to different concentrations of amino acids (AA). We tested four different concentrations of a solution containing the 20 AAs: 0x, 0.5x, 1x and 1.5x. Flies showed no difference in feeding between foods containing 0.5x, 1x and 1.5x AAs ($P=0.48$, linear model; Figure 5.2b), but fed at lower levels on food containing no amino acids. Consistent with each group of flies ingesting equal amounts of an increasingly protein-rich diet, egg laying increased for each increase in dietary amino acid concentration ($P<0.001$, linear model). It is possible that the three amino acid -containing foods tested all had too high an amino acid concentration to elicit any difference in feeding between them, perhaps because at all three concentrations flies were feeding the maximum volume that they physically could. So we extended the range of conditions to include an additional, lower amino acid concentration; 0.25x. This time we were able to resolve a difference in the rate of feeding between 0.25x and 0.5x AAs, where flies fed slightly more on 0.25x AAs than on 0.5x AAs ($P=0.03$, linear model, Figure 5.2c), but laid fewer eggs ($P<0.001$, linear model). Given that the difference in the rate of feeding between flies on these two conditions was not very large, the more pronounced difference in egg-laying may be expected. This is similar to the feeding and egg-laying responses to 1x yeast and 2x yeast, where there is only a slight difference in feeding between flies on these two conditions, but a disproportionally larger difference in egg-laying, where flies fed 2x yeast produced more eggs than those fed 1x yeast (Figure 5.1a, c).

We then asked whether either the ten essential amino acids (EAAs) or the ten non-essential amino acids (NEAAs) could account for the lowered feeding observed when all 20 amino acids were removed. To test this, we separately omitted EAAs or NEAAs from the food and measured the feeding response. Flies fed 30% less on medium lacking NEAAs, compared to a complete amino acid medium, but EAA deficiency produced a more pronounced difference in feeding than NEAA deficiency, with flies fed an EAA-deficient diet feeding 58% less than those fed all 20 amino acids. In fact, flies fed 40% less on the EAA-deficient food compared to the NEAA-deficient food ($P<0.001$, linear model; Figure 5.2d). Moreover, egg-laying was significantly compromised when either the EAAs or the NEAAs were removed from the food compared to that of flies maintained on the complete medium ($P<0.001$; linear model). Similar to the effect on feeding, EAA-deficiency had the greatest effect on egg-laying, with 82% fewer eggs laid, compared to 20% lower egg-laying upon NEAA-deficiency. Interestingly, flies lacking the 10 EAAs, while still having

the 10 NEAAs, produced fewer eggs than flies fed a medium containing no amino acids at all, suggesting that disproportionate amounts of the EAAs relative to each other is less favourable for egg-laying than the absence of all amino acids, highlighting the importance of amino acid balance.

5.2.2. The feeding responses of flies to single EAA deficiency

We tested whether the difference in feeding could be further resolved at the level of a single amino acid. Since the feeding effect was greater for EAA alteration than it was for NEAA alteration, and because several rodent studies implicate the importance of EAAs in animal foraging behaviour (Morrison et al., 2012), we focussed on the EAAs for subsequent investigations. We selected 4 EAAs for testing, covering a range of biochemical functional groups; namely arginine, tryptophan, leucine and methionine, representing cationic, aromatic, branch-chained and sulphur-containing groups, respectively. We singly omitted these EAAs from the food source and measured the feeding response of flies to these deficient diets.

We found that flies had significantly lower feeding on diets lacking each of the four EAAs that we tested, compared to flies maintained on food containing all 20 amino acids ($P<0.001$ for all comparisons; linear model; Figure 5.3a-d). However, the magnitude of this feeding difference varied for each amino acid omission, with tryptophan omission producing the greatest difference in feeding, followed by arginine omission, then methionine omission, with leucine omission displaying the smallest difference in feeding.

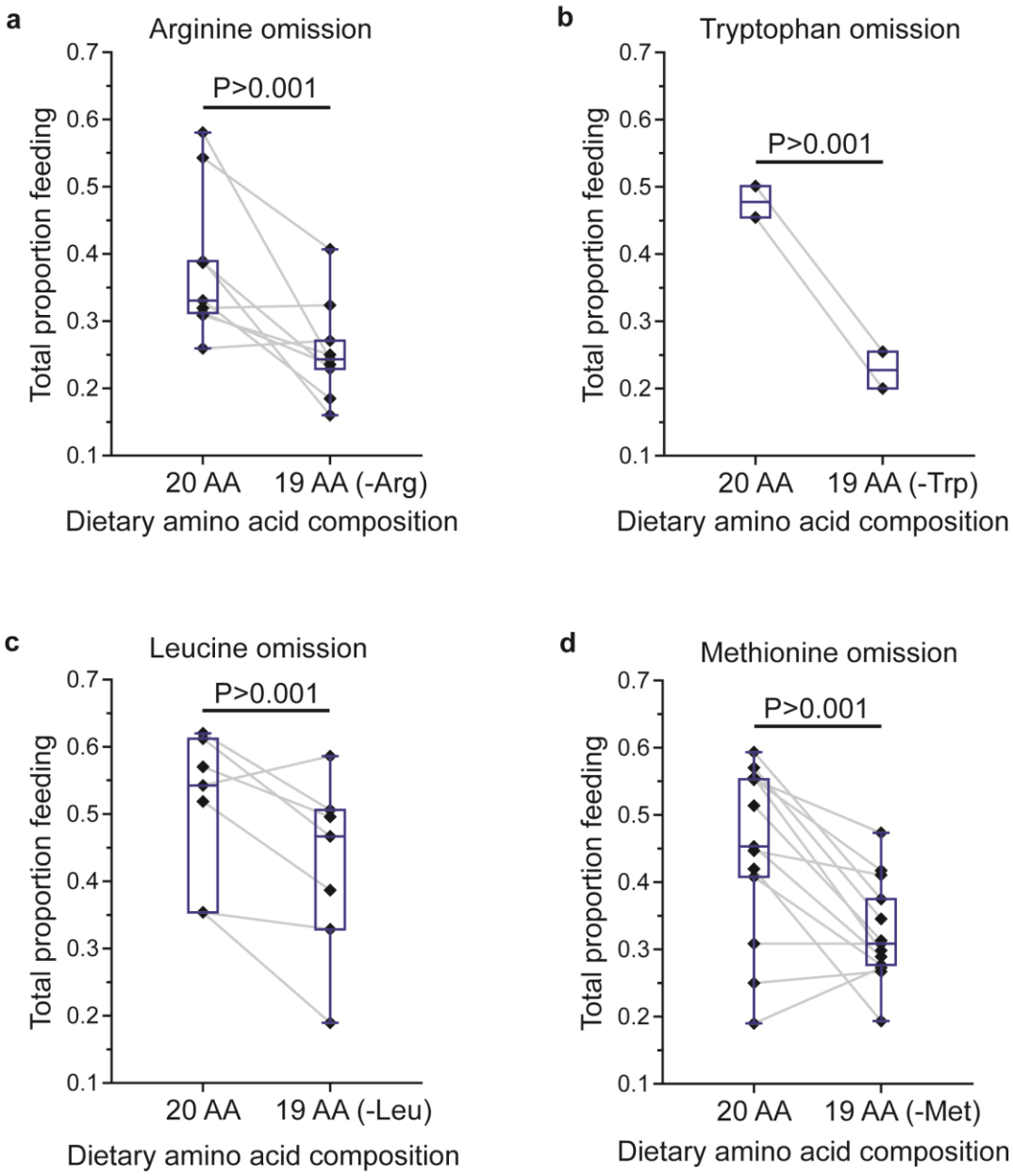
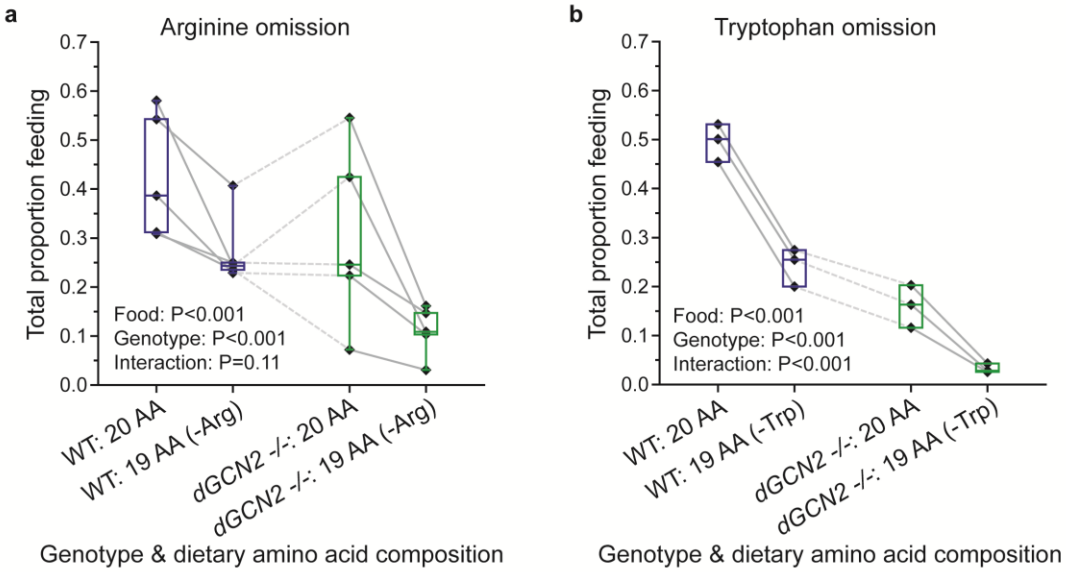


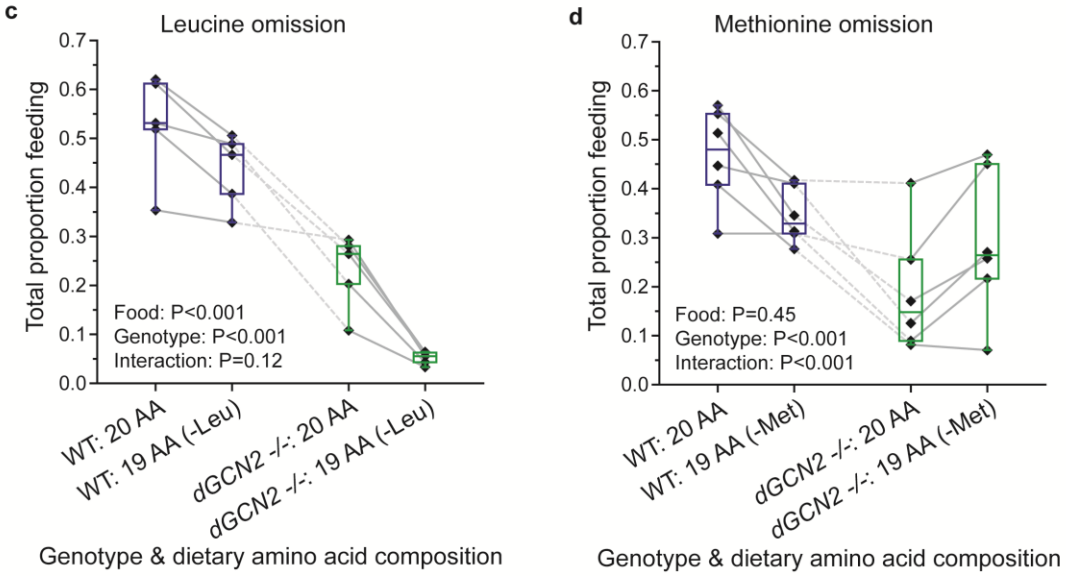
Figure 5.3. Feeding rates of wildtype female flies treated for 5 days on food media deficient in a single essential amino acid (EAA).

Omitting the EAAs **(a)** arginine (n=9 independent trials), **(b)** tryptophan (n=2 independent trials), **(c)** methionine (n=13 independent trials), and **(d)** leucine (n=7 independent trials) from a complete diet containing all 20 amino acids resulted in lower feeding in each case of the deficient diet. Scatter points represent the mean proportion feeding, with grey lines connecting cohorts in the same experiment. Box plots indicate the variation between trials. A linear model was used to assess the differences in proportion feeding, with P-values displayed on the graph.

5.2.3. The role of the GAAC pathway in mediating feeding responses to single EAA deficiency

We used *dGCN2* null flies to establish whether dGCN2 has a role in mediating the feeding responses to the single EAA dropout conditions tested earlier. We analysed these data using a standard linear model to establish an effect of food type on feeding, an effect of genotype, and an interactive effect between these variables, i.e. do *dGCN2* null flies respond to the single EAA dropout conditions in the same way that wildtype flies do? We combined data from various trials and accounted for this in our statistical model by assigning the experimental trial variable as a random effect. We found that across all single EAA omission groups tested there was a significant effect of genotype on feeding ($P<0.001$ for all comparisons, linear model; Figure 5.4a-d); *dGCN2* null flies exhibited generally lowered feeding across all conditions relative to wildtype flies. Similar to the response of wildtype flies, *dGCN2* null flies also exhibited lower feeding when either arginine, tryptophan or leucine were omitted from the diet, compared to their feeding on the complete food condition ($P<0.001$ for all comparisons, linear model). Unlike wildtype flies, however, there was no effect on feeding for *dGCN2* null flies of omitting methionine from the food (Figure 5.4d, $P=0.45$, linear model).


a
Arginine omission
Total proportion feeding
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Food: P<0.001
Genotype: P<0.001
Interaction: P=0.11
WT: 20 AA
WT: 19 AA (-Arg)
dGCN2 -/-: 20 AA
dGCN2 -/-: 19 AA (-Arg)
Genotype & dietary amino acid composition
b
Tryptophan omission
Total proportion feeding
Food: P<0.001
Genotype: P<0.001
Interaction: P<0.001
WT: 20 AA
WT: 19 AA (-Trp)
dGCN2 -/-: 20 AA
dGCN2 -/-: 19 AA (-Trp)
Genotype & dietary amino acid composition


c
Leucine omission
Total proportion feeding
Food: P<0.001
Genotype: P<0.001
Interaction: P=0.12
WT: 20 AA
WT: 19 AA (-Leu)
dGCN2 -/-: 20 AA
dGCN2 -/-: 19 AA (-Leu)
Genotype & dietary amino acid composition
d
Methionine omission
Total proportion feeding
Food: P=0.45
Genotype: P<0.001
Interaction: P<0.001
WT: 20 AA
WT: 19 AA (-Met)
dGCN2 -/-: 20 AA
dGCN2 -/-: 19 AA (-Met)
Genotype & dietary amino acid composition

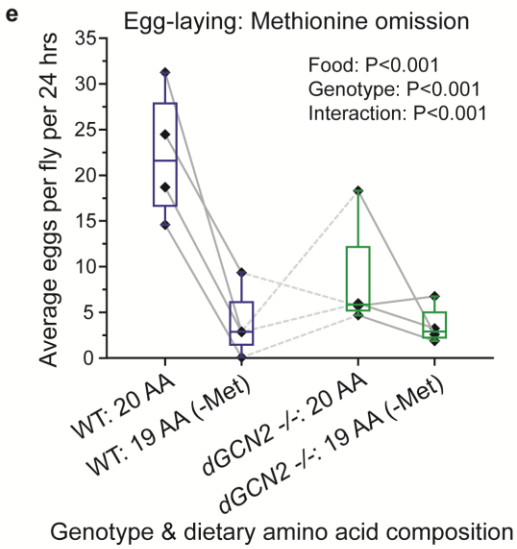

e
Egg-laying: Methionine omission
Average eggs per fly per 24 hrs
35
30
25
20
15
10
5
0
Food: P<0.001
Genotype: P<0.001
Interaction: P<0.001
WT: 20 AA
WT: 19 AA (-Met)
dGCN2 -/-: 20 AA
dGCN2 -/-: 19 AA (-Met)
Genotype & dietary amino acid composition

Figure 5.4. The effect of *dGCN2* deletion on the feeding rates of female flies in response to food media deficient in a single essential amino acid (EAA).

(a) *dGCN2* null flies responded in the same way as wildtype flies to a diet deficient in arginine (arg) (n=5 independent trials). **(b)** *dGCN2* deletion reduced the magnitude of the difference in feeding between a tryptophan (trp)-deficient diet and a complete diet (n=3 independent trials). **(c)** *dGCN2* null flies responded in the same way as wildtype flies to a diet deficient in leucine (leu) (n=5 independent trials). **(d)** *dGCN2* deletion completely attenuated the difference in feeding between a methionine (met)-deficient diet and a complete diet (n=7 independent trials). Scatter points represent the mean proportion feeding, with grey lines connecting cohorts in the same experiment. Box plots indicate the variation between trials. A linear model was used to assess the independent and interaction effects on feeding of food type and genotype, with the P-values displayed. **(e)** *dGCN2* deletion significantly altered egg-laying in response to met omission from a complete food medium (n=4 independent trials). Scatter points represent the mean egg-laying, with grey lines connecting cohorts in the same experiment. Box plots indicate the variation between trials. A linear model was used to assess the independent and interaction effects on egg-laying of food type and genotype, with the P-values displayed.

The interaction effect of genotype and each EAA omission on feeding was varied; *dGCN2* null flies did not respond any differently to arginine or leucine deficiency than wildtype flies (arginine, P=0.11; leucine, P=0.12, linear model), but there was a significant interaction effect in the cases of tryptophan and methionine omission (P<0.001 for both, linear model). Loss of dGCN2 resulted in an attenuation of the magnitude of the difference in feeding between the tryptophan-deficient medium and the complete medium. Interestingly, dGCN2 deficiency resulted in a reversal of the feeding response to dietary methionine omission, such that *dGCN2* null flies fed more on food lacking methionine. We measured egg-laying under these conditions to determine whether a there was a correlated egg-laying response. Both wildtype and *dGCN2* null flies had lower egg-laying when fed a methionine-deficient diet, compared to a complete diet (P <0.001 for both comparisons; linear model; Figure 5.4e). In light of the feeding response of *dGCN2* null flies on methionine-deficient foods, this egg-laying observation suggests an uncoupling of feeding and egg-laying responses to methionine deficiency. However, there was considerable inter-trial variability in the egg-laying response of *dGCN2* null flies to methionine deficiency, where some trials showed a decrease in egg-laying to varying degrees, while others showed no apparent difference between these conditions. Analysis of interaction between genotype and methionine deficiency across all trials suggests that, overall, loss of dGNC2 affects the egg-laying response to methionine deficiency (P<0.001; linear model).

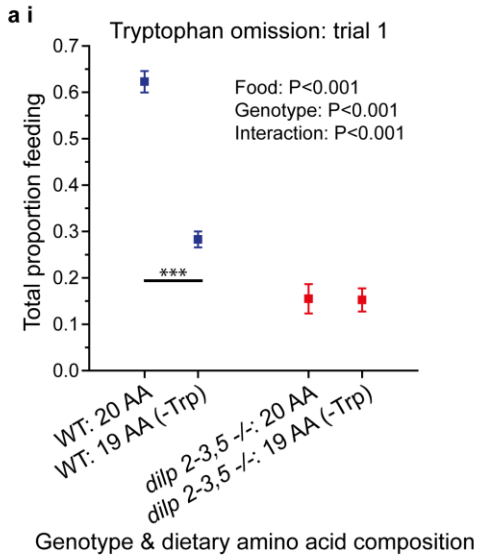
5.2.4. Testing a role for IIS in modulating the feeding response to single EAA deficiency

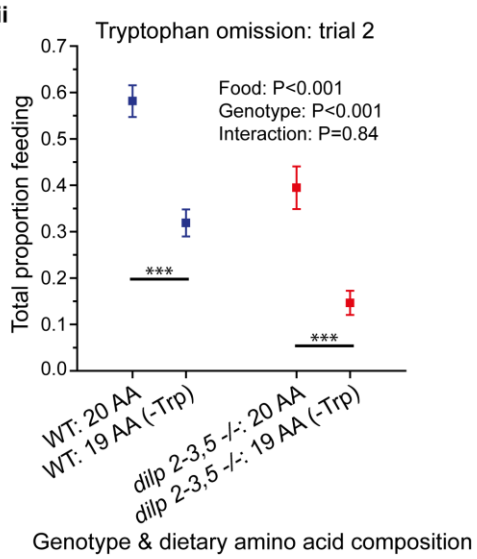
In addition to the GAAC pathway, other NSPs have the potential to modulate feeding in response to dietary change. To test whether the IIS pathway has a role in food-choice, we used flies with deletions of three of the eight *Drosophila* insulin-like peptide (DILP) genes, *dilp*2, *dilp*3 and *dilp*5 (Grönke et al., 2010). In one trial, deletion of the *dilp* genes attenuated the difference in feeding exhibited by wildtype flies on tryptophan-deficient and complete food media, such that *dilp 2-3,5* null flies fed at the same level on both foods (effect tests: food, P<0.001; genotype, P<0.001; food-genotype interaction, P<0.001; linear model; Figure 5.5ai). However, in another trial tryptophan omission resulted in a significant decrease in feeding, as seen for wildtype flies (effect tests: food, P<0.001; genotype, P<0.001; food-genotype interaction, P=0.84; linear model; Figure 5.5aii).

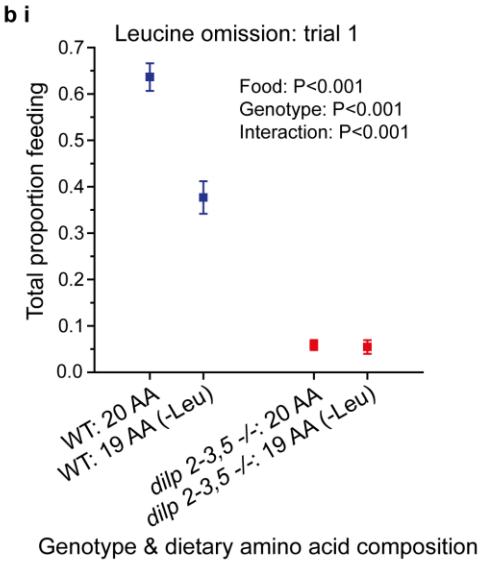
The same variability for *dilp2-3,5* null flies was also observed upon omission of leucine. In one trial there was no difference between *dilp2-3,5* null flies fed a complete diet or one lacking leucine (effect tests: food, P<0.001; genotype, P<0.001; food-genotype interaction, P<0.001; linear model; Figure 5.5bi), and in another trial *dilp2-3,5* null flies fed significantly less upon removal of leucine, like wildtype flies (effect tests: food, P<0.001; genotype, P<0.001; food-genotype interaction, P=0.34; linear model; Figure 5.5bii).

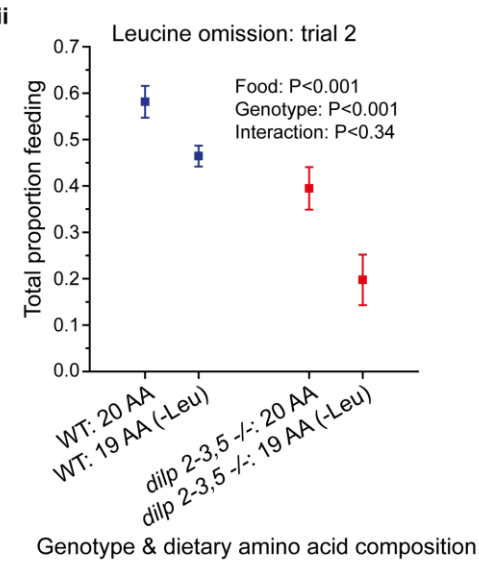
When we tested the feeding response of *dilp2-3,5* null flies to changes in dietary methionine we found no difference between a complete food medium and one lacking methionine, whereas wildtype flies did exhibit a difference in feeding on these conditions (effect tests: food, P<0.001; genotype, P<0.001; food-genotype interaction, P<0.001; linear model; Figure 5.5c). We have only conducted one trial testing the feeding response of *dilp2-3,5* null flies to dietary methionine manipulation, and as yet no trials have tested the effect of arginine omission for these flies.

In all cases, wildtype flies had significantly lower feeding on the EAA-deficient food compared to the complete food (P<0.01 for all comparisons; linear model). Given the variability we observed between trials in previous assays these values are likely to be a result of natural biological variation and characterisation of the behavioural response of IIS mutant flies to dietary EAA change requires further replication.


a i
Tryptophan omission: trial 1
Food: P<0.001
Genotype: P<0.001
Interaction: P<0.001
Total proportion feeding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
***
WT: 20 AA
WT: 19 AA (-Trp)
dilp 2-3,5 -/-: 20 AA
dilp 2-3,5 -/-: 19 AA (-Trp)
Genotype & dietary amino acid composition


ii
Tryptophan omission: trial 2
Food: P<0.001
Genotype: P<0.001
Interaction: P=0.84
Total proportion feeding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
***
***
WT: 20 AA
WT: 19 AA (-Trp)
dilp 2-3,5 -/-: 20 AA
dilp 2-3,5 -/-: 19 AA (-Trp)
Genotype & dietary amino acid composition


b i
Leucine omission: trial 1
Food: P<0.001
Genotype: P<0.001
Interaction: P<0.001
Total proportion feeding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
WT: 20 AA
WT: 19 AA (-Leu)
dilp 2-3,5 -/-: 20 AA
dilp 2-3,5 -/-: 19 AA (-Leu)
Genotype & dietary amino acid composition


ii
Leucine omission: trial 2
Food: P<0.001
Genotype: P<0.001
Interaction: P<0.34
Total proportion feeding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
WT: 20 AA
WT: 19 AA (-Leu)
dilp 2-3,5 -/-: 20 AA
dilp 2-3,5 -/-: 19 AA (-Leu)
Genotype & dietary amino acid composition

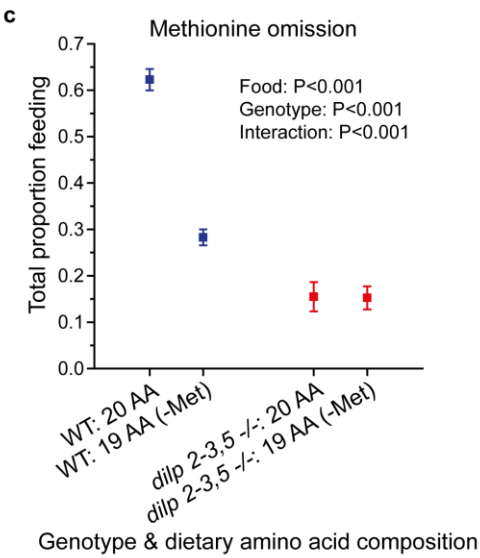

c
Methionine omission
Food: P<0.001
Genotype: P<0.001
Interaction: P<0.001
Total proportion feeding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
WT: 20 AA
WT: 19 AA (-Met)
dilp 2-3,5 -/-: 20 AA
dilp 2-3,5 -/-: 19 AA (-Met)
Genotype & dietary amino acid composition

Figure 5.5. The effect of *dilp2-3,5* deletion on the feeding rates of female flies in response to food media deficient in a single essential amino acid (EAA).

(a) i and **ii** represent independent trials of wildtype and *dilp2-3,5* null flies exposed to a complete food medium, and one lacking tryptophan (trp). In both trials, wildtype flies fed significantly less on trp-deficient food, but *dilp2-3,5* null flies had varied responses to trp deficiency between trials: in one trial (i) they fed at the same rate on a complete food medium and one lacking trp, while in a second trial (ii) they fed less on trp-deficient food.

(b) i and **ii** represent independent trials of wildtype and *dilp2-3,5* null flies exposed to a complete food medium, and one lacking leucine (leu). In both trials, wildtype flies fed significantly less on leu-deficient food, but *dilp2-3,5* null flies had varied responses to leu deficiency between trials: in one trial (i) they fed at the same rate on a complete food medium and one lacking leu, while in a second trial (ii) they fed less on leu-deficient food. **(c)** Wildtype flies fed significantly less on methionine (met)-deficient food compared to a complete food medium, while *dilp2-3,5* null flies fed at the same rate on a complete food medium and one lacking met. A linear model was used to assess the independent and interaction effects on feeding of food type and genotype, with the P-values displayed.

5.3. Discussion

We have shown that flies are able to discriminate between, and accordingly alter their behaviour, to diets of different nutritional values. Flies are sensitive to the presence or absence of even a single amino acid. We hypothesised that nutrient signalling pathways may have a role in regulating some of these behavioural responses to changes in the availability of dietary EAAs, and we have shown that, at least for methionine, the GAAC pathway is required.

EAA deficiency confers a greater effect on feeding than NEAA deficiency. It is unlikely that the feeding differences that we have observed between EAA and NEAA omission is the result of differences in the general nitrogen content of the food, since EAA or NEAA omission both result in a similar level of nitrogen loss from the diet used in these experiments (Piper et al., 2014). The difference in the level of feeding of flies maintained on these conditions is due to the identity of the amino acids themselves, and the implication is that fly feeding is largely motivated by the EAA, rather than the NEAA, content of the diet. Since the only source of EAAs is from the environment, we may expect that organisms have evolved adaptive behaviours that primarily seek out EAAs in the environment, more so than NEAAs which can be synthesised in the body. However, there is still an effect on feeding in manipulating dietary NEAAs, perhaps suggesting some precedent mechanism for seeking out total nitrogen. NEAAs may be costly to produce and therefore sought in the environment. Additionally, some EAAs serve as precursors for the biosynthesis of specific NEAAs, for example methionine is essential for the synthesis of the NEAA cysteine and the EAA phenylalanine serves as precursor for the NEAA tyrosine. Thus, reducing the levels of these NEAAs may indirectly create an EAA deficiency, and as such it can be expected that mechanisms that regulate NEAA ingestion may have been favoured for evolutionary selection.

One interesting observation that we made was that flies that were fed a medium containing no amino acids laid significantly more eggs than those fed a medium containing NEAAs but no EAAs. This is surprising, given that the latter food condition might be considered to be nutritionally more valuable than the former since it contains some amino acids, as opposed to no amino acids at all. The fact that the flies that were fed no amino acids laid more eggs than those given NEAAs only supports the notion that dietary amino acid

balance is as, if not more, important for egg-laying than dietary amino acid abundance. A signal indicating the presence of NEAAs without an accompanying EAA signal may indicate to egg-making mechanisms that there are insufficient resources to produce eggs, and so inhibit egg-laying. However, this latter explanation cannot account for the observed increase in egg-production when no amino acids are supplied. Alternatively, the presence of the NEAAs without the EAAs to balance/buffer may have been toxic to the fly, thereby compromising egg-laying.

A mechanistic question that has arisen from this work is whether the nutritional environment alters feeding behaviour to then in turn influence egg-laying (Figure 5.6, scenario a), or whether feeding behaviour changes as a response to the demand for egg production (Figure 5.6, scenario b). An alternative model is that feeding behaviour and egg-laying are influenced by the nutritional environment via distinct, parallel pathways, such that nutrients are signalled to regulators of egg-laying and feeding separately (Figure 5.6, scenario c). For most of the conditions we have tested, we observed a positive correlation between feeding and egg-laying, indicating that scenarios a and b are correct. Indeed, we would expect that egg-laying would change in response to feeding, since egg-laying potential is limited by the amount of nutrients ingested (Chapman & Partridge, 1996). However, *dGCN2* null flies, although had a higher feeding rate on the methionine-deficient diet compared to the complete diet, did not have an accompanying increase in egg-laying on the methionine-deficient diet, as would be predicted by scenarios a and b from the model in figure 5.6. Instead, it is likely that feeding and egg-laying are governed by separate pathways, which, since both are influenced by similar environmental factors, often results in correlated changes in feeding and egg-laying.

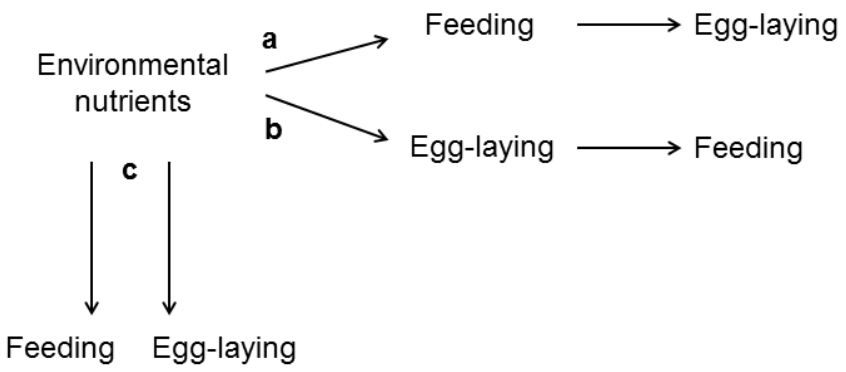


Figure 5.6. A model depicting three possible scenarios to explain the relationship between environmental nutrient availability, egg-laying and feeding in flies.
(a) Feeding changes in response to environmental nutrient availability, which in turn affects the availability of resources for egg-laying. **(b)** Changes in fecundity in response to perceived environmental nutrient availability alter feeding to support the resource demand for egg-production. **(c)** Environmental nutrient availability affects feeding and egg-laying through independent, parallel pathways.

The rate of feeding of *dGCN2* null flies was lower than wildtype flies across all food types tested, which, if we consider the role of dGCN2 to suppress translation, is perhaps unexpected since we might expect that a higher level of nutrient ingestion would be required to support a higher level of protein synthesis. One possibility is that *dGCN2* null flies lower their feeding in an attempt to control excess levels of protein translation. However, we have not experimentally verified an increase in protein synthesis for *dGCN2* null flies. We expect that deletion of *dGCN2*, being a central component of the GAAC pathway, would affect both upstream behaviours and downstream molecular changes in a way that may disconnect and desynchronise them, the consequences of which are difficult to predict. For example, *dGCN2* null flies may reduce their feeding because they can no longer sense environmental amino acids, and even though translation suppression may be decreased in these flies due to loss of eIF2α phosphorylation, translation may still be hindered by the lack of resources due to the suppressed feeding. Thus, if the control of feeding occurs upstream of translation, the molecular events taking place at the level of translation do not directly affect feeding behaviour. It is also likely that feedback mechanisms come in to play to balance some of these effects, and for the GAAC pathway such potential mechanisms have not yet been identified. Moreover, GCN2 is known to have broader functions, such as cell cycle control in yeast (Tvegård et al., 2007) and lipid metabolism in mice (Guo and Cavener, 2007), and so it is possible that the phenotypes that

we have observed for *dGCN2* null flies may be influenced by the effects of other molecular pathways that involve dGCN2.

The role of translation in influencing foraging behaviour could be tested by artificially downregulating translation, for example by the use of pharmacological inhibitors of translation. If wildtype flies begin to display aversive behaviour to a complete food medium then it may suggest that translation activation relays a message to the brain that promotes foraging behaviour as a means of obtaining the environmental resources required to support protein synthesis. However, downregulating translation could induce deleterious effects in the fly, making it sick, which may itself cause aversion to food.

dGCN2 null flies fed at a higher rate on food lacking methionine than on a complete medium, with feeding at a similar level to wildtype flies on a methionine-deficient medium. This suggests either/both that methionine deficiency partially rescues the low feeding rate exhibited by *dGCN2* null flies on a complete food medium, or that deletion of dGCN2 deafens the signal for methionine deficiency, thereby lessening the aversion to a methionine-deficient food source. The current mechanistic model for GCN2 provides support for the latter, where uncharged tRNAs (i.e. a lack of amino acids) signal to activate dGCN2 and cause behavioural aversion.

We find that not only is aversion to methionine deficiency reversed by dGCN2 deletion, but that *dGCN2* null flies feed at a higher level on food lacking methionine than on a complete medium. This implies that *dGCN2* deletion confers more than just a rescue of the aversive behavioural phenotype to methionine deficiency - it suggests that methionine deficiency, rather than methionine abundance, is the *dGCN2* effector signal, and this is in line with the current model of signalling through the GAAC pathway, where uncharged tRNAs (i.e. a lack of amino acids) signal to activate dGCN2. It is noteworthy, however, that GCN2 is thought to respond to all uncharged tRNAs, not just those that bind methionine. One interpretation of our data is that dGCN2 has a higher affinity for uncharged tRNAs that are specific to methionine. Mechanistically, uncharged methionine-specific tRNAs may activate dGCN2 more strongly than other types of uncharged tRNAs do, resulting in eIF2α phosphorylation, and subsequent inhibition of translation. Perhaps this inhibition of translation manifests behaviourally as a reduction in feeding, since protein synthesis is reduced and with it a decreased demand for resources. This may be

tested by the use of amino acid analogs, which are structural isomers of naturally-occurring amino acids that are of similar shape and size, but which have no biological value. They work by competing with natural amino acids, replacing them in proteins, thereby producing dysfunctional proteins, which may in turn interfere with protein synthesis itself. We might expect that methionine-deficient wildtype flies fed a methionine analogue would feed at a similar level to flies on a complete medium. If so, it may suggest that the signal that alters feeding in response to methionine abundance is upstream of translation, at the level of tRNAs, since the analogue would effectively be charging methionine-deficient tRNAs, depleting the amount of uncharged tRNAs that would otherwise activate dGCN2. Moreover, because the analogue would have no biological value, it may allow us to disentangle the effects on feeding of altered protein synthesis from upstream events, such as altered dGCN2 activity or altered eIF2α phosphorylation.

Evolutionarily, it would be beneficial for an animal, in the face of environmental nutrient deficiency, to conserve resource-costly egg-making efforts and seek out a more complete nutritional environment, thereby increasing its chances of longer-term survival for successful reproduction later. Our egg-laying data seems to support this theory - the magnitude of the difference in egg-laying between control and methionine-deficient flies is attenuated in *dGCN2* null flies, lending support to the hypothesis that dGCN2 serves to limit egg-production when a dietary imbalance is sensed, and because *dGCN2* null flies should be unable to detect the environmental imbalance, they do not accordingly reduce egg-production, continuing to produce eggs until the lack of methionine itself eventually limits egg production. This may be tested by measuring egg-laying at earlier time points, from the more immediate onset of methionine imbalance, in order to determine the rate at which egg-laying is reduced, compared to wildtype flies. Methionine restriction is known to limit egg-laying in flies (Grandison et al., 2009), which could explain the significance of an evolved adaptation to environmental methionine deficiency, since evolutionary selection for a trait can be driven by its ability to enhance reproductive fitness. If such a mechanism is specific to methionine availability, it would suggest that methionine is of particular importance for physiological maintenance or fecundity, or both, necessitating the conservation of the mechanism. Unless there are equivalent mechanisms that regulate the intake of other specific amino acids, this data may indicate that there is greater importance placed on the regulation of methionine intake specifically, compared to ingestion of other EAAs.

Why would such an adaptation have evolved around methionine availability, specifically? Methionine is important in metabolism, but it also plays a crucial role in translation. Its codon, AUG, is also a universal start codon that indicates the initiation of protein translation from mRNA in eukaryotes. Because most of the initiator methionine residues are removed by post-translational modification, it is believed that methionine's primary role is in translation initiation and not protein structure, albeit critical for disulphide cross links within proteins (Brosnan and Brosnan, 2006). Methionine is also a precursor of the amino acid cysteine, and these are the only sulphur-containing amino acids. Methionine is the amino acid that is the most susceptible to oxidation by almost all forms of reactive oxidative species, forming the oxidation product methionine sulfoxide (Vogt, 1995). Oxidation of methionine is countered by the activity of the enzyme methionine sulfoxide reductase, and impairment of this enzyme has been associated with reduced lifespan, age-related diseases and neurodegeneration (Cabreiro et al., 2006; Moskovitz, 2005). However, methionine oxidation has also been observed to be a functional activator of some proteins, and given the reversibility of methionine oxidation, it may be that methionine oxidation and reduction are a part of some refined mechanism that controls some form of homeostasis. Methionine is also a constituent of glutathione, an important antioxidant that prevents damage from reactive oxygen species, providing further incentive for an evolved system to regulate methionine levels. Moreover, methionine is an intermediate in the synthesis of taurine, one of the most abundant amino acids in animal tissues (Huxtable, 1989, 1992). The associated functions of taurine are varied – not only is it a major constituent of bile acid (Russell et al., 2003), but it also has a role as an antioxidant (Das et al., 2008; Green et al., 1991; Sinha et al., 2008), in osmoregulation (Moenkemann et al., 1999), in neurotransmission (Foos and Wu, 2002; Olive, 2002) and in membrane stabilisation (Birdsall, 1998). Thus, methionine has wide and varied biological roles, and is essential for important processes like translation and anti-oxidant defense. Given the biological significance of methionine, and its relatively low environmental abundance (Lochmiller et al., 1995; Müntz et al., 1998) it would be reasonable to expect that mechanisms that sense and seek out environmental methionine would be strongly selected for in order to ensure that the organism has an adequate supply of methionine to achieve reproductive success. Alternatively, flies may use methionine as an indicator amino acid to signal the presence of a high quality source of protein, in which case a methionine-sensing mechanism would also be warranted. I have provided evidence that the GAAC pathway

plays a role in methionine-sensing. Because NSPs are likely to be involved in DR-mediated lifespan extension, identifying the specific dietary components that they interact with could be useful in understanding how DR works to enhance longevity by providing clues as to the underlying physiological changes.

Published work on the GAAC pathway places great importance on the role of the brain as its main site of action. It would be interesting to explore whether the role of dGCN2 to control feeding behaviour can be pinpointed to specific areas of the fly brain. Indeed, in *Drosophila* larvae it has been shown that the dopaminergic neurons are an important site of dGCN2 activity; an amino acid imbalance input causes the activation of three dopaminergic neurons to elicit a behavioural aversion response to the diet (Bjordal et al., 2014). It would be useful to test whether this finding extends to the adult *Drosophila* brain, There are many brain-specific tissue-driver fly lines that cover an extensive range of brain regions that could be used to test the effect of genetically up- or downregulating dGCN2 on the behavioural responses to various dietary deficiencies.

In addition to investigating the role of the GAAC pathway in regulating fly feeding responses to EAAs, we also set out to characterise the roles of other NSPs, such as the IIS pathway. For the *dilp 2-3,5* mutants, repeat trials of the same experiments generated different results. Given the very low basal feeding rate of these flies, it is perhaps not surprising that they should exhibit so much variability between trials. It may be useful to test other IIS pathway mutants, such as *UAS-chico*, and *UAS-InR-DN*, which have mutations targeting the *Drosophila* insulin receptor and the substrate, respectively. It would also be interesting to test the feeding response to EAAs of TOR pathway mutants, especially to dietary leucine, given its ability to activate the TOR pathway (Proud, 2002).

We cannot say that amino acids are the sole dietary modulators of feeding as we have not tested the feeding response to manipulations of other dietary components. Moreover, it is important to note that changing the concentration of amino acids also changes their ratio with respect to all other nutrients in the medium. Thus, it is possible that it is this change in amino acid balance relative to other nutrients that may account, entirely or partially, for the phenotypes that we have observed. More extensive work will be required to determine the influences of each dietary component on feeding, and how these contributions change relative to changes in other dietary components.

Chapter 6 - General Discussion

6.1. Summary of findings

In this work, I set out to understand the mechanisms of lifespan extension due to dietary restriction (DR) in *Drosophila melanogaster*, with particular focus on the role played by nutrient sensing/signalling pathways (NSPs). To address this, I first characterised the physiological changes during DR to narrow the list of possible mechanisms for lifespan extension. I then tested whether NSPs could modify those same phenotypes in a way that correlates with extended lifespan under DR, in order to determine the likelihood that a signalling pathway could be the mediator of DR. Since NSPs relay environmental nutrient conditions to downstream effectors of physiology, I also investigated the role of NSPs in modulating the feeding response to nutritional imbalances.

In chapter 3 I took advantage of the recent finding that relatively high levels of essential amino acids (EAAs) in the diet account for the negative effect on lifespan of full-feeding (Grandison et al., 2009). I used this EAA-supplemented DR condition as the relatively high food control condition to which I compared DR effects on physiology. I found that DR and EAA-supplemented flies differed significantly in their responses to heat stress, starvation stress and in their levels of triacylglycerides (TAG). I then asked whether NSP mutants had alterations for these three phenotypes.

Long-lived insulin/IGF signalling (IIS) mutants showed no difference in their lifespan response to DR and EAA-supplementation and so I did not further pursue the IIS pathway as a mediator of lifespan extension under DR. In contrast, pharmacological down-regulation of the target of rapamycin (TOR) pathway altered the lifespan of EAA-supplemented flies as well as their response to heat stress and their TAG levels such that they responded more like DR flies than like EAA-supplemented flies, establishing a correlation between longevity, decreased heat stress resistance and increased TAG. A case for TOR in mediating lifespan extension under DR was reinforced by use of mutants with defects in dRagA, the amino acid sensitive upstream component of TOR signalling. Downregulating the TOR pathway in this way attenuated the harmful effects of EAAs on

lifespan, further indicating that TOR mediated longevity involves dietary EAAs specifically.

In chapters 4 and 5 I characterised the phenotypes of mutant flies null for *dGCN2*, a central component of the general amino acid control (GAAC) pathway that, to date, had not been explored in flies. *dGCN2* deficiency did not block the DR effect on lifespan, but *dGCN2*-deficient flies were longer-lived than wildtype flies under almost all nutritional conditions tested. I found that *dGCN2* deficiency modified the interaction between food and lifespan for changes in dietary yeast concentration, as well as in response to changes in the yeast to sugar ratio. It appears that dGCN2 serves to offer some protection against the detrimental effects on survival of single EAA deprivation, such that *dGCN2* null flies were shorter-lived compared to wildtype flies when deprived of dietary arginine, tryptophan or leucine, with the exception of dietary methionine deficiency, where dGCN2 compromised survival and egg-laying potential. In fact, the role of dGCN2 in sensitising flies to environmental methionine deprivation extended to feeding behaviour, such that *dGCN2* null flies fed at greater levels on food lacking methionine than on food containing a complete set of all 20 amino acids, which was the opposite of the response of wildtype flies to methionine deficiency, and of the response of both *dGCN2* null and wildtype flies to diets lacking the other EAAs tested.

6.2. Discussion

Our main goal for the work described in this thesis was to understand the molecular mechanisms by which DR extends healthy lifespan, using *Drosophila* as a model system. Our objectives were to explore this in the context of DR as defined by amino acid balance, to characterise the physiological profiles that define DR vs. fully fed flies in order to identify causation factors for lifespan extension, and to investigate how nutrient intake changes with the nutritional environment. The DR field has started to move away from the longstanding idea that calorie restriction *per se* confers lifespan and healthspan benefits, moving instead towards an outlook of DR that affords more importance to dietary balance - that is, the balance of nutrients in the diet relative to other nutrients (Mair et al., 2005). In particular, the balance of proteins, or amino acids, relative to other dietary components, as well as the balance of the individual amino acids relative to each other, have shown to be important in mediating longevity (Grandison et al., 2009), and the work in this thesis corroborates the importance of dietary amino acid balance in mediating lifespan changes under DR. The restriction of certain individual amino acids, such as methionine and tryptophan, have previously been demonstrated to promote longevity in mammalian models (Ables et al., 2014; Miller et al., 2005; Zimmerman et al., 2003), and it has been known that the availability of amino acids is sensed by NSPs, such as IIS, TOR and GAAC. However, little is known about how these nutritional signals are relayed through these molecular pathways to downstream effector pathways that influence longevity. It is possible that specific amino acids modulate the effects of different NSPs on lifespan. Indeed, some NSPs have a greater affinity for specific amino acids. For example the branched chain amino acids, and in particular leucine, stimulate the TOR pathway as effectively as does a combination of all amino acids (Efeyan et al., 2012; Kimball and Jefferson, 2006; Proud, 2002), whereas the GAAC pathway is thought to be stimulated by the absence of all amino acids.

TOR signalling has long occupied the spotlight as a primary candidate mediator of lifespan extension under DR (Kaeberlein and Kennedy, 2009; Stanfel et al., 2009; Vargas et al., 2010), and evidence for this is largely based on observations from yeast, worms and flies that DR does not further increase lifespan when TOR signalling is reduced, suggesting that DR and downregulated TOR signalling extend lifespan by common mechanisms. Moreover, in flies rapamycin has been shown to attenuate the negative effects of full

feeding on lifespan (Bjedov et al., 2010), further supporting a role for TOR in mediating the lifespan effects of DR. The data in this thesis adds to the mounting evidence that reduced TOR signalling under DR is responsible for its effects on lifespan. Uniquely, we show that the protective effects of rapamycin work via alleviation of the negative effects of full feeding as implemented by dietary amino acid imbalance, suggesting that it is the signalling of the EAAs, specifically, that accounts for the negative effects of increased TOR activity on fly lifespan, and this was supported by use of genetic models. We also took a novel approach to understanding the mechanisms behind these observations by asking how a downregulation of TOR signalling could alter DR-associated phenotypes in flies. By this method we were able to narrow the possible effects of DR on lifespan as relating to TAG regulation and thermotolerance, opening avenues for further exploration of the relationship between these physiological traits and TOR pathway activity under DR.

The link between fat and animal longevity is complex and not fully understood, and recent findings have propelled this topic to the forefront of DR research (Brock et al., 2007; Liu and Czaja, 2013; O'Rourke et al., 2009; Shmookler Reis et al., 2011; Vrablik and Watts, 2012). Counter to the common belief that fat is negatively correlated with health and lifespan, some long-lived rodent models have increased adiposity (Berryman et al., 2004; Coschigano et al., 2000; Flurkey et al., 2001), and genetically obese mice live longer than wildtype mice under DR (Harrison et al., 1984). In humans, counterintuitive correlations between moderate obesity and improved survival has been referred to as the 'obesity paradox' (Kalantar-Zadeh et al., 2003, 2004). It has been postulated that, in some cases of chronic disease, body fat may impart some protective benefit. This phenomenon has been commonly observed in wasting conditions such as congestive heart failure, end-stage renal disease, advanced malignancies, and AIDS, and it has been theorised that high fat levels in such conditions may serve as a metabolic reserve, providing nutrients to enhance survival under the stress of the illness (Habbu et al., 2006), but this has yet to be experimentally verified. Alternatively, methodological bias that do not control for the inherently higher mortality risk in patients with low BMI, who usually exhibit a more severe disease, may be accountable (Hu, 2008). A recent study following 10,500 patients with type 2 diabetes over 10 years found that although overweight and obese patients had a higher incidence of cardiac events (such as heart failure) compared to patients of normal weight, the overweight group lived longer than normal-weight patients, and that normal-weight patients had a mortality risk similar to that of obese patients. In fact, underweight patients

had the worst prognosis (Costanzo et al., 2015). Another study showed that not all obese people are metabolically unhealthy – some have higher fitness levels than others, do not suffer from common obesity-associated problems such as insulin resistance or high blood pressure, and have a 30–50% lower risk of mortality and morbidity compared to their metabolically abnormal obese peers (Ortega et al., 2013). Moreover, it has been speculated that different types of fat can have varied impacts on health. For example, it was reported that normal-weight patients with coronary artery disease had a higher risk of mortality if they had increased visceral fat, compared to obese patients with coronary artery disease who had a lower waist-to-hip ratio (Coutinho et al., 2013). Thus, the role of fat in lifespan regulation is emerging as a complex but potentially significant association warranting further study.

Our own findings suggest that TOR-mediated lifespan extension in flies occurs through regulation of TAG levels (chapter 3). We also report that GAAC pathway mutant flies have altered TAG levels under different nutritional conditions, but in a way that does not necessarily correlate with longevity (chapter 4). These inconsistencies do not necessarily rule out a role for TAG in regulating lifespan in response to changes in nutrient sensing – they may in fact be highlighting the complexity of the relationship between fat and lifespan. This relationship is likely to be complicated by the fact that there are different kinds of TAGs, varying in their fatty acid chain lengths and the stereopositions of these chains on the glycerol molecule. These structural properties determine the different functional properties of the TAGs as well as their specificity as substrates to different enzymes, which in turn determine how they are metabolised and their effects on cellular processes (Karupaiah and Sundram, 2007). Future work on the effect of DR on lifespan would benefit from characterisation of the TAG species profiles of DR and FF animals, and experimentally testing whether any differences between these profiles can account for the differences in lifespan. It may also be worth investigating how the expression of lipase enzymes change in response to the relative abundance of the different lipids, and how the nutritional environment and NSPs can influence these responses to affect physiology and lifespan.

The GAAC pathway plays an important role in coordinating a systemic response to nutritional stress, which involves a downregulation of global protein synthesis and a concomitant adjustment of gene expression patterns that shifts cells from a state of nutrient utilisation and growth to one of resource conservation (Spriggs et al., 2010; Wei et al., 2009). Indeed, activation of the GAAC has been shown *in vivo* to have survival benefits under conditions of various types of stresses, such as mitochondrial and hypertonic, and this protection is thought to be due to the attenuation of translation (Baker et al., 2012; Lee and Strange, 2012; Liu and Lu, 2010). Mammalian studies have shown that GCN2 activates key transcription factors, such as ATF4 and NF-kB, in order to manage stress (Wek et al., 2006). Our data supports a role for the GAAC in protecting against nutritional stress in flies, namely starvation stress and single amino acid deficiency, and further work is required to verify the hypothesis that this protection is attained through a downregulation of global protein synthesis. Such mechanisms of transcriptional and translational reprogramming are employed as a common cellular response to stress, and have been shown to be important in modulating stress resistance and lifespan extension in the context of other NSPs as well (Hansen et al., 2007; Kaeberlein et al., 2005; Rogers et al., 2011; Zid et al., 2009). The benefits to lifespan conferred by a reduction of translation may be due to the process of translation itself causing damage that increases the rate of aging, and/or because reduced translation induces a stress response that extends lifespan, for example by enhancing the ability to repair or prevent damage that may otherwise accelerate senescent decline (Syntichaki and Tavernarakis, 2006). Ageing is accompanied by alterations in protein synthesis, and because the NSPs play a major role in the regulation of global protein synthesis and have been implicated in the regulation of lifespan, they have become the focus of much investigation into the effect of transcriptional and translational changes on stress tolerance and ageing.

While the TOR pathway is an established regulator of ageing, less is known about how changes in the activity of the GAAC pathway mediate lifespan changes. Because the GAAC pathway senses nutrients, interest is focussed on the role that it might play in mediating lifespan in response to the nutritional environment. GCN4, a component of the GAAC pathway, has been shown to be involved in the extension of yeast replicative lifespan achieved by the reduction of 60S ribosomal subunit biogenesis (Steffen et al., 2008), and recently it was shown that *GCN*2 null *C. elegans* are more sensitive than control worms to amino acid limitation, with compromised survival under these conditions

(Rousakis et al., 2013). Loss of *GCN2* also supressed the extension of lifespan in a genetic model of DR in worms, and abolished the extended longevity of TOR pathway mutant worms. This study implicates the GAAC pathway as a mediator of lifespan under DR in worms, and in mediating the lifespan effects of altered TOR pathway activity. The impact of GCN2 function on lifespan had not yet been investigated in flies. Our work provides the first genetic evidence in flies that the GAAC pathway can alter the influence of diet on lifespan. In support of the data from worms, we show that *dGCN2* null flies are too more sensitive to amino acid limitation compared to controls, and although we have not observed an attenuation of lifespan extension in DR flies with loss of *dGCN2* our data does ascribe a complex role for the GAAC pathway in regulating the lifespan responses of flies across a wide nutritional landscape, including survival responses to single amino acid deficiencies. In particular, we describe a novel role for the GAAC pathway in mediating the lifespan, fecundity and feeding responses to methionine deficiency. In contrast to the data from worms, we did not observe a significant effect of dGCN2 in altering the lifespan response to TOR pathway downregulation as imposed by rapamycin treatment, which may reflect divergent functions of GCN2 in worms and flies. Since the GAAC pathway has an established role in the management of cellular responses to stress, it may be interesting to investigate how GAAC-mediated stress-induced reprogramming could affect lifespan, while being mindful that longevity is not always linked to increased stress resistance, as we have seen in this work (chapter 3). Such an association is likely to be dependent on genetic and/or environmental backgrounds (Pan et al., 2011; Ristow and Zarse, 2010; Sun et al., 2012). Further work to elucidate the relationship between GAAC-mediated stress resistance and longevity will require the identification of the specific downstream effectors of GCN2 and further investigation of any potential interaction with other NSPs.

The GAAC pathway has largely been studied in the context of feeding behaviour and has been established as an important modulator of food choice in rodent models, particularly in the regulation of responses to amino acid-imbalanced diets. Characterising how feeding decisions change with changes in the nutritional environment is important in understanding how animals regulate the intake of macronutrients to optimise different traits under different environmental conditions and how this can in turn affect overall lifespan. Characterisation of the metabolic and molecular adaptations induced by alterations in amino acid intake could provide clues about the physiological changes that give rise to the lifespan and health benefits of DR. The GAAC-mediated feeding response is activated in

response to a wide range of different amino acid deficiencies and is phenotypically characterised by behavioural aversion to the deficient food source. In this thesis we describe a more specific role for the GAAC pathway in sensing methionine deficiency to elicit a behavioural response, which complements our finding that the GAAC pathway has a specialised role in regulating physiological changes in response to methionine deficiency. We propose that the GAAC response is an adaptation to methionine deficiency specifically, which is an important finding given the growing amount of data demonstrating the lifespan and health benefits of methionine restriction in several laboratory animals.

It has been suggested that the effect of methionine restriction to extend lifespan is a result of an evolved adaptation to survive environmental methionine shortages, where short-term survival mechanisms are induced to enable the fly to endure until there is a renewed supply of methionine, thereby maximising reproductive success. The details of such a mechanism are not known. Increased lifespan tends to correlate positively with cellular stress resistance, and several studies suggest that such ability to tolerate stressors may underlie the beneficial effects of methionine restriction on lifespan; examples from various organisms suggest that methionine restriction induces general stress tolerance that is associated with enhanced survival – methionine-restricted organisms have shown increased levels of autophagy (Ruckenstuhl et al., 2014), resistance to heavy metal stress (Hwang et al., 2007; Singh and Sherman, 1974), decreased levels of reactive oxygen species (Sanz et al., 2006), increased glutathione levels (Richie et al., 1994), resistance to heat stress, reduced acid accumulation and upregulation of the retrograde response (Johnson and Johnson, 2014), upregulation of endocrine factors, and slowing of age-related changes in immunity (Miller et al., 2005). Additionally, methionine is metabolised through the trans-sulfuration pathway, and genetically upregulating the activity of this pathway has been shown to increase lifespan in flies (Kabil et al., 2011). Inhibition of the trans-sulfuration pathway attenuates the effect of DR to increase lifespan. Thus, it may be interesting to consider the trans-sulfuration pathway in further investigating the lifespan effects of the GAAC pathway, especially in the context of methionine restriction, which we have highlighted as being a particularly important activator of the GAAC pathway.

6.3. Outlook

If the TOR pathway is a central mediator of lifespan change under DR in humans, and if it does so through the regulation of physiological lipid content/dynamics, as our data suggests in flies, then characterising the basal TOR activity profiles and/or lipid profiles of an individual could determine the type of DR regimen that would be the most beneficial for them. As a long-term outlook, the efficacy of a DR diet for humans would benefit greatly from pharmacogenetic studies that could allow for the development of tailor-made diets according to an individual's genetic makeup. For example, genetic markers indicating the efficacy of NSPs and/or associated physiological markers of metabolism could determine the optimum dose of rapamycin, for example, or the required ratio of nutrients to maximize the beneficial effects of DR.

Our findings offer the potential for further development of interventions that target NSPs for treating age-related diseases in humans. Rapamycin serves as a pharmacological tool with which to manipulate TOR signalling. Recently it has been found that differential expression of TOR signalling is associated with ageing in humans (Harries et al., 2012; Passtoors et al., 2013), making the TOR pathway a relevant target for intervention in humans. Moreover, upregulation of autophagy in humans has been shown to have anti-aging effects, while decreased autophagy is associated with premature aging, neurodegenerative disorders, cancer and inflammation (Kuballa and Xavier, 2010; Madeo et al., 2010). Rapamycin is already approved by the Food and Drug Administration for human use as an immunosuppressant to prevent organ rejection in transplant patients and for the treatment of renal cell carcinoma. Remarkably, the longevity benefits of rapamycin may be reaped even when treatment is started at an advanced age, as demonstrated in mice (Harrison et al., 2009; Miller et al., 2011). Such an anti-ageing intervention that is effective when initiated relatively late in life could prove to be useful for clinical application as a means of treatment, as well as for prevention, of age-related decline in health. However, rapamycin has numerous reported side effects in humans, such as increasing susceptibility to viral and fungal infections, since it is an immunosuppressant (Mahe et al., 2005), and can cause alopecia, edema, apthuos ulcers, mucositis, rash, reduced fertility in males, hyperlipidemia and diabetes (Gyurus et al., 2011; Mahe et al., 2005; Zuber et al., 2008). Understanding the mechanism of action of rapamycin may not only offer insights into the underlying biology of the ageing process, but further pharmacogenetic research could

facilitate the development and refinement of rapamycin for use as an effective anti-ageing treatment for long-term use, so as to maximise its efficacy and reduce its side effects.

In light of my research, if TAG is a target of TOR-mediated longevity, and if an individual's lipid profile could be indicative of the rate at which they will age, then tailoring rapamycin dosage according to an individual's lipid profile may maximise the efficacy of the drug as a therapeutic treatment for ageing and age-related disease. The additional benefit of rapamycin as a therapeutic treatment for ageing is the relative ease with which it could be undertaken, compared to potentially rigorous DR regimes that may require a greater strength of will and extensive diet planning. Other drugs have also been shown to reduce TOR signalling, such as aspirin (Din et al., 2012; Gao et al., 2003) and metformin, which has been shown to directly inhibit the Rag GTPases (Kalender et al., 2010), and have reportedly fewer side-effects than rapamycin, making them candidate drugs for development for the treatment of ageing and age-related diseases. Moreover, supplementation of ω-6/ω-3 polyunsaturated fatty acids could potentially increase autophagy and elicit similar health and lifespan benefits to that seen in worms (Rourke et al., 2013).

The development of lifespan-prolonging dietary regimes for humans will prove to be challenging. DR, in the sense of a scale-down of total food intake at the level shown to extend lifespan in other animals, is a demanding regime to maintain long-term. Thus, dietary regimes where a specific dietary component is limited, like methionine restriction, may prove to be a more practical approach. A vegan diet, for example, is typically low in methionine, and is a diet that can be undertaken with relative ease. The concentration of methionine relative to other amino acids, as well as to other dietary components, will be an important balance to establish, and on which the success of a methionine restriction diet will depend. The safety of the diet will also depend on this balance as too little or too much methionine can be detrimental (Garlick, 2006; Pulikkunnel and Thomas, 2005). Moreover, this ratio balance may be different for different people, depending on several environmental and genetic factors, such as gender, weight, height, level of activity, disease state, etc, and this will be extremely difficult to work out without understanding the underlying mechanism of the lifespan-extending effects of methionine restriction at the physiological level at least.

Much work is still needed to uncover the molecular details of ageing, but progress has been greatly aided by the use of genetic mutants and pharmacological agents that target biological pathways implicated in the regulation of longevity. The DR paradigm holds much promise for developing interventions that enhance longevity and healthspan, and nutrient sensing/signalling pathways, such as TOR, IIS and GAAC, have shown great potential as targets for therapeutic treatment of age-associated diseases and disabilities. Understanding the biological mechanisms through which DR promotes health and longevity is an important requirement for developing DR mimetic drugs for human use. However, given the variability of the reported effects of DR interventions in laboratory organisms, achieving these objectives will be challenging and a greater research effort will be required to dissect the organism-specific effects of DR in a way that can be informative about DR for humans. The prospect of enhanced vitality in old age is one that warrants continued scientific effort, and the progress currently being made in the field inches us closer to this goal.

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Appendix

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Research Paper

Target of rapamycin signalling mediates the lifespan-extending effects of dietary restriction by essential amino acid alteration

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Abstract: Dietary restriction (DR), defined as a moderate reduction in food intake short of malnutrition, has been shown to extend healthy lifespan in a diverse range of organisms, from yeast to primates. Reduced signalling through the insulin/IGF-like (IIS) and Target of Rapamycin (TOR) signalling pathways also extend lifespan. In *Drosophila melanogaster* the lifespan benefits of DR can be reproduced by modulating only the essential amino acids in yeast based food. Here, we show that pharmacological downregulation of TOR signalling, but not reduced IIS, modulates the lifespan response to DR by amino acid alteration. Of the physiological responses flies exhibit upon DR, only increased body fat and decreased heat stress resistance phenotypes correlated with longevity via reduced TOR signalling. These data indicate that lowered dietary amino acids promote longevity via TOR, not by enhanced resistance to molecular damage, but through modified physiological conditions that favour fat accumulation.

INTRODUCTION

Dietary restriction (DR) is an intervention whereby a considerable reduction of food intake, just short of malnutrition, extends lifespan. This has been demonstrated to be effective in a wide range of evolutionarily diverse organisms, from yeast [1] to invertebrates [2] and mammals [3], and is considered one of the most robust environmental interventions to extend lifespan in laboratory organisms. Moreover, the longevity promoting effects of DR are accompanied by a range of health benefits. DR rodents had a delayed onset or a lesser severity of age-related diseases such as cancer, autoimmune diseases and motor dysfunction [4–6] and improved memory [7]. In *C. elegans*, DR was shown to reduce proteotoxicity [8]. DR rhesus monkeys were found to have improved triglyceride, cholesterol and fasting glucose profiles, and a reduced incidence of diabetes, cancer, cardiovascular disease and brain atrophy [9].

The molecular mechanisms underlying the physiological changes elicited by DR have yet to be elucidated, however, experimental data point towards nutrient signalling pathways as playing an important role. The evolutionarily conserved Target of Rapamycin Complex 1 (TORC1) pathway senses amino acid availability and signals to enhance translation via activation of S6 kinase-1 (S6K1) and inhibition of eIF4E binding protein-1(4E-BP1). TORC1 also regulates transcription and autophagy in response to a range of signals, including nutrient availability, cellular energy levels, and growth factors, in such a way that growth rates match resources [10]. Experimental validation of a role for TORC1 in determining lifespan has come from a range of laboratory organisms. Lifespan extension by inhibition of TORC1 pathway genes has been demonstrated in *S.cerevisiae* [11], *C.elegans* [12], *D. Melanogaster* [13] and in mice [14–19]. How TORC1 inhibition promotes longevity is unknown.

Another nutrient sensing pathway that is commonly associated with modified ageing is the insulin/insulin-like growth factor signalling (IIS) network. Mutations in components of the IIS pathway have extended lifespan in a host of model organisms [20]. Because the IIS pathway senses nutrients, considerable effort has been made to assess the role for IIS in modulating the longevity responses to DR. While IIS does not seem to be solely accountable for DR, some experimental data suggest overlapping mechanisms for IIS- and DR-mediated lifespan extension [21].

Recent work has shown that adjustments to the dietary amino acid balance can mimic the benefits to lifespan by DR in *D. melanogaster* [22]. Supplementing a DR diet with the ten essential amino acids (EAA) phenocopy the effects of full feeding (FF) on lifespan and fecundity, indicating that the beneficial effects of DR are a consequence of improved amino acid balance. Experimentally, the addition of EAAs to DR (DR+EAA) offers a sharper instrument with which to dissect the potential causes of lifespan change in response to nutritional balance than the FF condition, which is achieved by increasing the concentration of dietary yeast. Here we characterize physiological and metabolic parameters that define DR and fully fed flies with the aim of identifying candidate factors for causation of the lifespan response to DR.

RESULTS

TORC1 signalling but not IIS signalling is required for the effect of EAA on lifespan and fecundity

Dietary restricted (DR) flies are longer-lived than fully fed flies, but produce fewer eggs. The effect of full feeding to shorten lifespan and increase egg laying can be mimicked by the addition of the 10 essential amino acids (EAA) to DR food (Figures 1a-1c).

To assess the role of the longevity-associated nutrient signalling pathways as potential mediators of the effect of EAA on lifespan, we tested the response to DR of flies that are long lived due to deletion for genes encoding three of the *Drosophila* insulin-like peptides, (DILPs) *ilp*2, *ilp*3 and *ilp*5. We found no difference between the responses of wild type and DILP mutant flies to the addition of EAA to DR food, indicating that IIS is not required for the lifespan extension by DR (data not shown). In contrast, addition of the TORC1 inhibitor rapamycin extended the lifespan of flies on DR+EAA such that their lifespan was not shorter than those subjected to DR (Figure 2a). Rapamycin treatment also prevented the increase in egg laying seen for EAA addition to DR food, in fact egg laying was effectively blocked by rapamycin treatment. We also found that

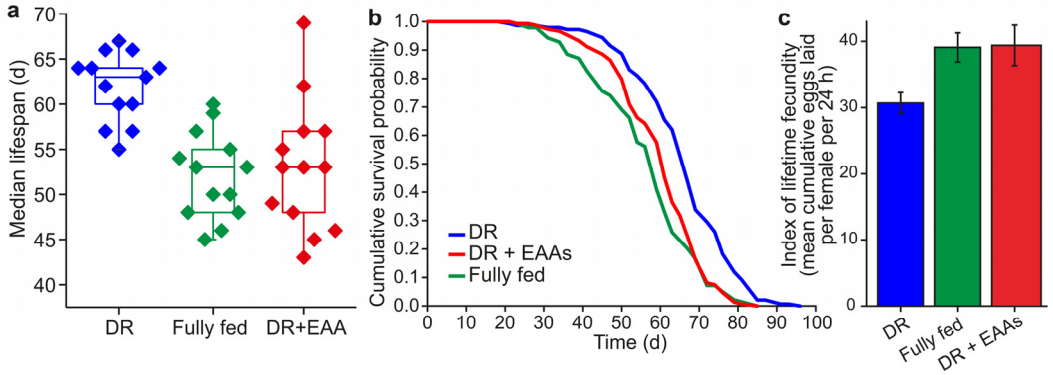

Figure 1. Amino acids mediate lifespan and fecundity changes under DR. (a) Summary of *Drosophila* median lifespans under dietary restriction (DR), full feeding (FF) and essential amino acid supplementation of DR (DR+EAA) (n=13 biological replicates; DR vs FF, P<0.001; FF vs DR+EAA, P=0.9383; DR vs DR+EAA, P=0.002; Wilcoxon rank-sum test) **(b)** A representative lifespan experiment: adding EAAs to DR food shortened lifespan (P< 0.001) to that of FF flies (P=0.194); n=150 per treatment; compared using the log-rank test. **(c)** Adding EAAs to DR food increased egg-laying (P<0.001) to that of FF flies (P<0.936). Fecundity: mean±s.e.m.; n=15; compared using the Wilcoxon rank-sum test.

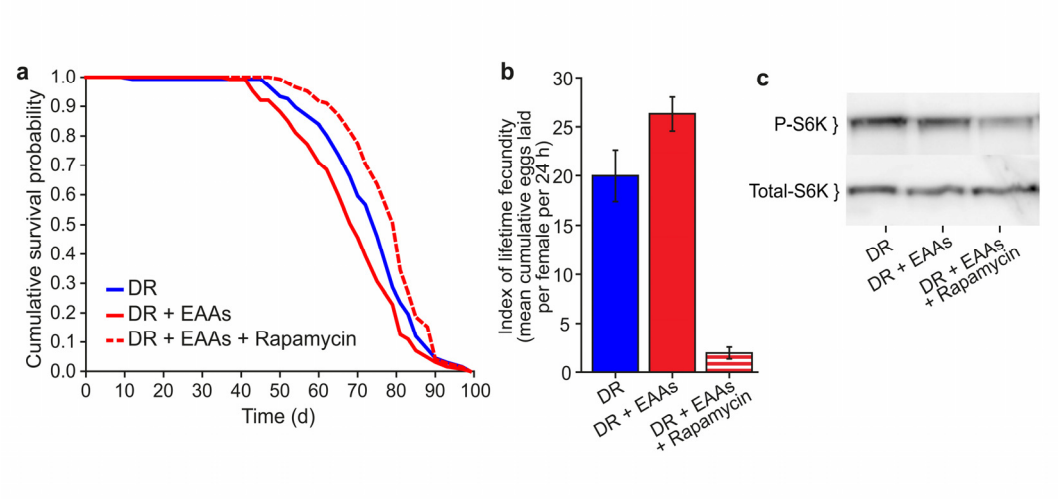

Figure 2. Effect of Rapamycin treatment on EAA-supplemented flies. (a) Rapamycin treatment extended the lifespan of DR+EAA flies beyond that of DR (DR+EAA vs DR+EAA+Rapamycin, $P<0.001$; DR vs DR+EAA, $P<0.012$). $n=150$ per treatment; log-rank test. **(b)** Rapamycin treatment decreased the lifetime fecundity of DR+EAA flies ($P<0.001$). Fecundity: mean±s.e.m.; $n=10$; Wilcoxon rank-sum test. **(c)** Levels of phospho-T398-S6K were measured from whole-fly protein extracts. Treatment with rapamycin for 7 days decreased phospho-T398-S6K levels in DR+EAA+Rapamycin flies relative to DR+EAA flies.

phosphorylation of the TORC1 target S6K was reduced by the addition of rapamycin (Figures 2b, 2c). Together, these data are consistent with TORC1 signalling playing a role in mediating the change in lifespan upon DR.

EAA supplementation alters responses of DR flies to H_2O_2 stress, heat stress, starvation stress, and TAG levels

We set out to identify phenotypic correlates of lifespan change under our dietary conditions in order to understand the causal mechanisms of increased lifespan under DR. Long-lived animal models often have an associated increase in the ability to resist environmental stresses and this is assumed to reflect a general increase in their health. Long-lived insulin/IGF-like signalling (IIS) mutant flies have been shown to be resistant to acute toxic doses of DDT, paraquat and hydrogen peroxide (H_2O_2) [23–25]. We tested whether long-lived DR flies are protected from the harmful effects of these compounds. We found that DR flies were significantly more resistant than DR+EAA flies to a toxic dose of H_2O_2, whereas no difference was apparent for paraquat (Figures 3a, 3b). Surprisingly, DR flies were more sensitive to a toxic dose of DDT than DR+EAA flies (Figure 3c), indicating that, at least for DDT resistance, DR does not protect against this toxin in the same way that lowered insulin signalling does.

Long-lived DR *C. elegans* have increased resistance to heat stress [26,27]. Upon testing the response of flies to heat shock stress, we found that DR flies were significantly less resistant than DR+EAA flies (Figure 3d), indicating that longevity associated with amino acid reduction comes at a cost to heat stress resistance.

Finally, we found that DR flies showed greater resistance to starvation than DR+EAA flies (Figure 3e), suggesting a possible mechanistic relationship between longevity and starvation resistance. Resistance to starvation stress could depend on the availability of enhanced energy stores within the fly. While we found no difference between groups in the levels of the storage carbohydrates glycogen or trehalose (Figure 3f, 3g) we did find that DR flies had significantly higher levels of triacylglycerides (TAG) than DR+EAA flies (Figure 3h). It is possible that this difference in TAG levels is causative of the longevity differences between DR and DR+EAA flies such that increased TAG confers some benefit to survival.

Increased TAG and decreased heat-stress resistance correlate with increased lifespan with DR

If the above phenotypes induced by DR are causally linked to longevity through reduced TORC1 signalling, it should be possible to reproduce the same

physiological outcomes by treating flies with rapamycin. We therefore tested the effect of rapamycin on DR+EAA flies for H_2O_2 stress resistance, starvation sensitivity, heat shock stress resistance and TAG levels (Figures 4a-d). Of these, heat stress resistance and TAG levels changed upon rapamycin treatment of DR+EAA flies, such that the responses became more similar to DR flies; Like DR, rapamycin treatment increased the sensitivity of EAA-treated DR flies to a 39ºC heat stress, and increased their TAG content. There was no effect of rapamycin on the response of EAA-treated flies to H_2O_2 stress or to starvation stress.

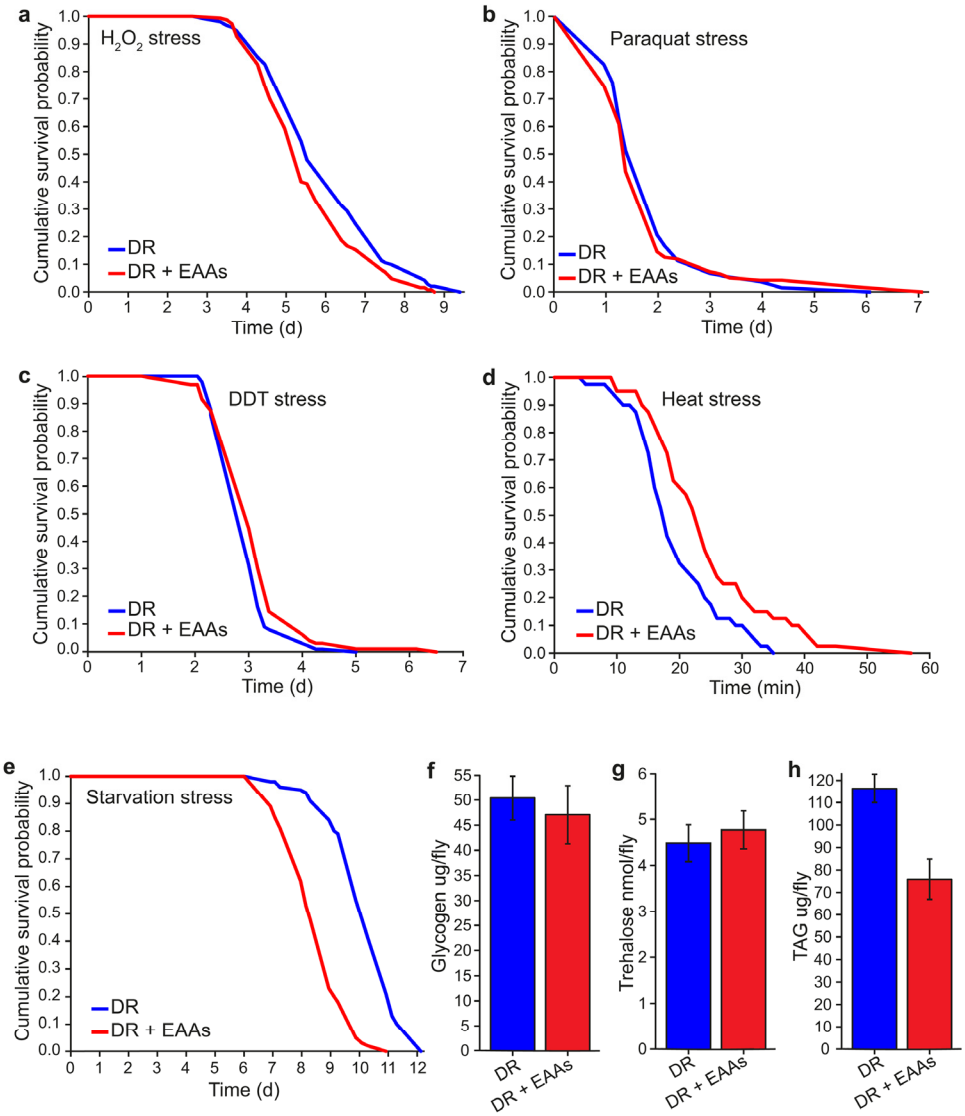

Figure 3. Phenotype comparisons between dietary restricted flies and those supplemented with EAAs. (a) DR+EAA flies showed a decreased resistance to hydrogen peroxide toxicity compared to DR flies (P=0.013; n=150 flies per condition). **(b)** There was no difference between DR and DR+EAA flies in their sensitivity to paraquat stress (P=0.517; n=150 flies per condition). **(c)** DR+EAA flies showed only a marginal, but significantly improved tolerance to DDT compared to that of DR flies (P=0.042, n=100 flies per condition). **(d)** DR+EAA flies were significantly more resistant to a 39ºC heat stress compared to DR flies (P<0.001; n=40 flies per condition). **(e)** DR+EAA flies were significantly more sensitive to starvation than DR flies (P<0.001; n=100 flies per condition). **(f)** After 7 days of treatment there was no difference in the amounts of glycogen measured for DR+EAA flies compared to DR flies (P=0.656; n= 6). **(g)** There was no difference in the levels of trehalose measured for DR+EAA flies compared to DR flies (P=0.630; n=6 flies per condition). **(h)** DR+EAA flies had significantly reduced levels of TAG compared to DR flies (P<0.001; n=6 flies per condition). For figures a-e, P values were calculated using the log-rank test. For figures f-h, P values were calculated by T-test, and error bars represent the s.e.m.

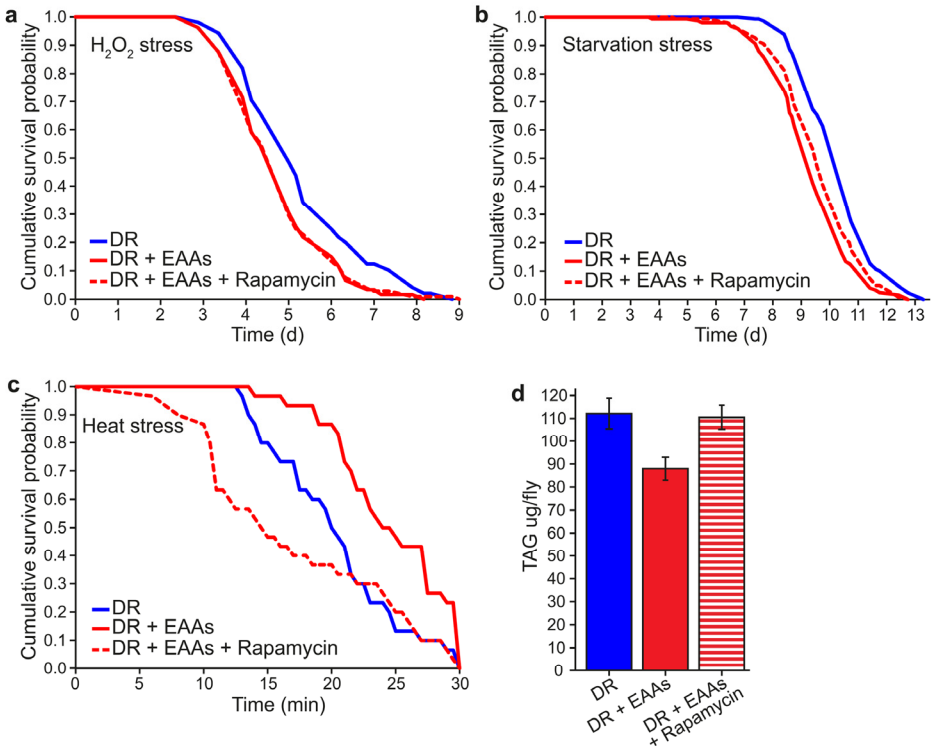

Figure 4. The effect of rapamycin to alter phenotypic differences between DR and DR+EAA flies. **(a)** Rapamycin had no effect on the sensitivity of DR+EAA flies to H_2O_2 stress (*P*=0.963; *n*=105 flies per condition). **(b)** Rapamycin had no effect on the sensitivity of DR+EAA flies to starvation stress (*P*=0.071; *n*=150 flies per condition). **(c)** Rapamycin, like DR, increased the sensitivity of DR+EAA flies to a 39ºC heat stress (*P*<0.001; *n*=30 flies per condition). For figures a-c, *P* values were calculated using the log-rank test. **(d)** Rapamycin treatment increased the triacyglyceride (TAG) levels of DR+EAA flies to the level of DR (*P*=0.011; *n*=6; T-test; error bars represent the s.e.m).

DISCUSSION

We have described the physiological and metabolic features that define long-lived DR flies in order to understand the mechanisms by which longevity is achieved. Our data indicate that dietary amino acids modify TORC1 signalling, which in turn alters lifespan outcomes. We also found that both dietary amino acid manipulation and TORC1 modification in flies alter TAG levels, such that higher body fat may play a causal role in enhancing fly lifespan in response to dietary restriction.

We found that the lifespan of insulin-mutant flies responded in a similar way to DR as wild-types, indicating that reduced IIS is not required for the lifespan-extending effects of DR. This appears to contrast previous studies that have reported interacting effects of IIS on DR, such that lifespan modification in response to yeast dilution is abolished in some IIS mutants [22,23,28]. These differences could be due to the fact that in the current study we modulated lifespan by adjusting EAA alone, rather than yeast. In doing so, we report a markedly different sampling of nutritional space than for yeast dilution, since we change the ratio of EAAs to all other dietary components, such as lipids, carbohydrates, non-essential amino acids, vitamins and trace elements. This may also explain why the phenotypes of our long-lived flies are somewhat different from those of other organisms subjected to DR. Interestingly, our experiments also showed that long-lived DR flies had decreased resistance to DDT, which is the opposite phenotype seen for IIS mutant flies, in which longevity is accompanied by dFOXO-

dependent DDT resistance [23,29]. Together, these data suggest that the beneficial effects on lifespan of DR can be achieved independently of IIS, similar to that reported by Tatar [21]. Moreover, it has been suggested that the effects of IIS on longevity are dependent on the status of TOR activity [30].

One of the strategies taken to understand the mechanisms by which dietary or genetic treatments enhance longevity is to seek out correlated physiological changes that may provide insights into the treatment's mode of action. A common mechanistic explanation for longevity requires enhancing systems to protect against the damaging side-effects of aerobic metabolism, such as that caused by oxidative stress or endogenous lipophilic toxins [31,32]. In our analyses, we found no evidence for broad-spectrum enhanced protection against stressors under DR. Thus, the mechanism for increased longevity under DR may not involve enhanced resistance to stress. Similar observations in studies on worms [33,34] has led to an alternative hypothesis that "hypertrophy" caused by inappropriate continuation of early-life growth programmes into later life is detrimental to an organism and causes ageing [35–38]. This explanation also implicates high levels of TOR signalling as its mechanism.

We found increased TAG levels correlated with longer life in our flies subjected to DR or rapamycin treatment. DR by yeast restriction in *Drosophila* has also been shown to increase lipid content [39–41], and several rodent studies show that higher fat levels correlate with increased lifespan [42–44]. In a recent study, Kapahi and colleagues showed that DR flies have increased TAG, and demonstrated an increased requirement for muscle-specific fatty-acid synthesis and breakdown in extending lifespan under DR [45]. Moreover, some long-lived TOR and IIS pathway mutants have increased fat levels [46–49]. Given that not all fat mutants are long-lived [50], it is likely that if fat levels are causally involved in extending life, the quality of fat accumulated is important. It would be interesting in future work to determine how lipid profiles change under different dietary conditions, to identify the specific types of lipids that are altered, and whether experimental manipulation can enhance lifespan.

EXPERIMENTAL PROCEDURES

General Methods

Standard laboratory food . Dietary restriction medium (1xSYA) contained100 g/l yeast (1x; MP Biomedicals, OH, USA), 50 g/l sucrose (Tate & Lyle, London, UK), 15 g/l agar (Sigma-Aldrich, Dorset, UK), and 30ml/l nipagin (Chemlink Specialities, Manchester, UK) and 3ml/l propionic acid (Sigma-Aldrich, Dorset, UK).This diet and its method of preparation is described in Bass et al., 2007 [51]. The fully fed medium (2xSYA) was prepared in the same way, except that it contained 200g/l yeast.

Experimental food. Rapamycin (LC Laboratories, MA, USA) was dissolved in ethanol and added to 1xSYA food at a final concentration of 200μM. Essential amino acids (Sigma-Aldrich, Dorset, UK) were dissolved in MiliQ water, and added to 1xSYA food at concentrations shown in Table 1. As control measures, ethanol alone was added to the food conditions that did not contain rapamycin, and water was added to food conditions that did not contain essential amino acids.

Table 1. Quantities of each of the essential amino acids added to 1l of 1xSYA food medium

Essential amino acid (Sigma-Aldrich)	Concentration in 1xSYA medium (g/l)
L-arginine	0.43
L-histidine	0.21
L-isoleucine	0.34
L-leucine	0.48
L-lysine	0.52
L-methionine	0.10
L-phenylalanine	0.26
L-threonine	0.37
L-tryptophan	0.09
L-valine	0.40

Fly stocks and husbandry. The wild-type Dahomey strain was originally collected in 1970 from Dahomey (now known as the Republic of Benin) and since maintained as a large outbred stock with overlapping generations at 25°C on a 12h light:12h dark cycle. These conditions allow for inter-generational breeding and the life expectancy of flies remain similar to that of newly caught wild flies [52]. Flies used for experimentation came from parental flies of the same age at egg laying, thereby controlling for the effects of parental age on lifespan [53].

Insulin-signalling mutant flies lacking the *Drosophila* insulin-like peptides (DILPs) *ilp*2, *ilp*3 and *ilp*5 were generated as described in Gronke et al., 2010 [23]. These flies were backcrossed into a control $white^{Dahomey}$ background stock, which was derived by backcrossing w^{1118} into the outbred wild-type Dahomey background [24]. All mutations were back-crossed into their control backgrounds for a minimum of 6 generations.

Lifespan. All experiments were conducted at 25°C on a 12h light:12h dark cycle, at a constant humidity of 65%. Flies were reared at a standard larval density of ~300 flies per bottle, and all experimental adults were collected within a 12 hour period after eclosion. Flies were allowed to mate for 48 hours after eclosion before the experimental females were separated out under CO_2 anaesthesia. Females were then randomly allocated to the experimental food treatments and housed in plastic vials containing food at a density of 10 flies per vial, with 15 vials per condition (n=150). Flies were transferred to a fresh food source 3 times per week, during which any deaths and censors were recorded.

Fecundity. Lifetime fecundity was measured as the cumulative total for days 7, 14, 21 and 28 of the mean number of eggs laid per female fly over each 24-hour period. Eggs in each vial were counted by eye using a light microscope after 18-24 hours exposure to flies.

Western blots. Protein extracts for western blot analysis were made from whole flies, sampled after 7 days of food treatment, using a TCA-based extraction protocol. 10µl of each sample was loaded into a 12% SDS-PAGE gel and blots were probed with anti-phospho-Thr398-S6K antibody (#9209, Cell Signaling Technologies, MA, USA), and total-S6K (re-made using a peptide sequence previously used to generate the total S6K antibody in Stewart et al., 1996 [54]). Both antibodies were used at a dilution of 1:12000 and normalised by probing with an anti-actin antibody at a dilution of 1:5000. Secondary antibodies conjugated to HRP (AbCam, Cambridge, UK) were used at a dilution of 1:5000, and the signals were detected by chemiluminescence.

Stress Experiments

Experimental flies were reared and housed as described for the lifespan experiment. Mated female flies were kept on the experimental food types for 7 days before being transferred to the stress conditions.

Paraquat, DDT and H_2O_2 Stress. The orally administered stressors were as made up follows: 1xSYA containing 20mM paraquat (Sigma-Aldrich, Dorset, UK), 1xSYA containing 0.03% w/v DDT (Supelco Sigma-Aldrich, Dorset, UK), 1.5% agar medium containing 5% H_2O_2 (Sigma-Aldrich, Dorset, UK) and 50g/l sucrose, or plain 1.5% agar medium for the starvation experiment.

Heat Shock. Experimental flies were transferred singly into dry empty 2ml glass vials, plugged with cotton wool and placed into a water bath set at 39°C. The time taken for each fly to fall onto its back and stop twitching (knockout) was recorded.

Metabolic measurements

Experimental flies were reared and housed as described for the lifespan experiment. Mated female flies were kept on the experimental food types for 7 days before being frozen in liquid nitrogen. 6 replicas of 5 flies per condition were used for all metabolic measurements.

Triacylglyceride measurement. Flies per condition were homogenised in 0.05% Tween 20 (Sigma-Aldrich, Dorset, UK) according to Gronke et al., 2003 [55]. TAG content was quantified using the Triglyceride Infinity Reagent (Thermo Fisher Scientific, Surrey, UK).

Glycogen measurement. Flies were homogenised in 200µl saturated Na_2SO_4 solution and centrifuged for 1 min. 80µl of each sample was transferred to new Eppendorf tubes and 800µl chloroform:methanol (1:1) solution was added. Samples were centrifuged for 5 minutes and the supernatant was removed. The remaining pellet, containing precipitated glycogen, was resuspended in 1ml anthrone solution (anthrone in 50 ml 70% H_2SO_4) and incubated at 90°C for 20 minutes. 200µl of each sample was dispensed into the wells of a flat-bottomed 96-well plate, and the absorbance in each well was measured at 620nm and compared against a set of glycogen standards ranging from 0-2µg/µl (protocol adapted from Van Handel, 1965 [56]).

Trehalose measurement. Trehalose levels were measured using the Glucose Infinity Reagent (Thermo Fisher Scientific, Surrey, UK), as described in Broughton et al., 2005 [24].

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Conflict of interest statement

The authors of this manuscript declare no conflict of interests.

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