The biology of ageing: pro- and anti-ageing signals from the reproductive system

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Abstract

This context of this thesis is that ageing is characterised by the accumulation of random cellular damage leading to a decrease in reproductive function and survival, which ends in death. The antagonistic pleiotropy theory for ageing suggests selection favours genes with positive effects on early life even if detrimental effects are observed in later life, indicating a trade-off between lifespan and reproductive output. Ageing, however, is a complex genetic trait and strong interactions between genes that influence longevity and environment including with diet, stress and pharmacological factors have been discovered. Consistent with this, recent work has shown that both genetic and dietary alterations can substantially increase the healthy lifespan of a range of organisms, across many taxa.

The research in this thesis aims to examine whether a major cellular protection mechanism, autophagy, can counteract the lifespan-reducing effects of reproduction in the model organism *Drosophila melanogaster*. Experiments were conducted on males and females with different reproductive and nutritional status. Effects of genetic and pharmacological activators of autophagy on lifespan and reproductive success were investigated, as were trade-offs between reproduction and survival. The pharmacological autophagy activator Torin1, when added to the diet extended survival. There was no evidence of a trade-off of extended lifespan with reduced fertility, indicating theses are controlled, at least in part, independently. Hence, lifespan extension was apparently cost free suggesting Torin1 may offer a potentially fruitful route for further studies of healthy lifespan extension. Autophagy gene 8 (*Atg8a*) overexpression and knockdown manipulations were used to determine effects of autophagy up- and down-regulation on lifespan and reproductive success. Neither manipulation had the predicted effect on lifespan, although *Atg8a* knockdown did compromise, to some extent, the survival response of females to diet restriction. In the final piece of research in the thesis, diet-deprived males lost the ability to respond adaptively to rivals. This effect was not protein-specific so is predicted to be autophagy independent.

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Chapter 1 General Introduction

Ageing

Ageing is a progressive decline in physiological function accompanied by a decrease in fertility and increase in mortality with advancing age (Kirkwood and Austad 2000; Hamet and Tremblay 2003; Vellai 2009). The proximate effects of ageing are thought to be driven by the intracellular accumulation of random cellular damage (Vellai 2009). This leads to decreased reproductive function and survival as age increases and ultimately ends in death (Flatt and Schmidt 2009). Ageing is considered to be a dynamic and malleable process, influenced by genes, environmental and epigenetic factors (Hamet and Tremblay 2003). Ageing and senescence are both terms that are often used interchangeably for the observed deterioration in an organism's physiological state late in adult lifespan, but they are not synonymous. Senescence usually refers to the decreased function measured as the loss of individual cell function, with a focus on the cellular mechanisms involved. Cellular senescence does not therefore lead inevitably to organismal senescence. Ageing, on the other hand, refers to a more widespread decline in organismal bodily function characterized by a potentially complex and progressive accumulation of damage in molecules, cells and tissues with the passage of time (Partridge and Barton 1996; Markaki and Tavernarakis 2011). The overall rate at which age-related damage accumulates can be regulated by pathways that control maintenance and repair, which in turn may be modulated by metabolic factors (Vellai 2009).

Though many diseases often show a strong age-related incidence, ageing is an ultimate product of evolution. There are ultimate reasons why virtually all organisms age, because there are evolutionary explanations for ageing (i.e. ageing is not a directly 'maladaptive' phenomenon, and ageing is instead likely to represent a side effect of other evolutionary processes). There are therefore important ways in which changes attributable to age can be distinguished from those related to disease. Unlike any known disease, age related changes occur in every human, given

sufficient time (i.e. ageing is 'ultimate' in an evolutionary sense). Ageing occurs in virtually all species so far studied and there is no disease that affects all members of a population only after they reach reproductive maturity (Hayflick 1998).

The process of ageing is therefore distinct from the expression of age-related diseases (Hamet and Tremblay 2003). However, despite this, some researchers aim to treat ageing as a disease in much the same way as other genetically influenced conditions (Longo, Shadel et al. 2012). Mutations that reduce lifespan may do so by causing serious disorders that result in death, but this can be relatively independent of ageing. Some authors such as Hayflick (1998) believe that human ageing is an artefact of civilization, because we have , through factors including improved health, cleaner environments and healthier diets, escaped the many causes of death in the post-reproductive period (Hayflick 1998). In nature, ageing may be rare due to competition, predation and/or parasitism (Flatt and Schmidt 2009). However, as both ageing and age-related diseases are driven by cellular damage, it is feasible that there is some overlap between the underlying causative pathways (Vellai 2009).

Explanations for the evolution of ageing are of great interest, a fundamental question is why, given that ageing impairs survival and fertility, does natural selection not eliminate it (Kirkwood and Austad 2000)? In some species there is little mortality or reduction in fertility as age advances, indicating that ageing cannot be defined as the inevitable result of wear and tear. Ageing is however ubiquitous, and the most current thinking is that it arises because of a combination of genetic, environmental and epigenetic factors, as described below.

Why ageing occurs

Early explanations of why organism's age included the idea that senescence was genetically 'programmed' to limit population sizes or to accelerate the turnover of generations, thus facilitating adaptation to changing environments. Evidence for this theory in wild populations is sparse but is

proposed to include the Pacific salmon in which death coincides with the end of a semelparous (once only) reproductive cycle (Kirkwood and Austad 2000). The suggestion is that salmon only reproduce once and so die to make way for the next generation. However, this strategy of programmed death cannot be selected outside a kin context, for which there is no evidence in the salmon example. Instead the high death rate that occurs in the salmon after reproduction is thought to be due to the enormous expenditure of energy that precedes mating in this species (Hayflick 1998).

During the early to mid-1900s it was believed that normal cultured cells were immortal and ageing was caused by extracellular events. However, it is only abnormal or cancer cells that are immortal (Hayflick 1998). Research suggests that normal cells have a finite capacity for replication, for example cellular senescence is shown in isolated cells that demonstrate a limited ability to divide in culture, a phenomenon known as the Hayflick limit (Hayflick and Moorhead 1961; Burnet 1974; Shay and Wright 2000). In mammals, features of DNA replication prevent cells from completely copying the ends (telomeres) of linear DNA, and so with each division the telomere is shortened until it can no longer divide. In invertebrates a similar, but mechanistically different situation exists, where the ends of chromosomes are protected by repeated sequences arising from transposable elements. One of the first reports that human telomeres might shorten over time appeared in 1986 and these initial findings supported the idea that telomere attrition limits normal cell proliferation (Cook and Smith 1986). It appears that ageing has not been programmed by natural selection during the course of evolution. However, it is also true that few or no organisms have unlimited lifespans. As discussed below, a reason for this could be that it is reproductive characters that are direct subjects of natural selection, with ageing occurring as a by-product. Traits expressed in early life are therefore more important to natural selection than those expressed in later life (Hamet and Tremblay 2003).

The ageing process (i.e. the ever increasing decline in reproductive capacity) begins after the start of reproduction. Natural selection to reduce ageing rate will generally be quite weak, certainly weaker than the selective processes that shape early development and sexual maturation (Hamet and Tremblay 2003). Natural mortality may arise primarily due to extrinsic hazards, infection, predation, starvation and cold stresses. Therefore organisms from wild populations may not often live long enough to grow very old, hence natural selection has a limited opportunity to exert any influence over ageing (Kirkwood and Austad 2000). This is particularly so as ageing is primarily manifested in the post reproductive period where the direct contribution of individuals to fitness diminishes significantly. There is some evidence for ageing in the wild in some species such as elephants. However, very aged individuals will generally form only a small proportion of wild populations, hence, selection to reduce ageing will again be very weak or non-existent. Any genes that 'programme' ageing are not expected to be maintained at a stable equilibrium because individuals carrying a mutation to inactivate such genes would be favoured (Kirkwood and Austad 2000). This would be true unless genes for accelerated or programmed ageing arose in an environment in which relatedness is high and therefore fitness benefits can be gained by helping kin. There are, however, a few paradoxical cases (e.g. tortoises, lobsters) in which ageing is reported not to occur. In these organisms the mortality rate apparently remains flat with increasing age. Whether this is really the case is not yet clear, but should the data hold up, one suggestion is that the explanation may be related to the fact that in these creatures there is no fixed adult size (Hayflick 1998). Other organisms that apparently escape the ageing process, such as hydra, have the capacity to regenerate all their cells (Alvarado 2000), hence whether a single hydra at two different points in time is the same 'individual' is debatable.

The idea outlined above, i.e. that ageing can be programmed in the absence of any kin selected benefits, has long persisted in the gerontological literature, even with much evidence to the contrary. A challenge for the kind of evolutionary approach taken in this thesis is therefore to correct this view

by providing evidence that ageing and lifespan are highly plastic and responsive to the prevailing environmental conditions.

Researchers have long sought general proximate explanations for the mechanisms underlying the increased decline in bodily function with age. Oxidative stress is one such mechanism that many now consider universal (Sohal and Weindruch 1996). Oxidative damage to macromolecules arising from the production of free radicals tends to accumulate with age and to be associated with life expectancy. Free radicals are produced as a normal by product of oxidative phosphorylation in the mitochondria, but the off target damage they can do increases with age, either because of increased frailty or because systems for neutralising free radicals become less efficient with age (Harman 1956; Droge 2002). However, there continues to be much debate about how strong and direct is the link between oxidative stress and ageing. Links between these two processes first emerged as the 'rate of living' theory (Hamet and Tremblay 2003) which was first suggested by the German physiologist Max Rubner in 1908. The theory states there is a relationship between body size, metabolic rate and longevity. It proposed that every organism is born with a limited amount of energy and that if this energy is used slowly, the rate of living would also slow and lifespan would be long. Put simply the theory was essentially that each organism had a finite number of heartbeats, breaths etc. and that just as a machine deteriorates with use so too would the body (Rubner 1982). The modern version of this theory is that organisms that process oxygen faster, i.e. have higher rates of oxidative phosphorylation and hence generate more oxidative damage, effectively live 'faster' lives and die younger. Examples would include small mammals that have rapid heartbeats and metabolize oxygen quickly and have short lifespans in comparison to tortoises that metabolise oxygen very slowly and have a long lifespan. However, there is little in this theory to help us to determine which individuals within a species will live the longest. In addition the relationship between metabolic rate and lifespan is tenuous, there are many organisms that live longer than they 'should' (e.g. birds are extremely long lived for their size and have a high metabolic rate) and, conversely, many that have shorter lifespans than they should (e.g. marsupials, with short lifespans and slow metabolism). In

general, then, the rate of living theory does not give a satisfactory account of variation in lifespan within or between species, and is therefore not currently widely supported in the study of ageing from an evolutionary perspective.

The evolutionary study of ageing has been very successful in providing ultimate explanations for the universality of ageing, regardless of the mechanism by which it occurs. There are two main, potentially overlapping theories: antagonistic pleiotropy (Williams 1957; Hamilton 1966) and mutation accumulation (Medawar and Medawar 1985). Both of these theories are based on the observation outlined above, that the power of natural selection wanes with age (Haldane 1957), hence deleterious phenotypes that are expressed only late in life and that contribute to ageing will not be strongly purged. The antagonistic pleiotropy theory for ageing postulates that selection will favour genes that have a positive effect in early life even if they have detrimental effects later in life (Williams 1966; Sozou and Seymour 2003). Much evidence for this theory has been gained from studies of artificial selection experiments in *Drosophila melanogaster* (Rose 1984; Partridge and Fowler 1993). In these experiments it is generally found that selection for elevated late life reproduction and long life, are linked to reduced early reproduction (Partridge 1987; Partridge and Barton 1993). These results are taken as important evidence of a trade-off between lifespan and reproductive output and to show that both cannot simultaneously be maximised, supporting the existence of antagonistic pleiotropy. More recent research in this area has revealed the nature of signals involved, as discussed later in chapter 6. The mutation accumulation theory for universal ageing (Medawar and Medawar 1985) similarly proposes that late life acting deleterious mutations will not be strongly eliminated by natural selection. Therefore, if such mutations are transmitted through the germ line, they will contribute to ageing. Again, supporting evidence has come from experiments with fruit flies that demonstrate increased variance in fitness with age, with no associated early life fitness benefits for short-lived individuals (Partridge, Gems et al. 2005; Kirkwood and Austad 2000).

With these basic evolutionary theories of ageing generally accepted, more recent research has sought to identify whether there are any fundamental cellular mechanisms involved in mediating these evolutionary processes (see also fig 1.1). These are thought to be, diet restriction i.e. under feeding without malnutrition, seems to mimic antagonistic pleiotropy by extending lifespan and reducing fertility, and can be explained by responses in the underlying life history traits. Nutrient sensing genes i.e. those members of the insulin-like growth factor (IIS) and Target of Rapamycin (TOR) pathways, described in more detail below. These highly conserved pathways are involved in sensing the amount and types of nutrients ingested in the diet, and may represent, in total or in part, the responses of organisms to diet, described above. Also autophagy, which is again a highly conserved cellular repair mechanism, (described in more detail below). The hypothesis is that stress such as nutrient deprivation can turn on protective mechanisms such as autophagy, which can then 'recycle' resources within cells in response to stress, by cleaning up damaged macromolecules and hence promote longevity.

The central topic of the research in this thesis is that the determination of longevity and extent of ageing is related to the level of activation of repair and protective processes such as autophagy. In the next section the specific relevant factors that have been shown to influence ageing, encompassing those factors listed above, are outlined.

Influences on ageing – pro- and anti-ageing signals

Understanding the ageing process and how it is regulated is an important and complex problem. There are a number of fundamental processes that affect how an organism ages, and many act in tandem with other processes. In figure 1.1 the outline of several important processes that can contribute to variation in ageing and lifespan are shown. These may underlie variability in accumulation of unrepaired damage and the natural decline in an organism's fitness.

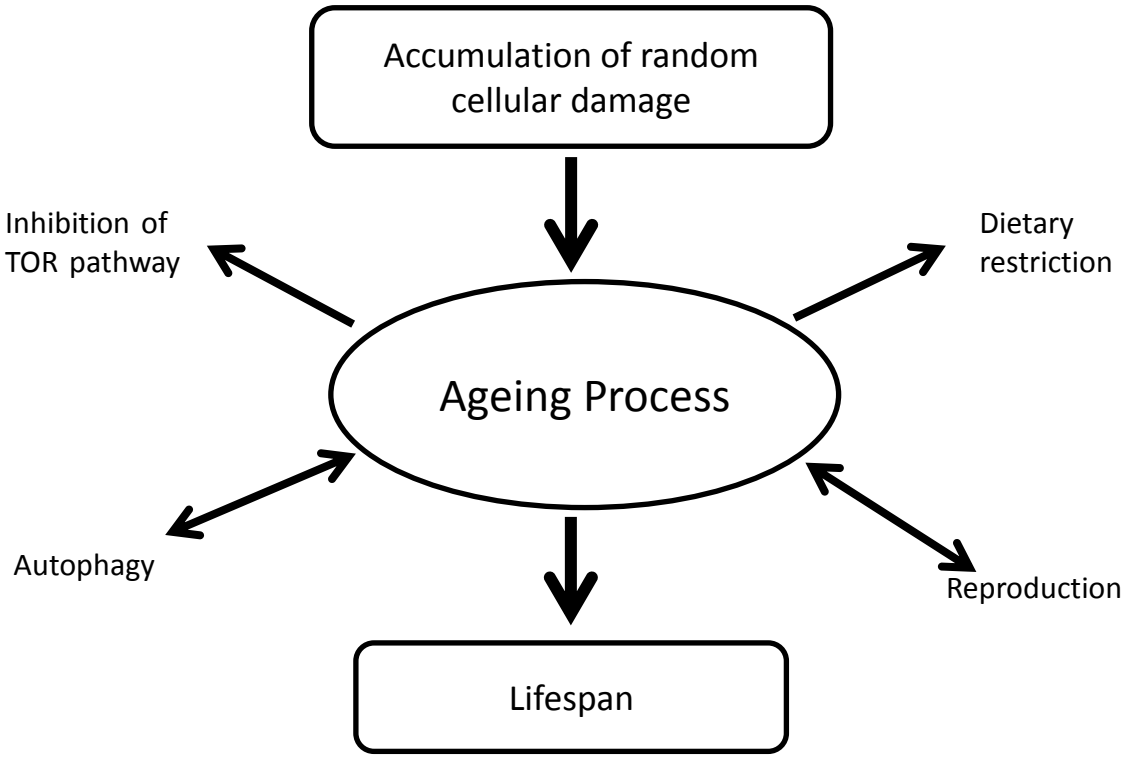


Fig 1.1 – Pro- and anti-ageing signals that determine organismal ageing (adapted from (Vellai 2009))

In the next section the evidence linking the processes identified above to variation in ageing and lifespan is described.

Ageing and nutrient signalling: Many mutations that are reported to extend lifespan are in genes that play key roles in nutrient signalling, the two major pathways are, insulin-like growth factor (insulin/Igf) and Target of Rapamycin (TOR) signalling (Fontana, Partridge et al. 2010). Genes in these pathways may in fact underlie the responses of organisms to different diets (see also next section, below) and are also linked to the cellular repair pathway of autophagy, also described below. The insulin-like growth factor (IIS) pathway influences the control of growth, stress resistance, reproduction and lifespan. Mutations that alter IIS can increase lifespan which indicates this pathway has a specific role in the ageing process. Klass (1983) discovered the first mutation of IIS, called *age-1* in *C. elegans,* it was later found to be located to a component of phosphatidylinositol 3-kinase (P13K) (Klass 1983; Giannakou and Partridge 2007). The initial steps in insulin signal transduction occur at the plasma membrane and lead to the activation of the class I

PtdIns3K. This enables promotion, recruitment and activation by the membrane of protein kinase B (PKB also known as AKT) through 3-phosophoinositide-dependent protein kinase 1 (PDK1), which has an inhibitory effect on autophagy. Note that this finding directly links nutrient sensing to autophagy, the central topic of investigation in this thesis, and could mediate, at least in part, the well-known sensitivity of autophagy to nutrient levels, along with input from the TOR pathway (see fig 1.1, fig 1.2 and below). The first experimental study to show autophagy affected lifespan was by Levine's lab which showed the knockdown of *bec-1 (C. elegans)* inhibits the lifespan-extending phenotype in mutants lacking the insulin signalling gene *daf*-2 (Melendez, Talloczy et al. 2003).

The second major nutrient sensing pathway that has been described is the TOR pathway (also called mTOR, the mammalian or mechanistic Target of Rapamycin). It is conserved across many species, including yeast, flies, worms and mice. Importantly for work in this thesis it has been shown to regulate lifespan in these organisms (Rusten, Lindmo et al. 2004; Mizushima 2007; Evans, Kapahi et al. 2011). It is linked to the IIS pathway above and plays a key role in the coordination of nutrient sensing signals overall. There are five signalling inputs that influence TOR: growth factor signalling, energy stress signalling, hypoxia signalling, nutrient signalling and Wnt signalling (Evans, Kapahi et al. 2011). TOR controls several fundamental cellular functions and is also a major regulator of cellular growth and proliferation. The P13K pathway regulates TOR via the tuberous sclerosis proteins TSC1 and TSC2 (Tuberous sclerosis complex 1 and 2) which act together to inhibit TOR which in turn mediates the signalling pathway that couples amino acid availability to S6 kinase (S6K) influenced translation initiation and growth. TOR therefore functions as a component of the P13K/Akt/TSC/mTOR/S6K (4E-BP) signal transduction pathway, which itself feeds into two protein complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2), (Strimpakos, Karapanagiotou et al. 2009) see fig 1.2.

TORC1 and TORC2 have distinct substrate specificities and are differentially sensitive to the antimicrobial chemical rapamycin (Liu, Kirubakaaran et al. 2012; Feldman, Apsel et al. 2009, see fig

1.2). TORC1 is rapamycin-sensitive, it includes several proteins, including the regulatory protein Raptor. Nutrients such as amino acids, glucose and oxygen enter the cytoplasm by passive diffusion through membrane pores and activate TORC1 either directly or through inhibition of TSC1 & 2. TORC1 promotes anabolism and inhibits catabolism by blocking autophagy through the phosphorylation of the ULK1-Atg13-FIP200 complex (Strimpakos, Karapanagiotou et al. 2009); (Laplante and Sabatini 2011). The most widely known targets for phosphorylation of TORC1 are the protein translation machinery, 4E-BP and S6K. At least four consistent effects are observed when TORC1 signalling is reduced. These are an up-regulation of mitochondrial oxidative phosphorylation (Zid, Rogers et al. 2009). An increased resistance to oxidative stress (Fabrizio and Longo 2003), a cell protective metabolic response (Mizushima 2007) and the initiation of autophagy (see below)(Rusten, Lindmo et al. 2004); (Mizushima 2007); (Evans, Kapahi et al. 2011).

TORC2 is rapamycin-insensitive and is less sensitive to nutrient levels than is TORC1. A major role of TORC2 includes the phosphorylation and activation of Akt (an AGC kinase). Overall the different functional roles of TORC2 are less well understood than those of TORC1 (Feldman, Apsel et al. 2009). It is thought that the sensitivity of the TOR pathway to growth factor signalling is modulated by the phosphorylation of the C-terminal motif of some AGC kinases such as Akt and SGK (Feldman, Apsel et al. 2009). TOR is also of interest in the study of age-related disease such as cancer, because deregulation of the P13K/Akt/TSC/TOR pathway is common in human tumours. Hence there has been much interest in the recent development of TOR inhibitors as a new class of anti-cancer drugs (Liu, Kirubakaaran et al. 2012). As TOR also affects lifespan, this is further evidence that complex traits such as ageing do not exert their influence in isolation, but do so in strong interaction with other factors.

Relevant to my thesis work, inhibition of the TOR pathway is reported to extend lifespan across the various model organisms so far tested (Kapahi, Zid et al. 2004); (Mehrpour, Esclatine et al. 2010). For example, rapamycin has a demonstrated capacity to increase lifespan, it also has significant

immunosuppressive activity, the significance of which isn't yet known (Hamet and Tremblay 2003; Bjedov, Toivonen et al. 2010).

The most prominent pathways associated with longevity are the insulin/Igf and TOR signalling cascades, both have critical roles in nutrient sensing, metabolism (Gelino and Hansen 2012) and have emerged as major regulators of growth, at least in *Drosophila* (fig 1.2). It is the inhibition of the TOR signalling by altering gene expression in this nutrient sensing pathway that extends lifespan in a manner that may overlap with known effects of caloric restriction on longevity (Giannakou and Partridge 2007). Despite the proximate complexity of ageing, recent work has shown that both genetic and dietary alterations can substantially increase the healthy lifespan of a range of organisms across many taxa, suggesting that the fundamental pathways underlying variation in ageing rates may be simpler than perhaps first thought (Fontana, Partridge et al. 2010).

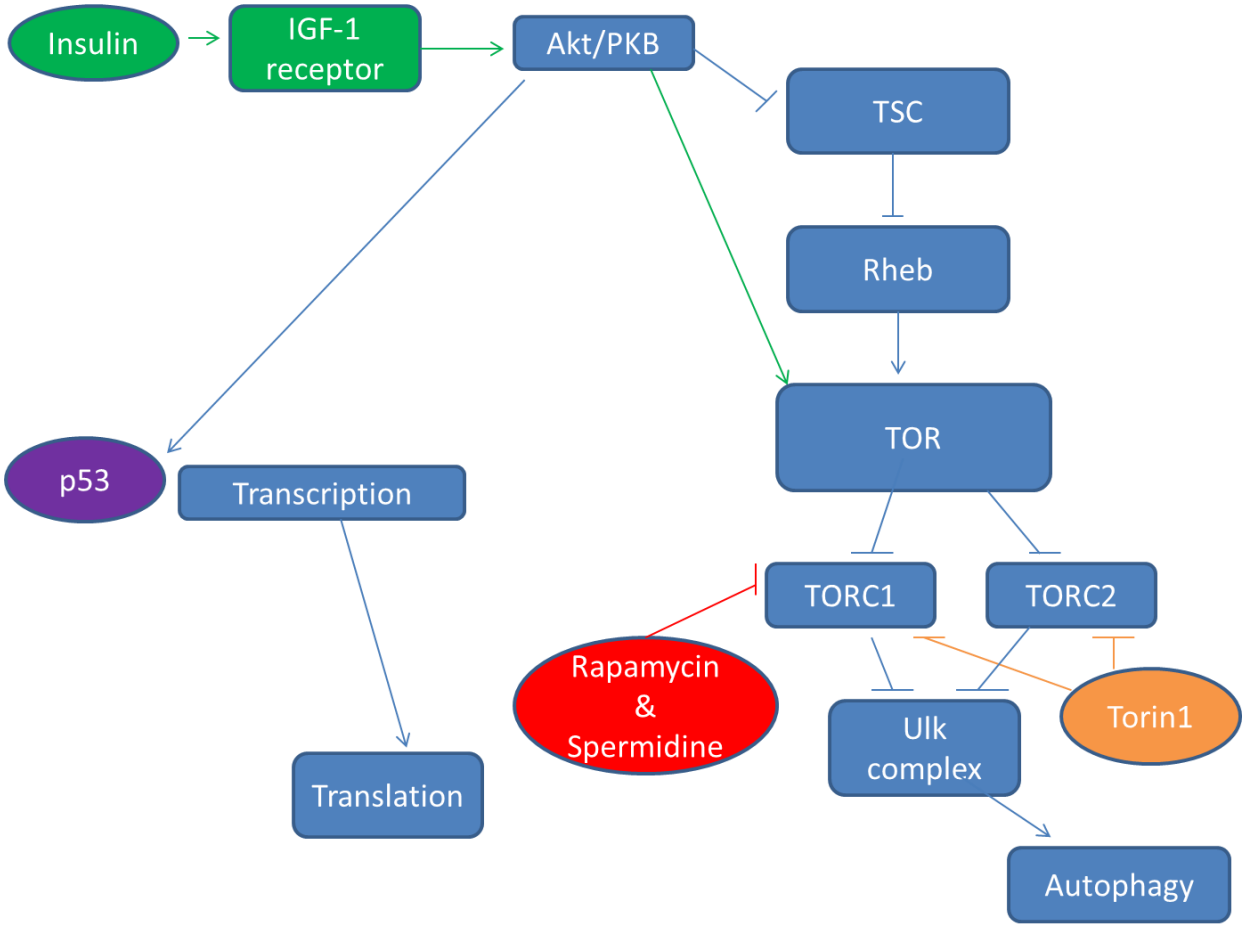


Fig 1.2 – Factors affecting activation of autophagy, extremely simplified to show where target of rapamycin (TOR) can be affected by pharmacological manipulations (adapted from (Klionsky 2007).

Ageing and Diet: Access to food for most species may be variable, because of this it is thought that cells evolved a mechanism to cope with the stress that can result from intermittent energy supply or storage. The variation in diet availability may have helped to shape cellular metabolism, tailoring it to break down or process food in a way to allow the body to cope with a long time lag between feeding events. It is thought that for humans in industrialised countries a steady and over abundant food intake may overwhelm cellular metabolism and the ability to clean up or neutralise harmful free radicals. This can contribute to the degradation and destruction of cells, causing a decrease in lifespan (Wattanapitayakul and Bauer 2001). In contrast there has been much work to investigate how caloric or dietary restriction can lead to an lifespan extension across many different taxa (Carey, Harshman et al. 2008; Piper, Mair et al. 2005; Kirkwood and Rose 1991). Of note is that the relation

between calorie intake and lifespan shows that food consumption above the optimal level progressively shortens lifespan, as suggested above. The usual practice of *ad libitum* feeding may promote obesity and other age associated pathologies in laboratory subjects, as does the wide variation in calorie intake among individuals from the same experimental cohort. It has been suggested therefore that laboratory subjects should be fed a controlled calorie intake for nutrition/ageing studies (Sohal and Weindruch 1996). However, it has also been shown that *Drosophila melanogaster* do not exhibit compensatory feeding, and eat at a relatively stable rate whatever quality diet is available (Fontana, Partridge et al. 2010). Hence, control of food intake is largely unnecessary in ageing studies conducted on this species.

Caloric restriction is defined as the restriction of nutrients whilst avoiding malnutrition and was first seen to delay ageing and disease in rats approximately a century ago (McCay, Crowell et al. 1935). Caloric restriction is usually a 20 to 40% reduction in calorie intake compared with an *ad libitum* diet but without compromising the maintenance of any essential nutrients (Piper and Bartke 2008). Extended longevity via caloric restriction has been consistently seen in model organisms, for example in rodents (Holehan and Merry 1986; Masoro, Shimokawa et al. 1991; Masoro 2005), and in the fruit fly *D. melanogaster* (Chapman and Partridge 1996). There have been multiple studies which indicate that effects of caloric restriction on lifespan extension can represent up to a 50% increase in maximum lifespan (Koubova and Guarente 2003). Even though the benefits of caloric restriction have been known for many years, the underlying mechanism is not yet fully resolved, though is likely to involve signalling through the IIS and TOR pathways, described above. Diet restriction has multiple effects, including metabolic, neuroendocrine and apoptotic, which vary in intensity between different species (Sohal and Weindruch 1996; Masoro 2005). As noted, it is believed that the IIS and TOR nutrient sensing pathways mediate the effects of caloric restriction however, it is not yet known to what extent they do so (Gelino and Hansen 2012). Consistent with the links between caloric restriction, nutrient sensing genes and lifespan, it is observed that

mutations in genes that encode for components of the insulin/insulin-like growth factor (IIS) result in lifespan extension. IIS is a key nutrient signalling pathway that coordinates traits such as development and growth with resource availability indicating that a reduction in IIS was a good candidate mechanism through which caloric restriction might extend lifespan (Giannakou and Partridge 2007). In the fruit fly mutations to *chico,* which encodes the insulin receptor substrate, lifespan was extended by 48% when nutrient intake was high. When food was limited *chico* flies were shorter lived than the controls as would be expected if the mutants were already partially restricted in their diet by their genotype (Clancy, Gems et al. 2001). However, experimental evidence to date suggests that lifespan extension by caloric restriction is not exclusively the product of a reduction in insulin signalling (Giannakou and Partridge 2007).

The attempts to modify longevity in animal models, often by dietary manipulation/caloric restriction, are due in part to a quest to find methods for humans to live healthier longer lives (Hamet and Tremblay 2003). As well as length of life *per se*, it is also of prime importance to consider how healthy might be the individuals subject to any extension to the normal lifespan. More generally, it has been suggested that it is crucial to consider the interactions between diet and genotype (i.e. interactions with apolipoprotein E genotype E4 (apoE4)), in the context of healthy ageing. The apoE4 genotype significantly interacts with dietary fat intake in healthy older people and is the most important genetic risk factor for Alzheimer's and other diseases. A protective effect of apoE4 is seen with lower dietary fat intake (Hamet and Tremblay 2003). It is believed that by lowering calorie intake this may minimise free radical interactions and so increase lifespan. It may also enhance the immune system and inhibit some forms of cancer (Gelino and Hansen 2012).

Because it is believed to be the lifelong accumulation of damage that drives the ageing process that leads to age-related disease and ultimately death, it is by enhancing or prolonging mechanisms that eliminate cellular damage that the rate of accumulated damage will decrease (Vellai, Takacs-Vellai et al. 2009). It is the pro- and anti-ageing signals via caloric restriction and pharmacological agents that

are important in this thesis, because a major part of this process is autophagy, an ancient conserved pathway, described below, that forms the major topic of investigation for this thesis.

Autophagy as a means to counteract ageing

Three types of autophagy have been identified, microautopahgy, chaperone-mediated autophagy (CMA) and macroautphagy (Klionsky 2005). Mircoautophagy is the least studied of the three. In this process, cytoplasm destined for destruction is sequestered by membrane invagination and/or septation. CMA is a secondary response to starvation and, unlike the other two processes, involves the direct translocation of targeted proteins across the lysosomal membrane (Klionsky 2005; Gelino and Hansen 2012). Macroautophagy, the best characterised, and hereafter referred to as autophagy, is a ubiquitous process in eukaryotic cells. It is activated in response to stress and results in the breakdown of damaged molecules and organelles within the lysosome, which allows the cell to adapt to environmental and/or developmental change (Klionsky 2005 & 2007). This stress related pathway removes damaged macromolecules, organelles and misfolded or damaged proteins from cells. During autophagy the damaged macromolecule is enclosed by a phagophore or isolation membrane (fig 1.3) and upon closure a double membrane-enclosed autophagosome is formed (Rusten, Lindmo et al. 2004; Mizushima 2007; Chang and Neufeld 2009). The term autophagic flux is used to denote the whole process of autophagy (fig 1.3), from delivery of the autophagic substrates to the lysosome to the degradation of the autophagic substrates inside the autolysosome (Mizushima, Yoshimori et al. 2012).

Regulating autophagy involves the de-repression of the TOR Ser/Thr Kinase discussed above, which inhibits autophagy by phosphorylating the first of many autophagy genes involved in this process, namely autophagy gene 13, *Atg13*, and it is this phosphorylation that leads to the dissociation of *Atg13* from a protein complex that contains *Atg1* and *Atg17* and so attenuates the *Atg1* kinase activity at the initiation phase. When TOR is inhibited the re-association of dephosphorylated *Atg13*

with *Atg1* stimulates its catalytic activity and so autophagy is induced. Vesicle nucleation (step 2 in fig 1.3) includes activation of a class III phosphatidylinositol 3-linase (P13K), which generates phosphatidylinositol-3-phosphate (PtdIns3P). P13K activation depends on the formation of a multi-protein complex in which *Atg6* participates. The vesicle elongation process includes two ubiquitin-like conjugation systems. One pathway involves the covalent conjugation of *Atg12* to *Atg5*, with the help of *Atg7* and *Atg10*. The second pathway involves conjugation of phosphatidylethanolamine (PE) to *Atg8* by the sequential action of the protease *Atg4, Atg7* and *Atg3*. Lipid conjugation leads to the conversion of the soluble form of *Atg8* called *Atg8-I* to the autophagic–vesicle-associated form *Atg8-II. Atg8-II* is often used as a marker of the extent of activation of autophagy because it is a limiting product on the autophagy pathway and because its lipidation and the specific recruitment to autophagosomes provides a shift from diffuse to punctate staining (which can be visualised) and increases its electrophoretic mobility on gels compared with *Atg8-I* (which can also be visualised, see chapters 3 & 4).

The *Atg9* complex potentially participates in the delivery of the membrane to the forming autophagosome, although this complex is poorly studied (He, Baba et al. 2009). Autophagosomes undergo maturation by fusion with lysosomes to create autolysosomes. In the autolysosomes the inner membrane, as well as the luminal content of the autophagic vacuoles, is degraded by lysosomal enzymes that act optimally within this acidic compartment. Importantly, pharmacological inhibitors are capable of inhibiting distinct steps of this process for example the rapamycin, spermidine and Torin1 inhibitors all block TOR, (fig 1.2) (Rusten, Lindmo et al. 2004; Mizushima 2007; Chang and Neufeld 2009; He and Klionsky 2009). Therefore, these compounds can be used to manipulate, as in this thesis, the extent of autophagy.

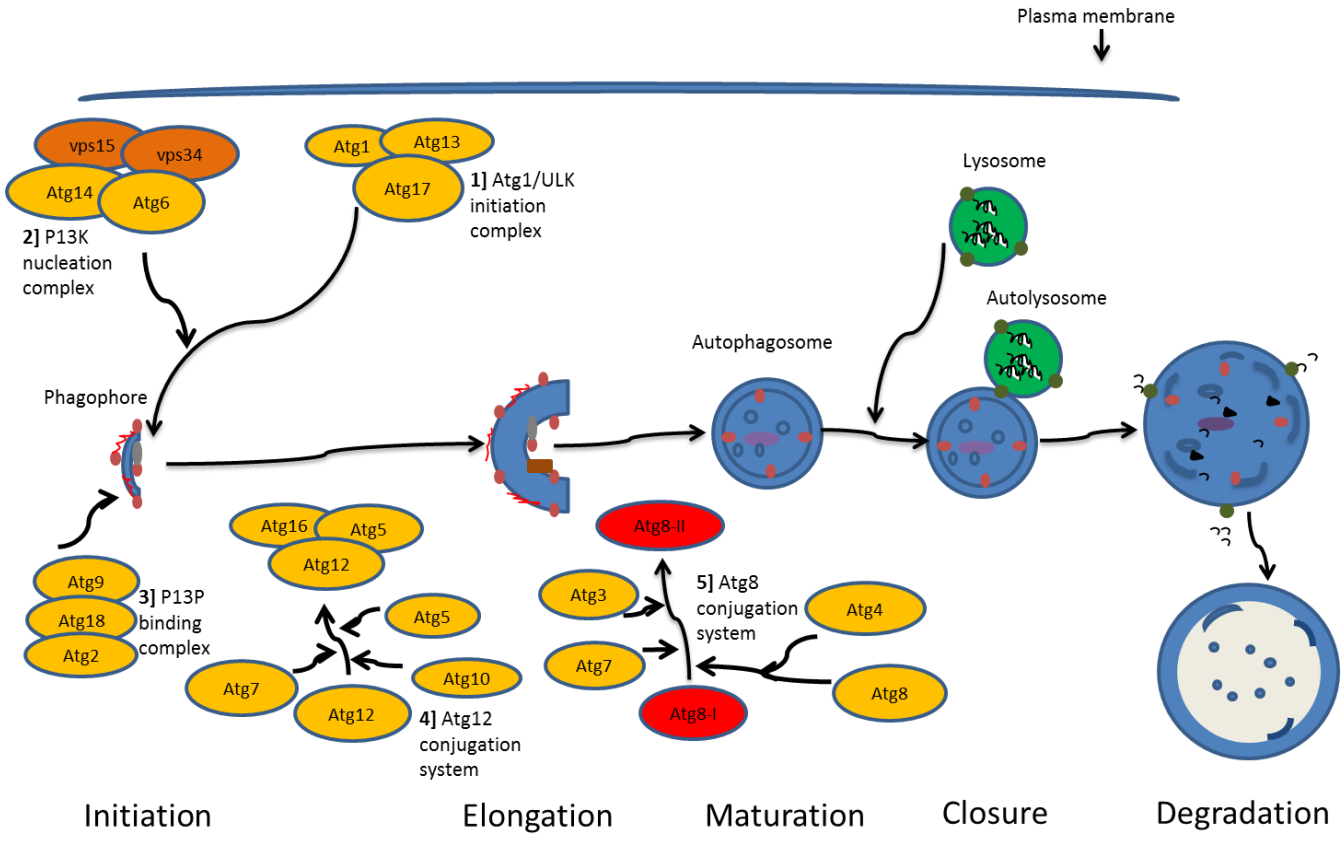


Fig 1.3 - Autophagy is initiated by the formation of the phagophore or isolation membrane. A damaged macromolecule is enclosed by a phagophore and upon closure a double-membrane autophagosome is formed. The outer membrane subsequently fuses with the lysosome and the inner membrane with its contents are degraded (adapted from (Yang and Klionsky 2010 ; Gelino and Hansen 2012))

It is known that autophagy has effects on lifespan, which can be triggered in response to environmental factors such as stress and nutrition (Eisenberg, Knauer et al. 2009; Rusten, Lindmo et al. 2004). Autophagy helps to maintain the balance between synthesis, degradation and the subsequent recycling of cellular products and, as stated, has been found to promote longevity (Eisenberg, Knauer et al. 2009). Rusten et al. (2004) used the *Drosophila* fat body as a model system to study the regulatory mechanisms of programmed autophagy, finding that ecdysone receptor signalling has the ability to promote autophagy through the down regulation of P13K signalling. Rusten et al. (2004) showed a complex regulation of programmed and starvation induced autophagy in the *Drosophila* fat body revealing a link between the hormonal induction of autophagy and P13K

signalling (Rusten, Lindmo et al. 2004). This shows there are other factors involved in the activation of autophagy rather than just the starvation response. P13K signalling suppresses autophagy in the fat body but during the last larval stages the negative regulation can be abolished by ecdysone signalling which allows autophagy to take place.

The expression of some autophagy genes in *Drosophila* is reduced in neural tissues as a normal part of ageing. Mutations in the *Atg8* gene result in a reduced lifespan. However, enhanced *Atg8* expression in older fly brains has been found to extend life by an average of 50%. It is suggested that the genetic or age-dependent suppression of autophagy is associated with the build-up of cellular damage in neurons and therefore causal in reduced lifespan. The age-dependent decrease in autophagy gene expression is paralleled by a pronounced accumulation of insoluble ubiquitinated proteins (IUP), which are often held to be useful as markers for cellular ageing. The maintaining of active autophagy prevents the accumulation of damage in neurons and promotes longevity. This suggests that the age-dependent suppression of autophagy may be a contributing factor in some human age-related disorders (Simonsen, Cumming et al. 2008). There is also evidence explaining the apparent paradox as to why autophagy which functions primarily as a cell survival pathway also has a function in tumour suppression. In apoptosis-defective cells autophagy prevents death from necrosis (a process that might exacerbate local inflammation and promote tumour growth). Stressed autophagy-defective tumour cells accumulate p62 (also called sequestosome 1), damaged mitochondria, reactive oxygen species (ROS) and protein aggregates which may cause DNA damage, oncogene activation and tumourigenesis.

The control of autophagy is nutritionally, hormonally and developmentally regulated through multiple signalling pathways. Autophagy gene 12 (*Atg12*), *Atg5* and *Atg8* (also known as *LC3* in mammals) are essential for the initiation and progression of autophagy. Some products of the *Atg* genes are small ubiquitin-like proteins. *Atg12* is covalently attached to *Atg5* which forms an *Atg12*-

Atg5 conjugate, which is a conserved requirement for the activation of autophagy from yeast to mammals. *Atg8* is conjugated to the lipid phosphatidylthanolamine (PE), which it is thought to allow membrane localization. Genetic analysis has shown that the *Atg12-Atg5* conjugate is needed for *Atg8* localization to the preautophagosomal structure (PAS) and that both conjugates are necessary for autophagy to progress (Rusten, Lindmo et al. 2004).

Throughout this thesis pharmacological, dietary and genetic manipulations have been used to activate autophagy and inhibit the TOR pathway. Previous studies suggest autophagy can counteract the deleterious effects of ageing, the central aim was to test this in a variety of different experiments. Dietary applications of spermidine and Torin1, which feed into the TOR pathway and alter autophagy, were used, as shown in fig 1.2. In fact, extension of lifespan using pharmaceuticals is often found to be dependent upon an intact autophagy pathway, reduced S6 kinase (S6K) activity and eukaryotic initiation factor 4E binding protein (4E-BP) and is also associated with a reduction in protein turnover (Eisenberg, Knauer et al. 2009; Rusten, Lindmo et al. 2004; Fontana, Partridge et al. 2010). Genetic manipulations of a limiting component of the autophagy pathway, *Atg8a* (fig 1.3) were also used. Dietary restriction experiments were also employed because this manipulation is known to lead to reliable responses in lifespan, with a central role for autophagy (Gelino and Hansen 2012).

To prolong length of life, investment is needed in somatic maintenance, which will, as a consequence, reduce resources that may otherwise be available for reproduction. Studies in different animal species have shown that reproduction can have a cost to longevity; even in humans (Westendorp and Kirkwood 1998). Much work has been done using *D. melanogaster* on damage caused by reproduction leading to shortened lifespan (Chapman, Arnqvist et al. 2003; Flatt 2011). Signals from the reproductive system are therefore implicated in modulating longevity.

Research has been conducted into the effects on lifespan of autophagy, but not much has yet been done on the suspected link between fertility effects and autophagy, on the magnitude of the potential trade-offs (see below).

Trade-offs

Life history resource allocation models have been developed, which demonstrate different life history traits are traded off against each other. One such trade-off is the cost of reproduction, where an elevated reproductive rate can lead to lower subsequent fecundity or survival later in life. Traditionally, the cost of reproduction has been interpreted as a consequence of the conflicting demands for resources between reproduction, growth and somatic maintenance (Reznick 1985; Barnes and Partridge 2002). Work in the model organism *Drosophila* has shown evidence that one cost of reproduction is an acceleration in the rate of ageing (Partridge 1987).

Current thinking is that natural selection declines with age, and extended longevity is acquired at some metabolic cost. For example, organisms may trade late survival for an enhanced reproductive investment in earlier life (Kirkwood 1997). There are a number of theories that suggest there is an established trade-off between survival and reproduction, for example the antagonistic pleiotropy theory (Williams 1957) and the disposable soma theory (Kirkwood and Holliday 1979). The abundant evidence for a trade-off between reproductive effort and lifespan suggests that animals must make a physiological choice about how their resources are used. In some species organisms die soon after mating or have a shorter lifespan as a consequence of mating (Kenyon 2010). It has been suggested that a high reproductive output correlates with a shorter lifespan while a lower reproductive output correlates with a longer lifespan (Hayflick 1998). High adult mortality rates favour the evolution of high early reproduction. Some animals such as humans and elephants may be able to invest in later age reproduction due to resistance to environmental hazards and/or better adaptation to the environment. These conditions can select for the evolution of a slow growth rate

and a slow reproductive rate and hence longer lifespan. In the absence of these factors however, natural selection will tend to favour traits that favour early enhanced fecundity against those that favour a greater longevity. The force of natural selection then is found to decline with age (Hayflick 1998). At least four general principles that underlie trade-offs have been identified (Flatt 2011). These are that survival and reproductive trade-offs are widespread throughout many organisms. The relationship between decreased fecundity and increased longevity can be uncoupled under certain circumstances. There are no robust alternative explanations for trade-offs when costs are not due to competitive resource allocation, and survival and reproductive physiological trade-offs do not always translate into evolutionary trade-offs.

Trade-offs are important in understanding the links between different life history traits and if/how they can be altered without fitness costs. It may be possible to manipulate trade-offs between survival and fecundity to extend lifespan and also give an insight into how much damage the reproductive process can cause. However, it is not always easy to determine the exact effect on the function studied. For example, lifespan extension by caloric restriction is not exclusively due to a reduction in reproductive costs (Carey, Harshman et al. 2008). Results of studies on humans have been conflicting, although results from Tabatabaie et al. (2011) suggest that a lower number of offspring and a pattern of delayed reproductive maturity may extend lifespan. This would indicate a trade-off between early fecundity and lifespan in humans (Tabatabaie, Atzmon et al. 2011). This has also been studied in an annual species of grasshopper (*Melanoplus sanguinipes*). The hypothesis was, females that lay fewer eggs, or that begin laying later in life, should live longer than those that lay more eggs or begin producing eggs earlier in the lifetime. However, no trade-off was found between these two factors, suggesting that survivorship, reproductive timing and offspring numbers may be controlled by undisclosed factors other than the simple relationship between lifespan and fecundity (Dean 1981). In nature, guppies that live with no predation have shorter lifespan and fewer progeny than those that live with a high level of predation. This has also been seen in guppies

where long-lived mutants have more progeny than their wild type counterparts, showing longevity doesn't always come at the cost of reproduction (Kenyon 2010). Mutations in the gene encoding components of the IIS pathway extend lifespan in the nematode *C. elegans*. The pathway was discovered in the nematode because it regulates the entry of developing nematodes into an alternative larval (dauer) stage when food is limited or conditions are crowded. This mutation under the right conditions can extend lifespan but also decreases fecundity, suggesting reallocation of nutrients from reproduction to somatic maintenance and repair. It was also found that a reduction in nutrients reduces reproductive rate but extends lifespan (Klass 1977). These findings are consistent with the idea of a trade-off between survival and reproduction based on the allocation of available nutrients. However, more recent suggestions are that the rate of reproduction can be uncoupled from that of survival, and that these can be independently controlled by a signalling mechanism rather than of direct competition for available nutrients (Hsin and Kenyon 1999; Patel, Knight et al. 2002; Barnes and Partridge 2002).

During periods of nutrition deficit cells and organisms can enter a 'standby mode' where cell division and reproduction are halted or minimized to allow for energy to be diverted for somatic maintenance rather than reproduction. This suggests that most species have developed these anti-ageing systems to overcome periods of starvation (Fontana, Partridge et al. 2010). Periods of famine often trigger what appear to be metabolic switches that paradoxically extend the normal life span, without sacrificing subsequent reproduction and survival when favourable conditions return (Kirkwood and Austad 2000). The hypothesis of this thesis is that this could, in part, be due to autophagy. Autophagy genes have been found to be enriched for expression in the reproductive system (Mizushima 2007) and in mice and humans autophagic activity is tightly regulated during development and the reproductive period (Vellai 2009). Recent work in *Drosophila* has identified a suite of hormone genes that are a potential source of pro-ageing signals with an enriched expression in reproductive tissues (Gerrard et al unpubl.). *Atg* genes found expressed at high levels in the reproductive tissues of *Drosophila* are: *Atg1, 2, 4, 5, 6, 7, 9, 12, 13* and *18.* Interestingly, very high

expression of *Atg8a* was found in the ovaries of females, while a very high level of *Atg8b* was found in the testes of males (Chintapalli, Wang et al. 2007). The finding of these genes in the reproductive tissues has prompted this study on the potential lifespan extending effects of autophagy, and specifically, whether this extension is possible because damage that occurs in the reproductive system under certain conditions i.e. full nutrition, and high reproductive rates, can be counteracted by the up-regulation of autophagy. It is thought that autophagy genes protect against the damage arising from elevated reproduction by targeting damaged proteins for degradation and so enhancing the fly's survival. This indicates that autophagy could be one possible mechanism by which the lifespan shortening effects of elevated reproduction might be countered.

This thesis examines the idea that ageing is driven by the accumulation of random cellular damage, leading to a decrease in reproductive function and survival which ends in death (Hamet and Tremblay 2003; Vellai 2009; Flatt and Schmidt 2009). The antagonistic pleiotropy theory for ageing suggests selection will favour genes with a positive effect on early life even if they have a detrimental effect on later life (Williams 1966; Sozou and Seymour 2003) indicating a trade-off between lifespan and reproductive output. Because our current understanding of survival costs of reproduction are limited, more work is required on understanding the genetics and physiology of these trade-offs and to uncover their mechanistic basis. In a complex genetic trait such as ageing genes interact strongly with the environment, for example diet, stress and pharmacological factors. This thesis aims to look at whether autophagy, an important but understudied pathway may be used to counteract the effects of reproductive damage in *D. melanogaster*. Despite the complexity of ageing, recent work has shown that both genetic and dietary alterations can substantially increase the healthy lifespan of a range of organisms across many taxa, suggesting that the fundamental pathways underlying variation in ageing rates may be simpler than perhaps first thought (Fontana, Partridge et al. 2010). To shed light on this I conducted experiments under different reproductive status on lifespan determining pathways such as nutrient availability and using genetic and pharmacological activators of autophagy. Any indication of trade-offs between reproduction and

survival is determined using lifespan, fecundity and fertility measures. The trade-off between increased lifespan and decreased fecundity can in certain cases be uncoupled (Gelino and Hansen 2012). However, signals from the reproductive system can influence ageing and this thesis means to test whether autophagy is one such signal.

The utility of the D. melanogaster model system

Robertson concluded in 1962 that some model species are better suited than others for tackling specific research questions because of differences in the life cycle. It was already realised even, well before huge increases in genetic manipulation techniques and genomic information now available that it is in *Drosophila* that genetic analysis can perhaps be taken the furthest (Robertson 1962). *D. melanogaster*, the fruit fly is one of the most studied organisms in the field of biology. The well-known genome sequence of this organism and its well characterized genetics, that have been studied for over 100 years, offer many advantages. The sequencing of the entire *Drosophila* genome was completed in 2000 and a large genomic database is available at http://flybase.bio.indiana.edu/. It is widely used for biological research in the study of life history evolution, developmental biology and genetics. There are many homologies between fundamental genes and processes (e.g. TOR and IIS, discussed above) between *Drosophila* and mammals including humans, suggesting the fruit fly has more utility to reveal fundamental and general principles of biology than is sometimes realised (Robertson 1962). *Drosophila* are also being used as a genetic tool for research into several specific human diseases such as Parkinson's and Alzheimer's as well as for general research into ageing, immunity, and cancer. Approximately 75% of known human disease genes have a gene match in the genome of *Drosophila* (Reiter, Potocki et al. 2001) and 50% of fly protein sequences have mammalian homologues (Chien, Reiter et al. 2002). An online database called Homophila is available for searching human disease gene homologues in flies and vice versa. (http://www.genomenewsnetwork.org/articles/06_01/Homophila_database.shtml). *Drosophila* has

long served as a model system for the investigation of many cellular and developmental processes common to higher eukaryotes, including humans (Gaillard, Rouquier et al. 2004). The *Drosophila* system is useful to investigate relationships between factors such as nutrients, autophagy, growth and development. It is easy to manipulate the diet of *Drosophila* under experimental conditions by simply altering the food medium contained within the food vials, rather than having to alter the access of the flies to the food. This is the case because caloric restriction of the *Drosophila* diet is not compensated for by an increased rate of feeding (Fontana, Partridge et al. 2010).

Apart from the vast store of accumulated knowledge concerning *Drosophila*, it is also easily cultured, has a short life cycle and one can obtain large numbers of experimental subjects over a short generation time (Roberts 2006). Relatively little equipment or space is required for their care and culture, hence the overall cost compared to other study systems is relatively low. Males and females are easily distinguished from each other and it is relatively easy to isolate virgin females when required, for setting up genetic crosses. *Drosophila* fruit flies are easy to handle, sort and move between culture vessels using ice anaesthesia or carbon dioxide. Under ideal laboratory conditions at 25^{o}C, there is a minimum generation time of approximately 11 days, which is the shortest generation time known for any *Drosophila* species. After eggs are hatched, the first larval moult occurs after 25 hours and the second 24 hours later. Puparia form 48 hours after that, and the formation of adult organs and structures in the pupal stage requires about 100 hours in total. After eclosion and the emergence of adult flies, it takes two to three days for the females to develop fully mature eggs (Powell 1997).

There are detailed bioinformatic data and genetic reagents available, for example overexpression or knockdown mutant stocks are available for most genes in the genome (approximately 13,000 genes) and specifically for key autophagy genes such as *Atg8* as used in this thesis. There are different lines of many of the relevant autophagy genes, some of which can be maintained against genetically

engineered 'balancer' chromosomes, which carry dominant visible markers, recessive lethal mutations and multiple inversions to prevent recombination. The use of balancers thus allows stocks of lethal alleles to be maintained in a heterozygous state. Visible genetic markers are commonly used in *Drosophila* research to detect the presence of balancer chromosomes or P-element inserts. Most of these markers are easily distinguishable with either the naked eye or under a microscope (e.g. the *Curly wing, Cy*, or *white* eyed w^{1118} markers).

Thesis outline

In this thesis the model organism *D. melanogaster* has been used to determine the effects of pro- and anti-ageing signals on survival, fecundity and fertility. The aim was to investigate the role autophagy played in the longevity and reproduction of *D. melanogaster* by using the experimental procedures outlined in the five data chapters of this thesis.

Chapter 2 provides general materials, methods and techniques used throughout. A great deal of the work on longevity is based on genetic analyses which have bought a wealth of understanding to the subject. However lifespan is only part of the picture and is unable to provide us with a complete understanding of the life history outputs. To gain a more detailed and mechanistic understanding of how lifespan is extended biochemical and molecular studies are required. This may also help to resolve inconsistent and contradictory data from the genetic manipulation studies of longevity. Methods for manipulating longevity pharmacologically, as employed here, can therefore be used to gain more understanding of the longevity process. In **Chapters 3 and 4** pharmacological activators of autophagy were used to test for effects on lifespan, fecundity and fertility in once mated females. Experiments using the same manipulations were then also extended to encompass both sexes and also a continually mated treatment. Predictions were that flies in which autophagy was activated through pharmacological dietary manipulation would have an extended lifespan. It is known that autophagy activation extends lifespan (Eisenberg et al. 2009). It was also predicted that flies fed

these activators with extended lifespan would trade-off against reproductive output, so egg and offspring numbers would be lower than for those individuals with no lifespan extension. More of the available resources would be allocated to extending lifespan, so less would be available for egg production.

Ageing is a complex process that at one time was thought to be beyond the reach of genetic manipulation. However with the combined use of model organisms gene alterations can be performed and the consequences mapped making ageing genetically accessible as with many other areas (Piper, Selman et al. 2008). **Chapter 5** used this approach and tested the overexpression of autophagy gene 8 (*Atg8a*) in three areas of the fly, the central nervous system, ovaries and ubiquitously to determine the effect of increasing *Atg8a* expression and so predicted autophagy activation on survival, fecundity and fertility of once mated females. In **Chapter 6** the *Atg8a* gene expression was knocked down and so it was expected autophagy would be inactivated. The prediction tested was that flies with decreased autophagy would show altered responses to caloric restriction in comparison to controls, because they would not be able to respond adaptively using autophagy. These tests were performed on once mated single females and fecundity and fertility data were also obtained. **Chapter 7** determined whether nutrition interacted with the ability of males to respond adaptively to males using manipulations of the amount of protein (yeast) and carbohydrates (sugar) available in the diet. Whether any such responses were autophagy dependent was examined.

The concluding **Chapter 8** then summarised the results and discussed the general themes that emerged from this work. The effects of ageing, reproduction, nutrition and any trade-offs discovered are discussed while potentially fruitful avenues for taking this research forward in the future are proposed.

Chapter 2 General Materials and Methods

2.1 Materials

All flies were maintained in a humidified room at 25°C on a 12:12 hour light:dark cycle.

2.1.1 Culturing equipment for maintaining flies (cages, bottles and vials)

Population cages holding wild-type *Drosophila melanogaster* measured 45x25x25cm. Each cage contained 12 glass bottles filled with 70ml of SYA food (recipe below). The cage populations were held under a regime of overlapping generations. Each week the oldest 3 bottles were removed and 3 new ones were added, allowing ample time for the flies to lay eggs, larvae to develop and for pupae to eclose. With egg to adult development taking 9-10 days at 25^{o}C this regime ensured that a full cycle of development could occur in each bottle put into the cage. Each bottle remained in the cage for 4 weeks.

Glass bottles (189ml, 1/3 pint) were used for culturing flies in the stock cages and also for maintaining mutant stocks. Mutant stocks were held on ASG medium (see recipe below) and were tipped over onto new food every two or three weeks, again giving ample time for flies to develop in the bottles. ASG medium was used for mutant stocks as it is softer than SYA and therefore more suitable for propagating the larvae of stocks less fit than the wild type.

Glass vials (73mm high x 23mm diameter) containing 7ml of SYA food (with differing amounts of yeast depending on the specific experiment) or agar only were used in longevity and mating experiments. Vials containing ASG medium were also used to maintain new stocks in quarantine in the 25°C incubator while establishing that the new stocks were healthy and free from mite infestation.

2.1.2 Food Recipes

Grape juice medium

The grape juice medium was used as an oviposition substrate to collect samples of eggs. The recipe to fill approximately 18 Petri dishes (8.5cm diameter x 1.5cm deep) is 550ml distilled water, 25g agar (Formedium Ltd, Hunstanton, UK), 300ml concentrated red grape juice (Solvino Ltd, London, UK) and 21ml Nipagin solution. The 500ml of the water and agar were mixed and brought to the boil. Red grape juice was added and brought to the boil again, then left to simmer for a few minutes. The remaining 50ml of water was then added and allowed to cool to about 60°C before adding the Nipagin preservative. This should be immediately dispensed into Petri dishes and left to cool at room temperature, then kept in a fridge until required for use.

Live yeast paste

Dried active yeast (DCL yeast limited) was used to supplement the grape juice medium used for egg laying, specifically to encourage females onto oviposition medium in order to lay eggs. Yeast granules were mixed with a few drops of water to form a paste and applied to the grape juice medium. Yeast granules or yeast paste (standardised volume) was also sometimes used, as specified, to supplement the standard food medium. ASG medium was made with 1l distilled water, 10g agar (Formedium Ltd, Norfolk, UK), 85g sugar (Brake Bros Ltd, Kent, UK), 20g brewer's yeast (DCL yeast Ltd, Scotland, UK), 60g maize meal (TP Drewitt, London, UK) and 25ml Nipagin solution. It was prepared by mixing agar and water in a saucepan and bringing to the boil. This was then taken off the heat and all dry ingredients stirred thoroughly. The mixture was allowed to cool to approximately 60°C before adding the Nipagin solution, then stirred and dispensed. SYA medium was prepared as above for ASG, except both the Nipagin and Propionic acid were added after cooling, and then dispensed. Ingredients are 970ml distilled water,15g agar (Formedium Ltd, Norfolk, Uk), 75g sugar (Brake Bros Ltd, Kent, UK), 100g brewer's yeast (DCL yeast Ltd, Scotland, UK), 30ml Nipagin solution and 3ml Propionic acid, (Sigma-Aldrich Co, UK).

Reduced yeast SYA media for nutrition dilution experiment (chapters 6 and 7)

The above SYA food medium recipe was used and amount of yeast was reduced as follows, 100% SYA was used as standard (100g brewer's yeast per litre of medium, DCL yeast Ltd, Scotland, UK). SYA at 50% (50g brewer's yeast per litre of medium) and 25% (25g brewer's yeast per litre of medium) were used as dilution diets. The agar only medium contained only 15g per litre of agar, with Nipagin and propionic acid added.

Medium containing spermidine and Torin1 activators of autophagy (chapters 3 and 4)

The required amount of spermidine or Torin1 was added to 10ml of water, 6g of yeast was then added and mixed to a paste. Specific details of the doses added are given in the relevant chapters. A standardised volume of this paste was then added to individual vials containing 7ml of agar only medium.

Comparison of food uptake as determined by dye ingestion across the different food delivery systems (appendix I and II)

Standard SYA food (as above) was delivered via three separate methods with 50mg dye (E133 Brilliant Blue) added per 100ml food (http://www.fastcolours.com/e133-brilliant-blue-fcf-granular-food-blue-2-ci-42090-23-c.asp).

DMSO toxicity(appendix III)

DMSO was used as the carrier for Torin1 (chapter 4). To complement these tests, DMSO toxicity was tested. DMSO was added to normal SYA (recipe above) in different amounts to determine the required dose to use to avoid confounding effects on lifespan arising from the DMSO itself.

10% w/v Nipagin solution (used in all food recipes as an antifungal agent)

This solution was made up using 1900ml 100% ethanol (Sigma-Aldrich Co, UK), 100ml distilled water and 200g Nipagin (Methylparaben, Clariant UK Ltd, Pontypridd, UK).

2.1.3 Experimental fly stocks

D. melanogaster wild-type

The *D. melanogaster* wild-type stock was originally collected from Dahomey, now the Republic of Benin, West Africa in 1970. These stocks have been maintained in the laboratory since collection in cages as described above.

Mutant stocks

Table 2.1 – Mutant fly stocks used in experiments from chapters 5 and 6.

Stock no.	Genotype	Description	Supplier
10107	*w^{1118} P{$w^{[+mC]}$=EP}Atg8a$^{[EP362]}$*	*UAS- Atg8a* stock for overexpression of *Atg8a*	Bloomington
30546	*P{w[+m*]=AplGAL4.G1a}1,y[1]w[*]; Mlf[Delta10]/CyO*	Expresses *Gal4* in the CNS	Bloomington
25378	*w*; P{GAL4-nos.NGT}40 P{lacO.256x}43 P{lacO.256x}50F P{lacO.256x}57A P{lacO.256x} 60AB/CyO; P{UAS- GFP.lacI}*	Expresses *Gal4* in the ovaries	Bloomington
30904	*w*; P{UASp-GFP.Golgi}1, P{tubP-GAL4}LL7/ TM3, Sb1*	Expresses *Gal4* ubiquitously	Bloomington
4414	*y1 w*; P{Act5C-GAL4}25FO1/CyO, y+*	Expresses *Gal4* ubiquitously	Bloomington
43096	*w^{1118}; P{GD4654}v43096*	*UAS-Atg8a*-inverted repeat for knockdown of *Atg8a*	Vienna
60000	*w^{1118}*	Isogenic host strain, control for *Atg8a* knockdown and genetic control strain for *Atg8a* overexpression	Vienna

Suppliers – Bloomington stock centre (http://flystocks.bio.indiana.edu/)
Vienna stock centre (http://stockcenter.vdrc.at/control/main)

2.2 Methods

2.2.1 Preparation of wild-type and mutant *D. melanogaster* for experimental use

Larval culture under standardised conditions

Petri-dishes containing grape juice medium (as above) were supplemented with a small amount of yeast paste added to the middle of the plate and then placed in one of the Dahomey cages for 12-24 hours to collect eggs for standard larval density culture. After removal from the cage the yeast paste was removed and the plate was incubated for 24 hours in the 25°C room to allow the first instar larvae to emerge. The first instar larvae were then picked using a mounted needle and placed in standard vials containing SYA food medium at a density of 100 larvae per vial. A standard volume of liquid yeast was added to each of the vials to allow larvae to develop under *ad libitum* nutrition.

Standardizing larval density is a useful tool for measuring adult fitness traits. A standard density equalizes competition between the larvae across different food vials and experimental treatments, and hence ensures that they all share the same environment. There are many environmental factors that can affect larval morphology or behaviour, for example larval density, temperature or nutrition can all affect body size so all must be controlled for (Gage 1995; Hosken, Blanckenhorn et al. 2000). Variation in body size should be avoided if possible, because studies in many species have indicated there are strong body size effects on fitness. For example, females prefer to mate with larger males (Partridge and Farquhar 1983; Partridge, Hoffmann et al. 1987) and males that have been deprived of nutrition during development will be smaller and less attractive (Friberg and Arnqvist 2003). Therefore, to minimise these potential confounding effects all flies were raised in standardised densities on full food during development, which results in minimal variation within experimental cohorts that might otherwise confound the fitness differences between treatments.

Maintenance of mutant stocks

Mutant stocks were grown up in bottles on ASG food. For crosses virgin females and males of the required genotypes were collected from the parental populations. These were then placed in a fresh bottle of medium at a density of 50 females and 50 males. Emerging flies with the correct phenotypic traits were then collected to use in experiments.

Targeting gene overexpression or silencing by use of the Gal4-UAS system

In this system, the gene of interest (*Atg8a*), the responder, is controlled by the upstream activating sequence (UAS) element. The transcription of the responder requires the presence of the *Gal4* transcription factor, so in the absence of *Gal4* the responder lines remain in a transcriptionally silent state. To activate transcription the responder line is mated to *Gal4* 'driver' flies expressing *Gal4* in a particular pattern. The resulting progeny then express the responder in a transcriptional pattern that reflects the *Gal4* pattern of the respective driver (Duffy 2002). The UAS is normally optimized to increase the number of *Gal4* binding sites in the UAS so as to increase the efficiency of over or under expression. If the UAS is upstream of an inverted repeat sequence of the gene of interest, then this will drive gene silencing through RNA mediated gene silencing.

2.2.2 Experimental procedures

Fly handling

Both CO_2 and ice anaesthesia were used to sort flies. To avoid damage and premature death, CO_2 anaesthesia was not used on flies less than 3 hours old.

Egg and offspring counts

Females were generally allowed to lay eggs in food vials for 24 hours and then transferred to fresh media. The vials were then retained to count eggs under a dissecting microscope. When measures of fertility were also required, either the number of hatched eggs 24h after the last egg had been laid was counted, or the egg vials were retained to allow the offspring to develop and eclose

(approximately 12 days later). If there was insufficient time to count the emerging offspring, the vials were inverted and frozen at 4^{o}C for counting at a later date. Vials containing offspring for counting were frozen upside down to prevent them from sticking to the food and hence to ease counting.

Egg laying ability was monitored (appendix II) to determine whether females fed yeast paste applied to an agar only plug was comparable to the fecundity of those kept on normal SYA.

Mating assays

For mating trials (chapter 7) vials were placed on a purpose built viewing rack, which allows large numbers of vials to be observed easily and simultaneously. When each fly was added to its prospective mate in the vial, the time of introduction, the time that mating began and finished was recorded. Copulations lasting less than 1 minute are considered possible pseudo-copulations, so only data from matings of 5 minutes and over was retained (Barron 2000). Virgin females were held in groups of 10 prior to testing.

2.3 Detecting the activation of autophagy

In order to determine if autophagy had been activated in experiments within this thesis, two methods were used. Western blot analysis was used to detect the processing of the *Atg8* protein into *Atg8-I* and *Atg8-II*, with detection of these cleavage products indicating the activation of autophagy (Klionsky, Abeliovich et al. 2008). This was an appropriate method for the experiments presented in chapters 3 and 4 in which flies were fed putative autophagy activators, and provides a better biological read out for autophagy status than changes in the expression of autophagy pathway genes themselves, e.g. they may have pleiotropic effects. This is because changes in gene expression *per se* do not necessarily indicate that autophagy activation state has been changed. For the experiments in which genetic manipulations were employed to cause a knockdown effect or

overexpression of *Atg8* in different tissues of the fly, qRT-PCR was used to validate the increased production of *Atg8* mRNA. This was useful to give a direct measure of the genetic manipulation to the *Atg8* gene itself. The product of *Atg8a* is a limiting reagent in the autophagy pathway, and plays no other known role. Of the autophagy genes known, its level of expression is therefore expected to provide a good indication of autophagy state.

2.3.1 Western blot analysis to detect autophagy activation

Lysis buffer (Barth, Szabad et al. 2011)

The lysis buffer used contained 120mM Sodium chloride (Sigma-Aldrich Co, UK), 50mM Tris (hydroxymethyl) aminomethane (Sigma-Aldrich Co, UK), 20mM Sodium Fluoride (Sigma-Aldrich Co, UK), 1mM Benzamidine (Sigma-Aldrich Co, UK), 1mM Ethylenediaminetetraacetic acid (Sigma-Aldrich Co, UK), 6mM Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (Sigma-Aldrich Co, UK), 15mM Sodium pyrophosphate tetrabasic (Sigma-Aldrich Co, UK), 1% Nonidet P-40 (Sigma-Aldrich Co, UK) and protease inhibitors (abcam, ab65621).

Resolving gel for denaturing SDS-PAGE (15%)

The ingredients for the resolving gel for denaturing SDS-PAGE were distilled water, 30% acryl-bisacrylamide mix (Sigma-Aldrich Co, UK), 1.5M Tris (pH 8.8, Sigma-Aldrich Co, UK), 10% SDS (Sigma-Aldrich Co, UK), 10% ammonium persulphide (Sigma-Aldrich Co, UK) and TEMED (Sigma-Aldrich Co, UK).

Stacking gel for denaturing SDS-PAGE (15%)

The stacking gel for denaturing SDS-PAGE was the same as above but with 1.5M Tris pH 6.8 (Sigma-Aldrich Co, UK).

For Western blots, whole flies were flash frozen in liquid nitrogen, and homogenized in 100µl lysis buffer (4 to 10 flies per sample). Proteins were then separated on a 15% SDS-PAGE gel and blotted

onto Immobilon FL transfer membrane (Millipore, http://www.millipore.com/). Protein presence was checked using Ponceau red (Sigma-Aldrich Co, UK) fig 2.1.

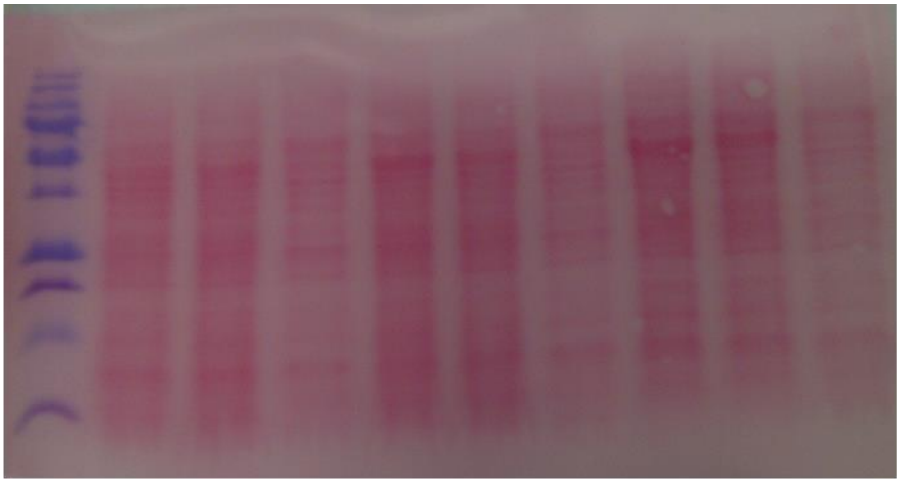


Fig 2.1 – Transfer membrane with protein bands stained with Ponceau red showing the presence of protein bands in each lane before the application of antibodies to detect *Atg8-I* and *II.* The gel shows an example of protein extracts from once mated single females (S♀), females exposed continually to males (Exp♀) and once mated single males (S♂). These flies were held on normal food (con), food with added Torin1 (Torin1) or held on agar only media (unfed). The membrane indicated that protein bands were successfully isolated and extracted from all samples.

To detect *Atg8-I* and *II* and the tubulin protein control the primary antibodies were anti-*dAtg8* 1:1000 (kindly supplied by K. Köhler,(Barth, Szabad et al. 2011)) and anti-tubulin1:10000 (Abcam plc, UK). Secondary antibodies were anti-rabbit 1:10000 (LI-COR, USA) and anti-rat 1:10000 (LI-COR, USA). For quantification of Western blot signals, image software odyssey (LI-COR, http://www.licor.com/) was used. I calculated the intensity value of the *Atg8-II* bands as a measure of autophagy activation (Klionsky, Abeliovich et al. 2008), with the intensity of *Atg8-II* being positively correlated with the degree to which autophagy was activated. To control for differences due to variation in loading intensity of the blot, *Atg8-I* and *Atg8-II* values were normalised by dividing by the intensity of the tubulin loading control and the ratio of *Atg8-II/Atg8*-I determined.

2.3.2 qRT-PCR analysis to detect overexpression of Atg8a

For validation of *Atg8a* mRNA levels by qRT-PCR, 4 to 10 flies per sample were flash frozen using liquid nitrogen. RNA was then extracted (as below), qRT-PCR was conducted using the qStandard provider (http://www.qstandard.co.uk/index.html).

RNA extraction

RNA was extracted using *mir*Vana™ miRNA Isolation Kit, (AM 1561, http://www.lifetechnologies.com). Fly samples were crushed while frozen, lysis buffer was added and the samples homogenized. Following extraction in chloroform, RNA was precipitated and then cleaned using the wash solutions provided and finally re-suspended.

Quantification and quality control

Total RNA (TRNA) was quantified in each sample using Nanodrop. The values obtained for the 260/280 wavelength ration varied from 2.1 to 2.25 λ yielding RNA concentrations between 258.8 to 1628 ng/μl.

To further assess the quality of each sample, I ran 1μl of each on an agarose gel (fig 2.2).

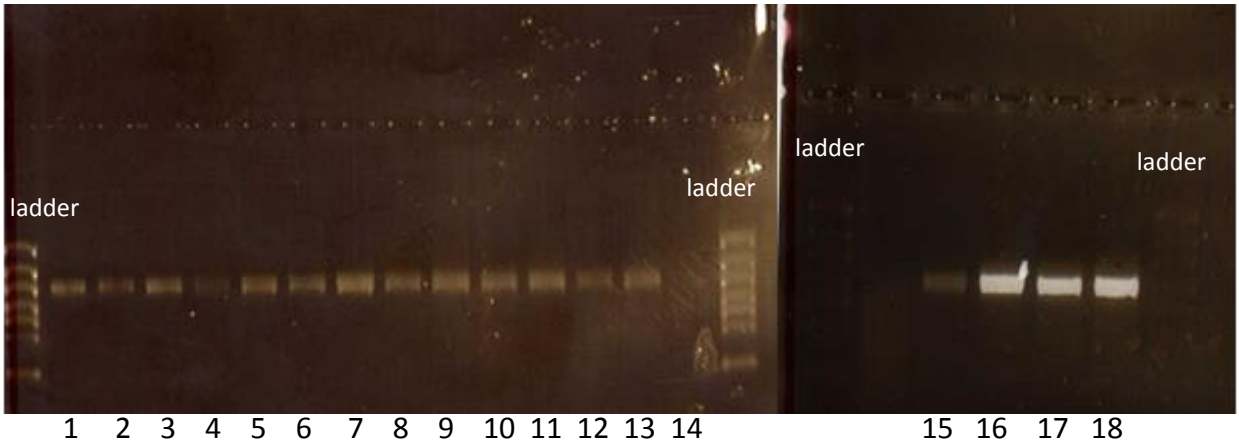


Fig 2.2 – Agarose gels of extracted total (T)RNA, to indicate quality of each sample. Samples were of single once mated female flies in different treatments (Samples are: 1= *UAS-Atg8a/UAS-Atg8a* on 50% yeast diet, 2= *Gal4/Gal4* on 100% yeast diet, 3 = *Gal4/Gal4* on 50% yeast diet, 4= *UAS-Atg8a/Gal4* knockdown on 100% yeast, 5= *UAS-Atg8a*/balancer on 100% yeast, 6= *UAS-Atg8a*/ovary Gal4 overexpression driver, 7= *UAS-Atg8a*/ubiquitous *Gal4* overexpression driver, 8= w^{1118}(isogenic host strain)/ovary *Gal4* overexpression driver, 9= w^{1118}/ubiquitous *Gal4* overexpression driver, 10= *UAS-Atg8a/UAS-Atg8a* on 100% yeast, 11= *UAS-Atg8a*/balancer on 50% yeast, 12= *UAS-Atg8a/UAS-Atg8a* on 25% yeast, 13= *Gal4/Gal4* on 50% yeast, 14= *UAS-Atg8a/Gal4* knockdown on 50% yeast, 15= *UAS-Atg8a/Gal4* knockdown on 25% yeast, 16= *UAS-Atg8a*/CNS *Gal4* overexpression driver, 17= *UAS-Atg8a/UAS-Atg8a* common control, 18= w^{1118}/CNS *Gal4* overexpression driver). Ladder = RiboRuler High Range RNA Ladder (www.fermentas.com).

For the quantification of *Atg8a* gene expression, *Atg8a* PCR primers were supplied by qStandard (table 2.2), and yielded one unique PCR product

Table 2.2 – *Atg8a* primers for qRT-PCR

Primer	Length	Start	End	Sequence
Forward	22	433	453	cgtcggtcagttctacttcct
Reverse	20	567	548	tcctcgtgatgttcctggta

qStandard qRT-PCR protocol:

Reverse transcription

500ng RNA for each sample was reverse transcribed using the Qiagen Quantitect reverse transcription in a 10µl reaction according to the manufacturer's instructions. This RT kit includes a

mandatory DNA wipeout step to remove genomic DNA. This step was extended to 4 min at 42^{o}C for these samples. Duplicate RT reactions were performed for two of the samples and the completed reaction was diluted 10-fold with 500ng/ml tRNA in water.

qPCR assay validation

Two microlitres of cDNA from each sample were amplified in duplicate 20µl reactions using Agilent Brilliant III Ultrafast SYBR green qPCR mix with each primer at a final concentration of 500nmol/l. The PCR products were then run on a 2% agarose gel to check that they yielded a single band of expected size. PCR products were then cleaned with microClean, quantified using a NanoDrop and the copy number calculated from the A260, and diluted to prepare a 10-fold standard dilution series. Standard curves were efficient (>95%, and linear over at least 7 log).

qPCR

Two microlitres of cDNA were amplified in duplicate 10µl reactions using Agilent Brilliant III Ultrafast SYBR green qPCR mix with each primer at a final concentration of 500nmol/l. The no-template control reaction contained 2µl of TRNA 500ng/ml. qPCR standards (10^{7}-10^{1} copies/reaction) for each gene were included in each run. Amplification parameters: 95^{o}C for 5 min followed by 40 cycles of 95^{o}C for 10 sec, 57^{o}C for 5 sec using a Rotor-Gene Q. Melt curves were checked for product specificity (single peak) and the presence of primer dimers.

Data normalization and analysis

The copy numbers/reaction were derived from the standard curves using the Rotor-Gene software. Two reference genes were used to normalise GOI (gene of interest) copy number (*Act5c* and *Gapdh2,*see tables 2.3 and 2.4 below). Melt curves exhibited a single peak at the expected temperature and coefficients of variation were calculated for the RT duplicates.

Table 2.3 – *Act5c* primers for qRT-PCR

Primer	Length	Start	End	Sequence
Forward	21	866	886	gttggagaagtcctacgagct
Reverse	21	956	976	caagcctccattcccaagaac

Table 2.4 – *Gapdh2* primers for qRT-PCR

Primer	Length	Start	End	Sequence
Forward	20	1050	1069	cactacccacccacactcta
Reverse	20	1100	1119	tctgaagtgtctcaccccat

Mean normalised copies of each reaction were used to calculate levels of *Atg8a* gene in each sample in order to allow comparison of normalised gene expression values for the gene of interest (*Atg8a*).

Chapter 3 Effects of activating autophagy via dietary delivery of spermidine on longevity, fecundity and fertility.

3.1 Abstract

The role of autophagy in the maintenance of cellular homeostasis is fundamental. It conserves resources and protects cells from damage during times of stress, such as when food resources become limited. The activation of autophagy can therefore have beneficial effects on cell, and hence organismal, homeostasis and longevity. Consistent with this, activation of autophagy is reported to increase lifespan in several model organisms including yeast (*Saccharomyces cerevisiae*), fruit flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*). However, it is not yet known whether this increased lifespan is accompanied by a trade-off in fertility. This is of interest in terms of whether potential extensions to longevity will inevitably be accompanied by decreased fertility. Here, I investigated whether I could activate autophagy by adding the natural polyamine spermidine to the diet. I then tested the hypothesis that activation of autophagy would extend lifespan while decreasing fertility.

I determined dose response effects of spermidine on lifespan and fertility, examined whether any such effects were observed in both sexes, and finally whether they interacted with reproductive status in females. Delivery of increasing amounts of 1M spermidine had a toxic effect, decreasing once mated female lifespan. Therefore for subsequent experiments a 1mM stock was used. At this concentration, autophagy did not appear to be activated by spermidine treatment or by diet restriction in single once mated females. However, autophagy was activated in both continuously exposed females and males fed spermidine, and also to a lesser extent in males held on a restricted diet. Therefore, the effect of spermidine on autophagy was context dependent, and the diet restriction led to elevated autophagy only in males. There was no significant extension of life in

response to spermidine in any of the experiments. Hence there was no evidence of effects of autophagy on lifespan in the treatments in which spermidine did activate autophagy (i.e. continuously mated females and males). There was also no evidence to show that treatment with dietary spermidine altered reproductive output. As expected, based on previous research, males lived longer than single, once mated females, which in turn lived longer than females continually exposed to males. Females continually exposed to males also produced more offspring than once mated females. The results suggest that cleaner and more reliable methods of activating autophagy are required, and also that it is important to validate the effects of any manipulations that are used.

3.2 Introduction

The role of autophagy in the maintenance of cellular homeostasis is fundamental as it allows energy reserves to be conserved when external resources are limited. More importantly for the research described here, autophagy plays an essential role in the removal of damaged proteins, organelles and potentially toxic protein aggregates (Levine and Kroemer 2008). Hence the safe and effective activation of autophagy could have therapeutic value through decreasing damage within cells, particularly as damage accumulates with increasing age. Rapamycin and rapalogs are the most effective clinically employed inducers of autophagy, but they are known to have severe immunosuppressive effects and hence have limited therapeutic application. This suggests a need to investigate alternative, nontoxic inducers of autophagy such as resveratrol and, as used in this chapter, spermidine (Morselli, Marino et al. 2011).

Spermidine is a polyamine that occurs endogenously, but which is also found at high levels in citrus fruits and soybean. It has roles in many different biological processes and is essential for cell survival via its effects on maintaining membrane potential, cell pH and hence cell volume. Spermidine was first isolated from semen in which it is found in large amounts and consistent with this, it plays a fundamentally important role in successful mammalian reproduction, in both parental and postnatal processes (Bauer, Carmona-Gutierrez et al. 2013). Polyamines such as spermidine are essential for successful spermatogenesis, oogenesis, embryogenesis, implantation or placentation. Spermidine may also be directly involved in fertilization, for example, it has been discovered that the seminal fluid of infertile males (humans) contains significantly lower levels of spermidine in comparison to normal fertile male controls (Calandra, Rulli et al. 1996). The efficiency of fertilization declines gradually with advancing paternal age and this is correlated with a decrease in intracellular polyamine concentrations. The decline in fertilization efficiency may therefore be associated with a reduced volume of semen, and sperm concentration as well as polyamine-dependent sperm motility

and morphology (Stewart and Kim 2011; Bauer, Carmona-Gutierrez et al. 2013). Bauer et al. (2013) demonstrated that spermidine is an essential requirement for successful mating in *S. cerevisae* and for effective fertilization in the nematode *C. elegans*. Interestingly the mechanism underlying these pro-fertilizing effects is autophagy independent (Bauer, Carmona-Gutierrez et al. 2013). Hence, spermidine may also play roles in essential reproductive processes separate from its effects that occur via the activation of autophagy.

As well as its roles in reproductive processes, spermidine is associated with lifespan extension in several different model organisms. For example, a decrease in the content of cellular polyamines has repeatedly been correlated with increased ageing in yeast (S. *cerevisiae*). Spermidine has been found to retard necrotic cell death and to rejuvenate old yeast cells (increasing replicative lifespan, i.e. number of daughter cells generated from one single mother cell). Unlike some of the effects of spermidine on fertilization described above, this extension in longevity is autophagy dependent (Eisenberg, Knauer et al. 2009). It has been shown that an exogenous supply of spermidine can lead to lifespan extension in yeast (*S. cerevisiae*), flies (*D. melanogaster*) and nematodes (*C. elegans*) (Madeo, Eisenberg et al. 2009). Addition of spermidine to fly food media can increase average lifespan by approximately 30%. Importantly, it has been shown that the exogenously supplied dietary spermidine is indeed ingested and metabolised (Eisenberg, Knauer et al. 2009), hence it can be assumed that the lifespan extension is a direct effect of the spermidine, rather than some off target effect. A less marked but still significant effect of dietary spermidine is also observed in *C. elegans* where lifespan is extended by approximately 15%. The spermidine effect on lifespan extension is independent of the sirtuin/resveratrol pathway, which is also proposed to activate autophagy and extend life (Morselli, Marino et al. 2011; Lionaki, Markaki et al. 2013). This means that there is potentially more than one pathway by which autophagy can influence lifespan. As well as effects on lifespan *per se* the addition of dietary spermidine in nematodes and fruit flies is reported to result in anti-ageing effects such as suppressing oxidative stress and necrosis (Eisenberg,

Knauer et al. 2009). Mice that had spermidine added to their drinking water manifested a reduction in age-associated protein oxidation, which is one of the markers of ageing. However, contradictory results are also reported, with data from Minios et al. (2012) showing no constant effect of spermidine on longevity in *D. melanogaster* (Minois, Carmona-Gutierrez et al. 2012). The contradictory results should add caution to the view that spermidine could be a universal anti-ageing drug (Madeo, Eisenberg et al. 2009).

As suggested above, the mechanism by which the anti-ageing effects of spermidine occur is thought to be via its activation of autophagy. Evidence for this comes from experiments in which genetic inactivation of genes required for autophagy inhibit the lifespan extending properties of spermidine (Eisenberg, Knauer et al. 2009; Madeo, Eisenberg et al. 2009). Additional supporting evidence comes from the observations that spermidine can induce autophagy in yeast, worms, flies and in human tumour cells (Eisenberg, Knauer et al. 2009; Madeo, Eisenberg et al. 2009; Morselli, Marino et al. 2011). The activation of autophagy leading to lifespan extension by spermidine is mimicked by the effect of dietary restriction. In fact dietary restriction can only prolong lifespan if the organism has an intact autophagy pathway. This points to both effects occurring via a common pathway. Taken together, these findings suggest that autophagy may be a common pathway that underlies all manipulations that can prolong lifespan (Madeo, Eisenberg et al. 2009). In sum, activation of autophagy via pharmacological agents, such as spermidine, are suggested to mediate cytoprotection, prolong lifespan and slow the effects of ageing (Morselli, Marino et al. 2011).

However, what has not yet been tested in manipulations of lifespan by activation of autophagy using exogenous agents such as spermidine is whether, if there is lifespan extension, it is achieved via a trade-off with fertility. This is of interest in terms of the potential for 'cost free' lifespan extension, and also in the context of whether trade-offs between lifespan and reproduction are obligate. There are numerous examples in nature where an inverse relationship between the extension of lifespan

and a decrease in fecundity can be seen (Carey, Harshman et al. 2008). If this is universally true, then any treatments to extend human lifespan would necessarily reduce fertility, which is likely to be an ethically unacceptable cost. However, it has also been shown more recently that the trade-off between lifespan extension and reduced fecundity can be uncoupled (Gelino and Hansen 2012). For example the removal of germline or germ precursor cells results in lifespan extension in nematodes and flies, but in *C. elegans* the removal of the entire reproductive system does not extend lifespan. Studies in mice also reveal that transplantation of ovaries from young to old mice can extend lifespan consistent with the idea that there are signals from the reproductive system that influence ageing (Cargill, Carey et al. 2003; Mason, Cargill et al. 2009). Overall, these findings suggest that lifespan extension arises from specific 'ageing signals' derived from the germline, and are not a simple consequence of sterility (Gelino and Hansen 2012). The general idea is that signals from the reproductive system can influence ageing and these may allow organisms to coordinate reproductive timing and ageing in a more integrated manner than by the existence of obligate trade-offs alone. Of relevance here to this emerging view of the more complex relationship between lifespan and reproduction, is the finding that spermidine affects reproduction and lifespan in yeast via autophagy independent and dependent processes, respectively (Bauer, Carmona-Gutierrez et al. 2013).

The aim of the research described in this chapter was therefore to test whether autophagy activation by addition of dietary spermidine leads to extended lifespan as reported by Eisenberg et al. (2009), but more importantly to test for associated effects on fertility, which have not previously been examined (Eisenberg et al. 2009). The effect of activating autophagy in once mated individuals of both sexes, and in females continually exposed to males was tested. This allowed me to determine if longevity and fertility were altered by spermidine fed individuals of differing sex and reproductive status. My overall hypothesis was that spermidine would activate autophagy and the

resulting repair of cellular damage that occurs might ultimately enable flies to mate at a high rate and maintain long life and to mitigate, at least to some extent, the effects of reproductive damage.

3.3 Materials and Methods

3.3.1 Effect of spermidine on once mated female survival, fecundity and fertility.

In this first experiment I tested the effects of different doses of spermidine on the life history of once mated females. Fly rearing was carried out as described in the general materials and methods, using standard density culturing of 100 larvae per vial, on standard SYA food medium (chapter 2). Wild type Dahomey females were allowed to emerge as adults from pupae and separated from males using CO_2 two days after eclosion. This ensured that at least one mating had occurred and that all females could therefore be considered 'once mated'. Females were than allocated randomly to one of six treatments in replicates of n=40 each. These treatments were carrier control (distilled water), 5µl, 10µl, 15µl, 20µl or 30µl spermidine. The food vials comprised a 7ml agar only base with a droplet of yeast paste (2mm diameter) containing spermidine or carrier control as indicated above. The live yeast paste for the spermidine treatments was made by adding 10ml distilled water, 6g live yeast granules (DCL yeast Ltd, Surrey, UK) and the relevant amount of spermidine (stock solution: 1M spermidine). This gave each individual female access to *ad libitum* yeast containing the different spermidine treatments. Females were given new food every second day. Eggs were initially counted twice a week until egg laying significantly declined. In the latter part of the experiment flies were placed on new food three times a week until all females were dead.

Female longevity was recorded daily until all females were dead. Individuals that became stuck in the food, escaped or were accidentally killed were entered as censors in the data. Eggs laid in the food vials over 24 hours were counted twice each week. The vials were then incubated and the number of unhatched eggs counted the following day to determine egg hatchability. Egg counts

ceased when half the remaining females in a treatment stopped laying eggs or there were less than 5 females remaining in a treatment.

I summed for each female in each treatment the total number of eggs produced from all samples over her lifetime. I did this separately for the total number of eggs laid, and those that were hatched and unhatched.

3.3.2 Effect of spermidine on once mated and continually mated female survival and fertility and on once mated male survival

I next conducted two replicate experiments to test whether any effects of spermidine might interact with sex and mating status. Informed by the results from the initial experiment above, I reduced the level of spermidine stock solution from 1M to 1mM. Fly rearing and handling was carried out as above in 3.3.1. Females and males were collected and separated using CO_2 three days following eclosion. Hence males and females could be considered to have mated once. Flies were then randomly allocated to one of 6 treatment groups in replicates of n= 40 each. Food recipes were as above, but this time I used a single dose of spermidine at a concentration of 1mM, versus carrier control (15µl of spermidine or carrier control was added to the yeast paste in each food vial). The treatments comprised (i) single once mated females, (ii) single once mated males and (iii) continually mated females kept together with 1 male, all held on either carrier control or spermidine food medium. In the continually exposed female treatment, males were replaced with fresh four day old males every 7 days to ensure reproductive activity remained high. To control for CO_2 exposure needed for the replacement of males in the continually mated female groups, the single female and male treatments were similarly anaesthetized each week. Flies were tipped onto new food daily until egg production significantly declined. From then on food vials were changed three times a week until all flies were dead.

Once mated female, once mated male and continually mated female longevity was recorded daily as above. Individuals that became stuck in the food, escaped or were accidentally killed were entered

as censors in the dataset. Eggs laid by once mated and continually mated females in the food vials over 24 hours were counted 2 times a week. The vials were then incubated and the number of unhatched eggs counted the following day to determine egg hatchability. Egg counts ceased when half the remaining females in a treatment stopped laying eggs or there were less than 5 females remaining in a treatment. For the once mated and continually exposed females I determined the productivity of eggs over each individual female's lifetime, those that were hatched and those that were unhatched were also recorded.

3.3.3 Western Blot analysis

Western blot analyses were used to determine whether autophagy had been activated by the dietary spermidine treatment. In order to demonstrate the activation of autophagy, cleavage of the *Atg8* gene protein into *Atg8-I* and *Atg8-II* was detected. The intensity and ratio of these bands was recorded and normalised as described in chapter 2. Ten flies were used per dose for the dose response experiment, for validation of autophagy activation. Flies were crushed after freezing in liquid nitrogen following 3 days of exposure to food media containing 15µl (1mM concentration) spermidine. Although not featured in the experiments described in this chapter, I also included an 'unfed' treatment to test whether spermidine could activate autophagy to a comparable extent to that of diet restriction. For the unfed treatments, flies were frozen as above following 3 days of exposure to agar only, (i.e. no food). Flies were homogenised in 100µl lysis buffer (120mM NaCl, 50mM Tris-HCl, 20mM NaF, 1mM Benzamidine, 1mM EDTA, 6mM EGTA, 15mM $NA_4P_2O_7$, 1% Nonidet P-40) containing protease inhibitors. Proteins were extracted from the flies and separated on a 15% SDS-PAGE gel and blotted onto Immobilon FL transfer membrane (Millipore, http://www.millipore.com/). Primary antibodies were applied overnight at 4^{o}C, anti-*Atg8* 1:1000 (kindly supplied by K. Köhler, (Barth, Szabad et al. 2011)), anti-tubulin1:10000 (Abcam ab160) and secondary antibodies for 1 hour, anti-rabbit 1:10000 (LI-COR 926-32211) and anti-rat 1:10000 (LI-COR 926-68076). For quantification of Western blot signals, image software odyssey (LI-COR) was

used to calculate the intensity value of the bands from the flies fed the different spermidine treatments.

3.3.4 Statistical analysis

Statistical analysis was performed using SPSS v 18 (SPSS 2009) and R (R Development Core 2008). Survivorship was calculated and compared for each treatment using Log Rank tests (SPSS 2009). The fecundity and fertility data were analysed using ANCOVA, to test for the treatments differences over time while accounting for within subjects repeated measures (R Development Core 2008). Repeated measures are used when one individual's, in this case, eggs are counted on consecutive days removing the likelihood of type 1 errors in the statistical analysis. I used the generalised linear model (glm) package to run the model in R as the data was not normally distributed, and a poisson error distribution which accounts for the error structure of the data, because it is count data. The model was simplified where appropriate. To probe for age specific differences in more detail, I also analysed age specific fecundity and fertility by using ANOVA or Kruskal Wallis tests, depending on whether the data were normal. I conducted tests on the data for each day and corrected for the number of tests using the sequential Bonferroni procedure (Rice 1989). This reduced the probability of Type 1 errors whilst allowing more insight into the nature of any differences indicated by the overall ANCOVA analysis. Age specific and fertility data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests.

3.4 Results

3.4.1 Effect of spermidine on once mated female survival, fecundity and fertility.

Survival

There were significant differences in lifespan between once mated females held on the different doses of spermidine (Log rank X^2=34.730, df=5, p<0.001, fig 3.1). However, there was no evidence for lifespan extension, in fact spermidine had a deleterious effect, causing a significant decrease in

longevity with increasing amounts of the 1M spermidine solution in the diet. This prompted the use of a lower concentration of spermidine in subsequent experiments.

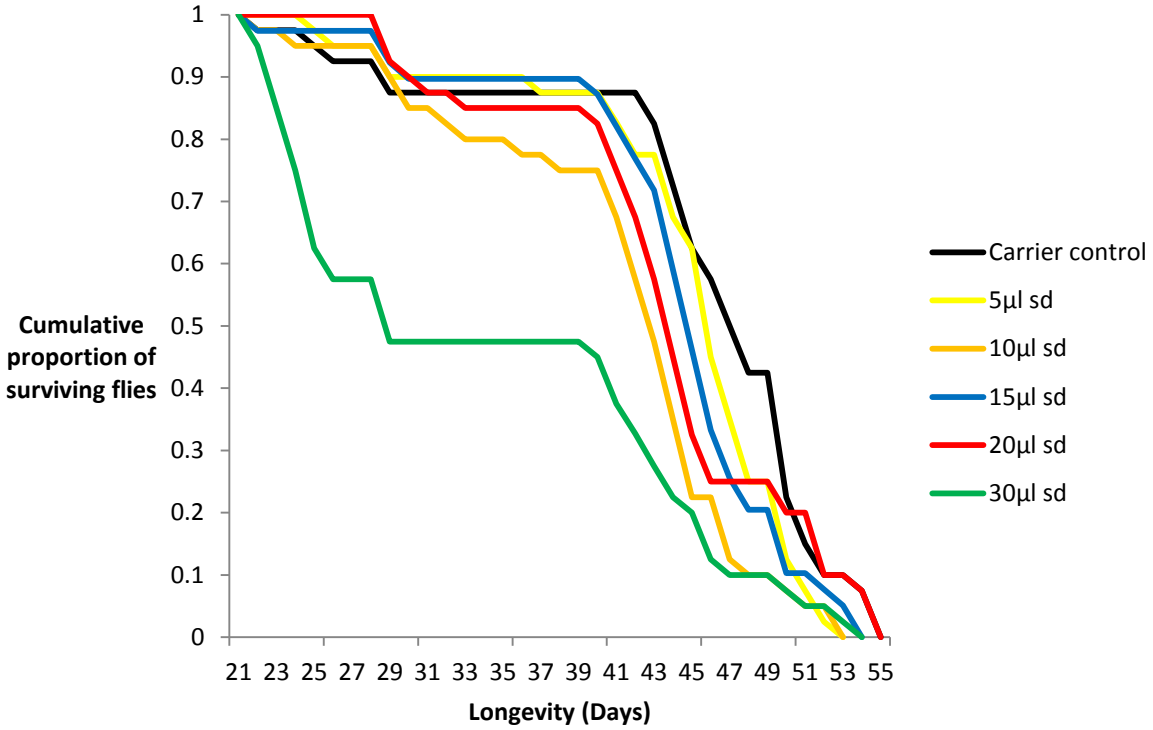


Fig 3.1 – Longevity of once mated females exposed to carrier control (H_2O), 5µl, 10µl, 15µl, 20µl or 30µl of 1M spermidine (sd) in the diet.

Age specific fecundity

The ANCOVA analysis showed, as expected, a significant decline in age specific fecundity across time ($p=0.001$, fig 3.2). Fecundity was generally lower, for the 15µl ($p=0.05$) and 30µl ($p=0.001$) spermidine females in comparison to the carrier control.

To determine with more precision where those differences primarily lay, I followed the ANCOVA analysis with tests on individual days, as described above. This showed that there were significant differences in the mean number of eggs laid on days 8, 15 and 17 of the experiment all at $p<0.05$. Once mated females given the highest amount of spermidine generally laid significantly fewer eggs on these three days than did females held on the other doses. This supports the idea that the

spermidine doses used in this first experiment was toxic and too high a concentration for any beneficial effects on female life history to be observed.

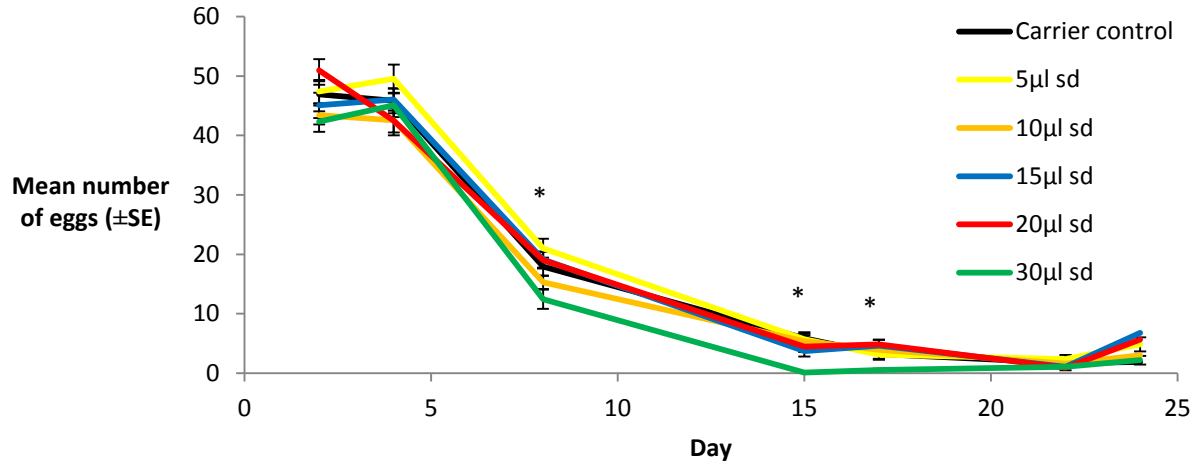


Fig 3.2 – Fecundity (mean number of eggs per female per 24 hours ±SE) of once mated females exposed to carrier control (H_2O), 5µl, 10µl, 15µl, 20µl or 30µl spermidine (sd) in the diet.

Age specific fertility

To analyse age specific fertility data (fig 3.3) I followed the same procedure as outlined above. The ANCOVA analysis showed that the 20µl spermidine females tended to have increased fertility over time, which differed significantly from the decline shown by the carrier control females ($p=0.05$). There was also a significant main effect of the 30µl spermidine treatment, which, surprisingly had significantly lower numbers of unhatched eggs (and therefore higher fertility) overall ($p=0.001$).

In general the ANCOVA analysis revealed an expected tendency for higher infertility in females held on the lower doses of spermidine. After Bonferroni corrections, there was a significant difference on days 8 and 17 in the number of eggs that were unhatched, with the highest dose of spermidine having the lowest level of unhatched eggs (all at $p<0.05$).

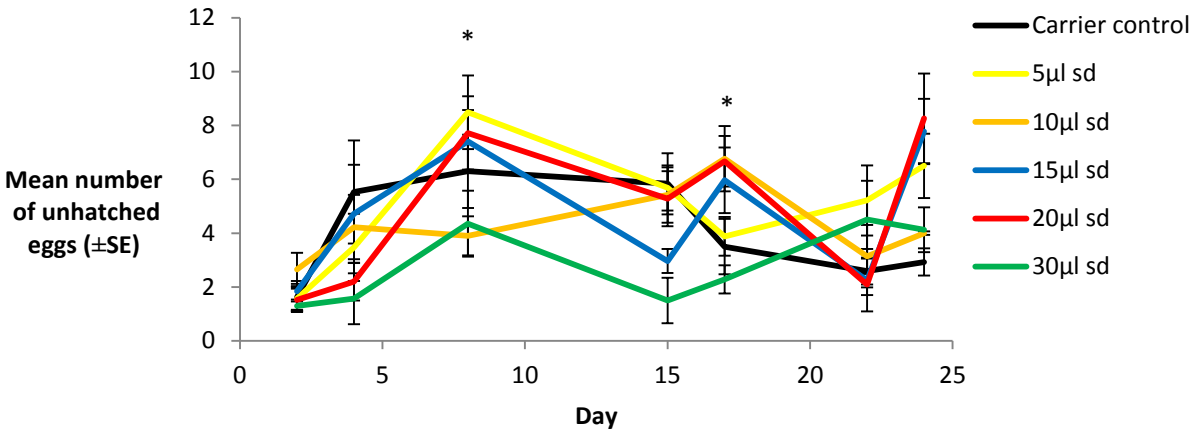


Fig 3.3 – The number of unhatched eggs (mean per female per 24 hours ±SE) of once mated female exposed to carrier control (H_2O), 5µl, 10µl, 15µl, 20µl or 30µl spermidine (sd) in the diet.

Lifetime egg productivity

Lifetime fecundity and fertility data (fig 3.4) showed a pattern that was consistent with the age specific data above, namely that spermidine treatment caused a significant decrease in female fecundity and, at the highest dose, a significant decrease in the number of unhatched eggs laid. Lifetime egg productivity was significantly different across the different treatments ($F_{5,232}$=6.484, $p<0.001$, fig 3.4a). Post hoc Tukey tests revealed that the 30µl spermidine treatment females produced significantly fewer eggs in their lifetimes than the control, 5µl, 15µl and the 20µl treatments (all at $p\leq0.026$). The 10µl spermidine treatment females also laid significantly fewer eggs over their lifetimes than the 5µl treatment ($p=0.032$).

The proportion of hatched eggs over the lifetime of the females was also significantly different across the different treatments ($F_{5,232}$=2.417, $p=0.037$) although this is not evident in the Post hoc Tukey analysis (fig 3.4b). Also significantly different was the proportion of eggs which did not hatch ($F_{5,232}$=5.133, $p<0.001$, fig 3.4c), post hoc comparisons using Tukey tests revealed that the 30µl spermidine treatment females produced significantly fewer unhatched eggs than all other treatments $p\leq0.015$ (fig 3.4c).

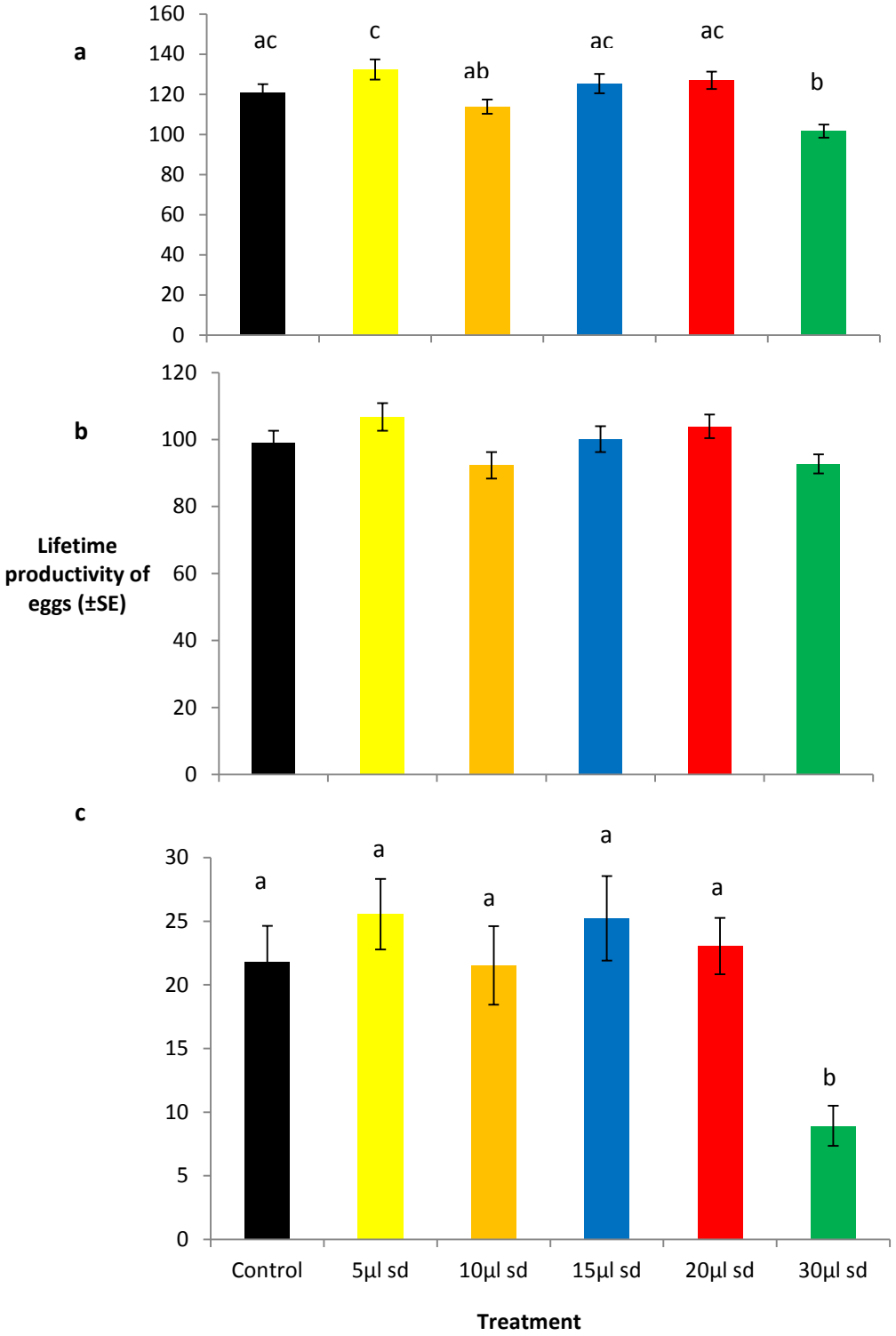


Fig 3.4 -Lifetime productivity of eggs laid by once mated females exposed to carrier control, (H_2O), 5µl, 10µl, 15µl, 20µl or 30µl spermidine (sd). Panel a shows the total lifetime egg production, b the lifetime number of hatched eggs and c the lifetime number of unhatched eggs. Letters not in common indicate a significant difference between the treatments as determined by Tukey post hoc tests ($p<0.05$).

3.4.2 Effect of spermidine on once mated and continually mated female survival and fertility and on once mated male survival.

Survival

In the first of the replicate experiments using the lower spermidine stock solution (1mM) and testing for interactions with sex and mating status, I observed significant differences in survival between the different treatments maintained as adults on spermidine versus control medium (Log rank X^2=104.792, df=5, p<0.001, fig 3.5a). There was again, however, no evidence for lifespan extension, and flies held on a spermidine diet had shorter lifespans than those held on the control diet. There were significant effects of mating status (i.e. single versus exposed treatment; Log rank X^2=79.456, df=1,p<0.001), with single flies of either sex living significantly longer than females continually held with a male, and a significant effect of diet with flies held on spermidine living significantly shorter lives overall (Log rank X^2 =4.230, df=1, p=0.040). The latter effect appeared to be due mostly to an effect of spermidine on lifespan in the once mated female group.

The second replicate experiment conducted also revealed significant differences in lifespan (Log rank X^2=31.305, df=5, p<0.001, fig 3.5b). However, as in the first replicate experiment, there was no evidence for any lifespan extension in the spermidine treated flies. In this replicate experiment, rather than a significant decrease in lifespan in spermidine fed flies there was no effect of the spermidine treatment (p>0.05). The results were otherwise consistent with the first replicate experiment in that there was a significant difference in survival between once mated and continually exposed individuals (Log rank X^2=28.551, df=1, p<0.001), with single flies living significantly longer. Males again lived significantly longer lives than did females (Log Rank X^2=14.261, df=1, p<0.001), and single females lived significantly longer than exposed females (Log Rank X^2=7.588, df=1, p=0.006).

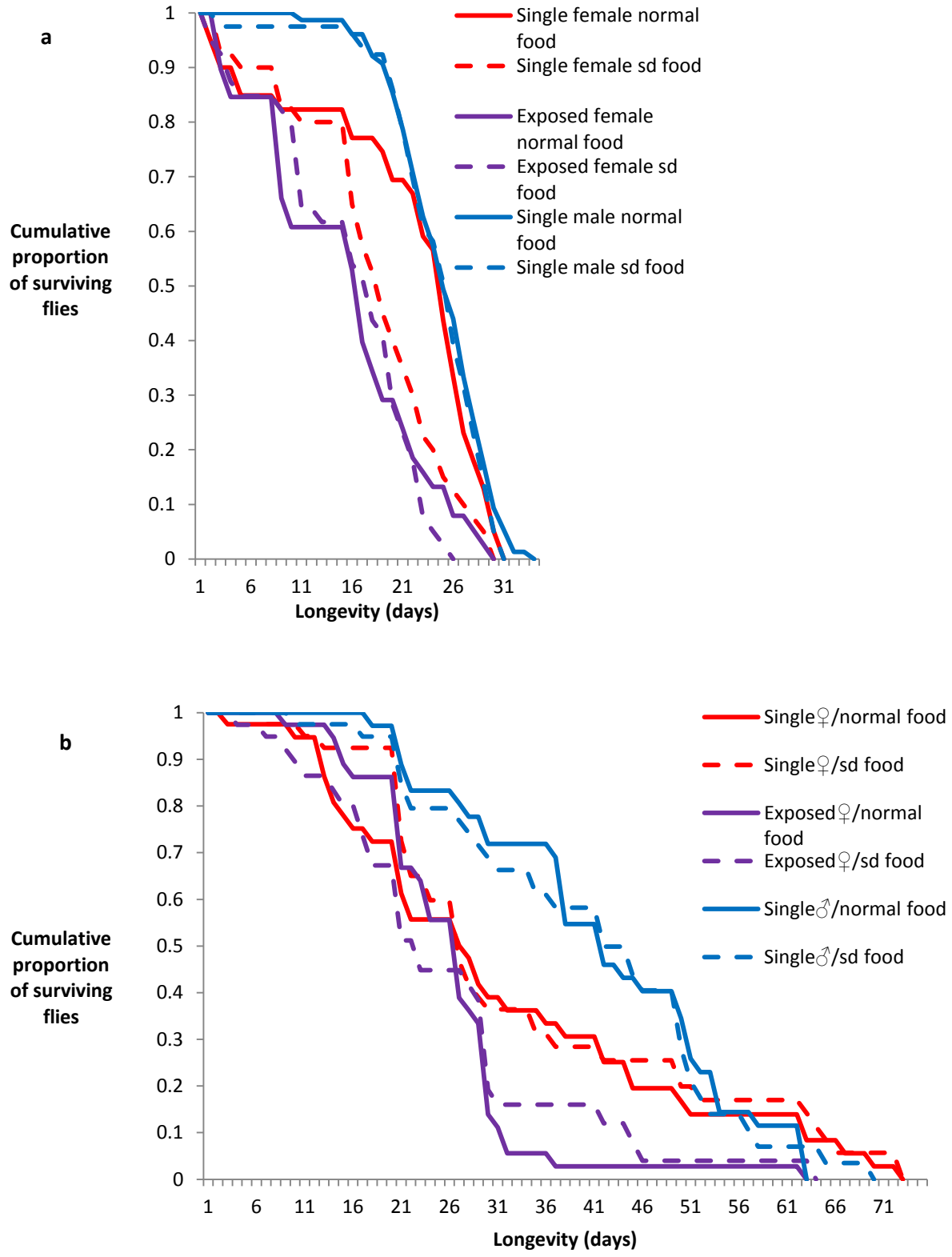


Fig 3.5a and b – Two replicate experiments (panels a and b, respectively) showing the survivorship of once mated female, (single female), continually mated females (exposed females) and males (single males) maintained on control food (yeast paste only) or 1mM spermidine (sd) diet (yeast paste+spermidine).

Age specific fecundity

In the first replicate experiment, the analysis of fecundity over time for all treatments, using ANCOVA, showed the expected significant decline in the number of eggs laid over time ($p=0.001$). There was also a significant interaction between female mating status and time ($p=0.05$), with exposed females generally showing a flatter pattern of egg production with age in comparison to the once mated treatments. The number of eggs laid by control females was initially significantly higher than the other treatments ($p<0.001$). To probe the differences in more detail, the age specific comparisons revealed that there were significant differences in the overall number of eggs laid only on day 20 ($p<0.05$), with the number of eggs laid by once mated females held on control food being significantly higher than for the other females (fig 3.6a). There was therefore no evidence for an effect of spermidine on fecundity.

In the replicate second experiment the ANCOVA again showed a significant overall decline in egg production over time ($p<0.001$). There was again an interaction between females mating status and time ($p=0.05$) and similar to above, the continually exposed females had a flatter pattern of egg production over time than did the once mated females. Initially the control females had lower fecundity ($p<0.001$) than the other groups and the spermidine treatment females had higher ($p=0.05$). More in depth day by day analysis revealed a significant difference in the number of eggs laid by females held singly versus those exposed to males on day 2, and on day 15 (all at $p<0.05$, fig 3.6b). As in the first replicate experiment, there was no evidence for an effect of spermidine on fecundity.

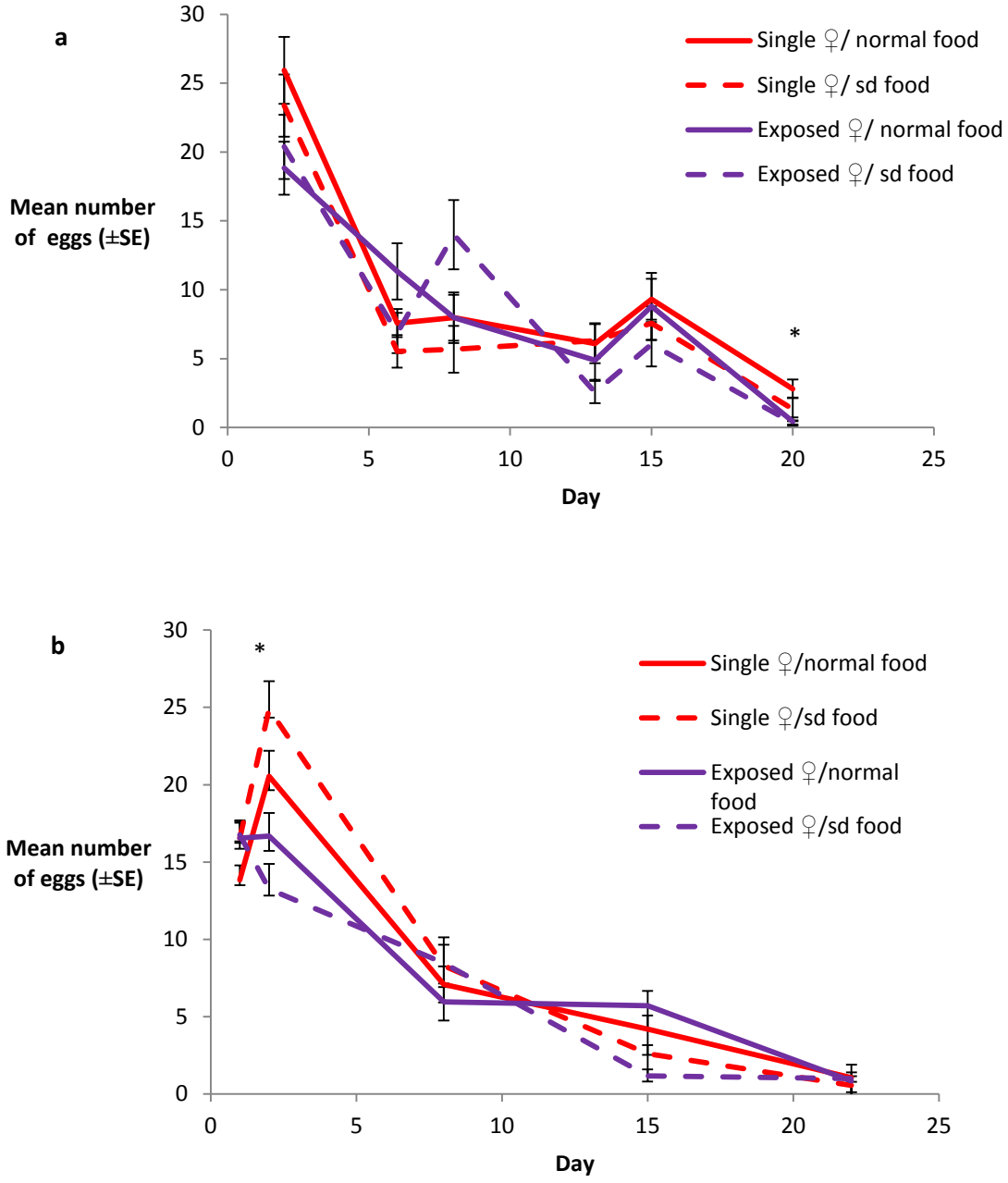


Fig 3.6a and b – Two replicate experiments (panels a and b, respectively) showing the fecundity (mean number of eggs per female per 24 hours ±SE)of once mated female and continually mated females maintained on control food (yeast paste only) or 1mM spermidine (sd) diet (yeast paste+spermidine). Significant differences in age specific fecundity are indicated by * ($p<0.05$).

Age specific fertility

ANCOVA showed that females exposed to males exhibited a significantly steeper decline in fertility over time ($p=0.05$, fig 3.7a) compared to the control (single ♀/ normal food). In further analysis, only day 15 showed a significant difference, with an increase in the number of unhatched eggs in the single females held on normal food ($p=0.05$).

ANCOVA analysis of the second replicate experiment showed, as above that females exposed to males exhibited a significantly steeper decline in fertility ($p<0.001$, fig 3.7b) in comparison to the control (single ♀/ normal food). Further analysis revealed significant differences on day 1 only, driven by higher numbers of unhatched eggs in the females continually exposed to males ($p<0.05$).

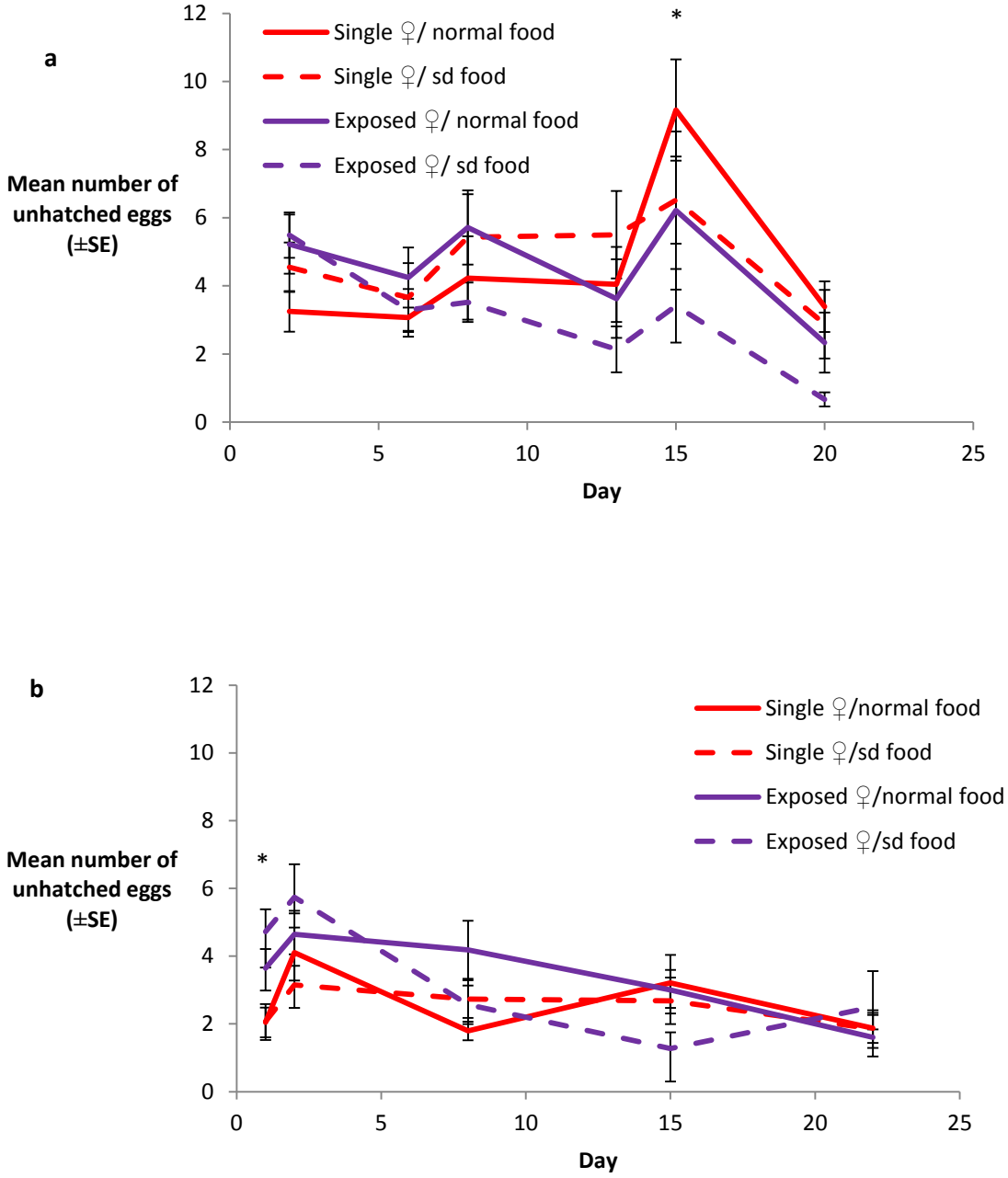


Fig 3.7a and b – Two replicate experiments (panels a and b, respectively) showing egg fertility (mean number of unhatched eggs per female per 24 hours ±SE) of once mated and continually mated females maintained on control food (yeast paste only) or 1mM spermidine (sd) diet (yeast paste+spermidine). Significant differences in age specific fertility are indicated by *($p<0.05$).

Lifetime productivity and fertility

Analysis of lifetime productivity for the first experiment (fig 3.8) revealed no significant differences in the lifetime number of eggs, the number of hatched or unhatched eggs.

In the second replicate experiment, however, once mated single females laid significantly more eggs in their lifetimes than did females exposed continually to males ($F_{1,153}$=8.063, p=0.005, fig 3.9a). There was also an interaction between food type and mating status, with spermidine treatment leading to increased fecundity in once mated females but decreased fecundity in continually mated females ($F_{1,153}$=6.298, p=0.013). The number of hatched eggs was also significantly different, with once mated single females producing fewer fertile eggs than those females that were exposed to males ($F_{1,153}$=15.019, p<0.001, fig 3.9b). There was again a significant interaction between mating status and food type, which followed the same pattern as described for total egg number ($F_{1,153}$=5.929, p=0.016). There was no effect of spermidine treatment on the number of unhatched eggs produced. However continually mated females laid significantly more unhatched eggs ($F_{1,153}$=4.889, p=0.029, fig 3.9c) suggesting that their fertility was compromised.

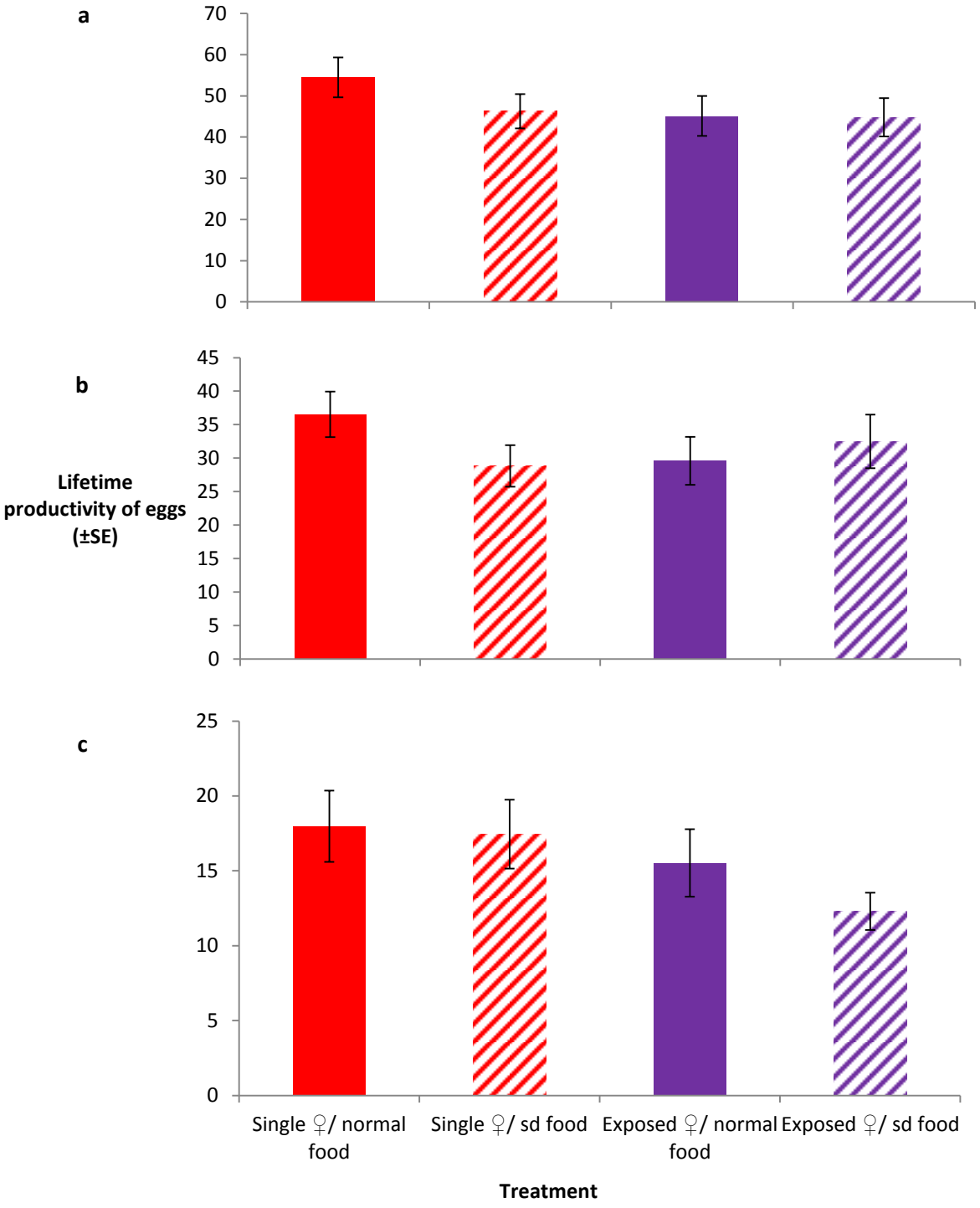


Fig 3.8 – Lifetime productivity of eggs in experiment 1 (mean per 24 hours ±SE) for once mated and continually mated females maintained on control food (yeast paste only) or 1mM spermidine (sd) diet (yeast paste+spermidine). (a) is the total lifetime egg production, (b) the number of fertile (hatched) eggs and (c) the number of unhatched eggs.

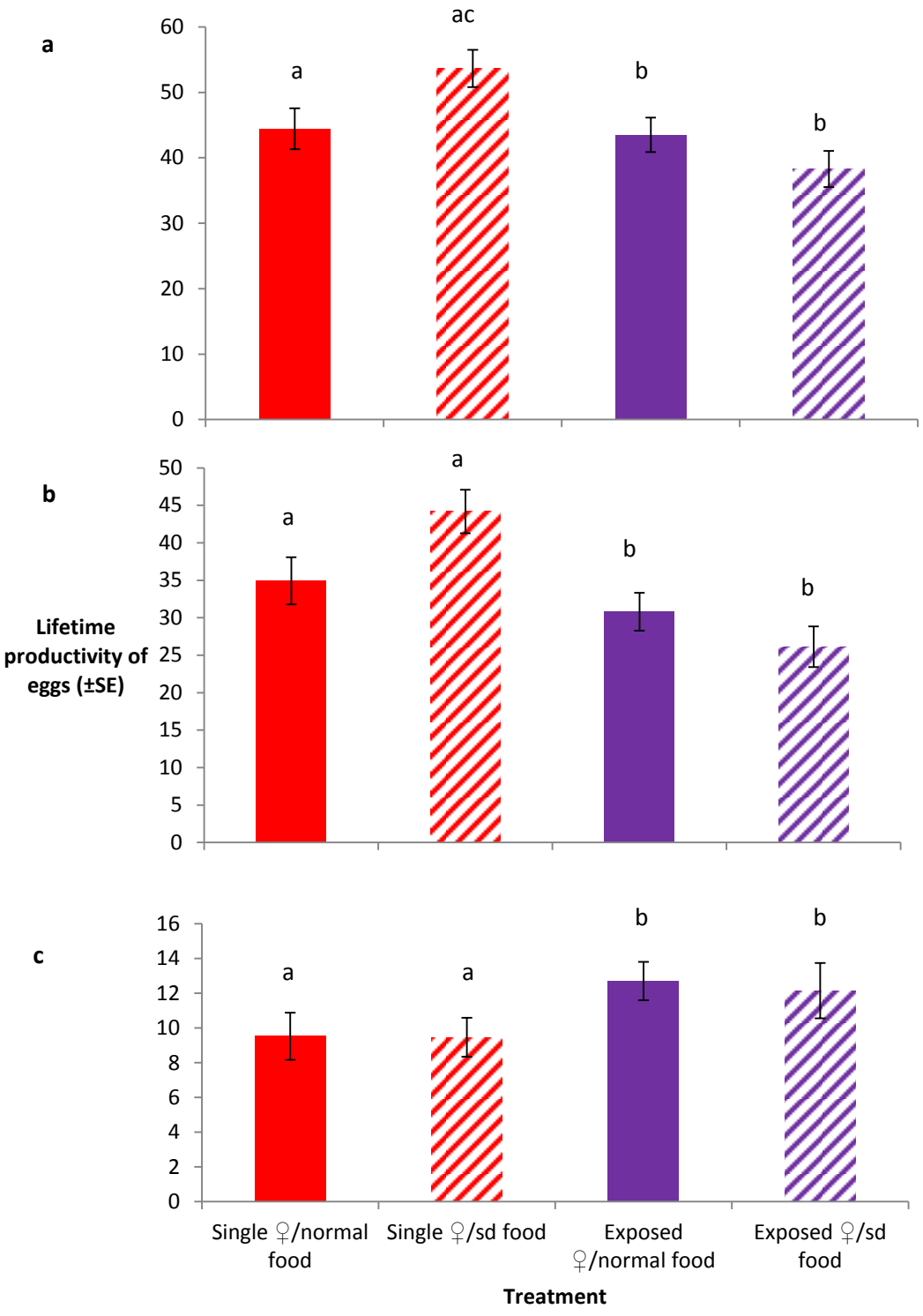


Fig 3.9 – Lifetime productivity of eggs in replicate experiment 2 (mean per 24 hours ±SE) of once mated females (single female) and continually mated females (exposed female) maintained on control food (yeast paste only) or 1mM spermidine (sd) diet (yeast paste+spermidine). (a) is the total lifetime egg production, (b) the number of fertile (hatched) eggs and (c) the number of unhatched eggs. Letters above the bars show the results of post hoc tukey tests. Different letters denote significant differences at ($p<0.005$).

3.4.3 Western Blot analysis

Western blot analysis was conducted to determine if 1mM spermidine led to the processing of the *Drosophila Atg8* protein into separate products *I* and *II.* Therefore the extent of the ratio of *Atg8-II* to *Atg8*-I indicated whether the activation of autophagy had been achieved in the different treatments. Processing can be seen in all the lanes below (fig 3.10). The extent of processing was determined by calculating the ratio between the intensity of *Atg8-II* and *Atg8-I* after both results were normalised against the tubulin the control (ratios can be seen in fig 3.10). Comparing between individuals with the same sex and mating status, this analysis indicates that autophagy was not activated by spermidine treatment or by diet restriction in single once mated females. However, autophagy was activated in continuously exposed females and in males fed spermidine, and also to a lesser extent in males held on a restricted diet. Hence the ability of spermidine treatment to activate autophagy appeared to be context dependent.

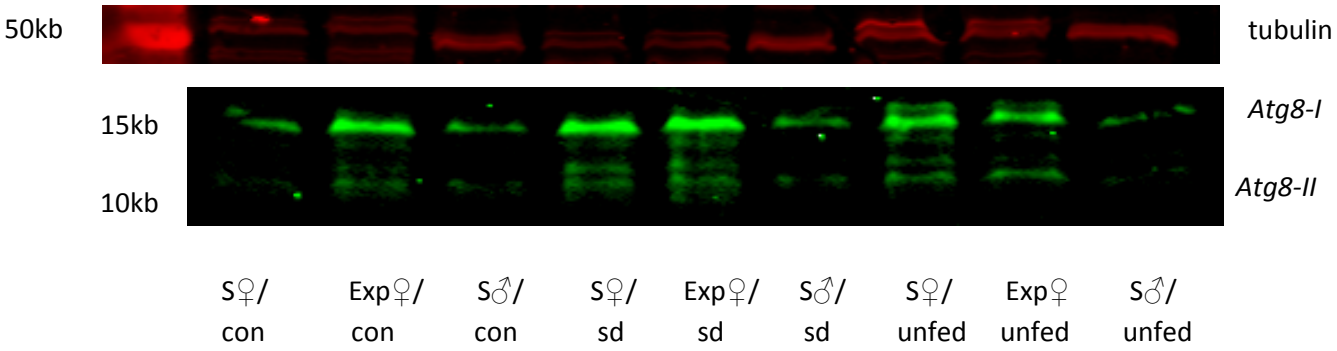


Fig 3.10 – Western blot showing *dAtg8-I* and *dAtg8-II* levels in once mated females (S♀), females exposed continually to males (Exp♀) and in once mated males (S♂) held on normal control food (con), food with added spermidine (15µl, 1mM concentration sd) or unfed. Cleavage of *Atg8* to *Atg8-I* and *Atg-II* indicates the activation of autophagy and the ratio of the two cleavage products (normalised to tubulin) gives the extent of autophagy activation. The ratios are: S♀/Con = 0.66, Ex♀/Con = 0.46, S♂/Con = 0.42, S♀/Sd = 0.51, Ex♀/Sd = 0.65, S♂/Sd = 0.51, S♀/Unfed = 0.49, Ex♀/Unfed = 0.44, S♂/Unfed = 0.50 (All intensities normalised against tubulin control for each lane).

3.5 Discussion

Overall the results of the experiments in this chapter indicate that, in contrast to previously published data (Eisenberg, Knauer et al. 2009) the pharmacological application of spermidine in the diet did not extend lifespan, and if anything tended to significantly decrease it. The addition of spermidine did activate autophagy. However, the extent to which this occurred depended upon sex and reproductive status. In females continually exposed to males, but not in once mated females, 1mM spermidine treatment caused a strong activation of autophagy. In males, both spermidine and diet restriction appeared to activate autophagy to a lesser extent, at least at the 1mM dose tested. Therefore any effects of spermidine on life history traits in males and females exposed to males can be attributed, at least in part, to the activation of autophagy. However, any effects of spermidine on the life history of once mated females are presumably mediated by other routes.

The results from the initial dose response experiment showed that 1M spermidine appeared to be toxic, resulting in a decrease in longevity as the amount of spermidine increased. In this experiment any beneficial effect of activating autophagy may therefore have been offset by the toxic effects of spermidine itself. In subsequent experiments the dose of spermidine was decreased by x3 orders of magnitude and tests were also performed on flies with differing reproductive status, i.e. once mated females, once mated males and females continuously exposed to males. However, these results also showed that the application of spermidine to the diet either had no or a detrimental effect on longevity. Overall, therefore, the effect of spermidine on lifespan was inconsistent. There was no evidence, therefore, to support the hypothesis that activation of autophagy, in the groups that experienced it, by the dietary application of spermidine increases lifespan. As expected, males lived longer than single females and single females longer than exposed females. This suggests that, in general, the life history traits were expressed as expected in the experiment, based upon previous studies (Carey, Harshman et al. 2008; Perez-Staples, Aluja et al. 2008; Eisenberg, Knauer et al. 2009).

Analyses of reproductive output for all the experiments indicated that there was, as expected, generally a significant decrease in female fecundity and fertility over time. Overall, however, there was little evidence for any consistent effect of the spermidine treatment on female fecundity and fertility. Spermidine treatment either had no effect on fecundity (e.g. fig 3.8), or slightly decreased (e.g. fig 3.9) or increased it (e.g. fig 3.9). Although differences were seen in numbers of eggs laid in the first experiment between those females held with and without males, lifetime productivity revealed no overall significant differences between any of the treatment groups. In the second repeat experiment the females exposed to males and held on food with added spermidine laid significantly more eggs, but not more fertile eggs than those on the normal food. There is therefore no evidence that treatment with dietary spermidine extended lifespan or altered reproductive output. There were also no interactions between female reproductive status and the extent of any effects of spermidine, hence there was no evidence that the extent of activation of autophagy by spermidine (in continually exposed but not once mated females) was correlated with the magnitude of any such differences that did occur.

Western blot analysis (fig 3.10) showed that autophagy had been activated in the continually exposed females fed spermidine, but interestingly not in the unfed females. In males, autophagy also appeared to be activated when males were fed spermidine and when those males were unfed. Continually exposed females laid fewer eggs when held on spermidine over their lifetime, which was also shorter suggesting no effects of the activation of autophagy and no trade-offs in this area.

My results show that, despite evidence that spermidine provided through the diet can activate autophagy, there is little evidence that this has any beneficial effect on lifespan. The unique contribution of the work presented is to show that this also applies to the effects of spermidine on fecundity and fertility. The lack of lifespan extension is in contrast to previous research (Eisenberg, Knauer et al. 2009; Madeo, Eisenberg et al. 2009), which therefore motivates an investigation of the potential reasons for the differences. However, a careful inspection of the available published

evidence does reveal that spermidine may not in fact be a very reliable or clean activator of autophagy, i.e. it often does have variable effects. For example, in contrast to the stated conclusion, Minois et al. (2012) showed no consistent effect of spermidine on longevity, in half of the experiments conducted there was no effect on lifespan (Minois, Carmona-Gutierrez et al. 2012). Hence, the results from the multiple experiments conducted in this chapter may be more consistent with the general picture from the literature than is currently realised.

There are several possibilities as to why these results contrast with published data that do show effects of spermidine on lifespan:

(i) The delivery of spermidine and essential food components to the flies could be variable. Equivalent delivery of spermidine and food to the different treatments depends upon a lack of variation in the rate and quantity of food ingestion across the different spermidine dose diets, across control and spermidine diets and between females and males. An implicit assumption is that there is no compensatory feeding, as the ability of flies to exhibit compensatory feeding seems limited (Piper and Bartke 2008), but the feeding behaviour of males and females is observed to be divergent (Magwere, Chapman et al. 2009). Hence autophagy may not always be activated in a way that perfectly corresponds to the amount of spermidine supplied in the food media. Further research could monitor feeding rates, maybe via the CAFÉ feeding method (Ja, Mak et al. 2007) and better document the extent of autophagy activation under various spermidine treatments.

(ii) Spermidine is a crude activator of autophagy. Hence spermidine may not cleanly activate autophagy and may have other toxic side effects, or collateral effects on other biological processes. For example one side effect may be that polyamines such as spermidine are required by most cells to enable optimal growth, however an accumulation will lead to an inhibition of cellular growth (Limsuwun and Jones 2000). Consistent with this, in rats it was found that dietary application of spermidine caused a decrease in body weight in conjunction with lower food uptake. Also a slight decrease in plasma creatinine, calcium and inorganic phosphate levels were observed which are

required for an organism to develop, for example, optimal growth, bone development and fertility (Til, Falke et al. 1997).

(iii) Spermidine also has pleiotropic effects upon reproductive processes, as discussed in the introduction. These may or may not be autophagy dependent. Therefore the application of spermidine to the diet may have competing and interacting effects on the same life history processes.

The possibility that autophagy activation interacts with female reproductive status is interesting. It may be the case that any benefits of autophagy are dependent upon the type of stress being experienced. An intervention that consistently causes an increase in lifespan from yeast to primates is caloric restriction (Fontana, Partridge et al. 2010). It is possible that the underlying mechanism involves the induction of autophagy and this possibility deserves much more study. Although the process by which lifespan is extended in response to diet is not well understood some researchers believe that it is the absence of specific dietary amino acids that mediate the effects of caloric restriction, rather than the actual restriction of calories themselves, (see chapter 6), (Grandison, Piper et al. 2009). Madeo et al. (2009) showed that lifespan extension by spermidine, rapamycin and caloric restriction in yeast, flies, and mice might be mediated by a common pathway (Madeo, Eisenberg et al. 2009). In order to test more rigorously whether the potential common pathway might involve autophagy, it is clear that better, cleaner methods for activating autophagy are needed (see chapter 4).

As discussed above, previous research shows that spermidine may have separate effects on reproduction through both autophagy dependent and independent pathways. For example spermidine controlled mating in yeast does not appear to depend on autophagy (Bauer, Carmona-Gutierrez et al. 2013). The external administration of spermidine can restore efficient mating in yeast cells even when they were autophagy deficient indicating autophagy is not involved in the positive effects of spermidine on mating and/or fertilization. Similarly, the essential role of

spermidine in the successful mating of *S. cerevisae* and effective fertilization in *C. elegans* are also autophagy-independent. Overall, this suggests that there are alternative pathways, which may involve a different molecular target of spermidine. A possible candidate could be the calcium channels that are regulated by spermidine (Bauer, Carmona-Gutierrez et al. 2013). Reduction of spermidine in *C. elegans* through the deletion of spermidine synthase (SPDS-1) led to a significant reduction in the total numbers of eggs produced, providing evidence for a trade-off between lifespan and fertility under the control of spermidine pathways. More work is therefore needed on this topic in flies, perhaps using better methods of manipulating spermidine, e.g. genetically.

Spermidine has been found to have multiple effects, which is why it is difficult to show how it interacts with all aspects of the cellular process. Spermidine is thought to activate autophagy via the activated protein kinase (AMPK) which in turn inhibits TOR and protein synthesis. TOR signalling is a critical pathway in longevity, as noted in the introduction. TOR proteins are sensors that link nutrient availability to cellular growth. In yeast, worms and flies the manipulation of TOR genetically or with pharmacological intervention can greatly improve longevity and similar effects have been seen in mice (Harrison et al 2009). The inhibition of TORC1 also has a major effect on protein translation, which could indicate any advancement of longevity may be autophagy dependent, but could also equally be the result of effects unrelated to autophagy (Madeo, Eisenberg et al. 2009; Rubinsztein, Marino et al. 2011). This could also be true of spermidine if it works in the same way.

Although the effects of spermidine appear unreliable in the experiments described here, previous research, for example Bauer (2013), shows that inhibiting autophagy by knocking out essential autophagy genes can abrogate the longevity extension effect of spermidine in yeast, flies and nematodes (Bauer, Carmona-Gutierrez et al. 2013). In contrast, a study on carnation flowers found no delay in senescence when spermidine was introduced, and in some treatments an advancement in senescence was seen (Downs and Lovell 2006). It may be that in certain instances when spermidine is added to the diet longevity extension may be seen if it can effectively and cleanly

mimic caloric restriction (Kaeberlein 2009). Caloric restriction has been shown to increase longevity at least in part by the inhibition of TOR and a primary function of TOR is to repress autophagy. It is not known if spermidine can directly inhibit TOR but it is known to interact with RNA to modulate mRNA translation, and it is estimated that approximately 60% of spermidine is bound to RNA. Several mutations that alter mRNA translation increase lifespan which could indicate another route by which spermidine could enhance longevity. Spermidine also suppresses inflammation and it is believed inflammation contributes to a variety of age-related diseases in mammals (Igarashi and Kashiwagi 2010). It has been suggested that humans could add spermidine to the diet via consumption of foods that contain high levels of it, including soy beans, tea leaf and mushrooms. However, the amounts required to be ingested to have any effect on longevity are estimated to be huge. Also in relation to mammalian ageing the oxidation of spermidine causes oxidative stress that can induce cell death and it has been shown that high levels of polyamines are associated with malignancy.

In conclusion, before recommending a policy that people should strive to obtain spermidine through dietary modifications, much more work needs to be undertaken in order to determine more clearly the consistency of any effects on lifespan and also to identify the full range of beneficial and deleterious side effects. The research described in this chapter suggests that a great deal of caution is needed.

Chapter 4 Effects of activating autophagy via dietary delivery of Torin1 on longevity, fecundity and fertility

4.1 Abstract

Autophagy is down-regulated as a normal result of increasing age, leading to the suggestion that manipulations that activate autophagy might be able to increase lifespan. The accumulating evidence indicates that pharmacological manipulation of autophagy through diet can result in an increase in longevity in various species. The TOR pathway can be inhibited, and hence autophagy activated, by inactivating TORC1 through dietary manipulation using various chemicals. Torin1 appears to be a cleaner and more robust chemical activator of autophagy than has previously been employed in this field (e.g. spermidine, chapter 3). I found that addition of Torin1 to the diet activated autophagy and significantly increased lifespan in both sexes. My data supports the hypothesis that dietary application of Torin1 exerts its effects on lifespan because of increased autophagy but there was no evidence of a trade-off between longevity and fecundity or fertility. Elevated egg production was seen when females were fed Torin1 but overall this did not result in higher egg hatching. The results show that lifespan can be extended without trade-offs in fertility and point to Torin1 as a promising agent with which to pursue anti-ageing research.

4.2 Introduction

Many animal and human diseases show a distinct increase in incidence or severity with age. Hence, it is of paramount importance to understand the molecular and cellular mechanisms that regulate the ageing process in order to minimise its health impacts. Research over the last two decades has shown that several growth and nutrient sensing pathways that are highly conserved across animal taxa are intimately involved in determining length of life. One such important pathway is the protein kinase 'target of rapamycin' (TOR) pathway, which when manipulated alters lifespan. For example down regulation of TOR is reported to increase lifespan (Hands, Proud et al. 2009; Wang, Lao et al. 2009). TOR is highly conserved across eukaryotes, and controls several fundamental cellular functions. It is a major regulator of cellular growth and proliferation (Feldman, Apsel et al. 2009; Hands, Proud et al. 2009; Liu, Kirubakaaran et al. 2012). TOR forms two protein complexes TOR complex 1 (TORC1) and TOR complex 2 (TORC2) which are differentially regulated. They have distinct substrate specificities and are differentially sensitive to the bacterial-derived inhibitor rapamycin (Feldman, Apsel et al. 2009; Liu, Kirubakaaran et al. 2012). TORC1 promotes anabolism and inhibits catabolism by blocking autophagy through the phosphorylation of the *ULK1-Atg13-FIP200* complex (Laplante and Sabatini 2011). While it is known that TORC2 is insensitive to rapamycin its functions are less well understood than those of TORC1 (Rusten, Lindmo et al. 2004; Feldman, Apsel et al. 2009).

Of particular relevance to the research described in this thesis is that one of the key processes regulated by TOR is autophagy. As discussed earlier in the introduction, autophagy is a conserved pathway found in yeast, *Drosophila* and humans. The autophagy pathway involves the sequential interaction of more than 20 proteins. One example is the *Atg8a* protein used in chapter 5 and 6 to manipulate autophagic state (Baehrecke 2003; Homma, Suzuki et al. 2010). Autophagy is naturally down-regulated as a normal result of increasing age. This has led to the suggestion that manipulations that activate autophagy might be able to increase lifespan. Consistent with this, over-

expression of autophagy genes has been shown to extend lifespan in yeast, flies, worms and human cells (Eisenberg, Knauer et al. 2009). It has been shown that the specific inhibition of TOR which activates autophagy results in extension of lifespan in various species (Mehrpour, Esclatine et al. 2010). Environmental factors including stress and nutrition can also affect the level of autophagy (Pletcher, Macdonald et al. 2002; Lee, Simpson et al. 2008). The TOR pathway can be inhibited, and hence autophagy activated, by inactivating TORC1 through treatment of cells with rapamycin or via nitrogen starvation. The extension of lifespan seen due to the resulting inhibition of TOR could be potentially via TOR's effects on protein synthesis. However, research on *C. elegans* suggests a more direct role of autophagy in the modulation of longevity because inactivating autophagy genes specifically prevents the inhibition of TOR activity from extending lifespan. This is an indication that the TOR pathway and autophagy act via the same signalling pathway to affect lifespan (Hands, Proud et al. 2009).

Feldman et al. (2009) reported the synthesis of pyrazolopyrimidines that could be used to selectively inhibit members of the P13k family including TOR. Two structurally distinct inhibitors of TOR, PP242 and PP30, have been shown to increase longevity in mice (Feldman, Apsel et al. 2009). Unlike rapamycin, these newer compounds target both complexes of TOR (i.e. TORC1 and TORC2). At the same time, Guertin and Sabatini (2009) reported the synthesis of a compound known as Torin1, which, like PP242 and PP30, was found to inhibit TOR with a higher degree of selectivity than other previously used protein kinases (Guertin and Sabatini 2009; Thoreen, Kang et al. 2009; Feldman, Apsel et al. 2009). Torin1 has a direct inhibitory effect on TOR and, as for PP242 and PP30 it is thought to effectively inhibit kinase function in both TORC1 and TORC2 complexes. It impairs cell growth and proliferation to a much greater degree than rapamycin and appears to be a better and cleaner inhibitor than spermidine (chapter 3). Thoreen et al. (2009) suggest that Torin1 suppresses the rapamycin-resistant functions of TORC1 that are necessary for the suppression of autophagy (Thoreen, Kang et al. 2009). Torin1, along with PP242 and PP30, is therefore one of the so called 'second generation' TOR inhibitors all of which are expected to be more effective than rapamycin as

mechanisms for manipulating TOR and hence autophagy itself. Torin1 was therefore selected for the experiments in this chapter to activate autophagy with increased selectivity in comparison to the spermidine treatments employed in chapter 3.

The main interest to date in pharmacological interventions affecting the TOR pathway has been to look for lifespan extension. However, it is essential that any associated effects on reproduction are also tested for. Increased lifespan associated with decreased function is not necessarily a desired outcome for lifespan interventions, but is a result that has been found on several occasions (Carey, Harshman et al. 2008; Bjedov, Toivonen et al. 2010). Reduced fecundity could invariably trade-off with extended lifespan, but there is no simple correlation with longevity (De Loof 2011). Extended lifespan can also be seen in sterile females also treated with rapamycin, which further confirms that lifespan extension caused by rapamycin treatment cannot be explained by reduced fecundity in those females (Bjedov, Toivonen et al. 2010).

Here I used the general experimental approach from chapter 3 to activate autophagy through addition of the Torin1 dietary additive. I tested for dose response effects of Torin1 on once mated female survival and fertility, for sex differences in response to Torin1 as well as whether the effects of Torin1 interacted with female reproductive status. I conducted experiments covering a range of different but physiologically relevant doses of Torin1.

4.3 Materials and Methods

4.3.1 Effect of Torin1 on once mated survival, fecundity and fertility.

Fly rearing was carried out as stated in the general materials and methods (chapter 2). Females were separated from males using CO_2 three days after eclosion. This ensured that at least one mating had occurred and that all individuals could therefore be considered 'once mated'. Single, once mated females were then allocated at random to one of five food treatment groups in

replicates of n=40 each. The treatments were carrier only (DMSO) control group (Con), 1μM, 10μM, 100μM and 1mM Torin1 (Torin1 was obtained originally from the Sabatini lab, Whitehead institute USA and then from Tocris http://www.tocris.com, stock number 4247). Two replicate experiments were performed using these doses and a third experiment then focused on the effect of lower doses of Torin1, using carrier control (Con), 0.5μM, 1.0μM, 5μM and 10μM Torin1. Each food vial comprised 7ml of agar only base (agar with no food added) with a drop of live yeast paste containing one of the Torin1 treatment doses or carrier control. Females were given a new food vial every 24 hours Monday to Friday until egg production ceased, and then three times a week until death.

The live yeast paste containing Torin1 or carrier control was made up by adding 5μl of the required dilution of Torin1 or carrier control to 10ml distilled water, and then adding 6g yeast. The paste was deposited as a droplet (2mm diameter) on the plain agar in the bottom of the vial. Flies were transferred during the experiment between vials without using anaesthesia. Following the first experiment it became clear that Torin1 has a low solubility and a tendency to 'settle' and that it is therefore crucial to mix extremely well within the DMSO carrier prior to use. In subsequent experiments extra care was taken to ensure that the Torin1 was well dispersed within the carrier control solution across the different dose treatments.

Female longevity was recorded daily, throughout each of the three experiments until all females were dead or until the experiment was terminated. Individuals that became stuck in the food, escaped or were accidentally killed were entered as censors in the data analysis.

Eggs laid in the food vials over 24 hours were counted 2 times each week. The vials were then incubated and the number of unhatched eggs counted the following day to determine egg hatchability. Egg counts ceased when half the remaining females in a treatment stopped laying eggs or where there were fewer than 5 females remaining in a treatment.

I summed for each female in each treatment the total number of eggs produced from all sample recorded over the lifetime. I did this separately for the total number of eggs laid, and those that were hatched and unhatched.

4.3.2 Effect of Torin1 on once mated and continually mated female survival and fertility and on once mated male survival.

Fly rearing and handling was carried out as in the experiment above. Females and males were collected from mixed sex cultures three days following eclosion using CO_2, and were therefore considered 'once mated'. Females and males were randomly allocated to one of the 6 treatment groups (n=40 for each). For this experiment a single dose of Torin1 (1μM) was used. The treatments were: single females, control versus Torin1 diet; single males control versus Torin1 diet and continually exposed females control versus Torin1 diet. In the continually exposed females, males were replaced with fresh 4 day old males every 7 days to ensure reproductive activity remained high. To control for the CO_2 exposure used for male replacement all females and males in the once mated treatment groups were also similarly CO_2 anaesthetized each week. Flies were tipped onto new food daily until week 4 and then three times a week until death.

Longevity was recorded daily. As above individuals that became stuck in the food, escaped or were accidentally killed were entered as censors.

Eggs laid in the food vials over 24 hours were counted 2 times each week. The vials were then incubated and the number of unhatched eggs counted the following day to determine egg hatchability.

For the once mated and continually exposed females I determined the total egg productivity for each individual over their lifetimes.

For the once mated male experiment male survival only was recorded.

4.3.3 Western Blot analysis

Western blot analyses were used to determine whether autophagy had indeed been activated in the Torin1 fed flies. As described in the experiments from the previous chapter (3) cleavage of the *Atg8* gene protein into *Atg8-I* and *Atg8-II* was used to demonstrate the activation of autophagy. Intensity of these separate cleaved bands was recorded and normalised against the intensity of the tubulin control bands for each lane. Ten flies were sampled from each of the doses used in the dose response experiment for validation of autophagy activation (doses were as stated above, carrier only (DMSO) control group (Con), 1µM, 10µM, 100µM and 1mM Torin1). Flies were crushed after freezing in liquid nitrogen 5 days after being placed on required dose of Torin1, and homogenised in 100µl lysis buffer (120mM NaCl, 50mM Tris-HCl, 20mM NaF, 1mM Benzamidine, 1mM EDTA, 6mM EGTA, 15mM $NA_4P_2O_7$, 1% Nonidet P-40) containing protease inhibitors. Proteins were separated on a 15% SDS-PAGE gel and blotted onto Immobilon FL transfer membrane (Millipore IPFL00010). Primary antibodies were applied overnight at 4^{o}C, anti-*dAtg8- 1*:1000 (kindly supplied by K.Ko¨ hler,(Barth, Szabad et al. 2011)), anti-tubulin1:10000 (abcam ab160) and secondary antibodies, anti-rabbit 1:10000 (LI-COR 926-32211) and anti-rat 1:10000 (LI-COR 926-68076) for 1 hour and 40 minutes. For quantification of western blot signals, image software from odyssey (LI-COR) was used to calculate the intensity value of the bands on the different doses of Torin1.

The same procedures were used for the once mated, continually mated females and the once mated males.

4.3.4 Statistical analysis

Statistical analysis was performed using SPSS v 18 (SPSS 2009) and R (R Development Core 2008). Survivorship was calculated for each treatment using the Kaplan Meier method. Survival data were analysed using Log Rank tests and Cox Regression analysis (SPSS 2009). Fecundity and fertility data were analysed using ANCOVA to test for the treatments differences over time while accounting for within subjects repeated measures (R Development Core 2008). I used the generalised linear model

(glm) package within R to account for the pattern of fecundity/fertility over time compared to the control group. I used a poisson error distribution, which is used for count data, to account for the error structure of the egg data and repeated measures. Repeated measure was treated for because the data is repeated egg counts over time from individual females. I used model simplification to generate, where possible, simplified models of the patterns of fecundity and fertility variation. Fecundity and fertility data were tested for age specific differences in more detail using ANOVA or Kruskal Wallis tests, depending on whether the data were normal. Age specific and fertility data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. I conducted tests on the data for each day and corrected for the number of tests (repeated measures as above) using the sequential Bonferroni procedure (Rice 1989). This reduced the probability of Type 1 errors whilst allowing more insight into the nature of any differences indicated by the overall ANCOVA analysis.

4.4 Results

4.4.1 Effect of Torin1 on once mated female survival, fecundity and fertility.

Survival

The initial experiment revealed no significant effect of Torin1 dose on once mated female survival (Log Rank X^2=4.913, df=4, p=0.29, fig 4.1a). There was therefore no lifespan extension or alternatively no evidence for dose dependent toxicity following Torin1 application. There was though, as noted above, a concern in this initial experiment about whether there was adequate dispersal of Torin1 within the yeast paste in the diet (T. Wileman, pers. Comm.). In the subsequent experiments below steps were taken to ensure Torin1 was better dispersed within the yeast paste therefore low/ variable availability of Torin1 to the flies in the initial experiment. In the second replicate experiment conducted using the same Torin1 dose treatments (fig 4.1b below) a significant

increase in longevity in the once mated females fed Torin1 was seen (Log Rank X^2=18.918, df=4, p=0.001).

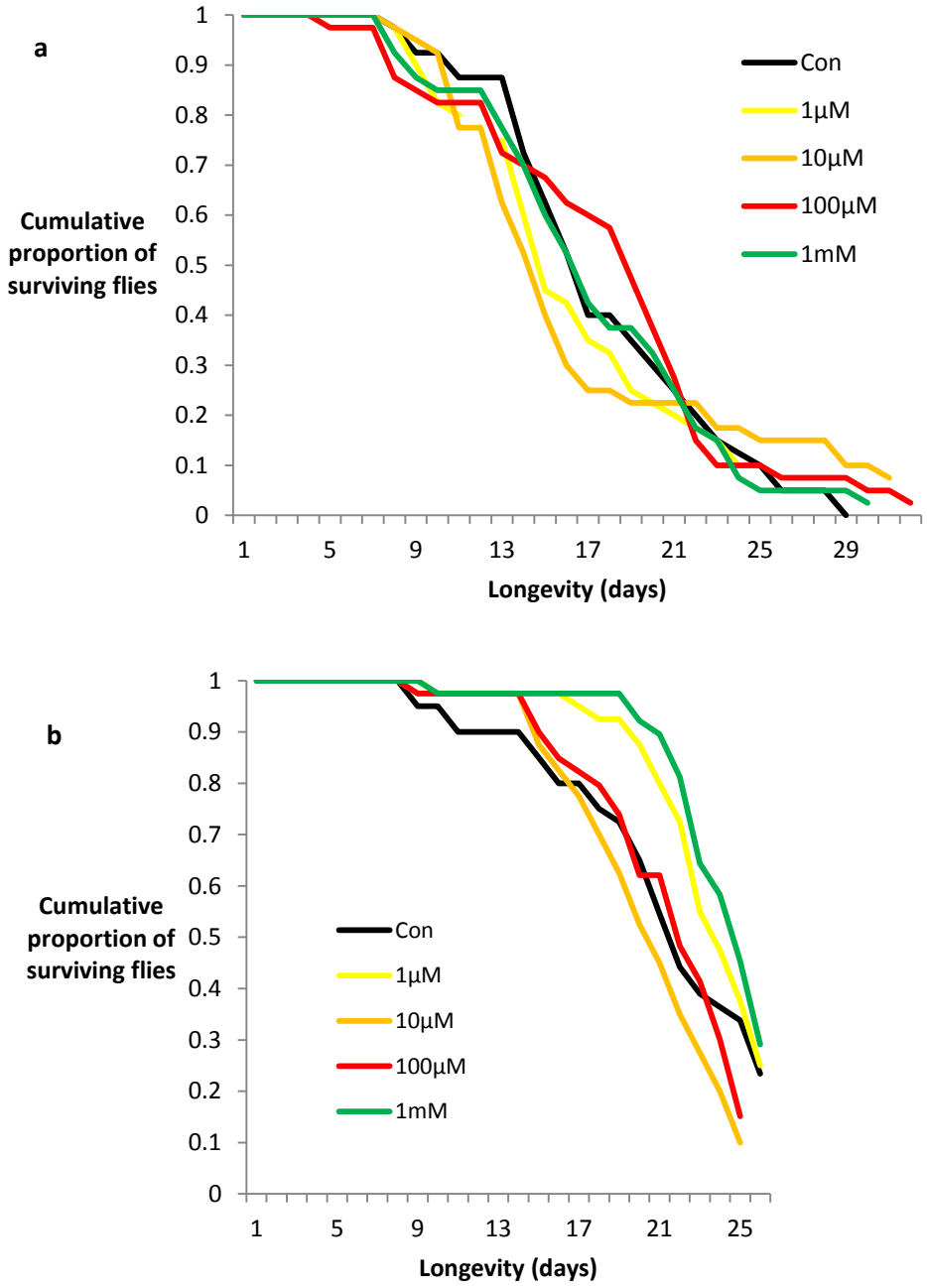


Fig 4.1 a and b– Survivorship of once mated females exposed to DMSO carrier control (Con), 1µM, 10µM, 100µM or 1mM Torin1 in the diet. Panels a and b show the two replicate experiments.

The third dose response experiment also showed a significant effect of Torin1 on once mated female lifespan (fig 4.2). There was evidence this time for a clear and consistent dose response, with a significant extension of longevity with increasing doses of Torin1 in the diet (Log Rank X^2=52.106, df=1, p<0.001).

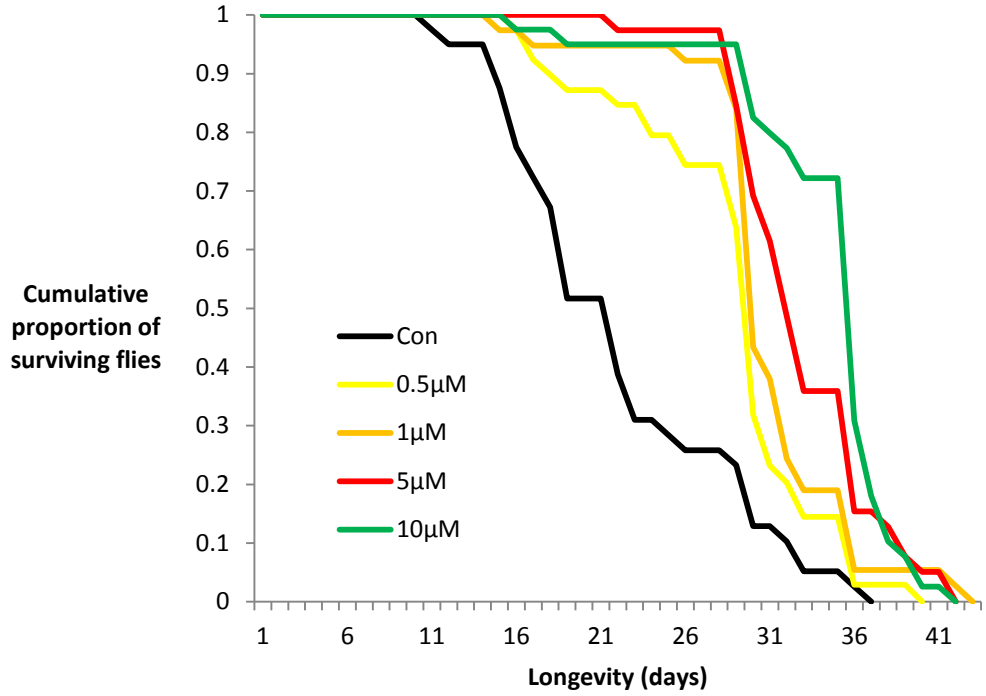


Fig 4.2– Survivorship of once mated females exposed to DMSO carrier control (Con), 0.5µM, 1µM, 5µM or 10µM Torin1 in the diet.

Age specific fecundity

To analyse age specific fecundity data (figs 4.3, 4.4) I first conducted ANCOVA, followed by tests on individual days, corrected for multiple comparisons by sequential Bonferroni tests. These analyses showed that there was no consistent effect of Torin1 on egg production. Detailed results are outlined below.

ANCOVA showed significant differences in the pattern of egg production over time and a significant interaction between food and time (both at p=0.001, first experiment). Consistent with this, the

second experiment also revealed significant differences in the pattern of fecundity over time ($p=0.001$) and a similar interaction between food and time ($p=0.05$).

To identify with more precision where any differences lay, the age specific data were analysed using ANOVA or Kruskal Wallis tests as appropriate, together with sequential Bonferroni corrections for multiple comparisons across different days. These tests showed that there were significant differences between treatments in the mean number of eggs on day 8 in the first experiment (fig 4.3a) and on day 8 and 10 in the replicate second experiment (fig 4.3b) all at $p<0.05$. On days where there were significant differences in fecundity, the data were further analysed using post hoc Tukey tests. These showed that in replicate experiment 1 on day 8, the 100µM treatment laid significantly fewer eggs than any of the other treatments ($p\leq0.013$, fig 4.3a). In the replicate experiment (fig 4.3b) the number of eggs laid by the control treatment females was significantly lower than the 100µM or the 1mM treatments ($p\leq0.001$) and on day 10 the control laid significantly fewer eggs than the 1µM treatment females.

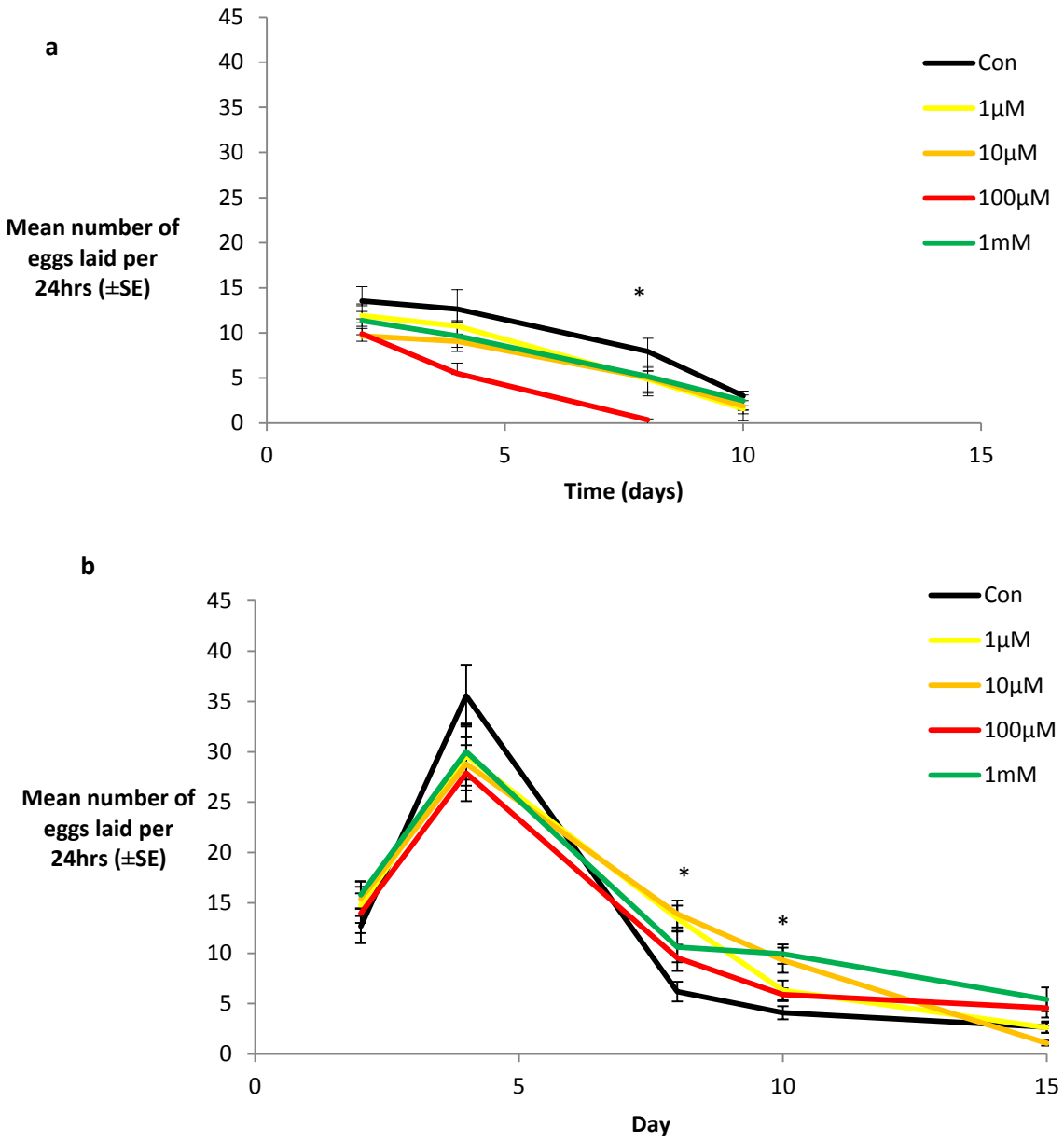


Fig 4.3a and b – Fecundity (mean number of eggs per female per 24 hours ±SE) of once mated females exposed to DMSO carrier control (Con), 1µM, 10µM, 100µM or 1mM Torin1 in the diet. Panels a and b show the two replicate experiments. * indicates a significant difference in age specific fecundity ($p<0.05$).

In the third experiment ANCOVA was again used to examine the effect of Torin1, at lower doses, on fecundity (fig 4.4). Significant differences in the pattern of egg laying between groups over time was again observed ($p=0.001$) and also significant main effects of diet ($p=0.05$).

In the age specific analyses, significant differences in fecundity were seen on all days tested (fig 4.4). Post hoc Tukey tests showed that on day 2 the 10µM Torin1 treatment females laid significantly more eggs than those females in the 0.5 and 1µM food treatments (p=0.011). On subsequent days after, the control laid significantly fewer eggs than the 10µM treatment (p=0.027, day 4), the 1, 5 and 10µM treatments (p≤0.016, day 8) and the 5µM treatment (p=0.036, day 16). On day 9 both the control and the 0.5µM treatments laid significantly fewer eggs than the 5 and 10µM treatments (p≤0.022).

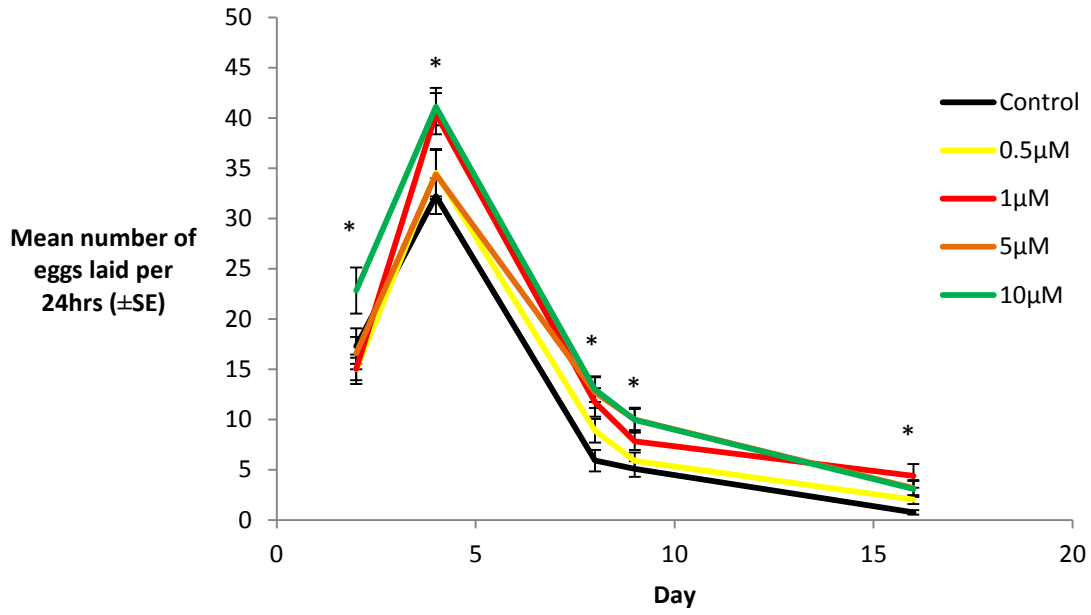


Fig 4.4 – Fecundity (mean number of eggs per female per 24 hours ±SE) of once mated females exposed to DMSO carrier control (Con), 0.5µM, 1µM, 5µM or 10µM Torin1 in the diet. * indicates a significant difference in age specific fecundity (p<0.05).

Age specific fertility

In the analysis of age specific fertility data (figs 4.5, 4.6) I followed the same procedure as outlined above. In general these analyses revealed a tendency for higher egg infertility in the Torin1 fed females. Futher details of the analyses are laid out below.

In the first experiment there was a significant effect of time, and a significant interaction between food and time (ANCOVA, p=0.01-0.05). In the second experiment there was also a significant interaction between type of diet and time (p=0.01-0.05).

In terms of fertility (the number of unhatched eggs) there was some evidence of elevated infertility (higher number of unhatched eggs) with increasing doses of Torin1. The first dose response experiment revealed no significant differences in fertility (fig 4.5a) but there was evidence for higher egg infertility in Torin1 fed over control females in the second replicate experiment (fig 4.5b), with significant differences in the number of unhatched eggs on day 8, 10 and 15 (all at $p<0.05$ after Bonferroni correction).

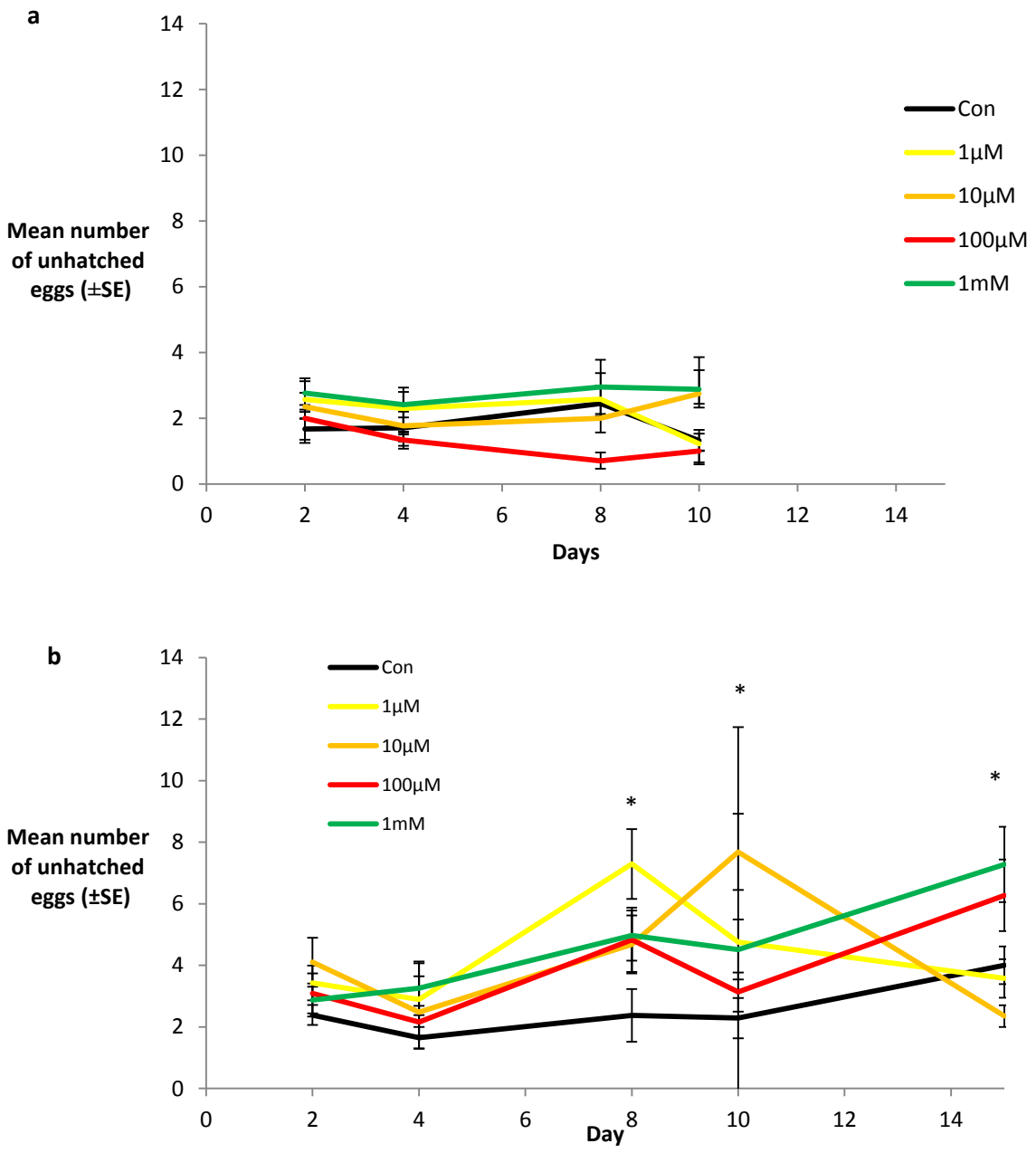


Fig 4.5a and b – The number of unhatched eggs (mean per female per 24 hours ±SE) of once mated females exposed to DMSO carrier control (Con), 1µM Torin1 (1µM), 10µM Torin1 (10µM), 100µM Torin1 (100µM) and 1mM Torin1 added (1mM). Panels a and b show the two replicate experiments). * indicates a significant difference in age specific fecundity ($p<0.05$).

In the third, lower dose, Torin1 experiment (fig 4.6) ANCOVA again revealed a significant interaction between food and time for egg fertility ($p=0.05$). Age specific analyses showed significant differences in egg fertility on day 8 and 9 ($p<0.05$ following Bonferroni correction).

Tukey analysis revealed that on day 8 control females laid significantly fewer unhatched eggs than did females held on the 5 and 10µM foods. On day 9 the 0.5µM treatment laid significantly fewer unhatched eggs than the 10µM treatment.

Overall, there was no evidence for systematic effects of Torin1 on age specific fecundity (figs 4.3, 4.4), but some evidence that higher doses of Torin1 in the diet led to higher egg infertility (figs 4.5, 4.6).

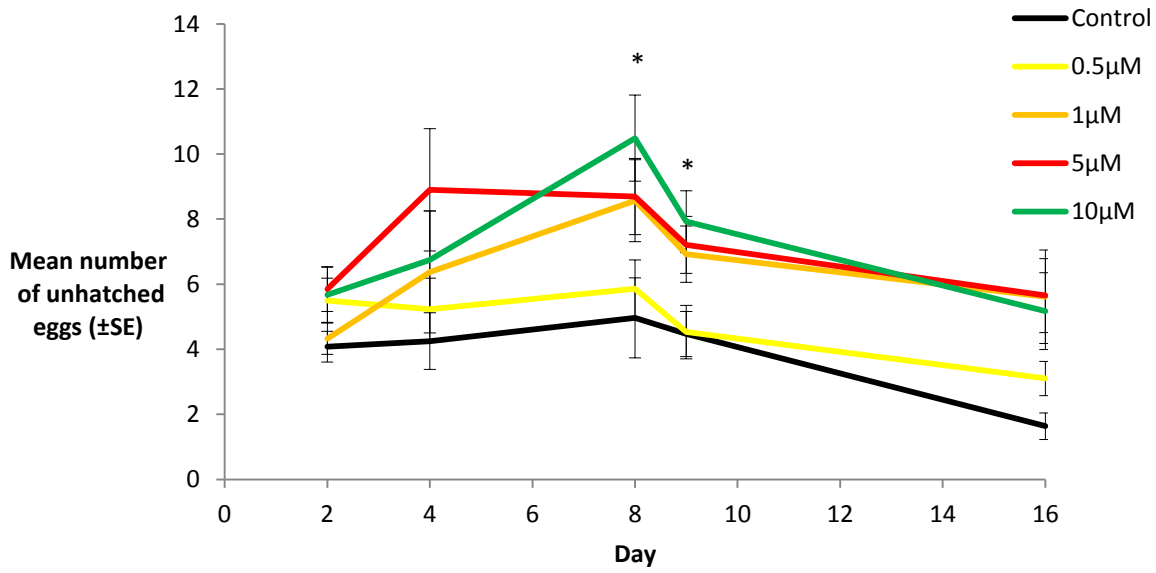


Fig 4.6 – The number of unhatched eggs (mean per female per 24 hours ±SE) of once mated females exposed to DMSO carrier control (Con), 0.5µM, 1µM, 5µM or 10µM Torin1 in the diet. * indicates a significant difference in age specific fecundity ($p<0.05$).

Lifetime egg productivity

Lifetime fecundity and fertility data for the three dose response experiments were compiled (figs 4.7 – 4.9). These analyses were consistent with the age specific data shown above, that Torin1 diet treatments had no consistent effect on egg production itself, but appeared to increase egg infertility.

In the first dose response experiment (fig 4.7), there were significant differences in lifetime egg productivity ($F_{4,195}=7.842$, $p<0.001$, fig 4.7a). There was no consistent effect of lowered egg production with increasing Torin1 dose ($F_{4,195}=7.692$, $p<0.001$, fig 4.7a), though a slight increase in infertile egg laying with increasing Torin1 dose ($F_{4,186}=4.702$, $p=0.001$, fig 4.7c).

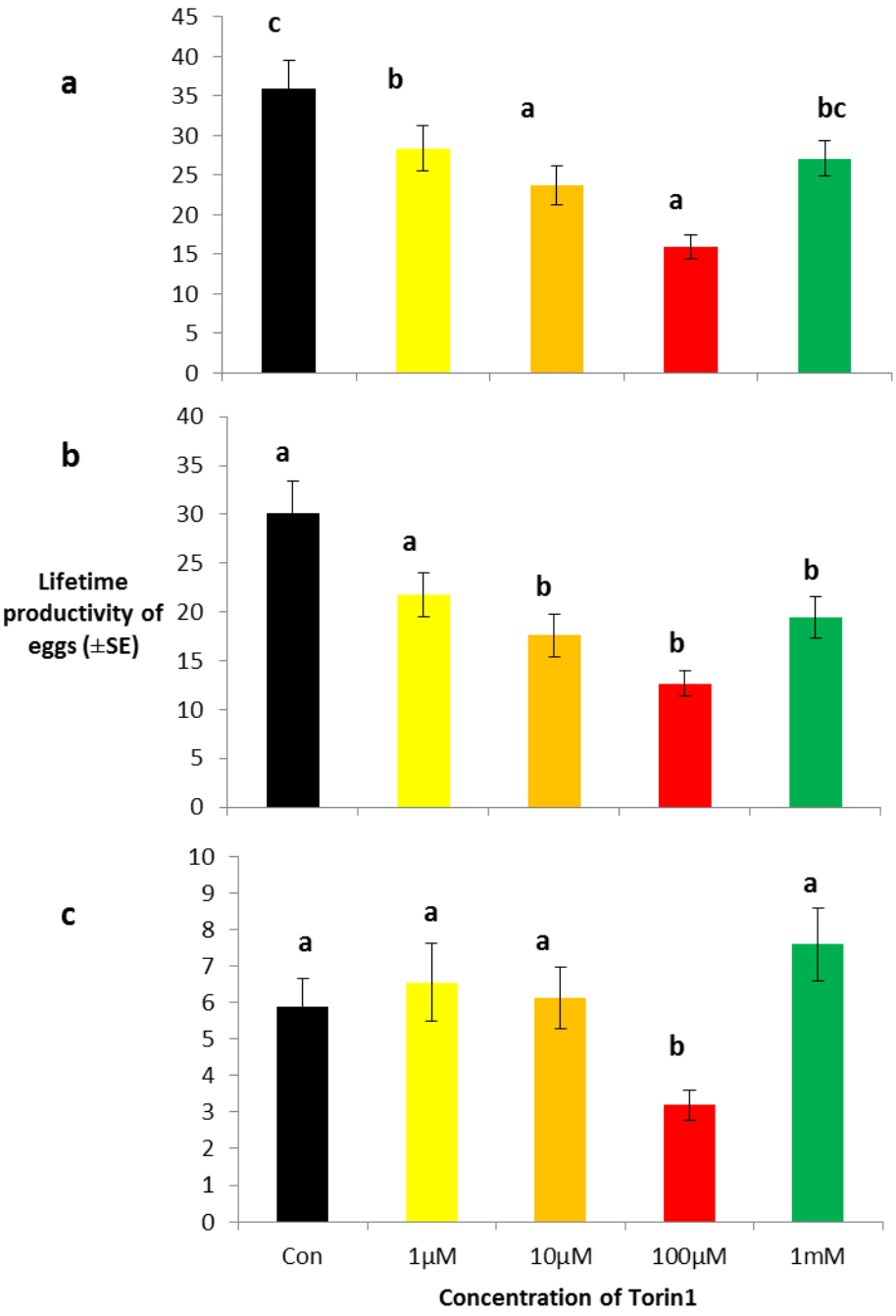


Fig 4.7-Lifetime productivity of eggs laid by once mated females exposed to DMSO carrier control (Con),1µM, 10µM, 100µM and 1mM Torin1. Bars at the top (a) are the total lifetime fecundity, the middle (b) the number of hatched eggs and the bottom (c) the number of unhatched eggs. Different letters above the bars indicate a significant differnce (Tukey post hoc test, $p<0.05$).

In the replicate dose experiment (fig 4.8), there was no significant effect of Torin1 on lifetime egg production ($F_{4,195}$=1.029,p=0.394, fig 4.8a) or for the number of hatched eggs ($F_{4,195}$=0.359, p=0.838,

fig 4.8b). However, as above, the number of unhatched eggs produced over the lifetime rose significantly with increasing Torin1 dose ($F_{14,195}$=4.554, p=0.002, fig 4.8c).

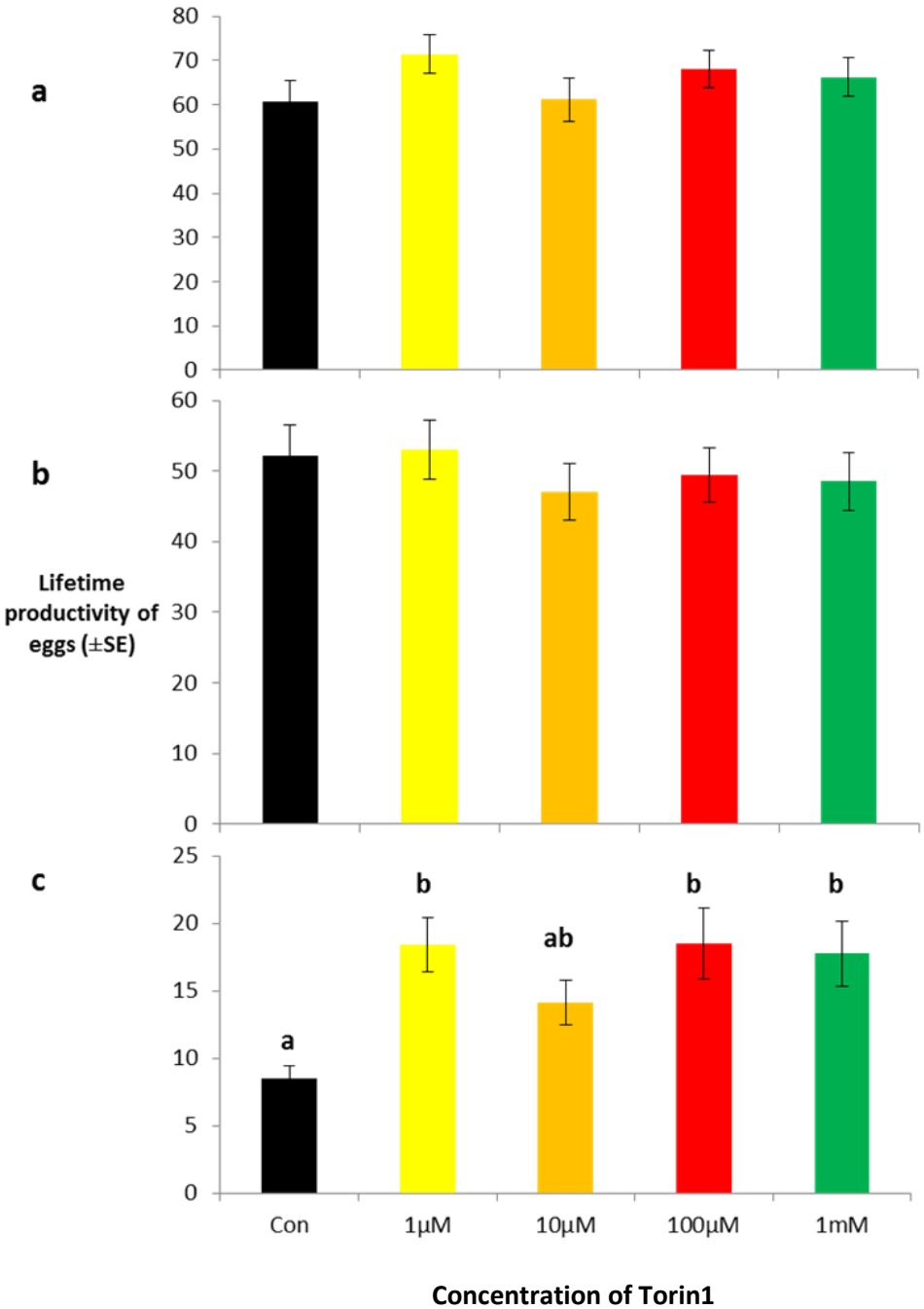


Fig 4.8-Lifetime productivity of eggs laid by once mated females exposed to DMSO carrier control (Con), 1µM, 10µM, 100µM and 1mM Torin1. Bars at the top (a) are the total lifetime fecundity, the middle (b) the number of hatched eggs and the bottom (c) the number of unhatched eggs. Different letters above the bars indicate a significant difference (Tukey post hoc test, p<0.05).

In the third dose response experiment (fig 4.9) there was a significant increase in the number of eggs laid as Torin1 dose increased ($F_{4,195}$=9.430,p<0.001, fig 4.9a) and also in the number of unhatched eggs ($F_{4,195}$=7.784,p<0.001, fig 4.9c). There was no significant difference was seen in the number of eggs that hatched between the different treatments ($F_{4,195}$=2.220,p=0.068, fig 4.9b).

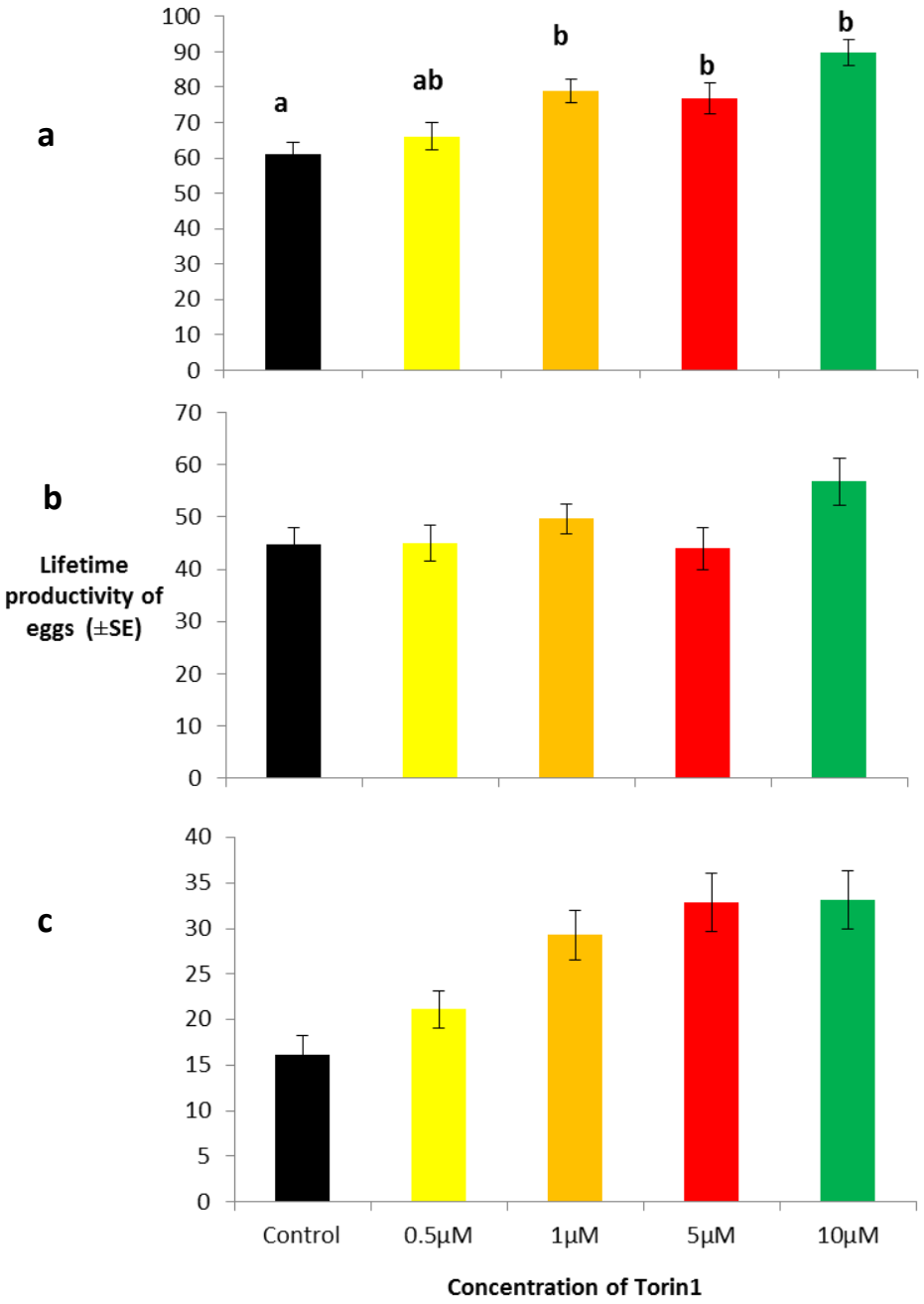


Fig 4.9-Lifetime productivity of eggs laid by once mated females exposed to DMSO carrier control (Con), 0.5µM, 1µM, 5µM and 10µM Torin1. Bars at the top (a) are the total lifetime fecundity, the middle (b) the number of hatched eggs and the bottom (c) the number of unhatched eggs. Different letters above the bars indicate a significant difference (Tukey post hoc test, p<0.05).

4.4.2 Effect of Torin1 on once mated and continually mated female survival and fertility and on once mated male survival

Survival

The final experiment of this chapter tested how any effects of Torin1 might interact with sex and mating status. In terms of survival there were significant differences between the different treatments (once mated females, continually mated females and once mated males) maintained as adults on Torin1 versus control medium ($F_{5,234}$=7.032, p<0.001, fig 4.10). There were significant effects of diet, with the flies fed Torin1 living significantly longer overall ($F_{1,234}$=4.075, p=0.045). There was also a significant effect of mating status, with flies maintained singly living longer than those maintained together with the other sex ($F_{1,234}$=5.134, p=0.24) and of sex itself, with males living significantly longer than females ($F_{1,234}$=10.517, p=0.001).

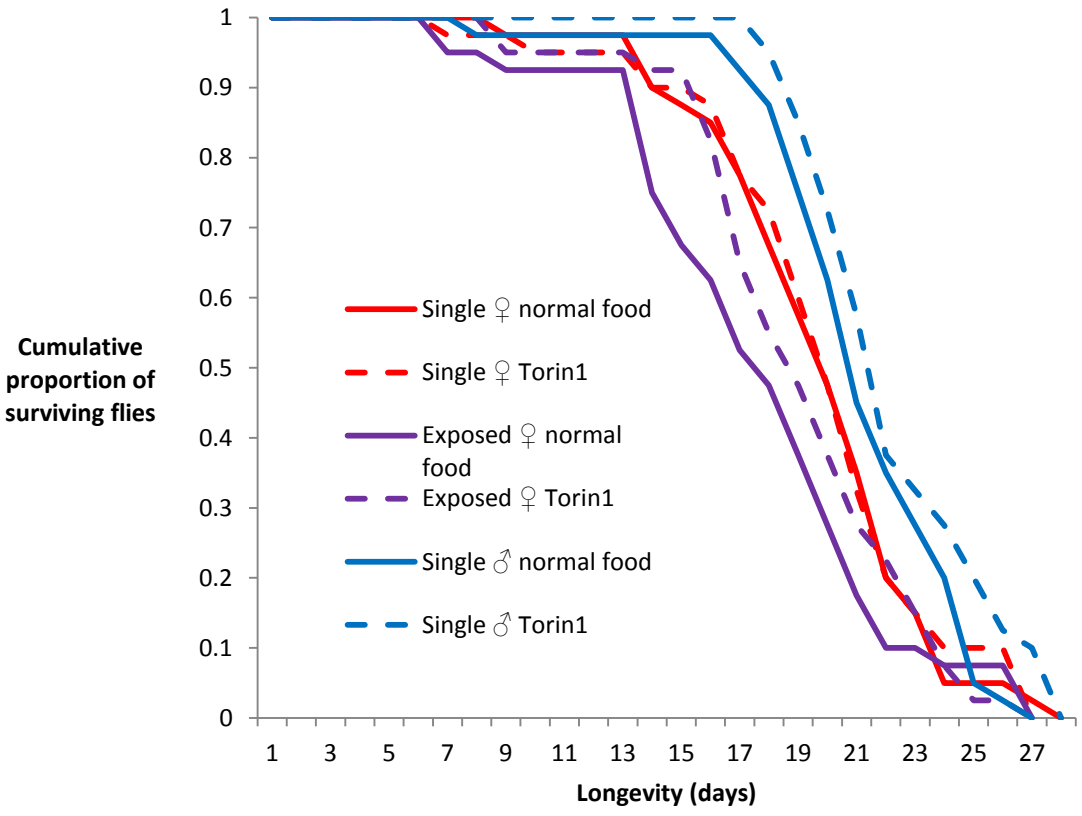


Fig 4.10–Survivorship of once mated females (single female), continually mated females (exposed female) and once mated males (single male) maintained on control (normal) or 1µM Torin1 diet.

Age specific fecundity

ANCOVA showed a significant difference in the pattern of fecundity over time for those females that were kept singly versus exposed to males (p=0.001). This was revealed as a significant interaction between mating status and time (p=0.01) and between food type and time (p=0.05).

To determine with more precision where these differences lay, I followed the ANCOVA above with analysis of age specific fecundity, corrected for multiple comparisons. These analyses revealed significant differences in the number of eggs laid on day 2 and 4 (p<0.05).

On day 2 there were significant differences in the number of eggs laid between single females and those continually exposed to males (p<0.001), and between those females held on the different diets (p=0.014). There was also a significant interaction between mating status and diet (p=0.001). On day 4 there were also significant differences in the number of eggs laid by females held on different diets (p<0.001).

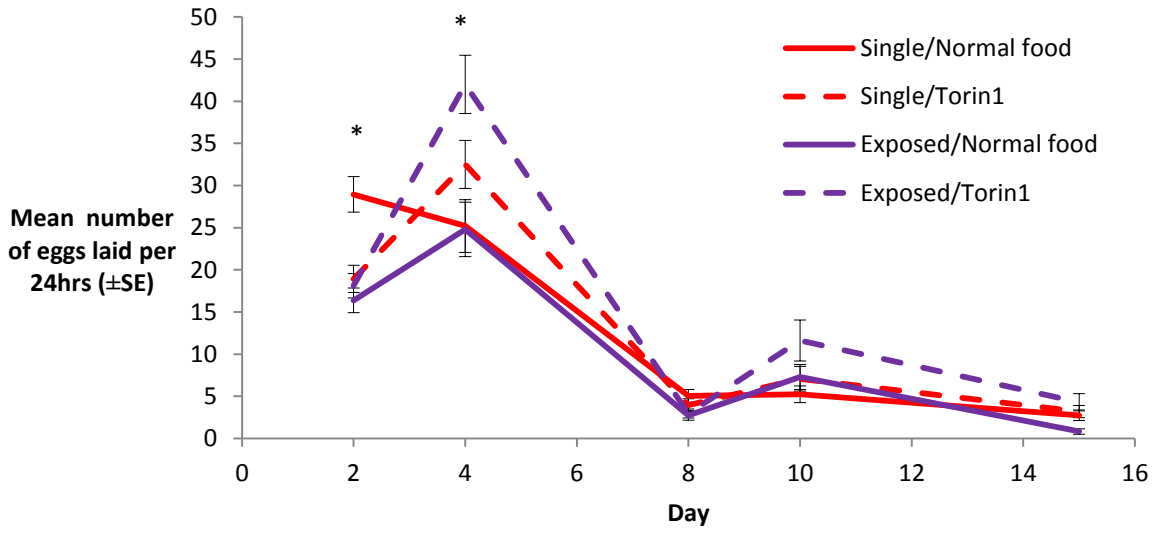


Fig 4.11– Fecundity (mean number of eggs per female per 24 hours ±SE) of once mated (single female), and continually mated females (exposed female) maintained on control (normal) or 1µM Torin1 diet.

Age specific fertility

ANCOVA analysis of age specific fertility (fig 4.12) revealed no significant differences between the different Torin1 treatments or female mating status treatments (p=0.069).

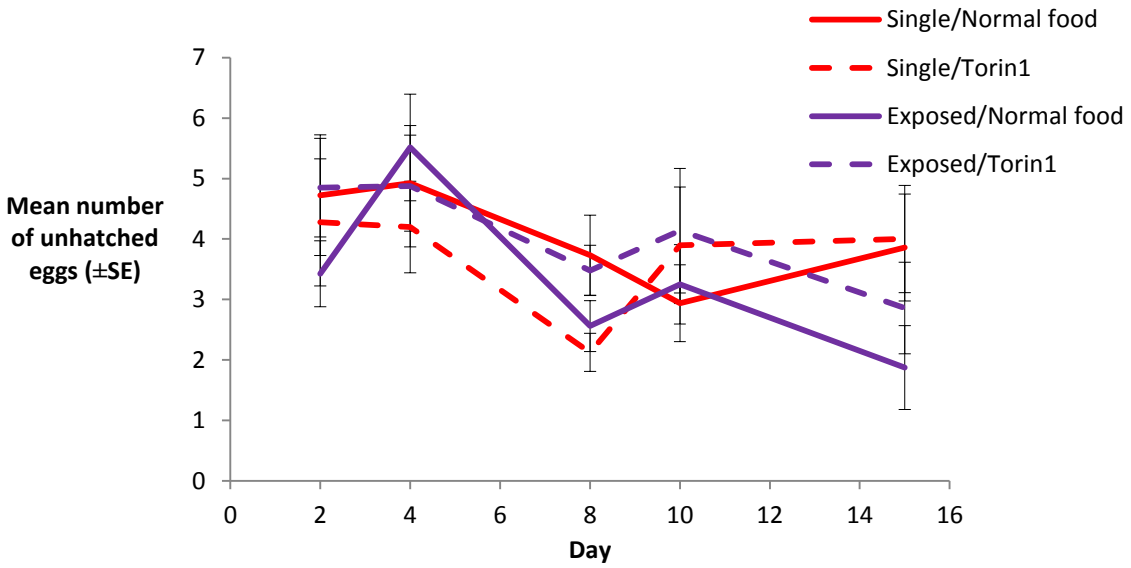


Fig 4.12–Egg fertility (mean number of unhatched eggs per female per 24 hours ±SE) of once mated females (single female), and continually mated females (exposed female) maintained on control (normal) or 1µM Torin1 diet.

Lifetime egg productivity and fertility

The final step was to analyse female lifetime productivity data. There was a significant interaction between diet and mating status ($F_{1,156}$=9.572, p=0.002) as well as a significant main effect of diet itself ($F_{1,156}$=7.150, p=0.008, fig 4.13a).

Post hoc testing using Tukey tests showed that continually exposed females kept on normal food produced significantly fewer eggs in their lifetimes than their counterparts maintained on Torin1 (p<0.001). There were no significant differences in the numbers of hatched or unhatched eggs in each treatment (fig 4.13b and c).

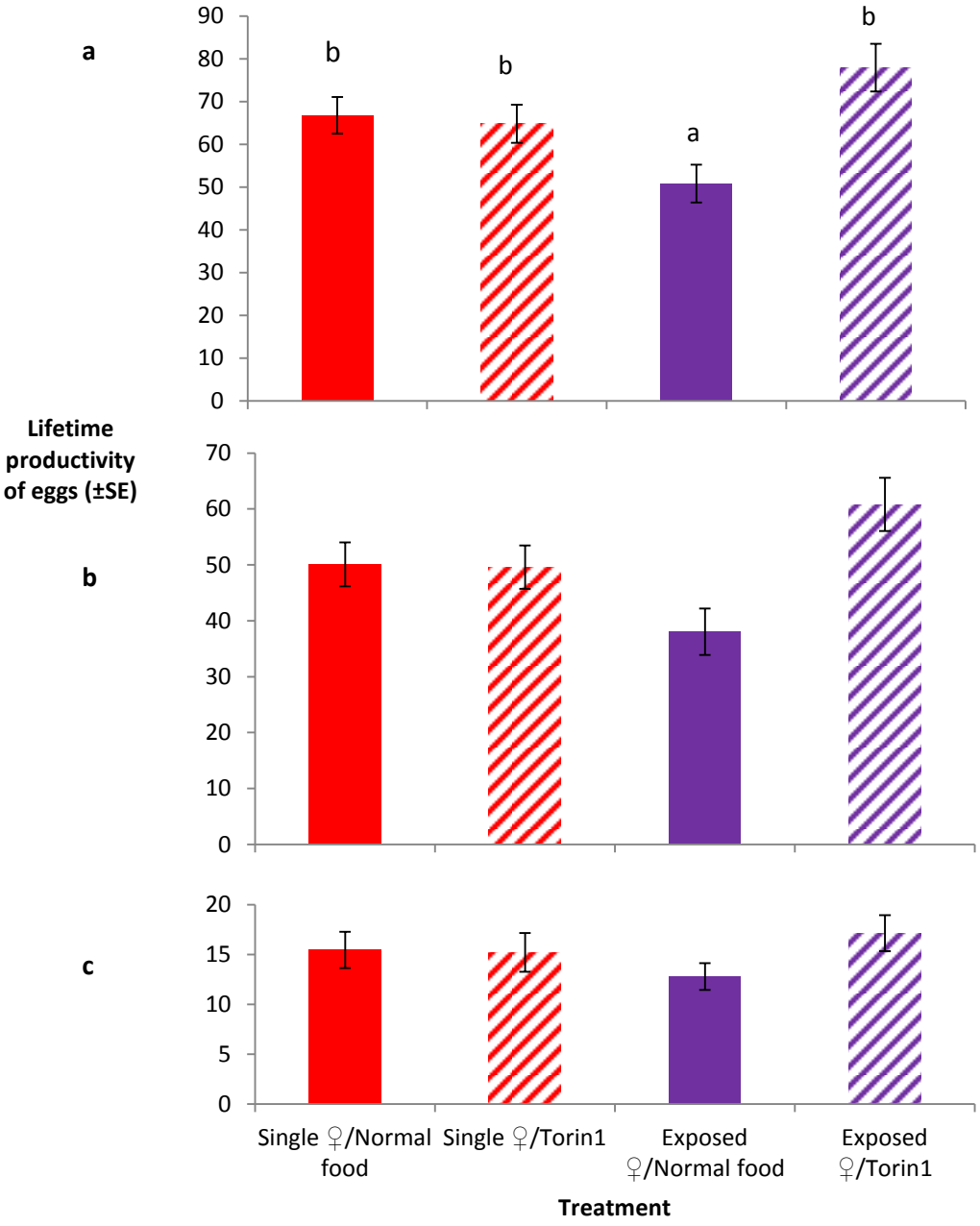


Fig 4.13-Lifetime egg productivity (mean per 24 hours ±SE) of once mated females (single female) and continually mated females (exposed female) maintained on control (normal) or 1µM Torin1 diet. Bars at the top (a) show the total lifetime egg production, in (b) the number of fertile (hatched) eggs and in (c) the number of unhatched eggs. Letters above the bars show the results of post hoc tukey tests. Different letters denote significant differences ($p<0.05$).

4.4.3 Western blot analysis

Western blot analysis was conducted to determine if the *Atg8a* protein had been processed into seperate products *I* and *II* to indicate if the activation of autophagy had been achieved, as measured by the extent of *Atg8-II*. Both *Atg8-I* and *Atg8-II* were normalised against the control tubulin (fig 4.16) and the extent of cleavage calculated by determining the ratio between the normalised values of *Atg8-II* and *I*.

The analysis was considered in two steps, firstly whether the dose of Torin1 affected the activation of autophagy and secondly whether reproductive status and sex affected the activation of autophagy.

Dose response of Torin1 on autophagy activation

This first analysis provides evidence that autophagy was activated in females fed different does of Torin1 in their diet, as the normalised ratios of *Atg8-I* to *II* increased with increasing doses of Torin1, before decreasing slightly again at the highest dose (fig 4.14).

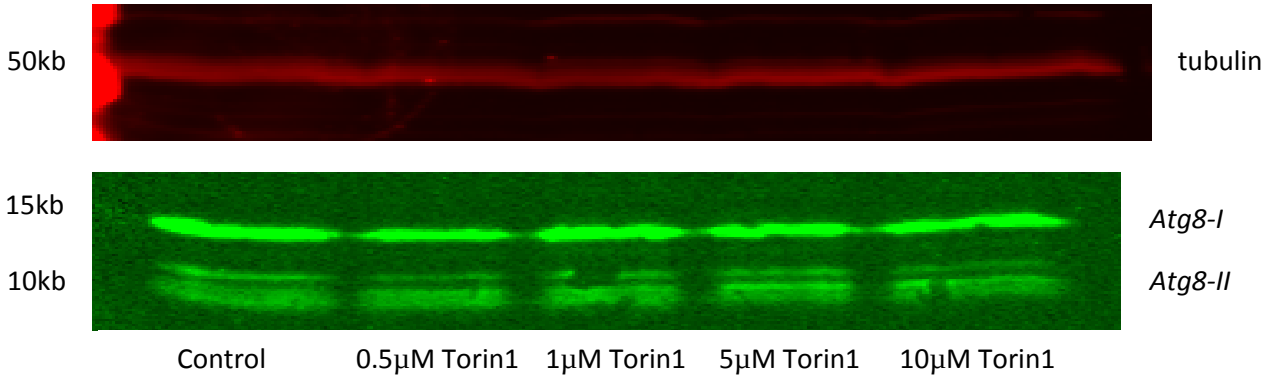


Fig 4.14- *Atg8-I/Atg8-II* ratios of once mated single females exposed to DMSO carrier control (0.38), 0.5µM (0.44), 1µM (0.58), 5µM (0.60) and 10µM (0.49) Torin1 in the diet (in green, tubulin control is shown above in red).

Effect of sex and reproductive status on autophagy activation

This second analysis also indicates, by consideration of normalised *Atg8-II* to *I* ratios, as indicated in fig 4.14, that Torin1 activated autophagy at a dose of 1µM in all three groups (single females, females continuously exposed to males and single males) at different levels. The un-fed single females and females exposed to males also had autophagy activated. But overall the single females had higher levels of *Atg8-II* than in the other two groups.

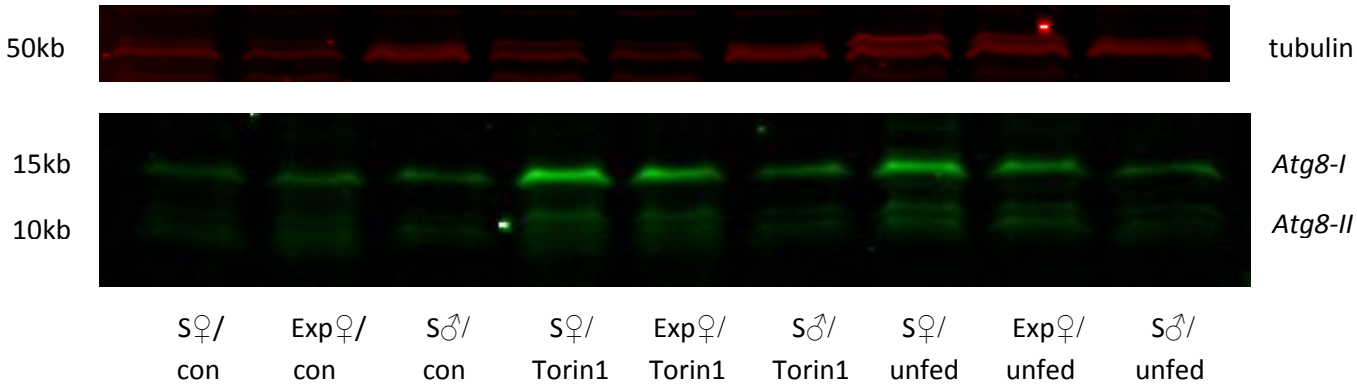


Fig 4.15- *Atg8-I/Atg8-II* ratios of once mated single females (S♀), females exposed continually to males (Exp♀) and single once mated males (S♂) on normal food (con), food with added Torin1 and those with no food (unfed) seen in green. Tubulin control is shown above in red. Cleavage of *Atg8* to *Atg8-I* and *Atg-II* indicates the activation of autophagy and the ratio of the two cleavage products gives the extent of autophagy activation: S♀/con 0.26, Exp♀/con 0.03, S♂/con 0.09, S♀/Torin1 0.34, Exp♀/Torin1 0.073, S♂/Torin1 0.25, S♀/unfed 0.40, Exp♀/unfed 0.20, S♂/unfed 0.11.

4.5 Discussion

Taken together my results show evidence for significant lifespan extension when autophagy is activated via dietary manipulation using Torin1. This effect was observed in females, males and females that were held continuously with males. A significant dose response was also seen as longevity increased as levels of Torin1 were increased (fig 4.2). Elevated egg production was seen when females were fed Torin1 but overall this did not result in higher egg fertility. There was therefore no evidence for a trade-off between longevity and fecundity or fertility. Overall the Torin1

treatments resulted in higher egg numbers but the same number of hatched eggs. My data support the hypothesis that dietary application of Torin1 exerted its effects on lifespan because of increased autophagy. The data from the Western blots showed that increased Torin1 in the fly diet results in increased cleavage of the *Atg8* protein and therefore increased rates of autophagy itself. Caloric restriction extends lifespan, but a trade-off is seen between this lifespan extension and fecundity (Masoro 2005; Carey, Harshman et al. 2008). The application of dietary Torin1 in contrast appears to also extend lifespan but without the trade-off effect on fecundity. It is possible that the dietary administration of Torin1 may have repaired damage caused by reproduction via the activation of autophagy, preventing any trade-off effects on fecundity.

Effects of dietary manipulation on lifespan

Extension in lifespan thorough dietary manipulations and application of dietary components has been seen in many different organisms. Some of this extension of lifespan is thought to arise because of increased protection via removal of damaged macro molecules through increased rates of autophagy. Previous research has sought to test this idea by activating autophagy through delivery of spermidine. Spermidine is a natural polyamine which declines in concentration during ageing in humans. Experimental data show that when added to cells of yeast, flies, worms and human immune cells spermidine can promote extended lifespan (Eisenberg, Knauer et al. 2009; Morselli, Galluzzi et al. 2009; Madeo, Eisenberg et al. 2009).

However, spermidine has been found to be an unreliable activator of autophagy, giving inconsistent results in longevity studies (chapter 3),(Minois, Carmona-Gutierrez et al. 2012). This could be due to the fact that spermidine inhibits only part of the TOR pathway, i.e. that involving TORC1, and even then this inhibition is not absolute. Resveratrol is another dietary additive that has been tested in lifespan studies in many species, and which has been proposed to exert its effects at least partly through autophagy. In mice resveratrol extended the lifespan in obese subjects, but there is no evidence that it can prolong life in healthy individuals. Several followup studies have also confirmed

that there is no increase in lifespan of healthy individuals when fed resveratrol (Agarwal and Baur 2011). Both resveratrol and spermidine therefore have inconsistent effects upon autophagy activation. However more recent reports indicate that spermidine and resveratrol given in combination achieve a more consistant induction of autophagy (Morselli, Marino et al. 2011). Further work is needed to assess the relevant mechanisms involved. As discussed in the introduction, rapamycin is another widely-used treatment for extending lifespan, potentially via activation of autophagy through inhibition of TORC1. However, it is reported that although there are effects of rapamycin on lifespan, they are not dose dependant (Bjedov, Toivonen et al. 2010), in contrast to the effects I report in this chapter for Torin1. Treatment with rapamycin reveals sexual dimorphism for lifespan extension in flies and mice (Bjedov, Toivonen et al. 2010; Fontana, Partridge et al. 2010). In my results males were seen to live significantly longer than females and also those fed Torin1 lived significantly longer than those held on a normal diet.

What is clear from the above studies is that there is a pressing requirement for a clean inhibitor of TOR that is reliable and consistent. Pyrazolopyrimidines such as PP242, PP30 and Torin1 appear to inhibit both TORC1 and TORC2 with a high degree of selectivity (Thoreen, Kang et al. 2009); (Feldman, Apsel et al. 2009). It is believed that although Torin1 inhibits both the TORC pathways it is the more complete inhibition of TORC1 that causes the more reliable activation of autophagy through blocking of phosphorylation of 4EBP1 (Feldman, Apsel et al. 2009). This was demonstrated by Moorman et al. who indicated that TORC2 is not affected by Torin1 and so it is the more complete inhibition of TORC1 that causes Torin1 to be a more reliable activator of autophagy (Moorman and Shenk 2010). My results that give evidence for dose response effects of Torin1 on lifespan via induction of autophagy suggest that Torin1 provides a significant advance on other techniques tried to date, including that of spermidine as employed in chapter 3.

Effects of dietary manipulation on fecundity and fertility

Some studies have shown evidence for a trade-off between an extension in lifespan and fecundity, but this is not borne out by my results above. Fecundity was actually seen to increase when autophagy was activated but the numbers of fertile eggs was lower, resulting in equivalent fertility when autophagy was activated via Torin1 treatment. Hence there was no evidence of a trade-off between lifespan extension and fertility upon treatment with Torin1.

Bjedov et al. (2010) found a dose dependent effect of rapamycin treatment on reduced fecundity. However, although lifespan was also extended by rapamycin in this study, this was not dose dependent, indicating that the effects of rapamycin on fecundity and lifespan were not closely linked. Further evidence which confirms this analysis is that the lifespan of sterile mutant females that could not lay eggs was also extended by rapamycin (Bjedov, Toivonen et al. 2010).

It has long been believed that reproductive rate and longevity are inversely related. A causal relationship is presumed to exist with current reproduction and decreasing future survival because limited resources are used for reproduction which otherwise would have been used for maintenance of the soma. Risks taken during reproduction can lead to bodily injury which may also result in decreased longevity (Dean 1981). My results indicate there is no obligate trade-off between reproduction and longevity. This finding fits with an emerging view that lifespan and reproductive rate are intergrated, but controlled independently of one another (Grandison, Piper et al. 2009). In fact my results showed that early fecundity was enhanced by the activation of autophagy by Torin1, although this did not translate to an increase in fertility. Other studies have found short lived organisms tended to reproduce earlier and have a higher reproductive output over their lifespan compared to long lived animals which tend to have lower levels of reproductive output over a longer period of time (Dean 1981). This suggests that survivorship, the timing and the amount of reproduction may be controlled by factors other than the simple relationship between longevity and

reproduction in the life history of many organisms. Other factors such as male-female interactions may be a more imortant determinant of female survival than reproductive rate itself.

The enhanced number of eggs laid by females fed Torin1 had no apparent fitness value to the female. I suggest that this may be a side effect of Torin1 not related to the effects on autophagy, as this result has not been reported in other studies. Future work on the mechanisms involved is therefore necessary. Consistent with this idea is that it has recently been shown that sperimidine is essential for mating in yeast as well as for egg fertilization in the nematode *Caenorhabditis elegans*. These effects were in both cases found to occur independently from autophagy activation state (Bauer, Carmona-Gutierrez et al. 2013).

It has been shown that cells fed Torin1 become subject to a regulatory feedback loop. In experiments employing low doses of Torin1 in an acute treatment of 1 to 2 hours, TORC1 and TORC2 can be inhibited, but this effect subsequently is nullified by an increase in P13K activity at longer time points of 5 hours and over. For this reason it is recommended that doses of 250nM at least are used for all long term experiments, otherwise this regulatory feedback loop negates any effect of the experimental manipulations on autophagy. Peterson et al. (2009) found that at a low dose of 50nM Torin1 inhibits both TORC1 and TORC2. However, after acute exposure Akt^{S473} phosphorylation recovers by 48 hours despite the fact that $S6K1^{T389}$ remains dephosphorylated (Peterson, Laplante et al. 2009; Guertin and Sabatini 2009). The findings suggested that 50nM Torin1 is not sufficient to inhibit all of the TORC2 complexes as suggested above. A higher dose of Torin1 inhibits TORC1 more completely and blocks Akt^{S473} phosphorylation after 48 hours (Guertin and Sabatini 2009). These results suggest that the choice of Torin1 dose is particularly important in order for predicted effects on autophagy to be observed.

I conclude overall that Torin1 appears to be a cleaner and more robust chemical activator of autophagy than has previously been employed in this field. The accumulating evidence indicates that pharmacological manipulation through diet results in an increase in longevity through the

stimulation of autophagy. My results above suggest further research should be undertaken to determine the role of autophagy in relation to ageing and longevity. It may be that a diet that is rich in natural pro-autophagic components including Torin1, spermidine or polyphenols such as resveratrol may promote health and postpone ageing to some degree in humans.

Chapter 5 Effect of overexpression of autophagy, via the manipulation of the Atg8a gene, on female survival, fecundity and fertility

5.1 Abstract

Limiting the activity of autophagy has been observed to lead to a shortening of lifespan, hence overexpression of autophagy genes is predicted, by the same token, to extend lifespan. Evidence for this prediction has been observed in that manipulations that decrease protein translation and hence, increase autophagy, can result in lifespan extension. In the work described in this chapter I tested for extended lifespan in females with an overexpressed autophagy gene, *Atg8a*. I overexpressed *Atg8a* in several different tissues, including pan neuronally to test if I could observe a previously reported lifespan extension. Along with testing for any lifespan extension in the different tissues, I was interested in any associated effects on fecundity and fertility, which have not previously been investigated. My predictions were that neuronally overexpressed *Atg8a* should extend lifespan, as previously reported. By the same reasoning, I concluded that ubiquitously overexpressed *Atg8* should also extend lifespan, provided that it leads to sufficient overexpression within the nervous system. Lastly, I predicted that, if autophagy plays a significant role in mitigating reproductive damage, ovary overexpressed *Atg8a* treatment females would also show longer lifespan, or increased fecundity/fertility. I tested for activation of autophagy by measuring the level of expression of *Atg8a* using qRT-PCR. This showed clear evidence for overexpression of *Atg8a* in the ubiquitously expressed females, but not in the other overexpression genotypes. Overall there was no consistent evidence for increased lifespan in *Atg8a* overexpression genotypes for any of the tissues tested. However, given that I was not able to validate elevated *Atg8a* expression in all manipulations, the potential involvement of autophagy is unclear. For example I saw no lifespan

extension in females in which *Atg8a,* was and was not, overexpressed. The sensitivity of the qRT-PCR test for very small changes in relative *Atg8a* expression in one or a few tissues when isolating total RNA is, however, likely to be very low. The *UAS-Atg8a* control genotype tended to have the highest fecundity, fertility and lifespan, indicating that the other genetic manipulations employed may have, in combination, had unknown deleterious effects.

5.2 Introduction

With an increase in age comes a decrease in autophagy, which is thought to result in an increase in the incidence of molecular, cellular and tissue damage (Vellai 2009; Markaki and Tavernarakis 2011). This in turn leads to an overall decline in organismal function and an increased vulnerability to disease and death. Many studies using model organisms such as *Saccharomyces cerevisiae* (yeast), *Caenorhabditis elegans* (nematodes) and *Drosophila melanogaster* (fruit flies) have resulted in the discovery of several signalling pathways that are believed to influence ageing (Guarente and Kenyon 2000; Bishop and Guarente 2007; Kenyon 2010; Lionaki, Markaki et al. 2013). As discussed in the introduction, autophagy is one of these fundamental processes, and represents an evolutionary conserved cellular recycling process. Autophagy removes unnecessary or damaged cellular components, and degrades and recycles them. During the autophagic process damaged cellular components are sequestered into the autophagosome, a structure with a double membrane, and its contents are delivered to the lysosome where they are degraded by acidic hydrolases (Gelino and Hansen 2012). Work in *C. elegans* and *D. melanogaster* has shown that limiting the activity of autophagy leads to a shortening of lifespan (Bjedov, Toivonen et al. 2010). Therefore, overexpression of autophagy should by the same token extend lifespan. There is indeed evidence for this as manipulations that decrease protein translation and so increase autophagy result in lifespan extension in *D. melanogaster* and *C. elegans* (Hands, Proud et al. 2009).

Genetic screening has determined many of the genes involved in the autophagic process (Klionsky and Emr 2000). One of the main molecular components involved in autophagy is *autophagy gene 1* (*Atg1*), an upstream regulator of autophagy that is under TORC1 regulation. The overexpression of this gene is sufficient to enhance autophagy in *D. melanogaster* (Scott, Juhasz et al. 2007). Another essential gene in this pathway is *autophagy gene 8* (*Atg8*), a lipid-conjugated ubiquitin-like protein, which is crucial for the formation of autophagosomes, i.e. the double-membrane vesicles that encapsulate the damaged macromolecules (Xie, Nair et al. 2008). There are two homologues in flies,

Atg8a and *8b*, however the lifespan extending effects seen so far are associated with *Atg8a* (Simonsen, Cumming et al. 2008), which is the gene investigated in this chapter. Flies with mutations in the *Atg8a* gene are reported to have a shorter than average lifespan, an increased sensitivity to oxidative stress and an accumulation of insoluble ubiquitinated proteins (IUP) (Simonsen, Cumming et al. 2008), which are well validated biomarkers of physiological ageing. Flies with elevated levels of *Atg8a* are reported to have a longer than average lifespan, an increased resistance to oxidative stress and an accumulation of ubiquitinated and oxidized proteins (Simonsen, Cumming et al. 2008). The specific overexpression of *Atg8* in the brain and central nervous system (CNS) of *D. melanogaster* is reported to increase lifespan by approximately 50%, which could either indicate that tissue-specific expression of this gene (*Atg8a*) can stimulate autophagy throughout the body or that autophagy within the central nervous system is a particularly crucial determinant of longevity (Madeo, Tavernarakis et al. 2010; Simonsen, Cumming et al. 2008).

In previous chapters (3 & 4) I showed that autophagy could, under certain circumstances, be activated in flies fed pharmacological activators (e.g.Torin1), and that this could extend survival (chapter 4). However, it is not yet clear from those experiments which are the specific tissues in which autophagy needs to be activated in order for these effects to be seen. The possibility of overexpressing autophagy genes in specific tissues provides the tools to address this question, and this is the approach taken in the research presented in this chapter. In a previous study increased lifespan in *D. melanogaster* was reported upon activation of autophagy via overexpression of the *Atg8a* gene within the nervous system only (Simonsen, Cumming et al. 2008). However, whether this is both necessary and sufficient for lifespan extension is not yet known. Similarly in *C. elegans* it has been shown that expression of the protein ins-7 in the intestinal tissue alone can extend lifespan (Murphy, Lee et al. 2007). The enhanced expression of the *Atg8* gene in the brains of old *Drosophila* leading to extended lifespan (Simonsen, Cumming et al. 2008; Hands, Proud et al. 2009; Rubinsztein, Marino et al. 2011) is also associated with increased resistance to oxidative stress and reduced accumulation of oxidized proteins (Hands, Proud et al. 2009). This suggests that the up regulation of

autophagy via specific neuronal pathways may have a beneficial effect on the organism as a whole and be sufficient to reduce pathological ageing. How this effect might be achieved is not known, it might be a neuronal-specific effect, or that the manipulation of autophagy in the neurons can somehow affect autophagy in peripheral tissues.

In order to determine whether manipulation of autophagy in the nervous system is sufficient for lifespan extension, and to test whether other tissues are involved, I tested here the effect of activating autophagy in three different tissues of the fly. I repeated the manipulation of Simonsen et al. (2008) to activate *Atg8* in the central nervous system (Simonsen, Cumming et al. 2008), and also tested for effects of autophagy manipulation in the ovaries and ubiquitously, throughout the body. I did this by using the *Gal4-UAS* system (Brand and Perrimon 1993) to drive the expression of *Atg8a* using tissue-specific expression of *Gal4*. I recorded not only longevity but also fecundity and fertility data, which have not previously been investigated in any similar study. Hence I could therefore determine the comprehensive effects of autophagy activation via overexpression of the *Atg8a* gene in the different tissues that were tested.

I predicted that, provided the genetic manipulations actually did activate autophagy, lifespan should be extended in the neuronal overexpressed *Atg8a* females, as this had been reported previously (Simonsen, Cumming et al. 2008). By the same reasoning, the ubiquitously overexpressed *Atg8a* females should also show extended lifespan, as they would show elevated *Atg8a* everywhere, including in the CNS. I predicted that if autophagy plays a significant role in mitigating reproductive damage, that the ovary expressed *Atg8* females would also show longer lifespan and might show increased fecundity and fertility over controls. The effect of any trade-off of increased lifespan with decreased fertility would be apparent in the fecundity and fertility data recorded.

5.3 Materials and Methods

5.3.1 The effect on survival, fecundity and fertility of *Atg8a* overexpression in once mated females

Fly rearing was carried out as described in the general materials and methods (chapter 2). Flies in these experiments were cultured in bottles, 25 females and 25 males per bottle, on SYA medium and then crossed to obtain the required genotypes. Virgin males and females were collected using ice anaesthesia. Three different *Gal4* drivers were used: ovary specific expression of *Gal4*, using the following genotype: *w*; P{GAL4-nos.NGT}40 P{lacO.256x}43 P{lacO.256x}50F P{lacO.256x}57A P{lacO.256x}60AB/CyO; P{UAS- GFP.lacI}*,(Bloomington stock number 25378). For the central nervous system (CNS) specific expression of *Gal4*, genotype: *P{w[+m*]=ApplGAL4.G1a}1,y[1]w[*];Mlf[Delta10]/CyO*, (Bloomington stock number 30546). For the ubiquitous overexpression of *Atg8a* ubiquitous *Gal4* expression was used, genotype: *w*; P{UASp-GFP.Golgi}1, P{tubP-GAL4}LL7/TM3, Sb1*, (Bloomington stock number 30904).

These drivers were used to drive the expression of *UAS- Atg8a* (Bloomington stock number 10107). To generate genetically matched controls, each of the *Gal4* drivers and the *UAS-Atg8a* strain were separately crossed to the same w^{1118} isogenic control strain (Vienna stock number 60000). Further details of the full genotypes for each *Drosophila* strain used can be found in the general materials and methods section, chapter 2.

I conducted two replicate experiments using the same background flies and conditions in each, as follows:

Three experimental lines were used in which *Atg8a* was overexpressed *UAS-Atg8a/CNS Gal4* driver, *UAS-Atg8 /ovary Gal4* driver and *UAS-Atg8a/ubiquitous Gal4* driver. I generated controls for each of the *Gal4* drivers used by crossing each separately to the control w^{1118} strain: w^{1118}*/CNS Gal4* driver, w^{1118}*/ovary Gal4* driver and w^{1118}*/ubiquitous Gal4* driver. Finally, I also generated a control common to all three overexpression treatments by crossing *UAS-Atg8a* to the w^{1118} line to generate *UAS-*

Atg8a/ w^{1118}. The use of the 4 different types of control accounted for any off target effects of the *Gal4* and the *UAS-Atg8a* transgenes aside from their target effects on autophagy.

The three overexpression and four control female genotypes were selected from the crosses by using the appropriate visible markers, and then mass mated with wild type *D. melanogaster* males for 24 hours. Thus all females in the experiments could be considered once mated. Females were then separated from males using ice anaesthesia and placed singly in vials containing normal SYA food medium. Survival data were collected daily throughout the experiment, and eggs were counted three times each week until egg laying ceased. Females were transferred between vials without using anaesthesia to new food daily until egg laying stopped, or when offspring stopped emerging. After this point all females were placed on new food three times a week until all females were dead. When offspring had fully emerged, I froze the vials for later counting of offspring to determine egg adult viability.

Individuals that became stuck in the food, escaped or were accidentally killed were entered as censors in the data.

I summed for each female in each treatment the total number of eggs produced from all samples recorded over the lifetime, similarly for the total number of offspring produced.

5.3.2 Validation of *Atg8a* overexpression manipulations by qRT-PCR

In order to verify that the genetic manipulations did indeed result in overexpression of *Atg8a*, I tested for increased levels of *Atg8a* mRNA, using qRT-PCR. For full results of quality control and primer sequences see chapter 2 general materials and methods.

Total RNA from 10 flies per cohort was extracted using the *mir*Vana™ miRNA Isolation kit (http://www.lifetechnologies.com). Samples were sent to qStandard for qRT-PCR analysis (http://www.qstandard.co.uk/index.html). 500ng RNA was reversed transcribed using Qaigen Quantitect reverse transcription following the manufacturer's protocol. Runs were performed in

duplicates for two replicates using Agilent Brilliant III Ultrafast SYBR green qPCR mix and melting curve analyses were performed. Data were analysed using the Rotor-Gene software. Relative expression ratios were normalised to *Actin5c* and *Gapdh2*.

5.3.3 Statistical analysis

Statistical analysis was performed using SPSS v 18 (SPSS 2009) and R (R Development Core 2008). Survivorship was calculated for each treatment and survival data were analysed using Log Rank tests. Fecundity and fertility data for each treatment and control group of females were analysed as in previous chapters using ANCOVA (as described in chapters 3 & 4). This was followed by tests on individual days using ANOVA and Tukey post hoc analyses, or non-parametric Kruskal Wallis tests, corrected for multiple comparisons by sequential Bonferroni tests. I tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. Analyses for the qRT-PCR were obtained by testing for relative differences in *Atg8a* expression levels using ANOVA.

5.4 Results

5.4.1 The effect on survival, fecundity and fertility of *Atg8a* overexpression in once mated females

Survival

In the first experiment there was a significant difference in the survivorship across the treatments (Log rank X^2=49.694, df=6, p<0.001. fig 5.1a). However, there was no obvious evidence for a clear effect of lifespan extension in the *Atg8a* overexpressed genotypes. In fact the common control (i.e. the *UAS-Atg8a*/w^{1118} control common to all treatment groups) had the longest lifespan overall. Interestingly ovary overexpressed *Atg8a* genotype females appeared to have a significantly extended lifespan in comparison to the other experimental lines but this was not significant.

In the second replicate experiment, there was again a significant difference in lifespan across the different treatments (Log Rank $X^2 = 79.459$, df=6, $p<0.001$, fig 5.1b).

Although there were some effects on lifespan, overall, there was no clear effect of lifespan extension in the overexpression genotypes over and above the control genotypes. In the first experiment, the ovary overexpressed genotype females lived longer than their *Gal4* controls, but not longer than the common control. In the second experiment this effect was not repeated, and in this case there was some evidence for the longest lifespan being seen in the CNS overexpressed females, as predicted on the basis of the previous work. However, given that this same genotype was amongst the shortest lived in the first experiment what the data really suggest is that the lifespan effects are not consistent and are somewhat stochastically determined. This should give caution in the interpretation of such experiments if they are un-replicated (Simonsen, Cumming et al. 2008).

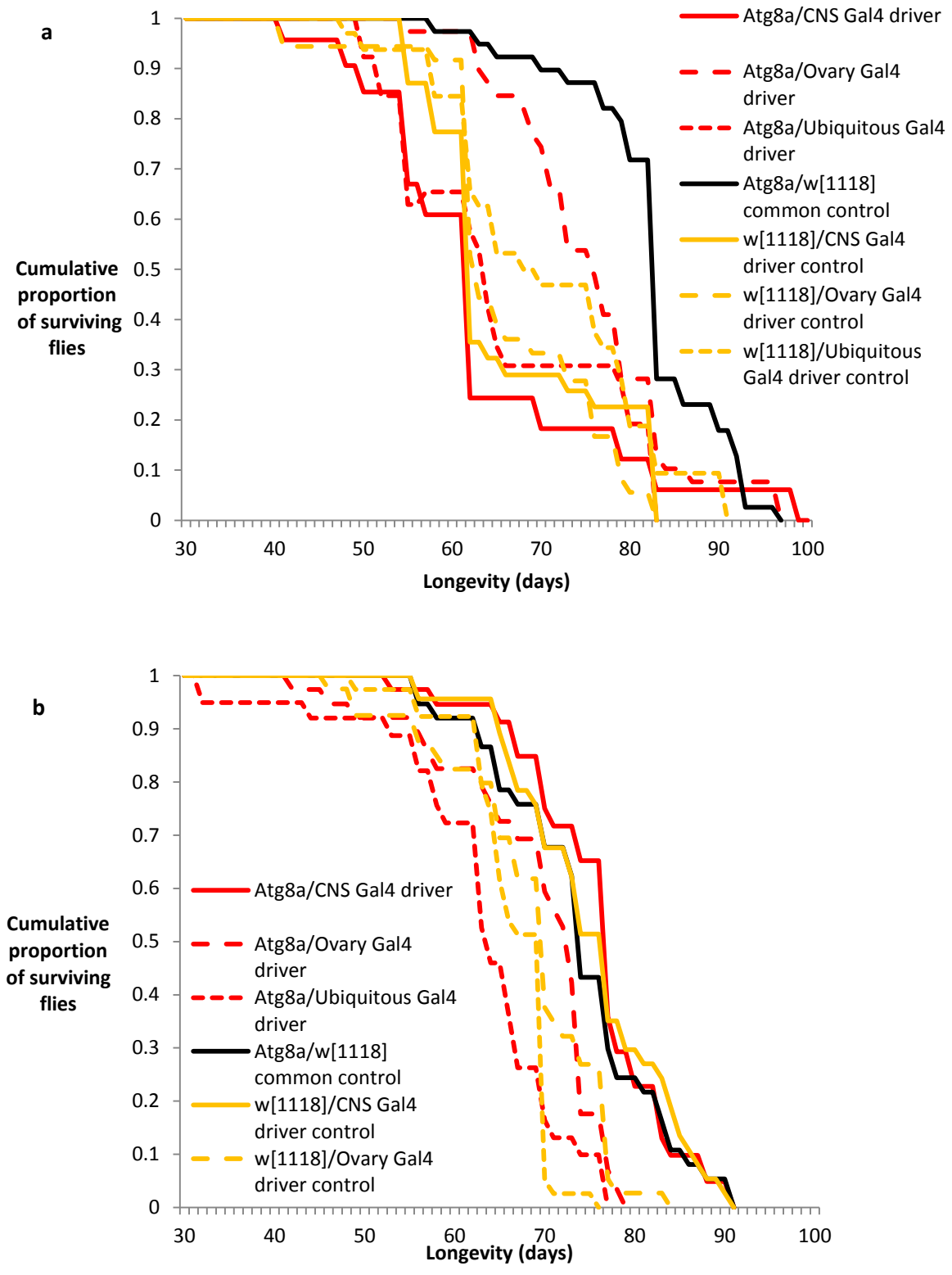


Fig 5.1a & b – Survivorship against time in days for two replicate experiments (a) and (b) for once mated females with autophagy overexpression genotypes. The treatments were: common control (*UAS-Atg8a/w^{1118}*), central nervous system control (*w^{1118}/CNS Gal4* driver), ovary control (*w^{1118}/ovary Gal4* driver), ubiquitous control (*w^{1118}ubiquitous Gal4* driver), and the overexpressed lines, central nervous system (*UAS-Atg8a x CNS Gal4* driver), ovaries (*UAS-Atg8a x ovary Gal4* driver) and ubiquitous overexpression (*UAS-Atg8a x ubiquitous Gal4* driver).

Age specific fecundity

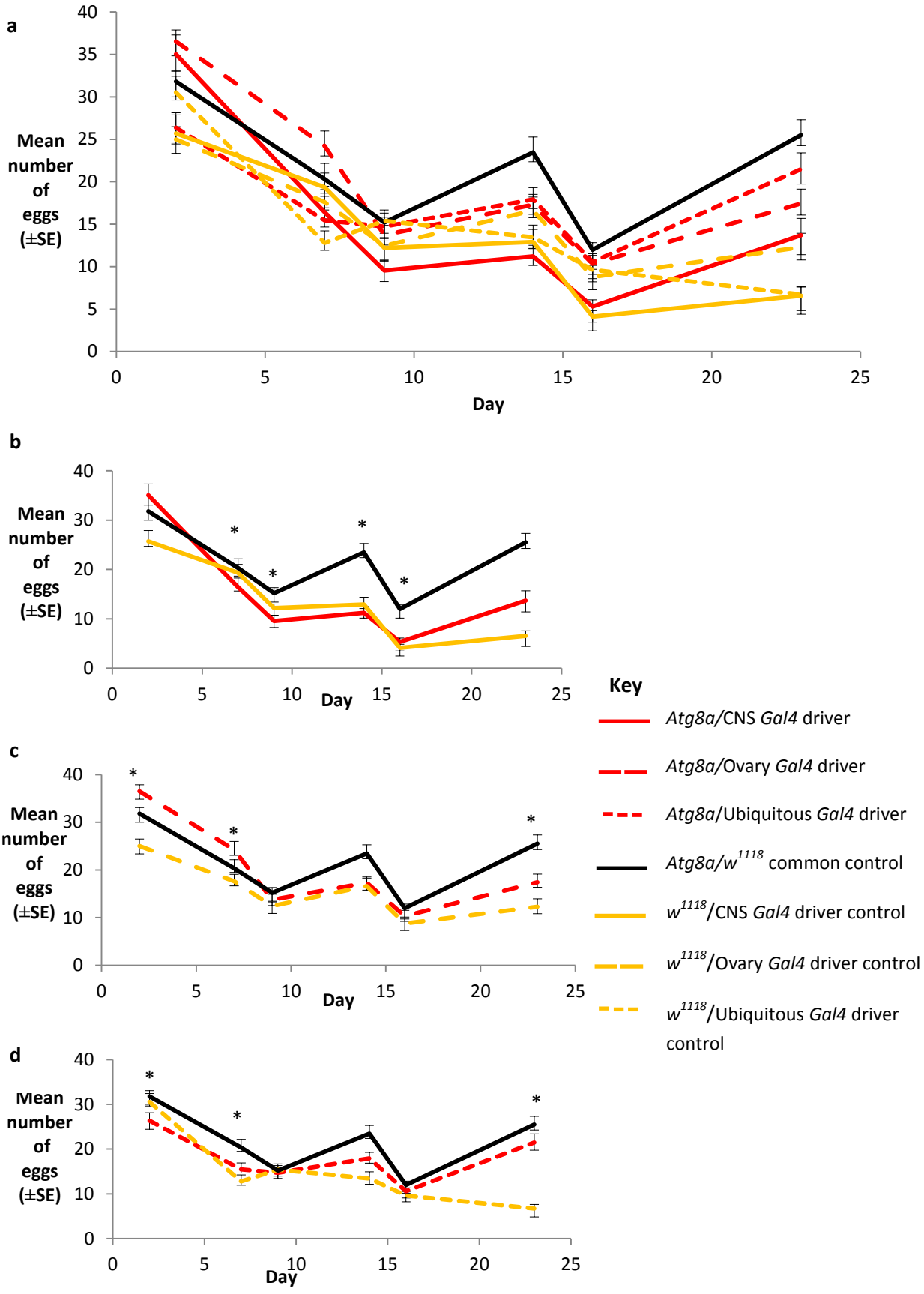
Analysis of differences in patterns of fecundity over time using ANCOVA, showed that *UAS-Atg8a/Ovary Gal4* driver, *UAS-Atg 8a/CNS Gal4* driver, w^{1118}*/CNS* control, w^{1118}*/Ovary* control, and w^{1118}*/Ubiquitous* control genotypes all exhibited a significantly steeper decline in fecundity over time ($p \leq 0.001$) in comparison to the *UAS-Atg8a/*w^{1118} common control. These differences were most clearly manifested as higher initial fecundity in *UAS-Atg8a/Ovary Gal4* driver females and a lower initial fecundity in w^{1118}*/CNS Gal4* driver control ($p=0.05$) in comparison to the *UAS-Atg8a/*w^{1118} common control (fig 5.2a). As expected, a significant effect of time itself was seen across all treatments, reflecting a significant general decrease in egg production over time.

To identify with more precision where the differences in egg production lay, the age specific data were then analysed using appropriate ANOVA or Kruskal Wallis tests together with Bonferroni corrections for multiple comparisons across the different days. There were significant differences in age specific fecundity on every day. Fig 5.2 panel (a) shows all the fecundity data from the whole experiment, but in order to more easily highlight the patterns seen, I also split the data into three panels, (b), (c) and (d), which each show the results for the different tissues in which *Atg8a* was overexpressed. For each of these panels I show each overexpressed genotype against its respective control and also against the common control. There was no consistent pattern of egg laying over time although the *UAS-Atg8a/*w^{1118} common control line females mostly laid higher numbers of eggs than the *Gal4* driver controls or the overexpression experimental genotypes. The one exception was that early in the reproductive lifespan of the females, the ovary *UAS-Atg8a* females had significantly higher fecundity.

In the replicate experiment, the ANCOVA revealed a significantly steeper decrease in fecundity over time in the, *UAS-Atg8a/Ubiquitous Gal4* driver, *UAS-Atg 8a/CNS Gal4* driver and w^{1118}*/Ovary* control genotypes in comparison to the common control ($p<0.001$). Initial fecundity was higher in the *UAS-Atg8a/Ovary Gal4* driver ($p=0.05$), *UAS-Atg8a/Ubiquitous Gal4* driver ($p<0.001$), *UAS-Atg 8a/CNS*

Gal4 driver ($p<0.001$), w^{1118}/*Ovary control* ($p<0.001$) and w^{1118}/*Ubiquitous* control ($p<0.001$) genotypes in comparison to the common control (fig 5.2e).

To identify with more precision where the differences in egg production lay, the same procedure was conducted as per above in the first experiment, by analysing the age specific fecundity separately for each day and visually by showing the data in three different panels for the three different tissues. Fig 5.2 panel (e) shows all the fecundity data from the whole second replicate experiment and panels, (f), (g) and (h) are given to highlight any patterns in each overexpressed genotype against its *Gal4* common control. Overall in this experiment there was greater variation in fecundity, but the general pattern was that as in the first experiment. The common control tended to have highest fecundity followed by the experimental overexpression genotypes and then the specific *Gal4* controls. There was no evidence this time for an initial fecundity increase in the ovary overexpressed genotype females.


a
Mean number of eggs (±SE)
Day
b
Mean number of eggs (±SE)
Day
c
Mean number of eggs (±SE)
Day
d
Mean number of eggs (±SE)
Day
Key
Atg8a/CNS Gal4 driver
Atg8a/Ovary Gal4 driver
Atg8a/Ubiquitous Gal4 driver
Atg8a/w1118 common control
w1118/CNS Gal4 driver control
w1118/Ovary Gal4 driver control
w1118/Ubiquitous Gal4 driver control

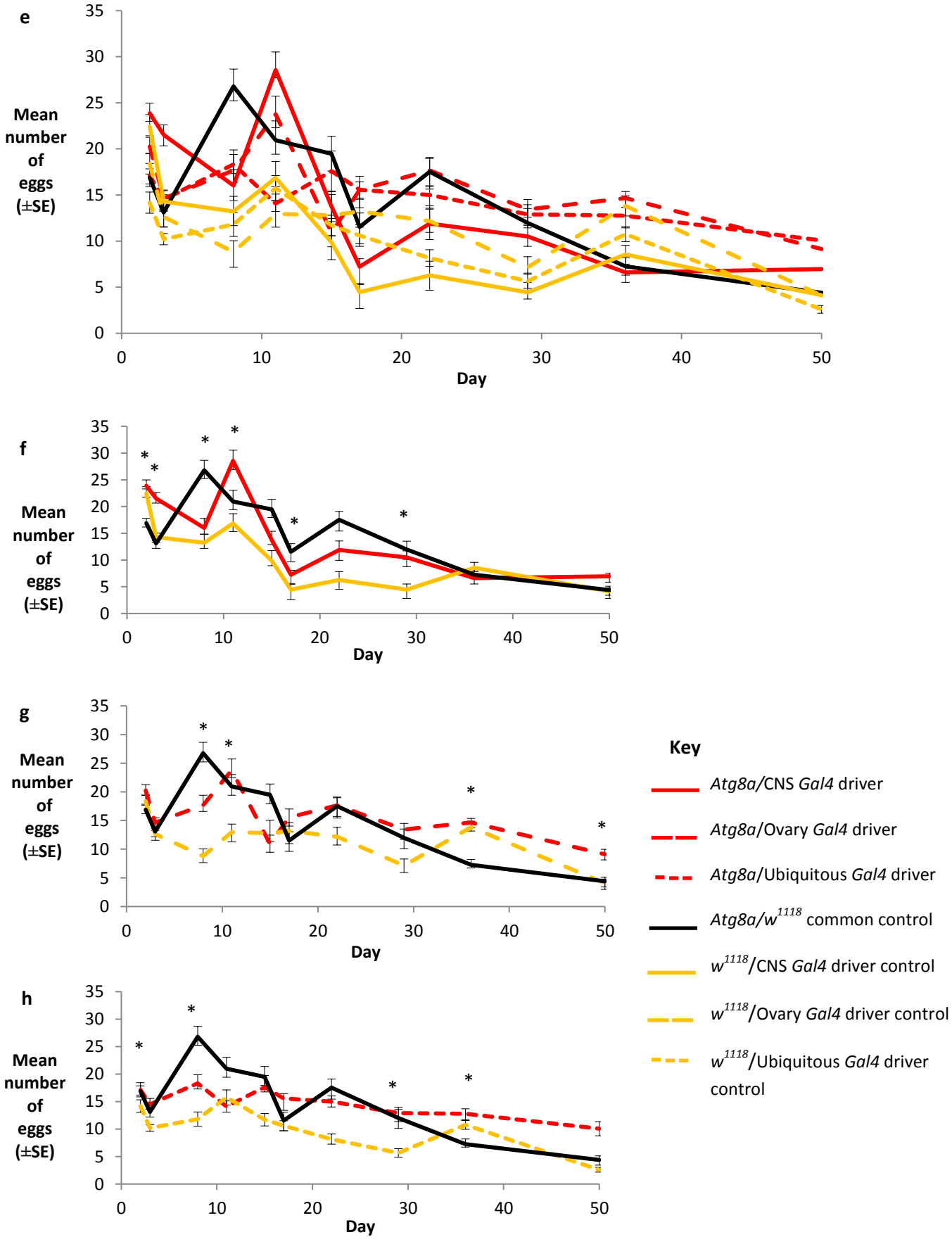
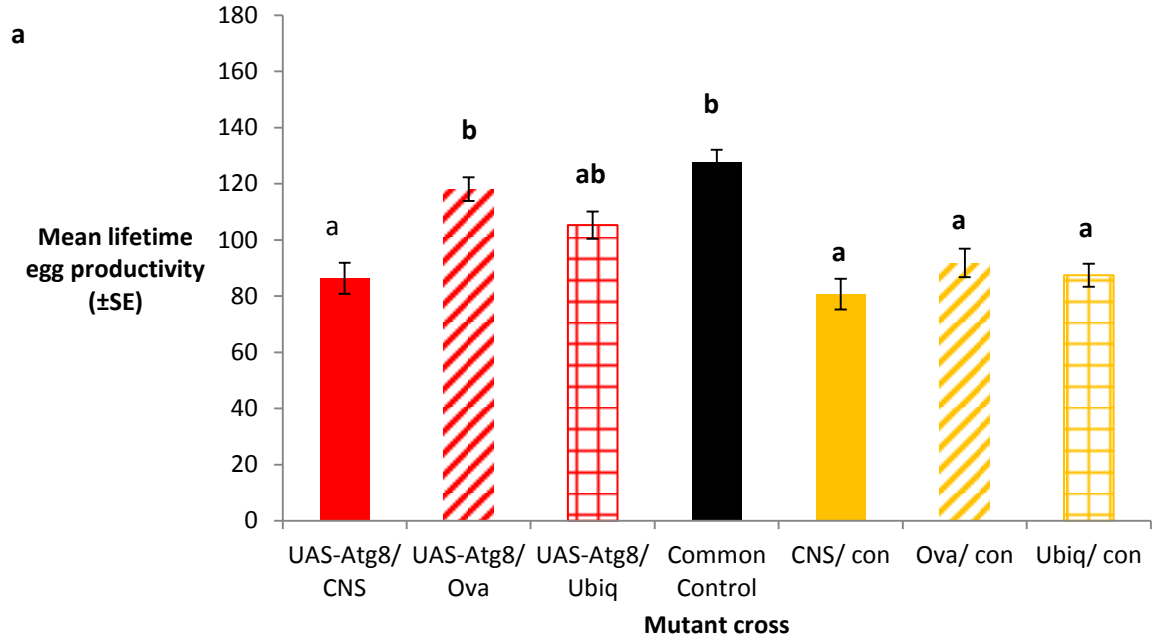

e
Mean number of eggs (±SE)
Day
f
Mean number of eggs (±SE)
Day
g
Mean number of eggs (±SE)
Day
h
Mean number of eggs (±SE)
Day
Key
Atg8a/CNS Gal4 driver
Atg8a/Ovary Gal4 driver
Atg8a/Ubiquitous Gal4 driver
Atg8a/w1118 common control
w1118/CNS Gal4 driver control
w1118/Ovary Gal4 driver control
w1118/Ubiquitous Gal4 driver control

Fig 5.2 – Mean number of eggs (±SE) laid against time by once mated females of given genotypes in the two replicate experiments panels **a** & **e**: common control (*UAS-Atg8a/W* ^{1118}), *Atg8a* overexpressed in the CNS (*UAS-Atg8a*/*CNS Gal4* driver), *Atg8a* overexpressed in the ovaries (*UAS-Atg8a*/*Ovary Gal4* driver), *Atg8a* over expressed ubiquitously (*UAS-Atg8a*/*ubiquitous Gal4* driver), the CNS control (*w* ^{1118}/*CNS Gal4* driver control), the Ovary control (*w* ^{1118}/*Ovary Gal4* driver control), the ubiquitous control (*w* ^{1118} X Ubiquitous *Gal4* driver control). **b** & **f**) common control (*UAS-Atg8a/w* ^{1118}), *Atg8a* overexpressed in the CNS (*UAS-Atg8a*/*CNS Gal4* driver) and the CNS control (*w* ^{1118}/*CNS Gal4* driver control). **c** & **g**) common control (*UAS-Atg8a*/*w* ^{1118}), *Atg8a* over expressed in the Ovaries (*UAS-Atg8a*/*Ovary Gal4* driver), and the Ovary control (*w* ^{1118}/*Ovary Gal4* driver control). **d** & **h**) common control (*UAS-Atg8a*/*w* ^{1118}), *Atg8a* over expressed ubiquitously (*UAS-Atg8a*/*Ubiquitous Gal4* driver) and the ubiquitous control (*w* ^{1118}/*Ubiquitous Gal4* driver control). * indicates a significant difference in the number of eggs laid ($p<0.05$).

Lifetime egg productivity

Analysis of lifetime egg productivity (fig 5.3a) from the first relicate experiment showed that the number of eggs laid by ubiquitous or CNS overexpression genotypes was lower than for the *UAS-Atg8a/w*^{1118} common control ($F_{6,270}$= 13.133, $p<0.001$). However, females with *Atg8a* overexpressed in the ovaries had the same numbers of eggs as those in the common control. Also, significantly more eggs were laid by the common control than the other three controls and the *Atg8a*/*CNS Gal4* driver (all at $p\leq0.040$). The same pattern was also seen for the *UAS-Atg8a*/*Ovary Gal4* driver.

Overall in the second experiment the overexpression experimental genotypes and the common control laid significantly more eggs over their lifetime than the other three *Gal4* control lines ($F_{6,268}$=14.773, $p<0.001$, fig 5.3b). The *Gal4* control lines, as in the first experiment, had consistently lower fecundity than the other groups ($p\leq0.017$).



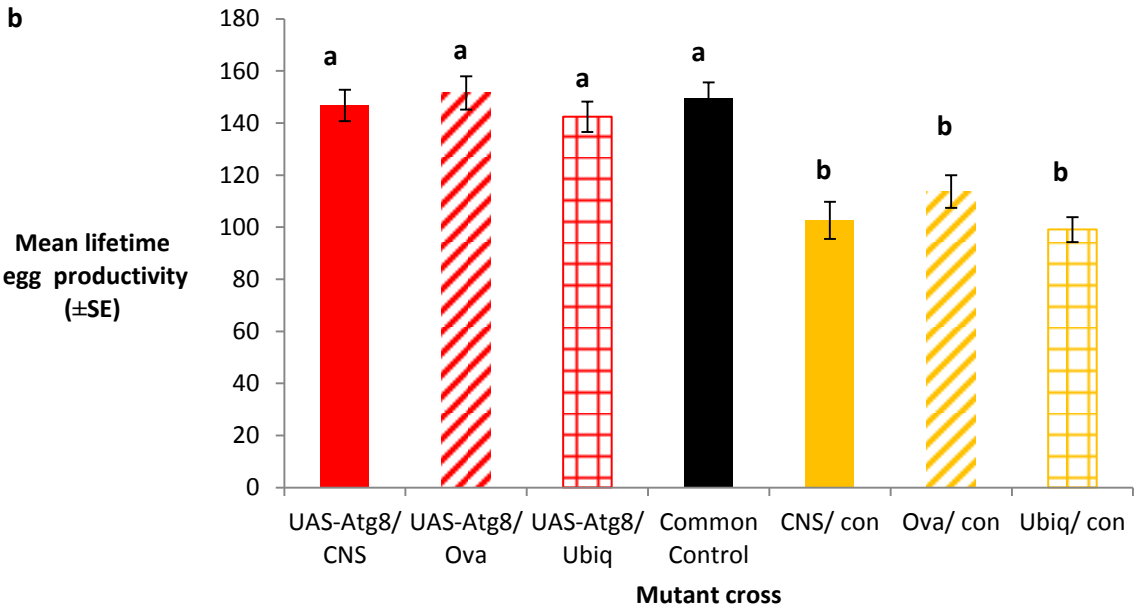


Fig 5.3a & b - Mean lifetime egg productivity (±SE) for once mated females with *Atg8a* overexpressed in the CNS (*UAS-Atg8a*/CNS *Gal4* driver), the ovaries (*UAS-Atg8a*/Ovary *Gal4* driver) and ubiquitously (*UAS-Atg8a*/Ubiquitous *Gal4* driver). For control females, CNS control (w^{1118}/*CNS Gal4* driver control), ovary control (w^{1118}/*Ovary Gal4* driver control), ubiquitous control (w^{1118}/*Ubiquitous Gal4* driver control) and common control (*UAS-Atg8aXW* ^{1118}). Panels a and b show different replicate experiments. Different letters above the bars indicate a significant difference at $p<0.05$.

Age specific fertility

ANCOVA analysis was conducted as above for the fecundity data, and showed a significantly steeper decline in offspring production over time in the *UAS-Atg8a*/*Ovary Gal4* driver ($p<0.001$), *UAS-Atg8a*/*Ubiquitous Gal4* driver ($p<0.001$), w^{1118}/*CNS* control ($p=0.001$), and w^{1118}/*Ubiquitous* control genotypes ($p<0.001$) in comparison to the common control. Fertility was initially higher in both the common control and the *UAS-Atg8a/Ovary Gal4* driver genotypes ($p<0.001$).

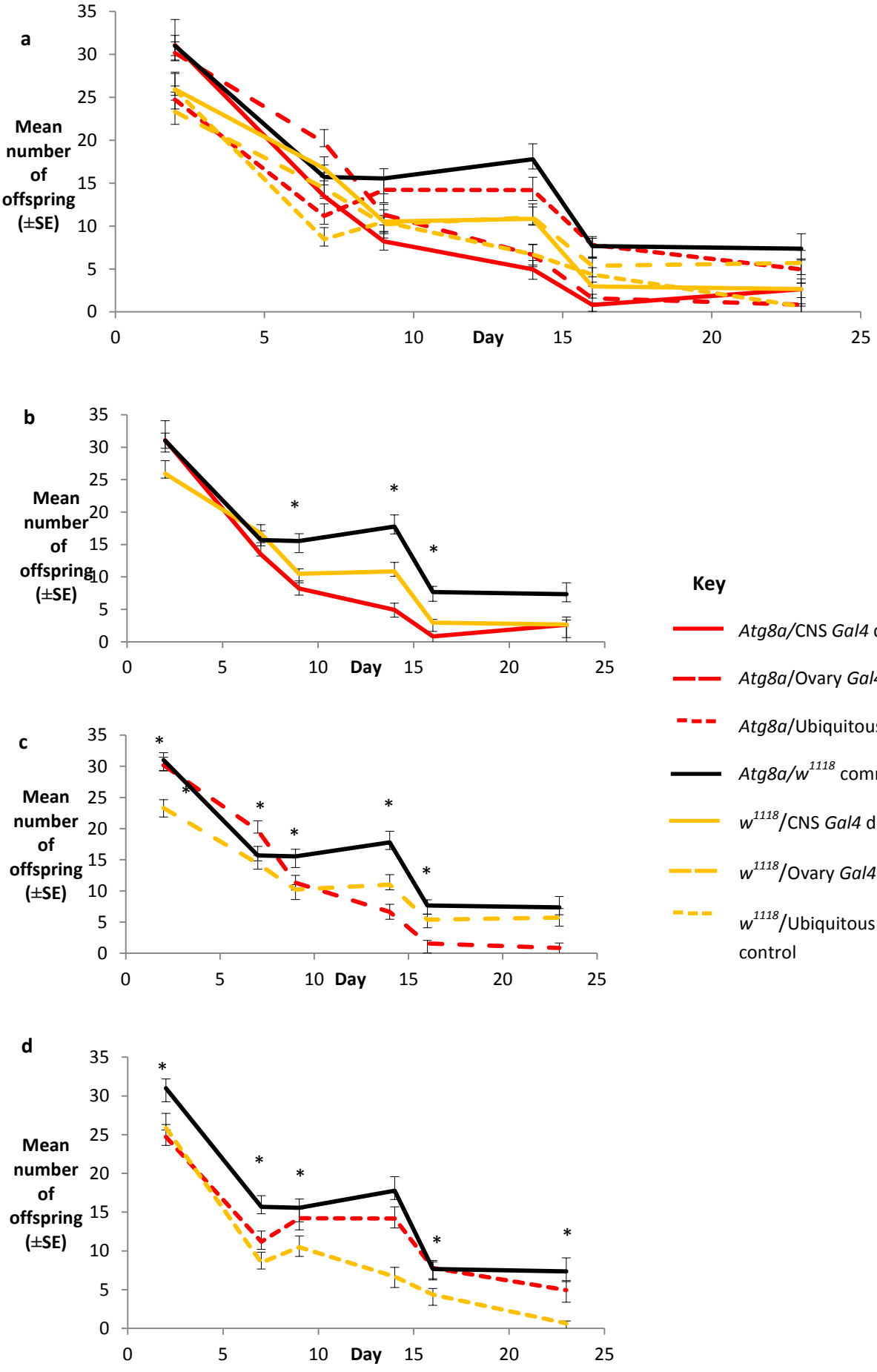
To identify with more precision where the differences in fertility lay, the age specific data were then analysed using appropriate ANOVA or Kruskal Wallis tests together with Bonferroni corrections for multiple comparisons across the different days. Panel (a) of fig 5.4 shows all the fertility data from the whole experiment, and panels (b), (c) and (d) the data for each of the tissues in which *Atg8a* was overexpressed, respectively. For each of the panels (b), (c) and (d) I showed each overexpressed genotype against its respective control and the common control.

Overall the common control had higher fertility than other genotypes. There was higher progeny production in the *UAS-Atg8a*/*Ovary Gal4* driver genotype, but this was a short-lived phenomenon. Towards the end of the data collection period the common control and the genotypes containing the ubiquitous *Gal4* driver maintained higher progeny levels.

In the second replicate experiment progeny production was more variable. ANCOVA showed that *UAS-Atg8a*/*CNS Gal4* driver and *UAS-Atg8a*/*Ovary Gal4* driver genotypes differed in their pattern of progeny production in comparison to the common control ($p=0.05$). The w^{1118}/*Ovary* control genotype exhibited higher initial fertility than the common control ($p=0.05$) while the w^{1118}/*Ubiquitous* control exhibited initial lower fertility ($p=0.001$).

To identify with more precision where the differences in progeny production lay, the same procedure was performed as above. Panel (e) of fig 5.5 shows fertility data from the whole experiment, panels (f), (g) and (h) show the data for each of the tissues in which *Atg8a* was

overexpressed. Overall the *UAS-Atg8a*/*CNS Gal4* driver had a higher fertility than the other treatments while the w^{1118}*/ubiquitous* control had a lower fertility. A surge in fertility was seen in the common control on day 8 and in the *UAS-Atg8a*/*Ovary Gal4* driver on day 11, but in all the lines fertility declined from day 11, and after day 17 there were no viable offspring in any of the treatments.


a
Mean number of offspring (±SE)
Day
b
Mean number of offspring (±SE)
Day
c
Mean number of offspring (±SE)
Day
d
Mean number of offspring (±SE)
Day
Key
Atg8a/CNS Gal4 driver
Atg8a/Ovary Gal4 driver
Atg8a/Ubiquitous Gal4 driver
Atg8a/w1118 common control
w1118/CNS Gal4 driver control
w1118/Ovary Gal4 driver control
w1118/Ubiquitous Gal4 driver control

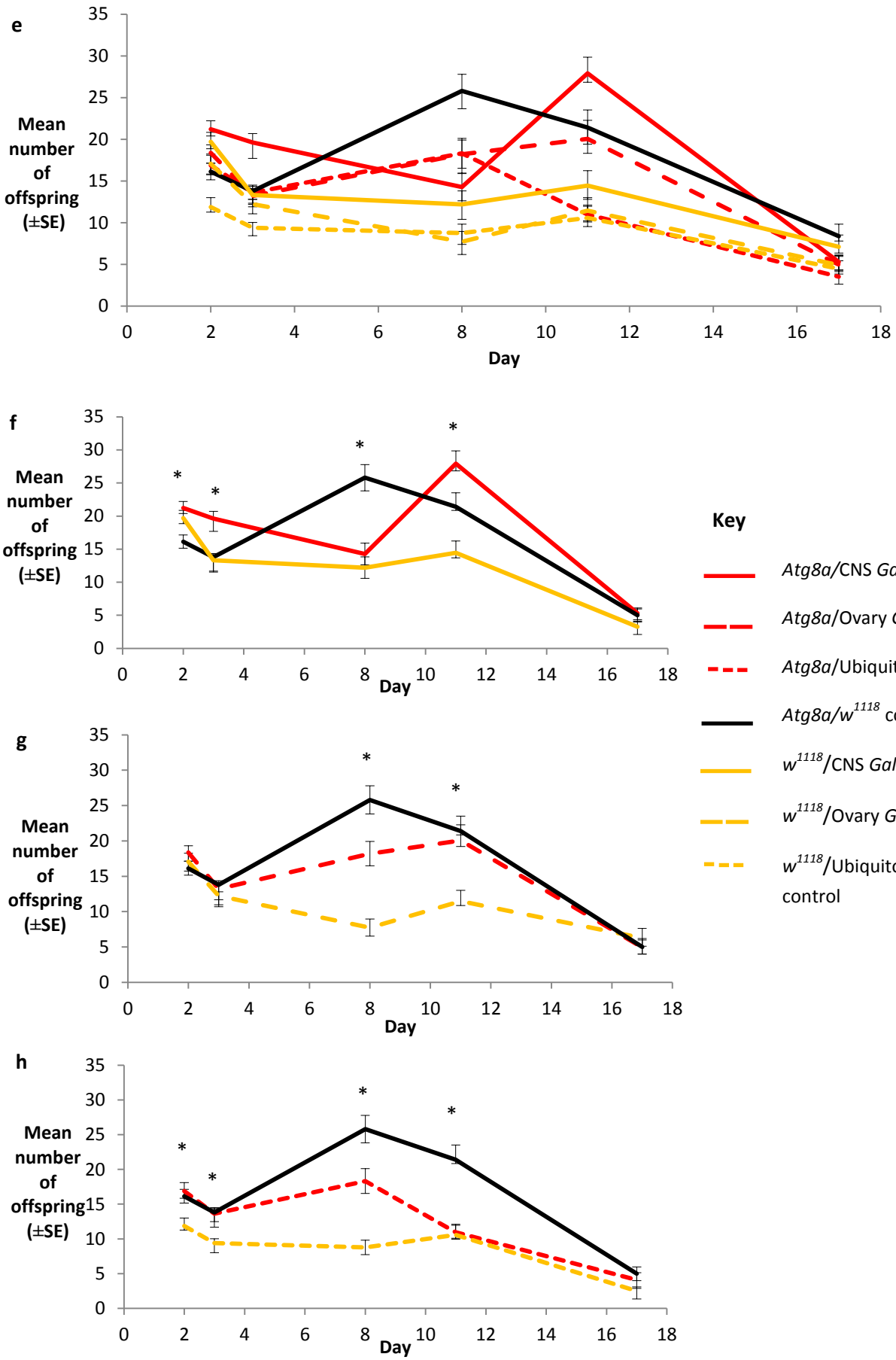
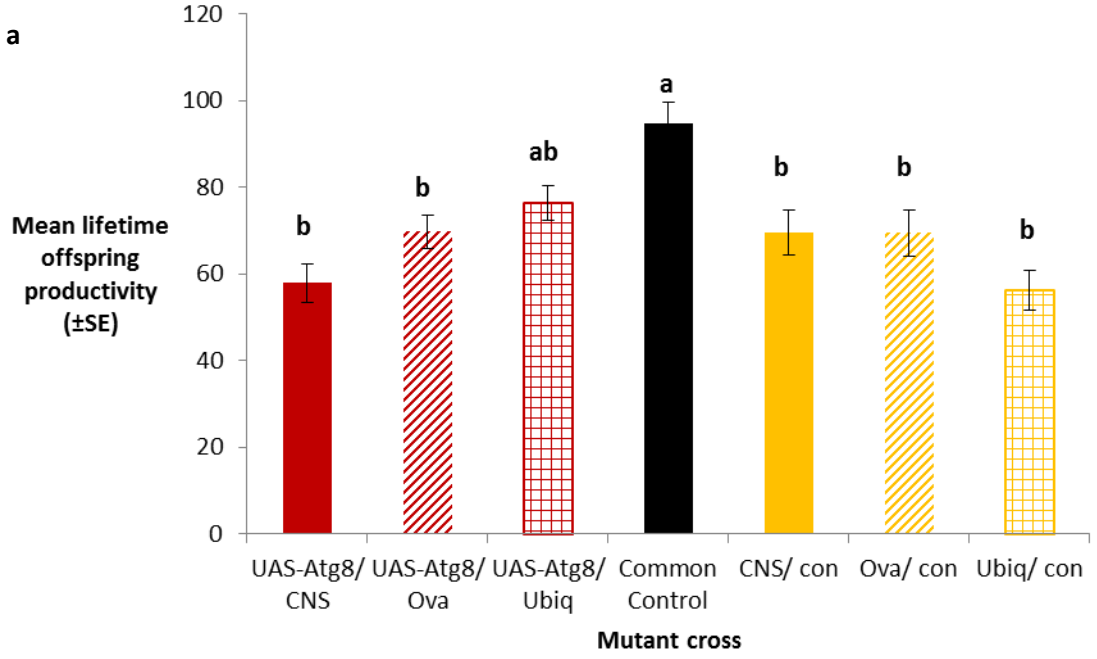

e
Mean number of offspring (±SE)
Day
f
Mean number of offspring (±SE)
Day
g
Mean number of offspring (±SE)
Day
h
Mean number of offspring (±SE)
Day
Key
Atg8a/CNS Gal4 driver
Atg8a/Ovary Gal4 driver
Atg8a/Ubiquitous Gal4 driver
Atg8a/w1118 common control
w1118/CNS Gal4 driver control
w1118/Ovary Gal4 driver control
w1118/Ubiquitous Gal4 driver control

Fig 5.4 – Mean number of viable offspring (±SE) of once mated females of given genotypes, panels **a** & **e**: common control (*UAS-Atg8a/w* ^{1118}), *Atg8a* overexpressed in the CNS (*UAS-Atg8a*/*CNS Gal4* driver), *Atg8a* overexpressed in the ovaries (*UAS-Atg8a/Ovary Gal4* driver), *Atg8a* over expressed ubiquitously (*UAS-Atg8a*/*Ubiquitous Gal4* driver), the CNS control (*w* ^{1118}/*CNS Gal4* driver control), the Ovary control (*w* ^{1118}/ *Ovary Gal4* driver control), the ubiquitous control (*w* ^{1118}/*Ubiquitous Gal4* driver control). **b** & **f**) constant control (*UAS-Atg8a*/w^{1118}), *Atg8a* overexpressed in the CNS (*UAS-Atg8a*/*CNS Gal4* driver) and the CNS control (*w* ^{1118}/*CNS Gal4* driver control). **c** & **g**) common control (*UAS-Atg8a*/*w* ^{1118}), *Atg8a* over expressed in the Ovaries (*UAS-Atg8a*/*Ovary Gal4* driver), and the Ovary control (*w* ^{1118}/*Ovary Gal4* driver control). **d** & **h**) common control (*UAS-Atg8a*/*w* ^{1118}), *Atg8a* over expressed ubiquitously (*UAS-Atg8a*/*Ubiquitous Gal4* driver) and the ubiquitous control (*w* ^{1118}/*Ubiquitous Gal4* driver control). * indicates a significant difference in the number of eggs laid (p≤0.05).

Lifetime offspring productivity

Analysis of lifetime offspring productivity (fig 5.5a) for the first experiment showed that females from the common control (*UAS-Atg8a/w*^{1118}) produced significantly more offspring than those from the other control lines (*w*^{1118}/*CNS Gal4* driver control, *w*^{1118}/*Ovary* driver control, *w*^{1118}/*Ubiquitous* driver control, $F_{6,270}$=8.341, p<0.001). CNS and ovary overexpression genotype females (*UAS-Atg8a*/*CNS Gal4* driver and *UAS-Atg8a*/*Ovary* driver, respectively) had fewer offspring than the common control (all at p≤0.025).

In the second experiment, overall the overexpression genotype lines and the common control produced significantly more offspring over their lifetimes than did the other three *Gal4* control lines ($F_{6,268}$=14.617, p<0.001, fig 5.5b). This was seen with *Atg8a* overexpression in the central nervous system (*UAS-Atg8a*/*CNS Gal4* driver), which also exhibited higher offspring production than the 3 respective *Gal4* control lines (all at p≤0.035).



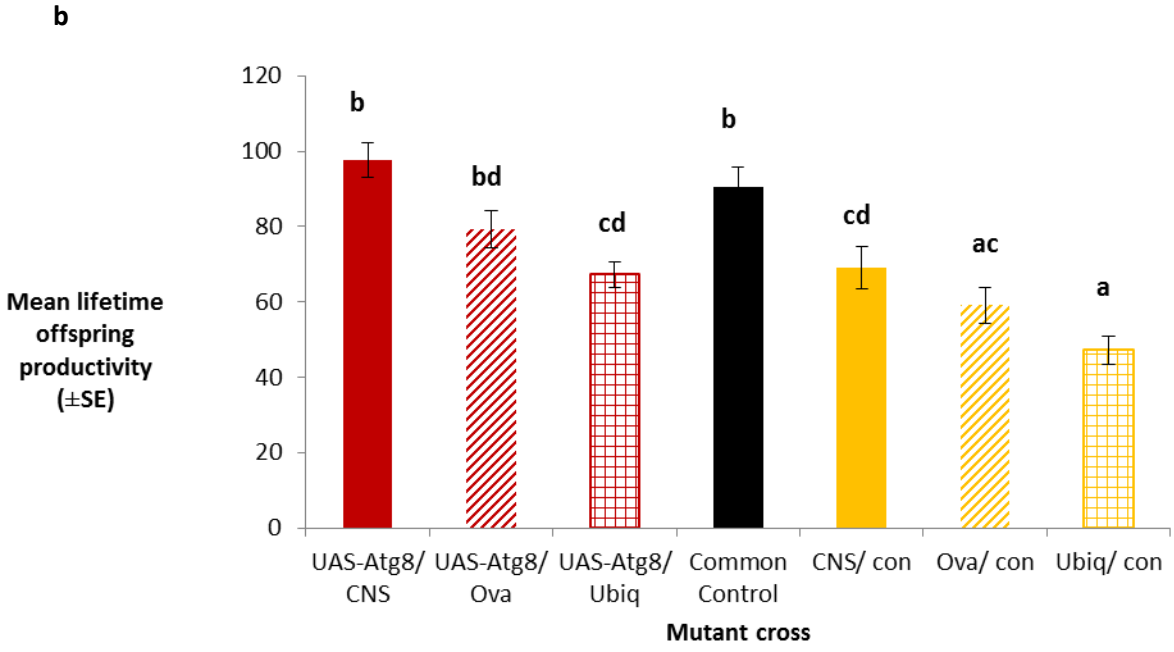


Fig 5.5a & b - Mean lifetime productivity (±SE) of viable offspring for once mated females with *Atg8a* overexpressed in the CNS (*UAS-Atg8a*XCNS *Gal4* driver), the ovaries (*UAS-Atg8a*XOvary *Gal4* driver) and ubiquitously (*UAS-Atg8a*XUbiquitous *Gal4* driver). For control females, CNS control (w^{1118} X CNS *Gal4* driver control), ovary control (w^{1118} X Ovary *Gal4* driver control), ubiquitous control (w^{1118}XUbiquitous *Gal4* driver control) and common control (*UAS-Atg8aXW* ^{1118}). Panels a and b show the two replicate experiments respectively. Different letters above the bars indicate a significant difference at $p<0.05$.

5.4.2 Validation of *Atg8a* overexpression manipulations by qRT-PCR

Relative *Atg8a* gene expression was significantly different across the different groups ($F_{6,15}$=115.451, p≤0.001). However, this was mostly due to significantly elevated *Atg8a* expression in the *UAS-Atg8a/Actin 5C-Gal4 ubiquitous* overexpression genotype (Fig 5.6). There was no evidence for significantly increased *Atg8a* gene expression in either of the other overexpression treatments (in CNS or ovary). However, the sensitivity to detect overexpression in these genotypes, especially in the CNS, is likely to be low, due to tissue swamping effects (Chintapalli, Wang et al. 2007). Nevertheless, the results should be interpreted in light of the fact that there is no evidence that the genetic manipulations employed to overexpress *Atg8a* in the CNS and in the ovary actually worked as expected. There was also some evidence for a slightly increased level of *Atg8a* expression in the central nervous system control line (*w*^{1118}*/CNS Gal4* driver), p≤0.013.

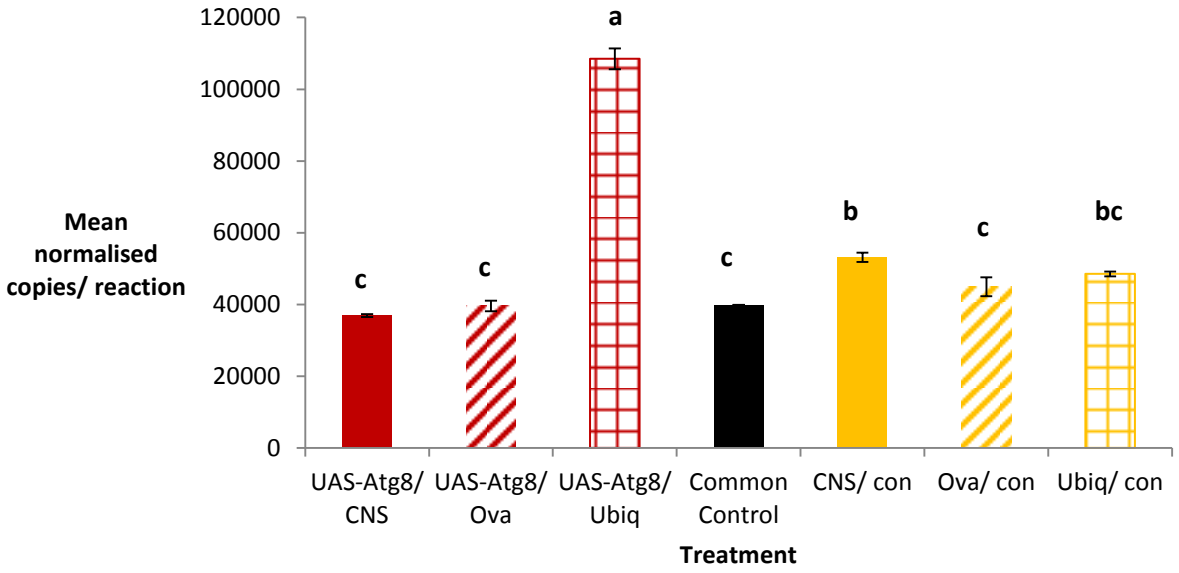


Fig 5.6 - Mean normalised copies/ reaction of Atg8a (±SE) for once mated females with Atg8a overexpressed in the CNS (UAS-Atg8a/CNS Gal4 driver), the ovaries (UAS-Atg8a/Ovary Gal4 driver) and ubiquitously (UAS-Atg8a/Ubiquitous Gal4 driver). For control females, CNS control (*w* ^{1118}*/CNS Gal4* driver control), ovary control (*w* ^{1118}*/Ovary Gal4* driver control), ubiquitous control (*w* ^{1118}*/Ubiquitous Gal4* driver control) and common control (*UAS-Atg8aXW* ^{1118}). Different letters above the bars indicate a significant difference at p<0.05.

5.5 Discussion

The analysis of relative *Atg8a* expression levels by qRT-PCR showed clear evidence for overexpression of *Atg8a* in the ubiquitously expressed genotype females, but not in the other overexpression genotypes. Overall there was no consistent evidence for increased lifespan in *Atg8a* overexpression genotypes for any of the tissues tested. This included the neuronal tissue in which lifespan extension had previously been reported (Simonsen, Cumming et al. 2008). However, I was not able to validate elevated *Atg8a* expression in that genotype, therefore this conclusion is tentative, although I also saw no lifespan extension in females in which *Atg8a* was ubiquitously overexpressed (i.e. in females in which the genetic manipulation clearly worked). The sensitivity of the qRT-PCR test for very small changes in relative *Atg8a* expression in one or a few tissues where RNA levels may be very low, when testing RNA extracted from whole fly samples can, however, be subject to tissue swamping (Chintapalli, Wang et al. 2007). Hence it is still possible that the genetic manipulations elevated *Atg8a* locally in a manner that was not detectable by the qRT-PCR. Further testing on individual tissues is needed to validate that possibility. However, even assuming that there was overexpression of *Atg8a* in the targeted tissues, there was no consistent effect on lifespan, fecundity or fertility in the overexpression genotypes. It was instead the *UAS-Atg8a* control genotype that tended to have the highest fecundity, fertility and lifespan. Restricting the conclusions to the validated ubiquitous overexpression of *Atg8a,* there was therefore no evidence that elevating autophagy via induction of *Atg8a* has any beneficial effects on lifespan or fertility. Indeed, the other genetic manipulations employed may have, in combination, had unknown deleterious effects.

The interpretation of the results from the two replicate experiments was also made difficult by inconsistent effects. For example in the initial experiment, the experimental *UAS-Atg8a/ovary Gal4* driver genotype females had significantly longer lifespans than the other experimental and *UAS-Atg8a* control lines (note though that the common control was the longest lived group in that

experiment by far). Whereas, in the second replicate experiment the experimental line *UAS-Atg8*/*CNS Gal4* driver overexpression and control (w^{1118}/*CNS Gal4* driver) genotypes were longer lived. This could indicate that overexpression of *Atg8a* somewhere (in the ovary or the CNS) can cause slight increases in longevity, perhaps indicating that the site of expression is not crucial. However, the problem with this interpretation is that the ubiquitously overexpressed genotype females were validated to express higher levels of *Atg8a* yet did not show any hint of an effect on lifespan. Unless the hypothesis is that there is degradation of *Atg8a* when expressed at high levels, then these results are as yet inconclusive. The longer lifespan in the CNS overexpressed genotype females in the second experiment is consistent with the previously published data (Simonsen, Cumming et al. 2008), but the finding that this effect was not replicable in my work should increase caution about the reported effect in general.

Although fecundity varied across the two experiments, in both, it was the *UAS-Atg8a/*w^{1118} common control females that consistently laid more eggs than the other genotypes. In fact the *Gal4* driver controls (CNS, ovaries and ubiquitous *Gal4* drivers) all laid consistently fewer eggs. The numbers of offspring were also significantly higher in the common control group in both experiments. In the second replicate experiment the *UAS-Atg8a*/*CNS Gal4* driver females also produced significantly higher numbers of offspring. There was no consistent pattern of increased fecundity and fertility in the overexpression genotype females. In the first experiment there was initially significantly higher fecundity in the ovary overexpressed females genotype, but this was not replicated in the second, and is therefore as yet an inconsistent finding. What did seem consistent was that the *Gal4* controls have lower egg and offspring numbers, suggesting that the expression of *Gal4* in the absence of any *UAS* target has detrimental effects on reproductive success. This could be tested by using control females carrying *UAS* upstream of a truncated/mutated transgene, and is a possibility that should be investigated further in order to facilitate improved gathering of unconfounded data from these types of experiments.

The expression of several autophagy genes is reduced as a normal part of ageing in *Drosophila* neuronal tissue. Insoluble ubiquitinated proteins (IUP) are a marker for neuronal ageing and degeneration, and accumulate when age-dependent suppression of autophagy occurs. Mutations in the *Atg8a* gene are reported to result in a reduced lifespan with an accumulation of IUP and an increased sensitivity to oxidative stress. But when *Atg8a* expression is enhanced for example, in older fly brains then mean lifespan is extended on average by approximately 56% (Simonsen, Cumming et al. 2008). Data from Simonsen et al. (2008) suggest that the genetic or age-dependent suppression of autophagy is closely associated with cellular damage build up in the neurons and so a reduced lifespan. Simonsen et al (2008) also suggest that if expression of autophagy is maintained it can prevent the age-dependent accumulation of damage in the neurons and promote longevity (Simonsen, Cumming et al. 2008). Neurons are important cells for autophagy dependent longevity and it has been shown the induction of autophagy in a specific tissue or cell type, in this case the neurons, may benefit the entire organism. Gelino et al. (2012) has also shown that timing of *Atg8* expression in the central nervous system might be critical for longevity associated effects with induction of *Atg8a* during adulthood but not during development, being essential for significant lifespan extension (Gelino and Hansen 2012).

Other autophagy genes when overexpressed are also reported to extend lifespan. For example, ubiquitous overexpression of *Atg5* extends the median lifespan of mice by 17% (Pyo, Yoo et al. 2013). This work demonstrated that the moderate overexpression of this gene enhanced autophagy. *Atg5* transgenic mice had anti-ageing phenotypes including leanness, an increase in insulin sensitivity and an improvement in motor function. It was suggested that both the leanness and lifespan extension resulted from increased autophagic activity (Pyo, Yoo et al. 2013). There can also be mixed effects, however for example ubiquitous overexpression of *Atg1* induces autophagy but also increases cell death (Gelino and Hansen 2012). This indicates that autophagy has to be harnessed under the right conditions in order to have 'clean' beneficial effects, and that it also has global effects on homeostasis as cell function declines with age. Damaged proteins and organelles accumulate with

age, a process which is accelerated in many human disorders and which may result in cellular and organismal death. Autophagy has in fact been linked to many age-related diseases such as cancer, muscle disorders, and also neurodegenerative diseases. The up-regulation of autophagy may enable the cell to endure stressful conditions by increasing the rate of damaged macromolecule turnover. It is also possible that autophagy promotes cell maintenance by removing accumulated toxic products and by using the recycled components as an alternative nutrient resource. This all suggests that autophagy induction favours longevity because an organism can recover more quickly from any stress induced damage (Gelino and Hansen 2012).

Recently researchers found autophagy is implicated in neurodegenerative diseases that affect humans (Gelino and Hansen 2012). If autophagy can be induced by the overexpression of a single gene (e.g. *Atg8*), addressing tissue specificity of autophagy induction targets for therapeutic purposes may be found. But obviously the effect of overexpressing a single gene may not be limited to one specific outcome, as is suggested by the results of the research described in this chapter. For example, overexpressing a gene such as *Atg8a* may extend lifespan through autophagy activation on one hand, but may also have deleterious effects through other affected pathways. Future research would therefore need to focus on specific and safe manipulations of only the lifespan-extending potential of autophagy.

Chapter 6 Effects of autophagy inactivation via knockdown of Atg8a on female survival, fecundity and fertility under normal and protein-limited diets.

6.1 Abstract

Autophagy is fundamentally linked to the process of ageing. Reduced activation of autophagy is reported to result in a reduction in the ability to repair the increasing amounts of damage that arise with age. Consistent with this, previous research shows that enhancing the expression of autophagy gene 8a (*Atg8a)* in the nervous system of *Drosophila melanogaster* can extend lifespan (however, see results of chapter 5 for contrary evidence). *Atg8a* plays a central and limiting role in autophagy and is therefore often used as an indicator of autophagy activation in cells and whole organisms (e.g. see also Westerns for autophagy in chapters 2, 3 and 4). Therefore, as *Atg8* has a specialized role in the autophagy pathway it has been proposed as a promising target for investigation in anti-ageing research. A second route by which lifespan can reliably be extended across many species is through caloric restriction without malnutrition. This effect may be connected to autophagy activation through the TOR signalling pathway, as described in chapter 1. Evidence for a link between caloric restriction and autophagy is that reduced diets activate autophagy and are associated with increased lifespan, and more directly that extended lifespan in response to diet in some species is shown to be dependent upon having a functioning autophagy pathway.

In this chapter I investigated the links between diet restriction, autophagy and lifespan, whilst also testing for trade-offs with reproductive rate, which have not previously been investigated.

I inactivated autophagy by using knockdown manipulations of *Atg8a* in once mated females and I also tested whether diet restriction could lead to extended life in wild type versus *Atg8a* knockdown

females. I predicted that those flies unable to activate autophagy would see no lifespan extension, in comparison to wild type controls, on a lowered protein intake. Egg production was also analysed in these females to determine if switching off autophagy would have any effect on fecundity or fertility in females held under normal or diet restricted conditions. In addition to this the results also allowed me to test for trade-offs in fecundity and/or fertility with any lifespan extension that was seen. The results showed that diet appeared to have more of an effect on lifespan and fecundity than genotype. Although autophagy may have been activated in certain fly cohorts this did not translate into a straightforward lifespan extension in these flies. I observed that both fecundity and fertility appear to be controlled, at least partly, separately from any lifespan extension drivers suggesting lifespan extension and fecundity may not be linked. Indications are that individuals on the restricted diet are induced to lay more eggs at the beginning of their life while those on the higher food are more conserved and more constant in their egg laying. A trade-off between lifespan and fecundity was found that appeared not be linked to autophagy activation but to reproduction to the detriment of survival.

6.2 Introduction

Autophagy is fundamentally linked to the process of ageing. The extent of autophagy is reduced with increasing age and this can result in a reduced ability to repair the ever increasing amounts of damage that arise with age due to, for example, increased oxidative stress. This can result in an increase in the number of severely damaged mitochondria reported with age (Vellai 2009; Hands, Proud et al. 2009; Markaki and Tavernarakis 2011). It is the accumulation of free radicals arising as a by-product of oxidative stress in the mitochondria that can lead to mitochondrial dysfunction, which is a common feature in ageing cells (Wallace 2005). The decrease in autophagic activity with age leads to a less frequent or inefficient clearance of damaged proteins and organelles in the cells, exacerbating the problem. Hence the decline in autophagy is believed to contribute centrally to functional deterioration in ageing organisms (Markaki and Tavernarakis 2011).

Gerontology research has produced several advances in understanding the genetic control of ageing. For example, genetic screening has been used in yeast to identify genes that are involved in the autophagic process (Klionsky and Emr 2000). Evidence suggests that autophagy can protect cells against many noxious agents and this can confer an extension in longevity when the protection is widespread across the whole organism. Therefore, an increase in autophagic turnover of cytoplasmic organelles or of long-lived proteins may be involved in many lifespan extending processes. Several genetic studies have shown that by decreasing protein translation lifespan can be extended (Hands, Proud et al. 2009) indicating autophagy genes are required in many long-lived organisms (Madeo, Eisenberg et al. 2009).

It has been shown in *D. melanogaster* that the inhibition of autophagy via the TOR signalling pathway shortens lifespan (Scott, Schuldiner et al. 2004). The fact that suppression of autophagy limits lifespan extension in both nematodes and flies is one of the key pieces of evidence that identifies autophagy as a longevity determinant (Scott, Schuldiner et al. 2004; Hansen, Chandra et al. 2008). However there are several aspects of the relationship between autophagy and ageing that

remain unsolved. For example, the loss of mitochondrial activity can induce autophagy and may slow ageing by maintaining mitochondrial quality and quantity (Jia and Levine 2007). In contrast it is also believed that autophagy can contribute to the removal of injured or damaged cells by facilitating the process of cell death (Samara, Syntichaki et al. 2008; Markaki and Tavernarakis 2011), in a comparable manner to the process of apoptosis. The mechanisms determining whether autophagy protects cells versus hastening their destruction if they are beyond repair are not well understood. However, what is clear is that there is considerable potential for reliable and effective manipulations of autophagy to provide significant interventions in anti-ageing research.

One of the key targets for manipulation of autophagy is to up and down regulate autophagy gene *8a* (*Atg8a*) (Tooze 2010). Importantly it has been shown that enhancing the expression of *Atg8* in the nervous system of *D. melanogaster* can extend lifespan (though see also results of chapter 5). *Atg8a* was overexpressed in the central nervous system (CNS) using *Gal4* driven by a CNS promoter, which resulted in significantly extended lifespan (Simonsen, Cumming et al. 2008; Hands, Proud et al. 2009). However, the effects on fecundity and fertility were not reported. In chapter 5 my data showed only limited evidence for lifespan extension following ubiquitous overexpression of *Atg8a* and no consistent effect on fertility. Consistent with the data showing that the over activation of autophagy genes can have beneficial effects, the opposite effect, namely decreasing the expression of autophagy genes such as *Atg1* and *Atg8* using knockdown genetic manipulations reduces lifespan (Simonsen, Cumming et al. 2008). Lowered expression of *Atg8* in *C. elegans* also showed suppressed lifespan in organisms that were autophagy deficient (Toth, Sigmond et al. 2008). Together these data suggest that manipulation of *Atg8a* might be a promising target for anti-ageing research, provided any trade-off with fertility is not too severe. A focus on *Atg8* is warranted because it has specialized and autophagy specific roles, whereas other autophagy genes show pleiotropic effects and additional phenotypes not related to autophagy (McPhee and Baehrecke 2009). *Atg* genes with such multiple effects would represent more complicated targets for potential interventions.

It is proposed that several different signalling pathways converge on autophagy in order to regulate lifespan in many diverse organisms (Markaki and Tavernarakis 2011). Studies in *Drosophila* and *C. elegans* suggest that autophagy may function downstream of other known longevity-influencing pathways such as insulin/IGF-1 and TOR signalling, caloric restriction and mitochondrial activity in order to regulate ageing (Markaki and Tavernarakis 2011). The well-established mechanism for promoting longevity of dietary or caloric restriction is known to induce autophagy, and has been demonstrated in many organisms including *D. melanogaster, C. elegans* and mice (Scott, Schuldiner et al. 2004; Piper and Bartke 2008; Hands, Proud et al. 2009). The idea is that autophagy can partly exert its beneficial effects on survival through removing damaged macromolecules or organelles while at the same time recycling nutrients and therefore conserving resources. In this manner it serves as an energy reserve which can be activated during starvation (Barth, Szabad et al. 2011). The current model, in which autophagy activation state is downstream of responses of the cell to nutrients, predicts that it should be possible to activate autophagy by experimentally inducing caloric restriction. Hence, it should be possible through dietary interventions to counteract the decline in autophagy activation with age, a prediction which has some empirical support (Cavallini, Donati et al. 2001; Hands, Proud et al. 2009).

That a reduction in food intake extends lifespan in many species is well known (Piper and Bartke 2008). Caloric restriction refers to decreased calorie intake, but without malnutrition. It is currently unclear, however, what is the lower calorie cut-off point below which autophagy can no longer be effective at reducing damage and conserving nutrients in response to diet restriction, this would be the threshold below which death occurs. The main nutrient sensing pathway to be associated with caloric restriction is the TOR pathway. The inhibition of TOR mimics the physiological effects of dietary restriction in yeast, flies and nematodes (Mair and Dillin 2008; Fontana, Partridge et al. 2010). It has also been seen that long-lived mice mutants (S6 mutants) exhibit gene expression patterns that are similar to those found in other organisms subjected to caloric restriction (Kenyon 2010). These mice have increased oxygen consumption, which suggests that the mechanism for lifespan

extension may be conserved. Translation is up-regulated in part by the ribosomal subunit S6 kinase through TOR in response to nutrients. The S6 mutant mice exhibit gene expression patterns similar to those that are triggered by caloric restriction, suggesting the TOR/S6 kinase pathway may also influence a response to caloric restriction in mammals (Kenyon 2010).

In this chapter, I investigated the interaction between diet restriction and autophagy in extending lifespan and tested for any associated effects on fertility. I tested whether diet restriction could lead to extended life in wild type once mated females and in those in which autophagy was down-regulated by knockdown of the *Atg8a* gene. I predicted that those flies unable to activate autophagy would see no lifespan extension, in comparison to wild type controls, on a lowered protein intake. Egg production was also analysed in these females to determine if switching off autophagy would have any effect on fecundity or fertility in females held under normal or diet restricted conditions. In addition, the results also allowed me to test for trade-offs in fecundity and/or fertility with any lifespan extension that was seen.

6.3 Materials and Methods

6.3.1 The effect on survival and fecundity of diet restriction and genetic knockdown of *Atg8a* in once mated females.

Fly rearing was carried out as set out in the general materials and methods (chapter 2). To obtain *Atg8a* knockdown females I used flies containing an *Atg8a* inverted repeat (IR) construct under the control of the upstream activating sequence (*UAS-Atg8a*-IR, Vienna stock number 43096) crossed to flies containing a ubiquitous Actin *5C-Gal4* driver (*Act 5C-Gal4/Cy*, Bloomington stock number 4414). Full genotypes for the crosses and further details are given in the general materials and methods section, chapter 2. Upon emergence, females were left for 3 days together with males from their own cohort to ensure mating. I then separated knockdown females (*UAS-Atg8a-IR/Act 5C-Gal4*)

from control females (*UAS-Atg8a-IR/Cy*) obtained from the same cross. The control and experimental females had therefore experienced exactly the same developmental environment as each other. Females of each genotype were then placed into one of two food treatments containing either 100% or 50% yeast. Females were given new food every 24 hours (mon-fri) until egg laying ceased and then three times a week until death. Females were transferred to new vials without anaesthesia.

Female longevity was recorded daily until all females were dead. Individuals that became stuck in the food, escaped or were accidentally killed were entered as censors in the data collection.

Every day, initially, and then approximately every 4th day during the females' lifetimes the number of eggs laid in the food vials over the 24 hour occupation periods was counted. Egg counts ceased when half the remaining females in a treatment ceased from laying eggs.

I also determined total egg productivity for each individual over their lifetime.

6.3.2 The effect on survival, fecundity and fertility of food dilution and genetic knockdown of *Atg8a* in once mated females.

I then replicated the above experiment but refined it further by constructing genetic controls for both the *UAS-Atg8a-IR* and the *Act 5C-Gal4* constructs and by including an additional diet treatment, to extend the lower end of the diet restriction. I also refined the mating procedure, upon emergence experimental and control females were held with wild type males for two days to standardise the identity of the males used for mating's across treatments. *Atg8a* knockdown females were obtained as above by crossing the inverted repeat (*UAS-Atg8a-IR*, Vienna stock number 43096) and ubiquitous *Gal4* diver (*Act 5C-Gal4/Cy*, Bloomington stock number 4414) lines to produce the *UAS-Atg8a-IR/Act 5C-Gal4* genotype. I generated the additional controls by crossing each of the parental lines separately to the w^{1118} Vienna stock centre progenitor line (stock number 60000). This gave *UAS-Atg8a-IR/*w^{1118} and *Act 5C-Gal4/*w^{1118} control genotypes. Following the 2

days of exposure to wild type males, females from different genotypes were placed on three different diets containing 100%, 50% or 25% yeast. Females were given new food every 24 hours (Mon-Fri) until egg laying ceased and then three times a week until death. Flies were transferred to new vials without anaesthesia.

Female longevity was recorded daily until all females were dead. Individuals that became stuck in the food, escaped or were accidentally killed were entered as censors in the data collection.

Approximately every 3 to 4 days during the females' lifetimes eggs laid in the food vials over 24 hours were counted. In this experiment I also counted offspring by incubating the vials for 12 days until offspring emergence. Egg counts ceased when half the remaining females in a treatment ceased from laying eggs.

I also determined total egg and offspring productivity for each individual over their lifetime.

6.3.3 Validation of *Atg8a* knockdown manipulations by qRT-PCR

In order to verify that the genetic manipulations did indeed result in a knock down of *Atg8a* in the experimental females, I measured *Atg8a* mRNA levels, using qRT-PCR. This also allowed me to test for the effect of diet restriction upon autophagic state as indicated by the level of *Atg8a* expression. For full results of quality control and primer sequences see chapter 2 general materials and methods. However, in sum, total RNA from 10 flies per cohort was extracted using the *mir*Vana™ miRNA Isolation kit (http://www.lifetechnologies.com). Samples were then sent to qStandard for qRT-PCR analysis (http://www.qstandard.co.uk/index.html). 500ng RNA was reversed transcribed using the Qiagen Quantitect reverse transcription following the manufacturer's protocol. Runs were performed in duplicates for two replicates using Agilent Brilliant III Ultrafast SYBR green qPCR mix and melting curve analyses were performed. Data were analysed using the Rotor-Gene software. Relative expression ratios were normalised to two control genes *Actin5c* and *Gapdh2*.

6.3.4 Statistical analysis

Statistical analysis was performed using SPSS v 18 (SPSS 2009) and R (R Development Core 2008). Survivorship was calculated for each treatment and survival data were analysed using Log Rank tests. Fecundity and fertility data for each treatment and control group of females were analysed as in previous chapters (3, 4 & 5) using ANCOVA. As previously, in order to probe for differences in more detail this was then followed up by analyses of age specific fecundity and fertility, while correcting for multiple testing using the sequential Bonferroni procedure. The data were first tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. Depending upon whether the assumptions were met, the data were then analysed either using parametric ANOVA and Tukey post hoc analyses, or non-parametric Kruskal Wallis tests.

6.4 Results

6.4.1 The effect on survival and fecundity of food dilution and genetic knockdown of *Atg8a* in once mated females.

Survival

In the initial experiment there were significant differences in survivorship between the different treatments (Log rank X^2=11.054, df=3, p=0.011, fig 6.1). There was a significant increase in lifespan for females kept on the 50% protein diet (Log rank X^2=5.406, df=1, p=0.020) and a significant effect too of genotype, with control females on both diets living longer than for the *Atg8a* knockdown females (Log rank X^2=4.189, df=1, p=0.041). However, there was no evidence that the *Atg8a* knockdown genotype females responded differently to the diet restriction, in comparison to the controls.

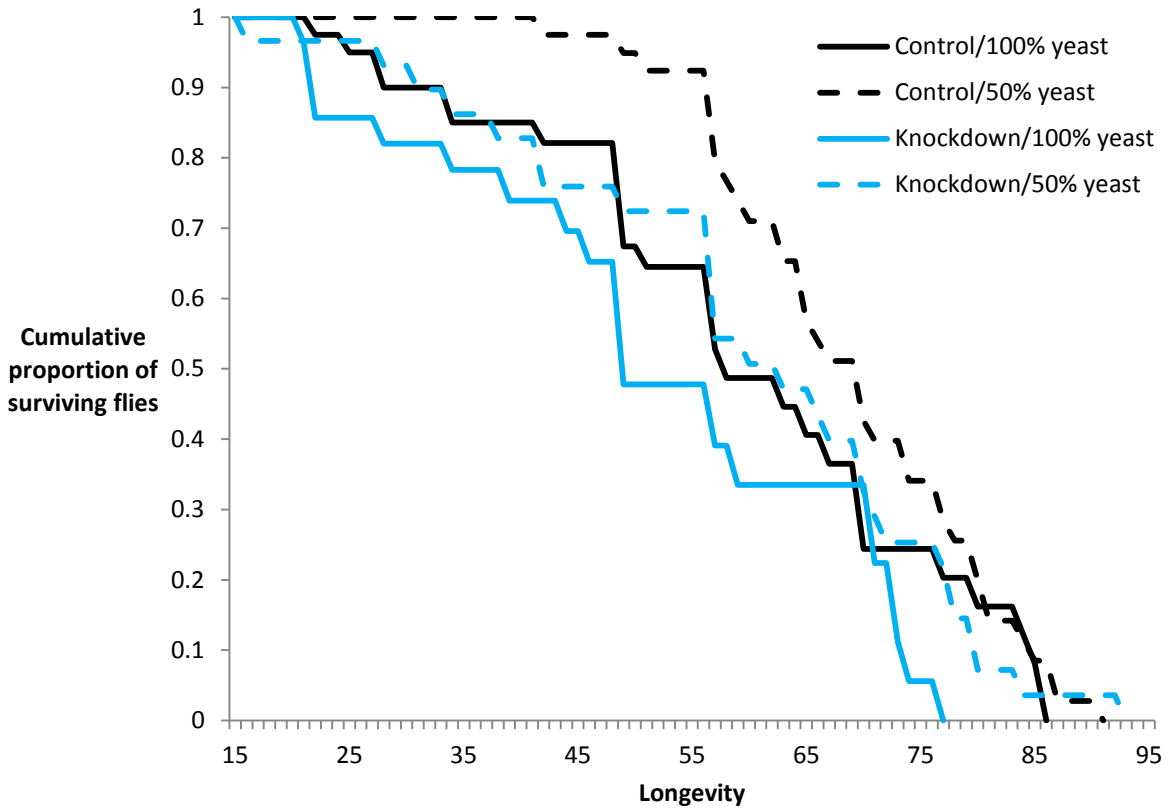


Fig 6.1- Survivorship of once mated *UAS-Atg8a-IR/Cy* control, or once mated *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females held as adults on diet containing 100% (solid line) or 50% (dashed line) yeast.

Age-specific fecundity

ANCOVA analysis testing for differences in fecundity patterns over time revealed, as expected, a significant decline in egg production over time. Those females on the normal 100% yeast diets showed a significantly different pattern of egg laying in comparison to those on the lower 50% diet. However, the pattern was somewhat unexpected, with fully fed females initially laying significantly fewer eggs and then laying more than the 50% yeast females ($p<0.001$). This was reflected as a significant interaction between diet and time ($p<0.001$). Overall, there was no evidence for a difference in egg production due to the different female genotypes.

The age specific tests showed that there were significant differences between treatments in the mean number of eggs laid on days 2, 11 and 26. Females held on the lower nutrition food initially laid significantly more eggs on day 2 but by days 11 and 26 this pattern had reversed ($p<0.05$, fig 6.2). Consistent with the ANCOVA, the main differences were due to diet and not to female genotype.

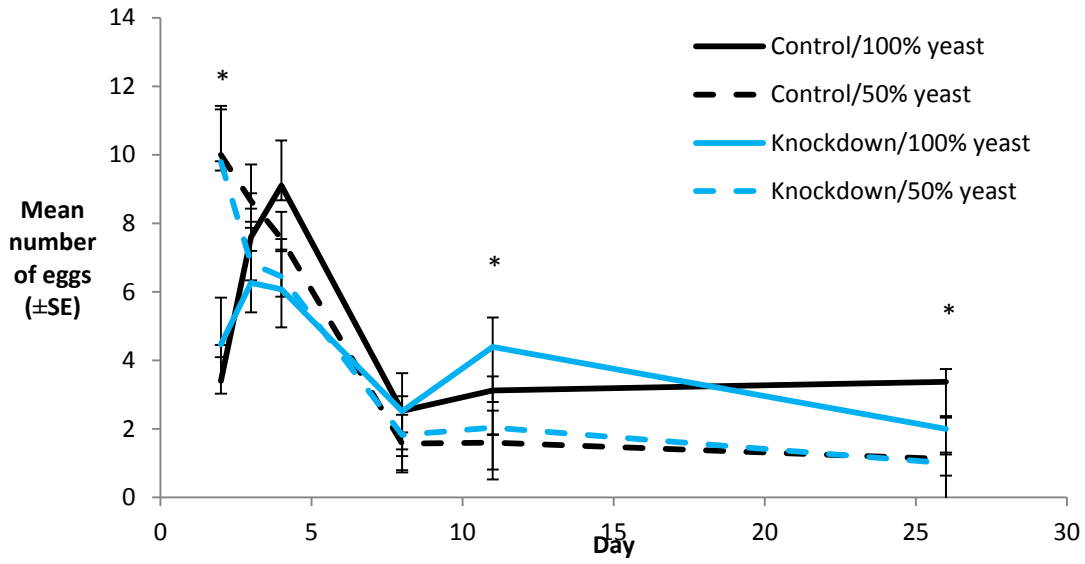


Fig 6.2- Mean number of eggs (±SE) laid against time of once mated *UAS-Atg8a-IR/Cy* control, or once mated *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females per 24hrs on diet containing 100% (solid line) or 50% (dashed line) yeast. * indicates a significant difference in the number of eggs laid by that treatment group all at $p<0.05$.

Lifetime egg productivity

Analysis of lifetime fecundity data (fig 6.3) showed that neither genotype ($F_{1,134}=0.201$, $p=0.654$) nor diet ($F_{1,134}=0.917$, $p=0.340$) had any significant effect on lifetime egg production. This was somewhat unexpected, as it had been anticipated based on previous work that females held on higher protein diets would have higher fecundity overall.

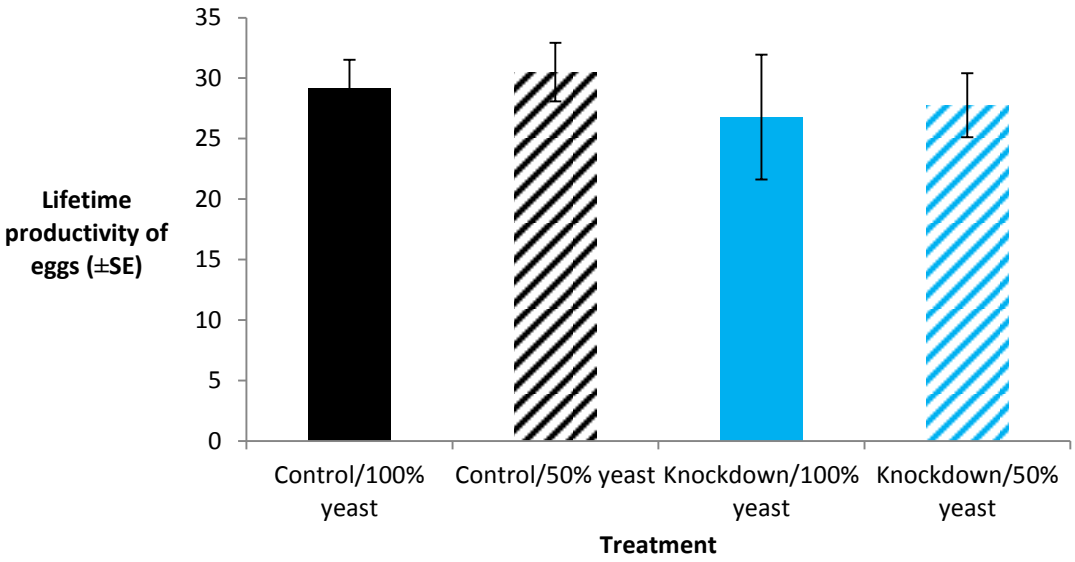


Fig 6.3 – Mean lifetime productivity of eggs (±SE) of once mated *UAS-Atg8a-IR/Cy* control, or once mated *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females held as adults on diet containing 100% (solid line) or 50% (dashed line) yeast.

6.4.2 The effect on survival, fecundity and fertility of food dilution and genetic knockdown of *Atg8a* in once mated females.

Survival

In this replicate but expanded experiment, there was a significant difference in survivorship between the different groups (Log rank X^2=82.849, df=8, p<0.001, fig 6.4). As above, there was a significant effect of the different diets (Log rank X^2=46.542, df=1, p<0.001) but no significant effect of female genotype (Log rank X^2=1.637, df=1, p=0.201). Therefore, as above, there was little evidence that *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females had a different pattern of lifespan responses to diet in comparison to control females. Lifespan was slightly higher at the 50% food level than at the 100%, which likely represents a slight caloric restriction effect on longevity, as has previously been observed. The lifespan of all genotypes was decreased significantly at the lowest food level.

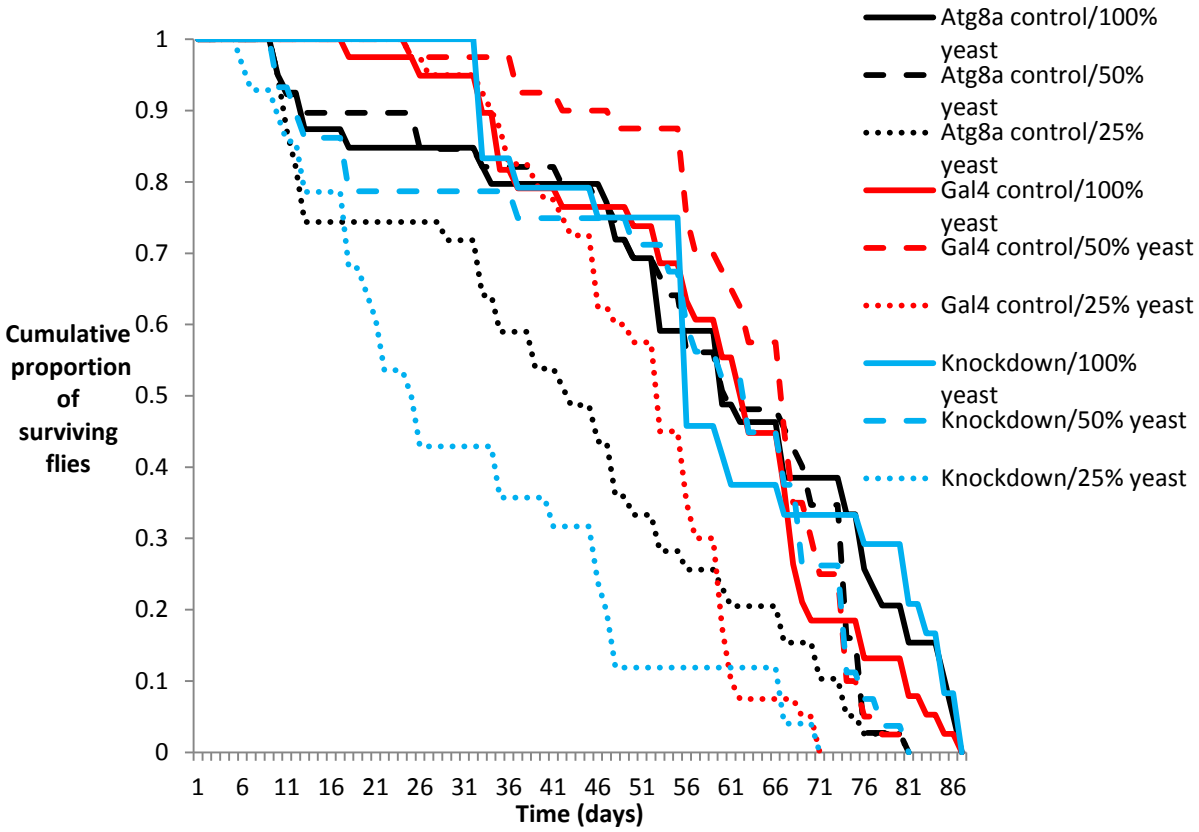


Fig 6.4- Survivorship of once mated *UAS-Atg8a-IR/w*^{1118} (*Atg8a* control)and *Act 5C-Gal4/w*^{1118} (*Gal4* control) and once mated *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females held as adults on diet containing 100% (solid line), 50% (dashed line) or 25% (dotted line) yeast.

As an alternative way of displaying the responses of the different genotypes to diet restriction I also plotted the median lifespan of females on the different food treatments (fig 6.5). This shows more clearly the slight increase in longevity for the females kept on 50% over the 100% diets and the major decrease on the 25% yeast diet. There is a trend for the decrease in lifespan to be more marked for the *UAS-Atg8a-IR/Act 5C-Gal4* knockdown genotype in comparison to the other groups especially the *Act 5C-Gal4/w*^{1118}, but this effect was not significant.

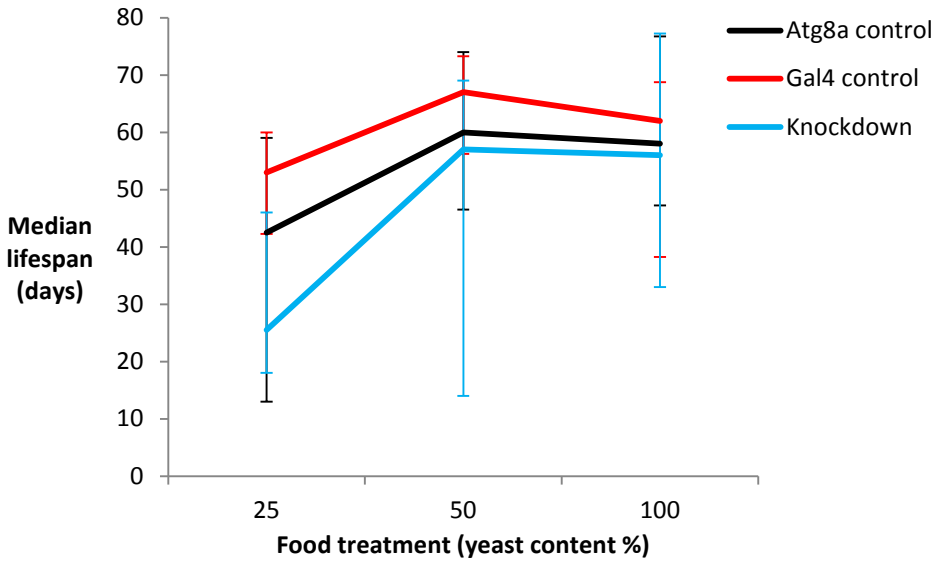


Fig 6.5- Median lifespan (±interquartile range) of once mated *UAS-Atg8a-IR/w*^{1118} (*Atg8a* control) and *Act 5C-Gal4/w*^{1118} (*Gal4* control) and once mated *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females held as adults on diet containing 100%, 50% or 25% yeast.

Age specific fecundity

Investigation of the fecundity data using ANCOVA (as described in chapters 3 & 4) reveals a complicated pattern of fecundity. There was a three way interaction attributable to an initial steeper decline in fecundity followed by a cessation of egg laying over time in *Atg8a* knockdown treatment held on the 25% yeast food ($p=0.05$). The most striking differences arose from interactions between food and time. In general the 50 and 25% diets showed a significant decline in fecundity in comparison to the 100% diet ($p<0.001$). There were also significant differences between the three *Act 5C-Gal4/w*^{1118} controls and the 3 *UAS-Atg8a-IR/Act 5C-Gal4* knockdown treatment groups relative to the *UAS-Atg8a-IR/w*^{1118} control. In addition there was a lower fecundity overall in the 50 and 25% food lines in comparison to the 100% controls.

Age specific analysis of fecundity showed that the protein concentration of the food had a significant effect on the number of eggs laid each day, with significantly more eggs laid by the 100% diet females over the 50% and then 25% diet females ($p=0.004$). There were also differences due to female genotype, particularly on the 100% diet, with the *Act 5C-Gal4/w*^{1118} control and *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females laying significantly more eggs than the *UAS-Atg8a-IR/w*^{1118}

control females (on days 1, 5 and 7, p<0.05, fig 6.6). Overall there was little evidence that *Atg8a* knockdown affected female fecundity in any consistent manner.

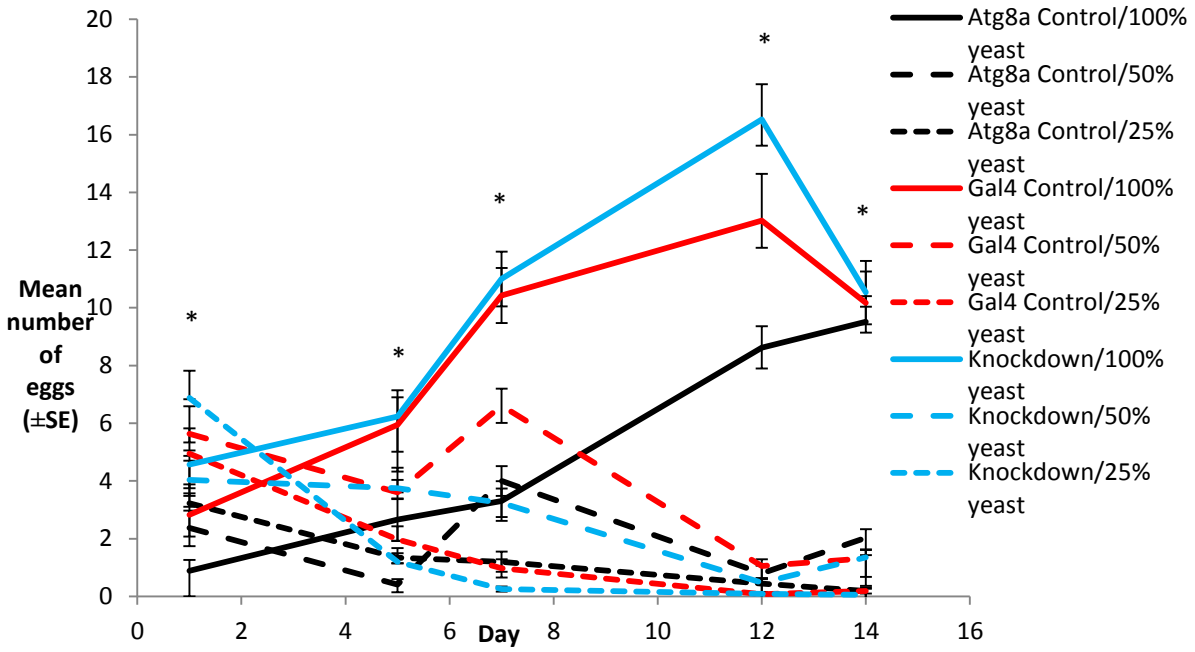


Fig 6.6- Mean number of eggs (±SE) laid by once mated *UAS-Atg8a-IR/w*^{1118} (Atg8a control) *and Act 5C-Gal4/w*^{1118} (Gal4 control) and once mated *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females per 24hrs held on diet containing 100% (solid line), 50% (dashed line) or 25% (dotted line) yeast. * indicates a significant difference in the number of eggs laid by that treatment group all at p<0.05.

Lifetime egg productivity

Analysis of lifetime fecundity data (fig 6.7) revealed significant differences across treatments ($F_{8,301}$=53.711, p<0.001). There was a significant interaction between diet and genotype ($F_{4,301}$=9.913, p<0.001) and also significant main effects of genotype. ($F_{2,301}$=22.471, p<0.001) and diet ($F_{2,301}$=174.102, p<0.001).

Analysis using Tukey post hoc testing showed that the number of eggs laid on the different foods were significant (p≤0.003), with more eggs laid on the 100% than the 50% and then the 25% diets. There were also differences due to female genotype particularly on the 100% diet as described above, with the *Act 5C-Gal4/w*^{1118} control and *UAS-Atg8a-IR/At 5C-Gal4* knockdown females laying more eggs than the *UAS-Atg8a-IR/w*^{1118} control females.

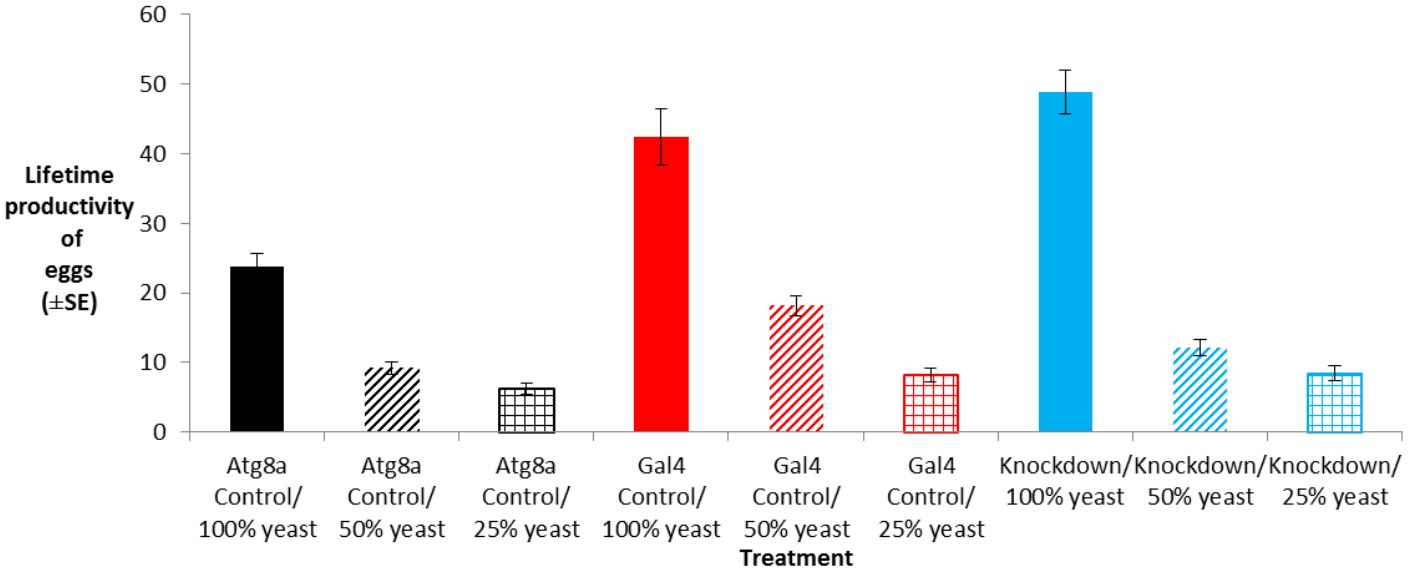


Fig 6.7 – Mean lifetime productivity of eggs (±SE) of once mated *UAS-Atg8a-IR/w*^{1118} (*Atg8a* control) and *Act 5C-Gal4/w*^{1118} (*Gal4* control) and once mated *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females held as adults on diet containing 100% (solid bar), 50% (striped bar) or 25% (checked bar) yeast.

Age specific fertility

The ANCOVA revealed a significant three way interaction, where eggs laid, the type of female (for example UAS-Atg8a-IR/w_{1118}) and time all interacted with each other. Females from the *Atg8a* knockdown treatment held on the 25% food having initially high but then a steep decline in fertility in control and *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females on the 100% food maintaining significantly higher fecundity over time than the *UAS-Atg8a-IR/w*^{1118} controls on 100% food ($p=0.01$).

Further analysis of the age specific patterns of fertility showed that protein concentration of the food had a significant effect on the number of offspring produced on days 5, 7, 12 and 14 (all $p<0.05$), with fewer offspring produced as the amount of dietary protein decreased. Female genotype was found to have a significant effect on the number of viable offspring produced on all days ($p<0.05$, fig 6.8). Overall, there appeared to be a significant fertility advantage for the *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females.

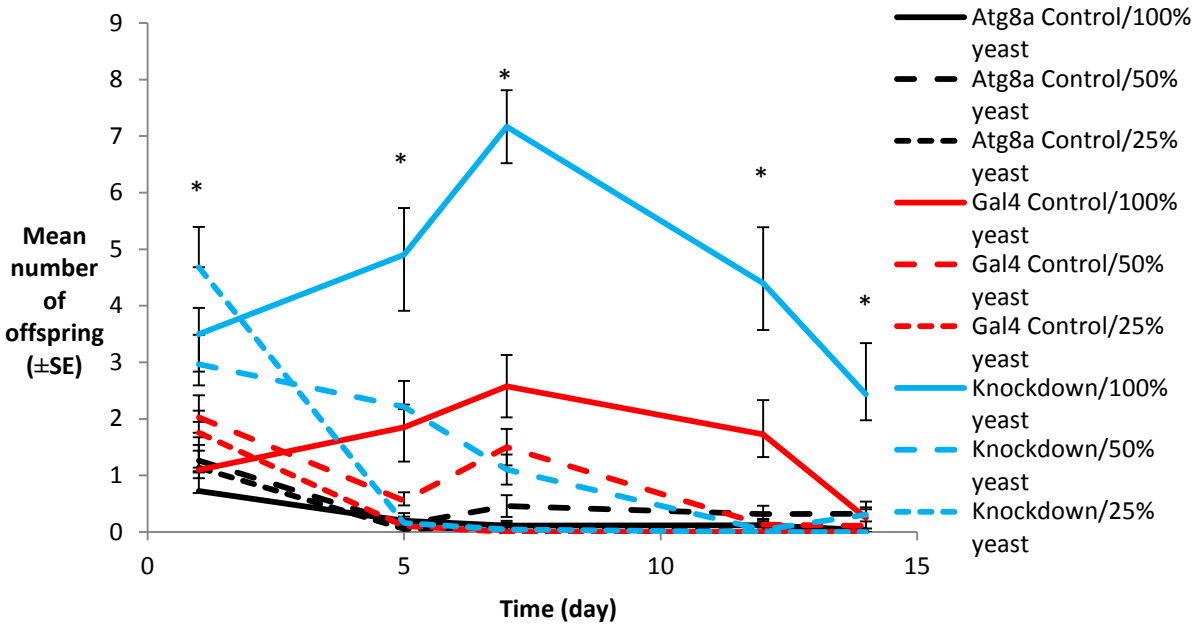


Fig 6.8- Mean number of viable offspring (±SE) from once mated *UAS-Atg8a-IR/w*^{1118} (*Atg8a* control) and *Act 5C-Gal4/w*^{1118} (*Gal4* control) and once mated *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females per 24hrs on diet containing 100% (solid line), 50% (dashed line) or 25% (dotted line) yeast. * indicates a significant difference in the number of eggs laid by that treatment group all at $p<0.05$.

Lifetime offspring productivity

Analyses of lifetime fertility data (fig 6.9) backed up the age specific patterns seen above, namely that *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females produced significantly more offspring (Log Rank X_2=12.585, df=2, $p\leq0.002$). Diet generally had the expected effect, with females on the highest protein food producing more offspring over their lifetimes (Log Rank X_2=76.028, df=2, $p<0.001$), however this was not seen in females with the *UAS-Atg8a-IR/w*^{1118} control genotype.

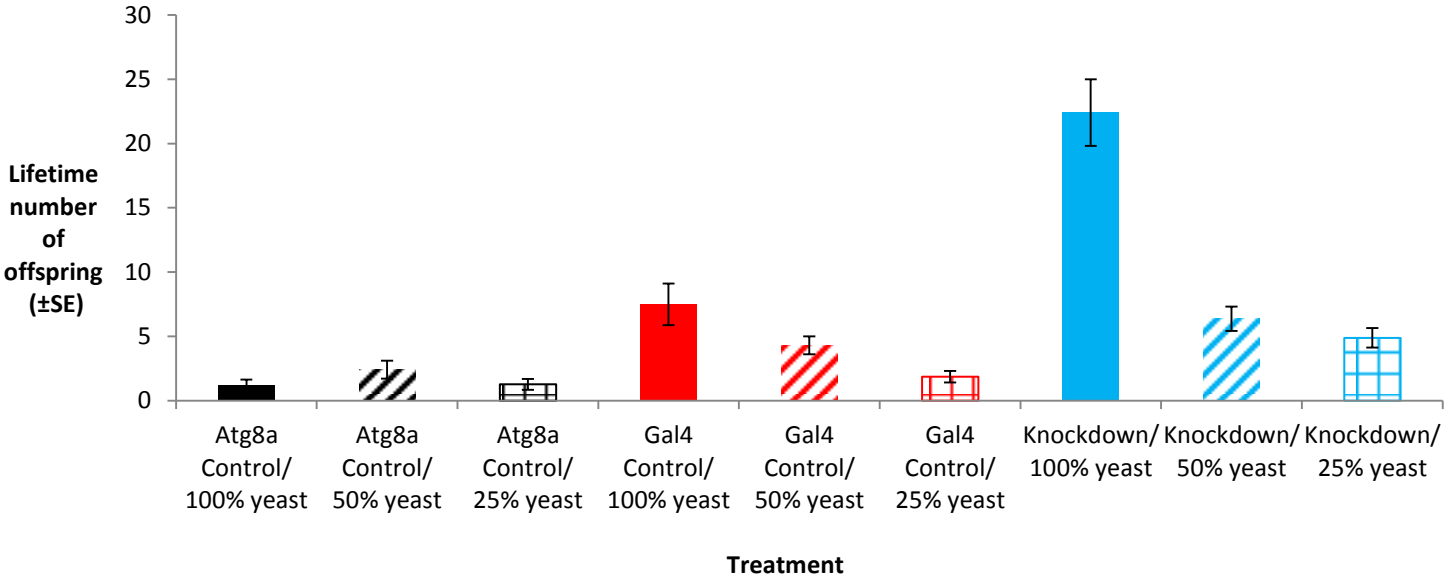


Fig 6.9 – Mean lifetime productivity (±SE) for once mated *UAS-Atg8a-IR/w*^{1118} (*Atg8a* control), *Act 5C-Gal4/w*^{1118} (*Gal4* control) and *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females held as adults on diet containing 100% (solid bar), 50% (striped bar) or 25% (checked bar) yeast.

6.4.3 Validation of *Atg8a* knockdown manipulations by qRT-PCR

I used qRT-PCR to test whether (i) the knockdown genetic manipulation would lead to a reduction in the level of *Atg8a* transcribed, and (ii) a reduction in the amount of yeast in the diet would lead to increased *Atg8a* expression. I assumed that the level of *Atg8a* would indicate autophagy state, with more *Atg8a* meaning more active autophagic processes.

The results from the qRT-PCR for the samples from the first experiment (fig 6.10) show that there was no reduction in the level of *Atg8a* in the knockdown treatment on either diet, relative to the controls ($F_{1,6}$=5.623, p=0.055). However, there was significantly higher *Atg8a* transcription on the 50% diet treatment ($F_{1,6}$=10725, p=0.017, fig 6.12).

In the second experiment there was evidence for significant differences in *Atg8a* expression levels across the different treatments ($F_{2,11}$=4.072, p=0.042, fig 6.11), and there was a significant interaction between treatment and diet ($F_{4,11}$=4.468, p=0.022).

There was a significant effect of diet ($F_{2,11}$=7.766, p=0.008), however this was driven by differences between the *UAS-Atg8a-IR/w*^{1118}control treatments on the different diets (post hoc Tukey tests

p=0.017). Reducing the protein content of the diet therefore had no effect on the level of *Atg8a* transcript detected across the *Act 5C Gal4/w*^{1118} controls and *UAS-Atg8a-IR/Act 5C Gal4* knockdown treatments. There were reduced levels of *Atg8a* transcript in the *UAS-Atg8a-IR/Act 5C Gal4* knockdown females in comparison to the three *Act 5C Gal4/w*^{1118} controls and also in the *UAS-Atg8a-IR/w*^{1118}control on 50% yeast. The *UAS-Atg8a-IR/w*^{1118} females on 100% food unexpectedly, had the lowest levels of *Atg8a* transcript overall. The evidence for *Atg8a* knockdown was therefore subtle and slightly contradictory. The knockdown treatments had lower levels of *Atg8a* transcript than all other controls, except the *UAS- Atg8a-IR/w*^{1118} females on 100% food. Females kept on food containing lower levels of protein tended to express higher levels of *Atg8a,* as expected, but this was only significant among the *Atg8a-IR/w*^{1118} control females.

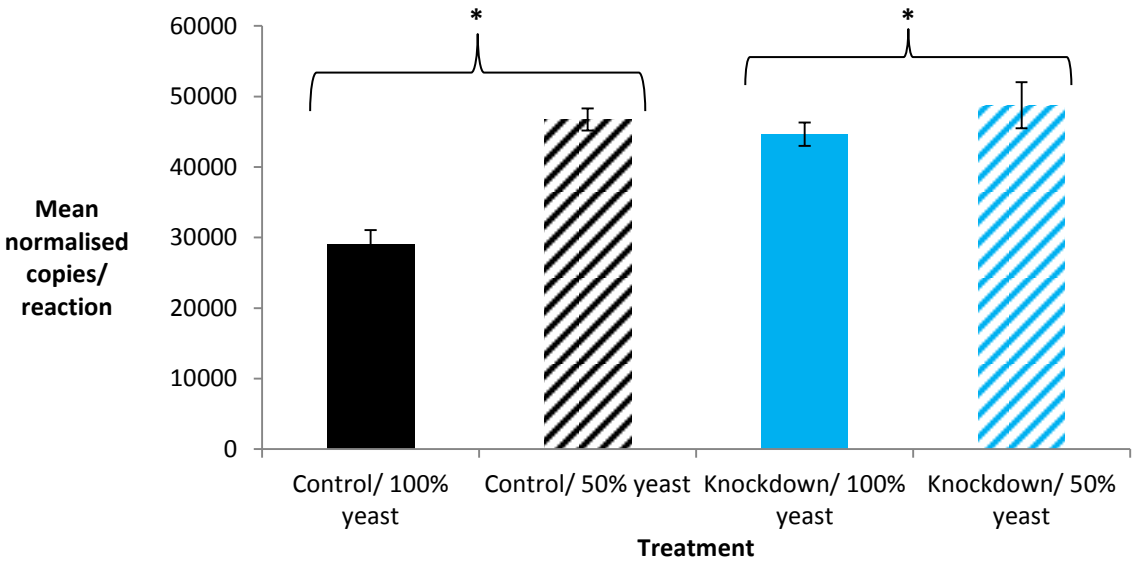


Fig 6.10 – Mean normalised copies/reaction of *Atg8a* (±SE) detected in the qRT-PCR, for once mated *UAS-Atg8a-IR/Cy* control and *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females held as adults on diet containing 100% or 50% yeast. * indicates a significant difference at p<0.05.

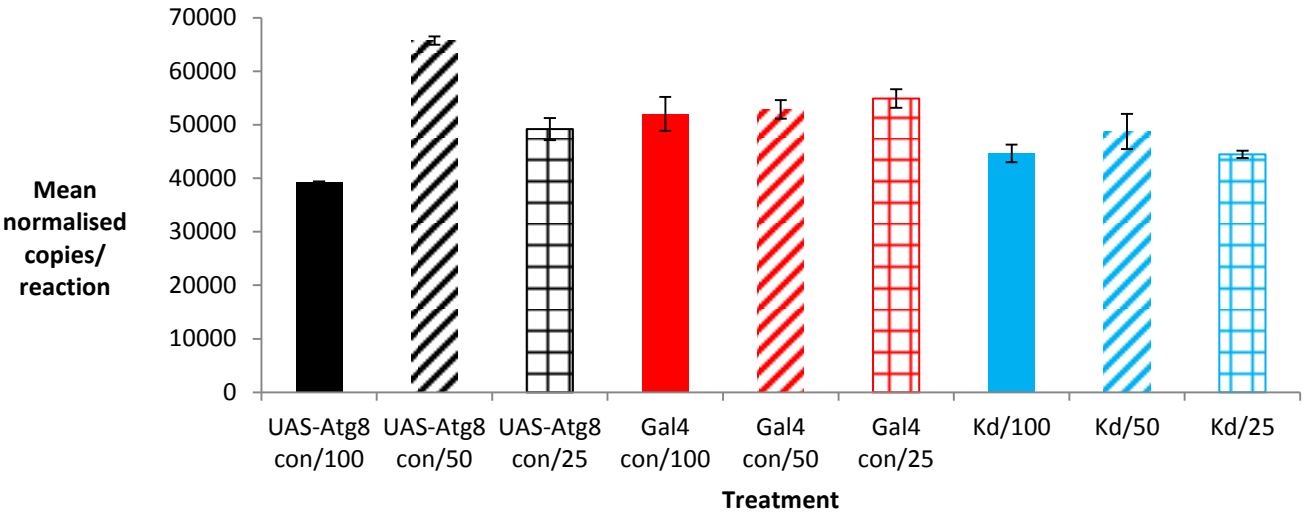


Fig 6.11 – Mean normalised copy number of the *Atg8a* transcript (±SE) for once mated *UAS-Atg8a-IR/w*^{1118} (*Atg8a* control), *Act 5C-Gal4/w*^{1118} (*Gal4* control) and *UAS-Atg8a-IR/Act 5C-Gal4* knockdown females held as adults on diet containing 100% (solid bar), 50% (striped bar) or 25% (checked bar) yeast. Different letters above the bars indicate a significant difference at $p<0.05$.

6.5 Discussion

The results of the initial experiment indicate that lifespan was extended via caloric restriction (females on 50% yeast) and not due to the genetic background of the female or levels of *Atg8a* activation. However, lifespan was extended when levels of *Atg8a* were elevated in the lower yeast diets, lending support to the lifespan extending properties of autophagy activation. The lack of lifespan decrease in the *Atg8a* knockdown females was not unexpected given that genetic manipulations did not apparently lead to any decrease in *Atg8a* expression in this first experiment. In fact the analysis of relative *Atg8a* expression here showed an up-regulation of *Atg8a* in the knockdown females (*UAS-Atg8a-IR*/*Act 5C Gal4*) compared to the control (*UAS-Atg8a-IR/Cy*) indicating autophagy was not knocked down, and was even slightly enhanced. Interestingly, it was the females held on the 50% yeast diet in this experiment that laid more eggs initially but with a greater decline in egg production over time than those on a full 100% yeast diet. Neither the background of the females nor the food made any impact on egg numbers produced overall.

I propose that levels of autophagy may have already been high in the knockdown females due to some internal stress of the genetic manipulation and that the caloric restriction upregulaed both genotypes from the standard level (higher in the knockdown) significantly (to a higher level in the control) and lifespan was extended in both. This autophagy activation may also have caused the surge in egg laying at the beginning of the lifespan in females held on a caloric restricted diet but with a steeper decline in numbers over time.

In the expanded second experiment diet again affected lifespan but this time a reduction in longevity was seen due to caloric restriction on the 25% yeast while females kept on the 50 and 100% diets showed no significant difference in lifespan. Overall the *UAS-Atg8a-IR/Act 5C Gal4* knockdown treatments had the shorter lifespan, irrespective of diet, and this is consistent with the idea that they generally had lower *Atg8a* expression levels than the majority of the controls. Next in survival were the *Atg8a-IR* controls and then the *Gal4* driver control females (fig 6.4), although these differences were not significant. The qRT-PCR analysis revealed highest levels of expression in the *UAS-Atg8a-IR/w^{1118}* control females held on 50% food, but there was some evidence nevertheless for subtle *Atg8a* knockdown in comparison to the majority of the control, though this was partly contradicted by the finding that the lowest levels of *Atg8a* expression overall was found in the *Atg8a-IR* control females on a 100% yeast diet. Egg production for females on the normal diet of 100% yeast was greater than those in the other two lower food treatments. Overall females maintained on the 100% yeast diet laid the most eggs, but it was the *UAS-Atg8a-IR/w^{1118}* control females that had the lowest egg production. The results support the idea that longevity and fecundity are not obligately linked as, independent of *Atg8a* levels, the egg laying pattern was the same. Numbers of offspring, measured in the second experiment were significantly affected by food and genotype. It was anticipated that egg numbers would be low because of the lower diet, females would be expected to put more resources into staying alive than laying eggs. But those females held on the lower calorie diet (25% yeast) produced more offspring than those on the 50% diet, with the lowest numbers from females on a normal diet (100% yeast). The *UAS-Atg8a-IR/Act 5C-Gal4*

knockdown females had higher numbers of offspring than either of the *Act 5C-Gal4/w*^{1118} or *UAS-Atg8a-IR/w*^{1118} control females.

Females held on 25% yeast in all treatment groups had significantly shorter lifespan than those held on the other diets indicating that food on its own has a significant effect on survival. These females also had significantly higher egg and offspring production even though there was no evidence for autophagy activation. It appears that on 25% food a trade-off was made and egg and offspring production was maximised to the detriment of survival but without any impact from autophagy, indicating fecundity and autophagy can be uncoupled as suggested above. However, some evidence of knockdown associated with shortened survival was seen in the *UAS-Atg8a-IR*/*Act 5C Gal4* knockdown females on 25% diet.

The *UAS-Atg8a-IR/w*^{1118} control females on a 50% diet had autophagy activated but lifespan extension was minimal compared to the other groups on 50 and 100% diets. It is only in this control that autophagy has been noticeably activated by diet. Significantly the *UAS-Atg8a-IR/Act 5C-Gal4* knockdown treatment appears not to have been affected by diet which was as expected although levels of *Atg8a* were higher than expected. This could be due to some base line autophagy activation as in the control on 100% diet.

In the initial experiment the control females had the same background as the manipulated females. These flies were then mated to males from the same cohort, which would have included both control (*UAS-Atg8a-IR/Cy*) and knockdown (*UAS-Atg8a-IR*/*Act 5C Gal4*) males. The numbers of eggs laid were low and offspring numbers were almost zero indicating that many of the females may still have been virgin. The extent of the knockdown may have been connected to female reproductive status, hence the results would not have been a robust indication of lifespan in these two treatments. This was taken into account and in the second experiment all females from the different treatment groups were mated with wild type Dahomey males to, as far as possible, ensure

a mating in all the treatment groups and that this was standardised. Offspring numbers were greater in this experiment than the previous one and allowed data collection to take place.

Indications are that although autophagy may have been activated in certain fly cohorts this has not translated into a regular lifespan extension in these flies. But what has been seen is that both fecundity and fertility appear to be controlled, at least partly, seperately from any lifespan extension. Indications are that individuals on the restricted diet are induced to lay more eggs at the beginning of their life while those on the higher food show a more consistent level of egg laying. In the second experiment the females from all the backgrounds lay continually and even increased egg laying as time passed when held on the control food (relevant in comparison to females on 50% food). This again suggests that lifespan extension and fecundity may not be linked.

Diet appears to have had more of an effect on lifespan than the genetic background of the fly. Previous caloric restriction experiments have suggested that the autophagy extending lifespan pathway may be separate to any efffects on fecundity while others have them linked as a trade-off, lifespan extension through autophagy in exchange for a reduction in fecundity of the organism (McPhee and Baehrecke 2009). The restriction of calories in the diet may lower the level of oxidative stress and thus the level of damage, this would decrease age-related declines in function and extend lifespan. Oxidative stress and damage increase during ageing due to an increase in the rate of generation of reactive oxygen species (ROS) a decrease in the mopping up of free radicals and hence an overall increase in the susceptibility of tissues to oxidative damage. The fact that the extension of lifespan is seen across many species when subjected to caloric restriction shows there is a broad relationship between energy intake and ageing. Caloric restriction extends lifespan but it has also been linked to a large array of physiological alterations in rodents which include glucose intolerance and learning, behavioral and immune responses (Sohal and Weindruch 1996).

Interestingly it appears that those flies that had limited autophagy potential didn't live as long as those that were left in the normal state. Although one strain of control flies (*Atg8a*) appeared to

have no extension of lifespan on the 50% diet, and yet up-regulated levels of *Atg8a*, while the other control (*Gal4)* on 50% diet had lifespan extension but no up-regulation of the *Atg8a* gene. Although the overall production of eggs in both these groups were lower than those on the control food (100%) offspring numbers were different. The *Atg8a* control on a 50% diet had up-regulation of *Atg8a* experession with no exension of lifespan and lower egg production but it did have slightly more offspring in comparison to the same genotype on 100% a diet. This suggests that although autophagy activation didn't extend lifespan it may have increased the number of viable offspring instead suggesting autophagy can target other areas not just lifespan extension. This is the first indication that autophagy may not just extend lifespan but may in certain circumstances divert nutrients to reproduction to enhance offspring survival. The *Gal4* control on a 50% diet had no up-regulation of *Atg8a* expression with an extended lifespan and lower egg and offspring production compared to the same genotype on a 100% diet suggesting that lifespan extension was due to a trade-off between fecundity/fertility and survival. It may be that autophagy is unable to be up-regulated in this genotype due to the *Gal4* insertion.

I have shown here indications that a trade-off between lifespan and fecundity may at times not be linked to autophagy activation (first experiment) and that sometimes autophagy may be linked to reproduction to the detriment of survival (second experiment) although further work on both issues is necessary.

Research in the organism *C. elegans* has shown that accelerated ageing of tissues and a reduced lifespan is a consequence of a loss of function mutation in some of the autophagy genes (Toth, Sigmond et al. 2008). This appears to show the importance of autophagy in regulating a normal lifespan. Also in *C. elegans* the knockdown of *Atg7* was shown to reduce lifespan. However, other experiments in which autophagy genes have been knocked down have reported no significant effects on lifespan (Hansen, Chandra et al. 2008; Jia and Levine 2007). This indicates that other factors may be present that can prevent autophagy from activating or that can silence the effects of

autophagy via other pathways. Negative findings could also be due to residual activity of *Atg8a,* which may still produce a significant autophagic flux. Autophagic flux refers to the complete process of autophagy which may still be active to some extent in *Atg8a* knockdown genotypes. Females who have had *Atg8a* knocked down in their system could still have enough *Atg8a* signal to allow autophagic flux, so that lifespan extension is enabled and egg production enhanced. As seen in the initial experiment knockdown *Atg8a* females had higher levels of *Atg8a* than expected indicating autophagy was still able to take place even if at a lower rate.

Although mutations in the *Atg7* gene allow the fruit fly to develop as normal strong *Atg8* hypomorphic mutations are known to be semi lethal. The genetic manipulations shown above may have left heterozygous *Atg8a* mutants in a hypomorphic state and so affected the robustness of the results. *Atg8* mutants are also hypersensitive to starvation and oxidative stress and as a result have in some instances had a shorter lifespan (McPhee and Baehrecke 2009) so treatments on flies to limit lifespan that already suffer from genetic factors that make them hypersensitive and open to shorter lifespan could also affect results. Another effect of this could be that the *UAS-Atg8-IR/w*^{1118} control may have had reduced fertility as seen above.

Also although lifespan extension using *Gal4* drivers has been seen regularly Kapahi et al. (2004) showed that some overexpression of TOR using *Gal4* drivers can prevent eclosion to adulthood (Kapahi, Zid et al. 2004). The down regulation of SK6 may also limit autophagy rates when under conditions of TOR inactivation or extended starvation in order to protect cells from potentially damaging effects of unrestrained autophagy (Scott, Schuldiner et al. 2004). The *Gal4* driver in the *Gal4* control from the second experiment could have affected the fertility of females from this treatment which could be why numbers of offspring in this control were severely limited.

It is proposed that a loss of autophagy controlled by TOR will accelerate ageing, this is expected because autophagy is important in maintaining a healthy cellular environment where damaged proteins and organelles are eliminated (Hands, Proud et al. 2009). However, the experiments

described in this chapter show that autophagy is not consistently turned on or off and may be regulated by a plethora of different factors including genetics and diet.

These two experiments should be used to build upon, they suggest there is some influence of autophagy activation and that caloric restriction doesn't work to up-regulate autophagy when *Atg8a* levels are down-regulated and autophagy is already inhibited. Diet cannot reverse genetically induced inhibition of autophagy. Future work in this area should include experiments where the control and knockdown line is backcrossed to make them more viable and nearer the wild type. qRT-PCR validation should be done at different time points throughout the experiment to see if autophagy levels alter as time passes and fecundity slows or if autophagy is high when fecundity is high at the beginning. Other validation measures could also be used to determine autophagy activation including Western blots as seen in chapters 3 and 4.

Chapter 7 Effect of adult male nutrition on a male's ability to respond to rivals

7.1 Abstract

Research conducted on invertebrates has demonstrated significant effects of diet on reproductive activity in both sexes. Much progress has been made towards understanding the mechanisms underlying the effects of diet and reproduction on survival, much of which has been achieved by studying female *Drosophila melanogaster.* However, there is far less information known for males. In previous chapters (chapters 3, 4, 5 and 6) I investigated the effects of diet and autophagy activation on lifespan and fertility. Here I focused this topic in related tests on males by testing for effects of diet, and diet-induced changes to autophagy, on male reproductive success. The major output I measured to determine male reproductive success was the ability of males held on different diets to respond adaptively to rivals by extending mating duration. I found significant effects of diet on male reproductive performance, which was consistent with results from previous research. The major finding was that males held on a starvation diet (containing agar but no protein or carbohydrate) lost the ability to extend mating following exposure to rivals in comparison to males held on other diets in which protein and/or carbohydrate components were varied. I concluded that both dietary protein and carbohydrate are necessary in the diet in order for males to respond adaptively to rivals. The effect appeared to be largely independent, however, of autophagy as it was not directly linked to the presence or absence of dietary protein, which was the key autophagy activator tested.

7.2 Introduction

Quantitative and qualitative variation in nutrition has a significant influence on reproductive physiology, behaviour and overall reproductive success (Thompson 1999) and specific dietary components are known to be essential for normal metabolism and development (House 1962). Many studies of invertebrates demonstrate effects of diet on reproductive activity of both sexes. For example, both male and female Scorpion flies *Panorpa cognata* copulate more often when fully fed on a non-limiting diet (Engqvist and Sauer 2003). Significant progress has been made towards understanding the effects of diet and reproduction on survival by studying female *D. melanogaster* (Chapman and Partridge 1996). For example on good quality diets a female fruit fly will mate more, produce more eggs, but have a shortened lifespan (Chapman and Partridge 1996; Chippindale, Leroi et al. 1993; Chippindale, Leroi et al. 1997). In male *D. melanogaster*, diet quality and quantity can impact on the ability to gain a mating, and an optimal level of nutrition is required to maximise reproductive success (Fricke, Bretman et al. 2008). Diet can also have direct effects on a male's ability to mate and to transfer sperm. For example, males of the medfly *Ceratitis capitata* fed a low protein diet mate at a lower frequency than those fed on higher protein diets. Protein deprived males transfer more sperm during mating, however the females with which they are paired are quicker to re-mate. This results in reduced reproductive success for the males on the low protein diet, indicating diet is a major factor in determining a male's reproductive success (Blay and Yuval 1997). Diet quality can also influence important reproductive characteristics such as pheromone production and blend, e.g. as shown in the Caribbean fruit fly *Anastrepha suspensa* (Sivinski and Heath 1988).

The effects of diet on reproductive processes are often also linked with changes in other life history traits such as longevity. A huge body of research across many different taxa reveals that a reduction in the amount and type of food consumed can, within certain limits, lead to an extension in lifespan, which is usually accompanied by a reduced rate of reproduction (Kirkwood and Rose 1991; Piper,

Mair et al. 2005; Carey, Harshman et al. 2008). This effect has been consistently observed in work using model organisms such as rodents (Holehan and Merry 1986; Masoro, Shimokawa et al. 1991; Masoro 2005) and *D. melanogaster* fruit flies (Chapman and Partridge 1996). Crucial to a male's overall reproductive success in addition to the ability to mate and transfer sperm described above, is his ability to respond adaptively to the presence of rival males (Bretman, Gage et al. 2011). For example, a male Norway rat (*Rattusnorvegicus)* will ejaculate more sperm in the presence of another male (Pound and Gage 2004) in order to gain higher fertilization success. In an environment in which there is sperm competition as seen in male crickets (*Acheta domesticus* and *Gryllodes supplicans*), sperm transfer to the female is also observed to increase in response to the presence of rival males (Gage and Barnard 1996). Bretman et al. (2009) used *D. melanogaster* to investigate male responses to rivals and found that when males were kept with other males prior to mating, the duration of their subsequent mating increased significantly (Bretman, Frickle et al. 2009). Increasing the absolute number of rivals had little effect on mating duration. However the length of exposure to rivals had a significant effect, the longer the period of confinement with rivals, the longer the males' subsequent mating duration (Bretman, Frickle et al. 2010). It was also shown that males detected the presence of rival males using a combination of auditory, visual, olfactory or tactile mechanisms (Bretman, Westmancoat et al. 2011). Whilst these effects are known to be important in determining a male's fitness, nothing is yet known about how they interact with diet, which is the topic of investigation in this chapter. In addition given that lack of nutrients, particularly proteins, is associated with the induction of autophagy (Kaeberlein and Kennedy 2008), I hypothesized that any effects of diet on reproductive success in males might be mediated via changes in the activity of autophagic processes.

Diet could interact with a male's ability to respond to rivals via the effects of any trade-offs that may be present. This could happen if a reduction in dietary resources leads organisms to shift energy to survival at the expense of reproductive processes, thereby limiting the options for optimal allocation

of resources to reproduction. The frequent reports of evolutionary trade-offs between reproduction and survival, for example in *D. melanogaster* (Chippindale, Leroi et al. 1993) and in rodents (Holehan and Merry 1985), means that such mechanisms could, in theory, be common. The data are consistent with this idea, i.e. that a shift in resources away from reproduction to the survival of adults under dietary restriction may alter the degree to which a male can respond to rivals (Aluja, Jacome et al. 2001; Fricke, Bretman et al. 2008). Therefore I predicted that restriction of dietary nutrients generally would have a significant negative impact on the ability of a male to allocate resources towards a range of different reproductive processes.

I tested for the effects of adult nutrition on the ability of males to respond to rivals by extending their mating duration. I predicted that nutrient shortage would lead to a diminution in this male response. I then tested for effects of specific protein and carbohydrate dietary components (yeast versus sugar, and a starvation diet) on the responses observed. This also allowed me to determine whether autophagy was associated with any reproductive benefits to males. Autophagy-mediated benefits should be specific to treatments that varied the presence or absence of protein. I therefore predicted that if reduction in the amount of protein specifically affected a male's ability to respond to rivals, that this effect might be mediated by autophagy.

7.3 Materials and Methods

7.3.1 The effect of variation in dietary protein and carbohydrate on male responses to rivals after 7 days of diet exposure

Experiments were conducted to determine if there were differences in male mating success and male responses to rivals when major components of their diet were manipulated. Both protein and carbohydrate levels were altered by varying the amount of yeast and sugar, respectively in the diet. Starting concentrations of protein (yeast) and carbohydrates (sugar) used in the standard diets were:

100% yeast (100g yeast per 1 litre of food) and 100% sugar = 75g sugar per 1 litre of food.

Fly rearing was carried out as in the general materials and methods (chapter 2). Males and females were collected as virgins at eclosion using ice anaesthesia. Females were housed in groups of 50 on a normal yeast diet, supplemented with added yeast granules until use in the experiment. Males were collected in groups of ten and housed on a diet of normal SYA for 2 days to allow their reproductive systems to fully develop. This procedure was adopted because I aimed to test the effect of diet-mediated resource allocation decisions, not effects of the diets on the failure to fully develop an intact reproductive system following eclosion. Males were then randomly allocated to one of four different diet treatments and placed either singly or with rivals, in groups of 4, for 7 days until mating.

For the mating tests, females were aspirated into vials containing agar. For the single male (no rival treatments) then one male per vial was used for the mating tests, for the plus rival treatment, one male of the four housed together was randomly chosen for the mating tests. An agar only diet was used as the medium in the mating tests vials in order to prevent any immediate responses of males held on poor diets prior to mating to a better quality diet during the mating tests themselves.

The effect of variation in protein and carbohydrate intake on male mating success and response to rivals was determined by altering the male's dietary intake of yeast and sugar, respectively. In the first experiment males were kept in 4 different nutritional environments to test the effect of varying protein (yeast) concentration in the absence of a carbohydrate (sugar) source: the control (100% yeast & 100% sugar), a diet with normal yeast but no sugar (100% yeast & 0% sugar) a diet with low yeast and no sugar (20% yeast & 0% sugar) and an agar only diet (0% yeast & 0% sugar).

Males were allocated at random to one of the 4 food treatment groups and placed in vials either singly or with rivals for 7 days until mating.

In the second experiment males were placed in one of 4 nutritional treatments to test the effect of varying carbohydrate (sugar) concentration in the absence of a protein (yeast) source: the control (100% yeast & 100% sugar), a diet with normal sugar but no yeast (100% sugar & 0% yeast), a low sugar diet with no yeast (20% sugar & 0% yeast) and an agar only diet (0% sugar & 0% yeast).

As above, males were allocated at random to one of the 4 food treatment groups and placed in vials either singly or with rivals for 7 days until mating.

For the mating tests in both of the above experiments, a single male and female were aspirated into agar only mating vials. Flies that didn't mate within 3 hours were discarded as were matings which lasted less than 5 minutes. The introduction time and start and finish times of mating were recorded to the nearest minute.

7.3.2 The effect of variation of dietary protein and carbohydrate on male responses to rivals after 5 days of diet exposure

Mortality was high when the males in the two above experiments were held with no food (i.e. agar base only) for 7 days, so an additional experiment was also conducted in which males were held for 5 days in their respective diet treatments. In addition, unlike in the two experiments above, this third experiment tested the effect of varying either yeast or sugar levels against a standard quantity of the other dietary components. Males were therefore allocated randomly to one of 4 dietary treatments: the control (100% yeast, 100% sugar), a diet with no yeast (0% yeast, 100% sugar), a diet with no sugar (100% yeast, 0% sugar) and an agar only diet (0% yeast, 0% sugar).

The same procedure was conducted for the mating tests as described previously. A single male and female were aspirated into vials containing agar. Flies that didn't mate within 3 hours were discarded as were matings which lasted less than 5 minutes. The introduction time and start and finish times of mating were recorded to the nearest minute.

7.3.3 Statistical analysis

Statistical analysis was performed using SPSS v 16& 18 (SPSS 2009). Mating latency and mating duration data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. If data distributions were not normal, the data were logged to improve normality (Log x10). Data from mating duration and mating latency were then subjected to univariate ANOVA. Correlations between mating duration and the time to mating using the Pearson's correlation test were tested for.

7.4 Results

7.4.1 The effect of variation in dietary protein and carbohydrate on male responses to rivals after 7 days of diet exposure

In the first experiment, which varied the amount of protein in the diet against a background of no sugar, there was a significant effect of diet on mating latency ($F_{3,227}$=4.489, p=0.004, fig 7.1a). Males held on normal yeast levels but with no sugar took significantly longer to mate. There were no significant differences in mating latency for males kept with or without rivals ($F_{1,227}$=0.097, p=0.755)

There was no significant effect of diet in this first experiment on overall mating duration ($F_{1,227}$=0.898, p=0.443, fig 7.1b). However, as expected, males exposed to rivals prior to mating mated or significantly longer overall ($F_{1,227}$=8.418, p=0.004). The one exception to that, however, was for males maintained on the no food (agar only) diet prior to mating. Hence males kept on diets containing with no protein or carbohydrate prior to mating did not respond to the presence of rivals (fig 7.1b).

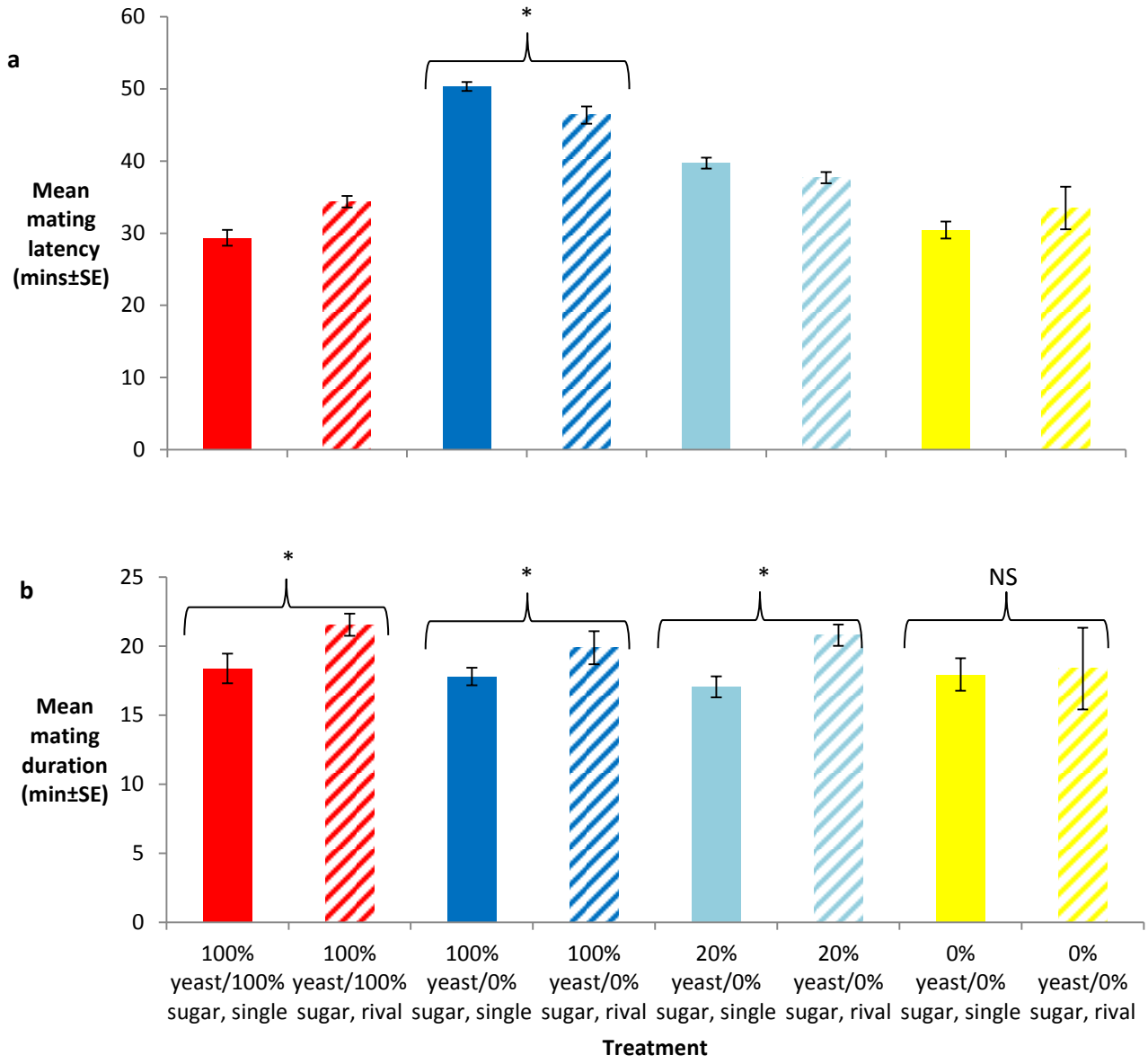


Fig 7.1 - Mean mating latency (a) and duration (b) (±SE) of males exposed for 7 days to variation in protein and carbohydrate components of the diet. In this first experiment, the effect of varying protein (yeast) against a background of no dietary carbohydrate (sugar) was tested. Diets were **1.** 100% yeast+100% sugar, **2.** 100% yeast+0% sugar, **3.** 20% yeast+0% sugar, **4.** 0% yeast+0% sugar. Males on each diet were kept either in the presence (striped bar) or absence (filled bar) of rivals prior to mating. (*) indicates a significant difference p=0.004, (NS) indicates no significant difference.

In the second experiment, which varied the amount of carbohydrate (sugar) in the diet against background of no protein (yeast), there was no effect of varying diet on mating latency ($F_{3,164}$=1.249, p=0.294, fig 7.2a). Male mating latencies when held on agar only diets were not significant ($F_{1,164}$=0.161, p=0.689).

There was, as above, no overall effect of variation in diets on mating duration ($F_{3,164}$=0.874, p=0.456). Mating duration was again significantly extended following exposure to a rival prior to mating ($F_{1,164}$=20.571, p<0.001). As above, the exception was for males held on an agar only diet, though the effect was more subtle than in the first experiment. Consistent with this, interaction between diet and rivals treatment were not significant ($F_{3,164}$=2.236, p=0.086). Hence as above, males maintained on diets containing no protein or carbohydrate prior to mating showed diminished responses to the presence of rivals (fig 7.2b).

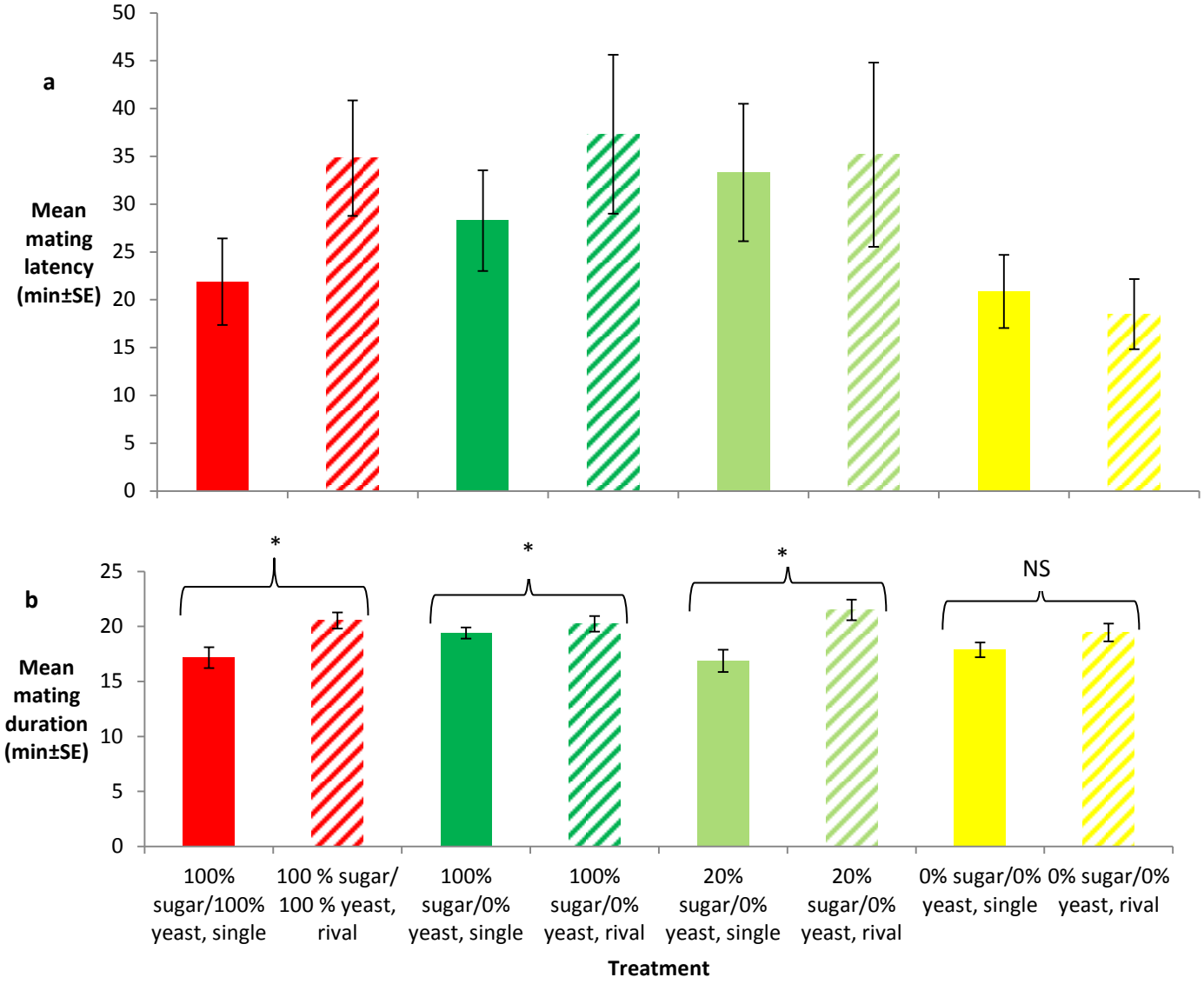


Fig 7.2 – Mean mating latency (a) and duration (b) (±SE) in males held for 7 days prior to mating on diets varying in protein and carbohydrates. In this second experiment, the effect of varying carbohydrate (sugar) against a background of no dietary protein (yeast) was tested. Diets were **1.** 100% yeast+100% sugar, **2.** 0% yeast+ 100% sugar, **3.** 0% yeast+20% sugar, **4.** 0% yeast+0% sugar diet. Males were held on each diet either in the presence (striped bars) or absence (filled bars) of rivals prior to mating. (*) indicates a significant difference $p<0.001$, NS indicates no significant difference.

The results of these two experiments suggest that there is variation in mating latency when males are held on different diets, but that this formed no consistent pattern with respect to diets or the presence or absence of rivals prior to mating.

There was also no overall effect of diet on mating duration. However those males held on agar only diets prior to mating lost the ability to extend mating duration following exposure to rivals.

7.4.2 The effect of variation in dietary protein and carbohydrate on male responses to rivals after 5 days of diet exposure

In this third experiment the presence and absence of protein and carbohydrate were varied against standard levels of other dietary components. Overall, there were no significant differences in mating latency between the 4 different diet treatments when males were held for 5 days on the different diets ($F_{3,289}$=0.303, p=0.824, fig 7.3a). There was also no overall significant difference in mating latency dependent on whether the males were held singly or kept with rivals prior to mating ($F_{3,289}$=1.113, p=0.292). On the normal diet, latency appeared shorter when males had been exposed to rivals prior to mating, but this effect was not significant and there was no overall interaction between diet and rival treatment ($F_{3,289}$=1.063, p=0.365). Consistent with the first two experiments above, there was no significant effect of diet on overall mating duration ($F_{3,289}$=2.785, p=0.41).

There was however, as expected, a significant overall effect of rival treatment ($F_{1,289}$=25.923, p<0.001, fig 7.3b), except, again as above, for the agar only treatment (significant interaction between diet and the rivals treatment, $F_{3,289}$=3.016, p=0.030). Consistent with the above results, therefore males held on the agar only diet lost the ability to respond to rivals by extending mating duration.

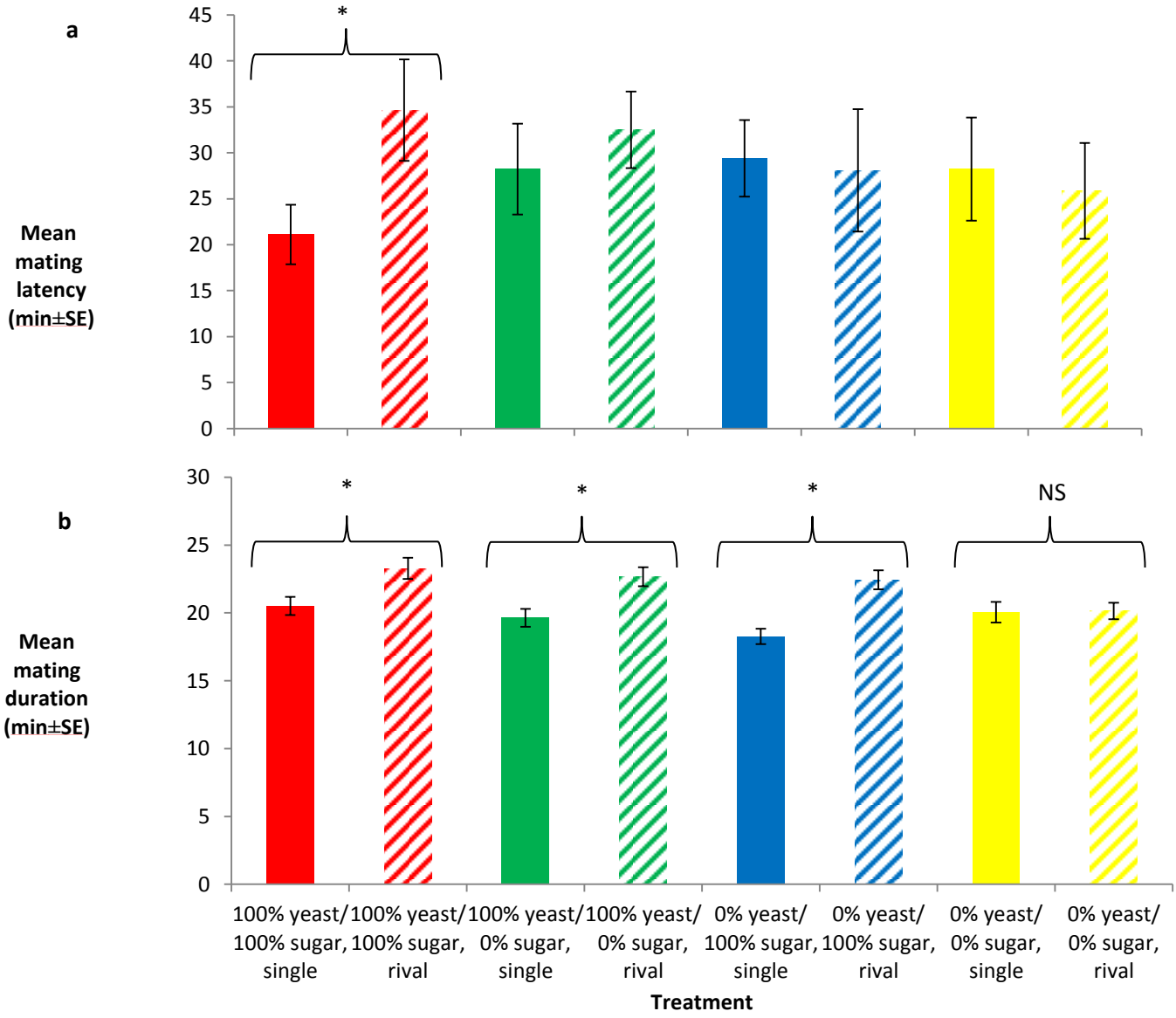


Fig 7.3 - Mean mating latency (a) and mating duration (b) (±SE) of males held for 5 days on diets varying in protein and carbohydrates. In this third experiment, the effect of varying protein (yeast) and carbohydrate (sugar) against a background of standard levels of other dietary components was tested. Diets were **1.** 100% yeast: 100% sugar, **2.** 100% yeast: 0% sugar, **3.** 0% yeast: 100% sugar, **4.** 0% yeast: 0% sugar diet. Males on each diet were kept either in the presence (4 male per vial, striped) or absence (1 male per vial, block colour) of rivals prior to mating. (*) indicates a significant difference $p<0.05$, NS indicates no significant difference.

7.5 Discussion

The consistent finding from the three experiments presented in this chapter is that males held on a starvation, agar only, diet for at least 5 days prior to mating did not respond to the presence of rivals by extending mating duration as occurred on all the other diets tested. A male's reproductive success depends on this ability to respond adaptively to rival males, prolonged copulation enhances male fitness by increasing success in sperm competition and by decreasing a mate's receptiveness to rival males (Bretman, Frickle et al. 2010). Males maintained on poor diets prior to mating presumably had few resources to allocate to reproduction, or what resources they had were traded off against the need to enhance survival.

I also observed significant effects of diet on reproductive performance, males held on normal yeast levels but with no sugar took significantly longer to mate, consistent with results from other species. For example, Mediterranean fruit flies fed low levels of protein copulate less frequently than those fed a higher protein diet (Blay and Yuval 1997). In some circumstances prey deprivation or an inadequate diet can lead to a partial or complete cessation of mating activity (Anderson and Franks 2001; Perez-Staples, Aluja et al. 2008). Adult Tephritid fruit flies need to ingest carbohydrates and water constantly to survive, and protein is required to attain sexual maturity (Aluja, Jacome et al. 2001). In the Mormon cricket *Anabrus simplex* the number of sexually active males decreases when males are held on a nutritionally poor diet (Gwynne 1993). Food intake has also been shown to have a positive effect on testis size and to influence mating duration in yellow dung flies, (*Scatophaga stercoraria),* (Ward and Simmons 1991). Males of the calliphorid fly (*Phormia regina*) have a high reproductive success rate when fed high levels of protein comparison to protein deprived males (Stoffolano, Tobin et al. 1995). Blay and Yuval (1997) concluded that diet is an important factor determining a male's reproductive success in the Mediterranean fruit fly (*Ceratitiscapitata*) (Blay and Yuval 1997). Shelly et al. (2002) suggests that diet can even affect the attractiveness of male Mediterranean fruit flies by altering their pheromone signalling (Shelly, Kennelly et al. 2002).

Nutritional studies have been performed on *D. melanogaster* using both yeast and sucrose. Yeast extract has been found to be detrimental to female fecundity and lifespan in high concentrations (Bass, Grandison et al. 2007). Dietary restriction has been shown to give rise to increased longevity but also reduced fecundity, and hence has strong effects on female reproductive traits (Chippindale, Leroi et al. 1997). Although there has been a lot less work done on the effect of nutrition in males in general, it has been reported that there is an increase in reproductive output with increasing quality of diet (Carey, Harshman et al. 2008). The results of my investigations here support the idea that diet has important effects on male reproductive success. I showed that diet can affect the ability of males to respond to rivals and that exposure of males held on normal levels of yeast and sucrose significantly slows the time taken to start mating.

The first two experiments exposed males to varying diets for 7 days prior to mating. This resulted in high mortality and hence fewer males remaining in each cohort to test. This prompted changes to the protocol in the third experiment where males were exposed to the diet treatments for just 5 days. The observation that, males were near to starvation at the time of the mating tests in the males exposed to the different diets for 5 or 7 days is consistent with the idea that, on the agar only diet there are few resources to allocate to different reproductive processes. Hence the lack of adaptive responses to rivals most likely arises due to the lack of resources in the system rather than reproduction losing out in a trade-off of nutrients with survival. Many males from the initial two experiments that survived on poor diets died immediately following copulation, which is also consistent with the reported detrimental effect of copulation on starvation resistance (Zwaan, Bijlsma et al. 1991).

Important to many species in which there is competition between males is the ability to succeed in sperm competition. Examples of traits selected in this context include nuptial feeding and sperm partitioning between different females. However a male whose food source is restricted will have

limited options in terms of energy to invest in copulation, sperm competition or offspring production (Bass, Grandison et al. 2007). Consistent with this, male Mediterranean fruit flies fed low levels of protein have a lower frequency of copulation and their mates have reduced fecundity. However, although protein deprived males transfer more sperm to their mates than those on the higher protein diets their mates re-mated again more quickly. This indicates that diet is a major factor in determining a male's reproductive success in competitive interactions with other males (Blay and Yuval 1997; Bass, Grandison et al. 2007). Responding to rivals in general will have costs and males that have no resources will be less able to carry those costs even if they could potentially respond adaptively.

A lack of protein in the diet is associated with the induction of autophagy (Yang and Klionsky 2010) (see also my analysis of autophagy in unfed males from chapter 3). However the differences I observed due to diet were not dependent on the specific presence or absence of protein, hence it is unlikely that any effects observed in this chapter were due to the activation or inactivation of autophagy. It has been shown that although autophagy can free up resources for the cell, there are other factors required for the autophagic process to activate. Autophagy can be activated via caloric restriction in the absence of malnutrition, but in these experiments the flies in some treatments (e.g. 5-7 days without food) would probably have been malnourished and so unable to be rescued by the activation of autophagy (Hansen, Chandra et al. 2008). Interestingly it is these malnourished flies that could not adjust to the presence of rivals prior to mating. Effects of diet on mating latency could be due to male or female derived effects (Bretman, Westmancoat et al. 2013). Diet could interact directly with male courtship behaviour, or males held on different diets could be more or less attractive to females. Males exposed to rivals may become less attractive as males because these males will produce and transfer more seminal fluid during mating, which can be costly to the female (Wigby, Sirot et al. 2009). In contrast Bretman et al. (2013) show that extended mating

duration in response to rivals is primarily determined by male-derived effects (Bretman, Westmancoat et al. 2013).

In future work it would be interesting to determine if the transfer of seminal fluid is equivalent in the agar only treatment group to that which occurs in the other diet treatments. It would also be interesting to determine the longevity, fecundity and fertility of males held upon different food treatments. In the scorpionfly (*Panorpa cognata*) higher nutrient availability for males can increase copulation frequency, and cause females to become reproductively active at a younger age (Engqvist and Sauer 2003). Further studies need to be conducted to investigate the effects of different diets and dietary components across the entire life cycle, *Anastrepha ludens* (Mexican fruit fly) reared in an environment with low availability of adult food resources develops a different metabolism, or an efficient way of conserving energy which results in a lack of effect on copulation (Aluja, Jacome et al. 2001). It would also be useful to determine if, over generations, there are any evolutionary changes in behaviour to compensate for the prevailing diets experienced.

Chapter 8 Conclusion

The main themes of this thesis were to determine whether pro- and anti-ageing signals from the reproductive system could mitigate damage occurring due to reproduction and so extend healthy lifespan. Firstly the thesis examined whether autophagy was activated using pharmacological activators and genetic manipulations, it then determined, if autophagy was activated, whether this affected survival. This thesis also determined if either of these manipulations affected fecundity and fertility, if trade-offs were made between survival and reproduction and how nutrition affected survival, fecundity and fertility.

Next this work determined whether the overexpression of autophagy gene *Atg8a* could extend lifespan and its effects on fecundity and fertility. This was followed by an experiment to knockdown expression of *Atg8a* to determine whether autophagy could subsequently be activated in flies with limited *Atg8a* expression. Diet was also manipulated to determine whether caloric restriction could rescue these flies and extend their lifespan. Trade-offs between survival and nutrition were also examined. Finally examined was a male's ability to detect and respond to rivals on different nutritional diets to determine if a male's response to a rival can be affected by nutrition. Below the main conclusions from each subject area are outlined followed by the overall conclusion, and a discussion of the wider context and potential future work.

Effects of pharmacological activators of autophagy on survival, fecundity and fertility

The effects of activating autophagy via the pharmacological application of spermidine through dietary intake were inconsistent (chapter 3). Overall spermidine administered in this way was found to have no effect on lifespan and at times was seen to decrease it. The effect of dietary spermidine was dependent on the reproductive status of males and females. Effects on life history traits in once mated males and in females continually exposed to males were considered at least in part

attributable to the activation of autophagy, which was observed to have been activated in the Western blot analysis. However, any effects of spermidine on the life history traits of once mated females were mediated by other pathways. This also indicates that damage incurred as a result of reproductive activities in the continually mated females might potentially be rescued by autophagy.

Activating autophagy via dietary manipulation using the pharmacological application of Torin1 showed evidence of significant lifespan extension (chapter 4). The effect was seen in all experimental groups, in once mated females and males, and females that were held continuously with males. A significant dose response was also seen as longevity increased as levels of dietary Torin1 were increased. Autophagy was activated in all groups with higher levels of autophagy observed in once mated females. It appears that those females exposed to males continuously had autophagy activated but not to the same extent as in once mated females, indicating a partial recovery of damage for this cohort from reproduction when autophagy was activated using dietary Torin1. Overall there was no evidence for a trade-off between longevity and fecundity or fertility in any of the experimental groups. The enhanced number of eggs laid by females fed Torin1 appeared to have no fitness value to the female (as it was not accompanied by increased fertility). So there was no higher fertility in Torin1 fed females but no trade-off either.

Rapamycin was the first chemical found that affects the TOR pathway, hence the name (Target of Rapamycin). Rapamycin potentially induces autophagy but it may also affect ageing though its capacity to supress inflammatory and autoimmune processes that have a negative effect on longevity (Rubinsztein, Marino et al. 2011). This may also be the case for spermidine as chapter 3 has shown inconsistent results in longevity when using this in the diet. The fact that spermidine may at times increase but at others decrease lifespan, and that Torin1 has been shown to extend lifespan in some instances (chapter 4) suggests that introducing chemicals into the body can affect many different biological processes. It appears these can be both beneficial and detrimental so I suggest much more research is needed before the miracle drug for a longer healthier life can be unveiled.

Effects of the overexpression of autophagy via genetic manipulation of the autophagy 8a gene on survival, fecundity and fertility

Atg8a was overexpressed in three different tissues to determine if lifespan could be extended via the activation of autophagy (chapter 5). In the different tissues I also tested for associated effects on fecundity and fertility, which have not previously been investigated.

No lifespan extension was seen in the neuronally *Atg8a* overexpressed flies but I was also unable to validate, using qRT-PCR, the overexpression of *Atg8a* in this group. No lifespan extension was seen in ubiquitously overexpressed *Atg8a* females although *Atg8a* was seen to be up-regulated following qRT-PCR tests. Because autophagy plays a significant role in mitigating reproductive damage it was expected that, ovary overexpressed *Atg8a* treatment females would show longer lifespan, or increased fecundity/fertility. These effects were not seen, however, this group also showed no *Atg8a* overexpression and therefore there was limited evidence that autophagy had been activated. The *UAS-Atg8a* control genotype tended to have the highest fecundity, fertility and lifespan.

Effects of knockdown in expression of the autophagy 8a gene and dietary manipulation on survival, fecundity and fertility

The interaction between diet restriction and autophagy in extending lifespan and any associated effects on fertility was investigated (chapter 6). The *Atg8a* gene was down-regulated via a knockdown *Gal4* driver to determine whether caloric restriction could lead to extended life as has been seen in wild type females. Predictions were that females unable to activate autophagy would see no lifespan extension, in comparison to wild type controls, on a lowered protein intake. Effects on fecundity and fertility were also analysed using egg production to determine if switching off autophagy would have pleiotropic effects.

There was evidence that autophagy was activated as expected in some treatment groups but not all. However, this was not translated into lifespan extension in these flies. Both fecunduty and fertility appear to be controlled, at least partly, independently from lifespan, as often increased fertility combines with extended lifespan. It appears those females held on a restricted diet are induced to lay more eggs at the beginning of their life while those on the control food were more consistent in their egg laying over time. It appears that the diet and not the genetic background of the fly had an effect on lifespan. Data analysed here agrees with previous caloric restriction research, that the autophagy extending lifespan pathway may be separate to any efffects on fecundity (McPhee and Baehrecke 2009). However there is still the possibility that there may be a trade-off between longevity and fecundity, as independent of autophagy activation those females held on 25% food had shorter lifespan but enhanced reproduction.

Effects of adult male nutrition on mating success and the ability to respond to rivals

The results of my experiments in chapter 7 indicate that males held on a starvation diet with no protein or carbohydrate for at least 5 days prior to mating did not respond to the presence of rivals as they did on other diets. This suggests that males require both protein and carbohydrate in their diet in order to make the adjustment in behaviour to rivals. In addition males were slower to mate when held with rivals but only when maintained on a full food diet containing both protein and carbohydrate.

Overall conclusions

Autophagy is a fundamentally important, conserved and complex process, with links to many different cellular pathways. Overall, autophagy was quite difficult to manipulate cleanly and consistently, I suggest that much more research into the different effectors of autophagy could usefully be undertaken. Pharmacological activators in some instances have been shown to work

successfully when added to the diet of *D. melanogaster*. Spermidine has recently been shown to be essential for mating in yeast as well as egg fertilization in *C. elegans* (Bauer, Carmona-Gutierrez et al. 2013) but has shown itself to have unreliable effects on lifespan, including some that are detrimental (as shown by my results). Lifespan was extended with Torin1, which appeared to be a reliable activator of autophagy and also appeared from these few experiments to show no trade-off effects with fecundity or fertility. The results observed for the spermidine activator of autophagy were inconsistent, from no effect to a detrimental effect. I have concluded that spermidine is an inconsistent 'dirty' activator of autophagy while Torin1 is a reliable 'clean' activator of autophagy. Obviously much more investigation is required, as to my knowledge, my research here is the first to deliver Torin1 to the whole multicellular organism. The enhanced number of eggs (although not more fertile eggs) laid while females were held on Torin1 suggests that although there is no fitness value for the female as the lifespan extension is achieved without noticeable damage, future work in this area is necessary. The Torin1 work should be followed by testing on fecundity and fertility implications of adding Torin1 to the diet whether the results are autophagy dependent or independent. If the effects of Torin1 on fecundity or fertility are not linked to autophagy then it may be used to manipulate fecundity without a detrimental effect on lifespan. These manipulations may have fertility treatment implications, such as the enhancement of fertility in an organism without damaging lifespan expectation and without up regulating the autophagic pathway.

Interestingly overexpression of the *Atg8a* gene didn't appear to extend lifespan. The control genotype tended to have the highest lifespan, fecundity and fertility suggesting that other genetic manipulations employed may have, in combination, had unknown deleterious effects.
Caloric restriction, but not knockdown of *Atg8a* expression was found to affect fecundity. Females held on the highest calorie diet attained higher numbers of eggs overall, but it was the lower calorie diet of 25% yeast that had the higher numbers of viable offspring. This seems to indicate that genetic manipulation of the *Atg8a* gene has no effect on fecundity and fertility but that dietary

manipulations do. Male nutrition was also found to be important to mating ability but was not due to any activation or inactivation of autophagy. A trade-off between survival and reproduction is indicated between males on a starvation diet as no response to rivals was seen.

Data presented here underscores the idea that the induction of autophagy is universally required for lifespan extension by caloric restriction and by the pharmacological agent Torin1. Further study is needed to determine the efficiency of pharmacological agents to activate autophagy and the side effects of each, whether beneficial or detrimental. For example, my work suggests that when autophagy is activated reproductive damage may be mitigated to a certain extent via the pharmacological intervention of Torin1. Overexpression and knockdown models are required to determine which *Atg* genes are important for autophagy and at which point, although more research on achieving consistent activation is needed. These results suggest further research is required to determine which, if any, genetic manipulations designed to increase longevity do so independently of autophagy.

Conclusions set in a wider context

As the human population reaches ever older ages, the study of ageing is becoming increasing prominent across many areas of research. The demographics of the world are changing rapidly with an increasing abundance of elderly people in the population and a declining number of working age individuals to support them. The population as a whole is getting older and with this increase comes increases in chronic diseases and physical and mental issues that negatively impact their quality of life. It is ageing itself that is the leading risk factor for many diseases that increasingly dominate the older generation. Hayflick wrote in 1998 that no one over the age of 80 will actually die from what is written on their death certificate. He believed that the accumulation of the usual increase in molecular disorder or age changes, that people have lived long enough to incur, would be what killed them. It is these changes that have increased their vulnerability to whatever is recorded on

the death certificates (such as heart failure) that is the ultimate cause of death. Hayflick goes on to state that this can even apply to many accidental causes of death in this age group where diminished eyesight, hearing or decrease in reaction time would be the direct cause of death (Hayflick 1998).

If researchers can understand and modify the rate of ageing, so not just increase the length of life but extend the length of healthy lifespan then a reduced risk of diseases may be seen. This in turn could lead to an increased healthier lifespan allowing older people to keep working and avoid high health care costs (Longo, Shadel et al. 2012). Work in the ageing field is not just bringing us closer to a longer healthier life, but also treatments for disease and illness in the ageing population. Drugs for cancer have, or are being, developed from chemicals that were first thought to only extend lifespan.

This thesis suggests autophagy can extend lifespan via its cytoprotective mechanisms such as, to clear away defective proteins and to maintain nutrient and energy homeostasis during starvation. However, the activation of autophagy can also be harmful, excessive autophagy may cause undesirable cell death or allow some cancer cells to become resistant to chemotherapy. Thus, further work on the definitive roles of autophagy within a healthy body and in specific disease contexts are needed. To investigate if the stimulation or inhibition of autophagy is beneficial to the cell/organism a greater understanding of how autophagy works is needed, for example in identifying regulatory pathways controlling autophagy, how *Atg* proteins act in autophagosome formation and the mechanism of sequestering vesicle formation (Yang and Klionsky 2010).

Work in the future

Work on ageing has not always been undertaken as it was considered that it was an inevitable process that could not be altered. However, the ageing process is like many other biological processes that are subject to regulation by signalling pathways and transcription factors. Many pathways were first discovered in model organisms such as yeast, nematodes and flies, and were then found to be homologous to pathways in mammals. This indicates the important role these

model organisms have played in fundamental discoveries and the even more important role they still have to play in the identification of new pathways, how external forces affect behaviour e.g. longevity, and the future development of treatments to combat ageing and age related disease. It was believed that extending lifespan by the slowing of ageing would result in death from diseases such as Alzheimer's but what has actually been discovered so far is that many mutations that slow ageing also postpone age-related diseases. This is because although autophagy influences ageing it also influences cell growth, division and survival by the interaction of signals that control these processes. This is why there is a link between longevity and cell loss to the same autophagy pathway, and why the general consensus is that most animal species become more susceptible to cancers and other diverse degenerative disease as they age. If autophagy can be used as a potential target for de-accelerating the ageing process by prolonging autophagic activity, one possibility for this is via pharmacological means, in the elderly population the limits of healthy life may be extended (Vellai, Takacs-Vellai et al. 2009). So by combating ageing we may also be combating many age-related diseases.

The observations seen in this thesis and in other related work indicate the complexity of autophagy induction at the organismal level and highlight a need for further investigation into the mechanisms by which the induction of autophagy may be sufficient to increase longevity. Research suggests that the induction of autophagy could be useful in treatments for bacterial and viral infections (Orvedahl and Levine 2009) as well as some cancers (Hoyer-Hansen and Jaattela 2008). Many suspected autophagy inducers (such as rapamycin) have off target effects or undesirable side effects (such as caloric restriction). Research suggests it may not just be about how much is eaten but also when it is eaten which may influence which lifespan extending pathways are activated in the body. There is a wide interest in natural food components that may promote autophagy, and which urgently need to be explored. It is also possible that there are as yet undiscovered regulators of autophagy that influence its induction in response to different stimuli and the fly model system is well suited to

making such discoveries. Studies in *Drosophila* have been informative in addressing aspects of autophagy for example in tissue specificity of autophagy induction which could be important for targeting autophagy for therapeutic purposes. *Atg8* overexpression has been used to induce autophagy in neurons and has shown a marked increase in survival (though this was contradicted by my results in chapter 5). These types of studies reveal why we need to understand which tissues are important for autophagy dependent longevity. If the induction of autophagy is beneficial in selective tissues then it will be necessary to design autophagy targeting therapies that act in a tissue specific manner (Gelino and Hansen 2012). A challenge for researchers working in the autophagy field will be how to turn autophagy gene-dependent survival and death pathways on and off for the treatment of different clinical diseases.

Further research using laboratory manipulations of autophagy and autophagy genes is required as to the evolutionary causes of longevity. If future mutations could in effect give us longer lifespans then it should be possible to develop drugs that will mimic these effects and extend survival rates in this way. Results suggest that the supplementation of rate-limiting components of the autophagic pathway could also be beneficial for the health and maintenance of the human body in a variety of stressful conditions such as old age. However, more work needs to be undertaken on the pharmacological activators of autophagy and the implications especially of Torin1 and its application to a whole organism to test for any side effects deleterious or otherwise.

It appears that with time and patience the application of autophagy modulators to adjust rates of ageing and preserve healthy life may one day be possible.

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Appendix I Comparisons between food delivery systems on longevity, fecundity and fertility of Drosophila melanogaster

A1.1 Introduction

Studies of feeding behaviour in *Drosophila melanogaster* are limited, with a lack of rigorous methodology. The Capillary Feeder method or CAFE assay is one method that has been used, in which the food is placed in capillary tubes suspended above the vials containing the flies (Ja, Carvalho et al. 2007). The experiment reported here in appendix I was conducted to compare different feeding methods that deliver small quantities of food, which could at a later date be supplemented with chemicals. When using diet to deliver different chemicals to the fly, and to test for associated effects on lifespan it is particularly important to avoid food restriction or starvation conditions. These might themselves lead to reduced lifespan (if starvation is very severe), or might induce caloric restriction (malnutrition without starvation) leading to extended lifespan and reduced fertility (Grandison, Piper et al. 2009; Partridge 1987). These effects could themselves confound the results and interfere with the interpretation of the effects of the chemicals delivered on life history. Therefore in order to examine the effects of any chemicals or regents on life history it is important to start from a set of conditions that are most closely related to normal feeding conditions experienced by un-manipulated wild type flies.

Here I compared different methods to see which of them would replicate the lifespan and fecundity of flies held on normal standardised food. This then ensured that any chemicals or other reagents could be given to flies that are experiencing a standard set of nutritional conditions, for example, inducing starvation.

The aim of this experiment was therefore to determine how the female life history is affected by different food delivery systems and to determine which method results in feeding, egg production and offspring viability most comparable to the un-manipulated wild type culturing conditions.

A1.2 Materials and Methods

Flies used in the experiment were from the wild-type Dahomey strain. The experiment was carried out as laid out in the general materials and methods (chapter 2). Larvae were raised at a density of 100 per vial. Males and females were collected at eclosion using ice anaesthesia and were housed in groups of 10 on a normal yeast diet of 100% brewer's yeast per litre medium consisting of 970ml distilled water, 15g agar, 75g sugar, 100g brewer's yeast, 30ml Nipagin solution and 3ml Propionic acid, for 48 hours prior to experiment (full details chapter 2).

Females were anaesthetized using CO_2 and randomly assigned one per vial to one of six treatment groups. Each vial contained an agar plug upon which the different diets were placed. Control groups were those fed 20µl liquid food per 24 hours (normal SYA food with no added agar placed on the top of the agar plug). All experimental groups were fed liquid food, as in the control group but with added food dye to assess food uptake (50mg dye (E133 Brilliant Blue)/100ml food, http://www.fastcolours.com/e133-brilliant-blue-fcf-granular-food-blue-2-ci-42090-23-c.asp). Food was administered by three different methods, on a small filter paper disc placed inside the vial, directly pipetted onto the surface of the agar inside the vial or retained inside the pipette tip which was suspended in the vial. In each set of control and dye fed flies there were six individual groups, virgin and once mated females (mated 48 hours after eclosion). The treatment groups used are set in the table below:

Table A1.1 – Identification used for treatments and description of food delivery methods.

Id	Description
DiscVCon	Virgin females fed control food via a paper disc.
DiscVDye	Virgin females fed control food with added dye via a paper disc.
DiscMCon	Mated females fed control food via a paper disc.
DiscMDye	Mated females fed control food with added dye via a paper disc.
PipVCon	Virgin females fed control food via a pipette tip.
PipVDye	Virgin females fed control food with added dye via a pipette tip.
PipMCon	Mated females fed control food via a pipette tip.
PipMDye	Mated females fed control food with added dye via a pipette tip.
DropVCon	Virgin females fed control food dropped directly onto agar in vial.
DropVDye	Virgin females fed control food with added dye dropped directly onto agar in vial.
DropMCon	Mated females fed control food dropped directly onto agar in vial.
DropMDye	Mated females fed control food with added dye dropped directly onto agar in vial.

Flies were placed in new vials every 48 hours and old vials were stored (frozen) to enable eggs to be counted at a later date. In order to check whether vacated vials could be supplemented with food that would ensure all the eggs laid in it would hatch and all larvae would develop, I supplemented all vials, once the females had been removed, with liquid SYA food.

Comparison of food uptake as determined by dye ingestion across the different food delivery systems.

Ten flies from the end of the food trial were picked randomly from each treatment group (mated and single females combined). These females were then photographed under a widefield microscope in order to compare the intensity of blue within the abdomen of the flies.

Statistical analysis

Statistical analysis was performed using SPSS v 18 (SPSS 2009). Data were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests. Longevity was analysed using Cox regression analyses. Fecundity and fertility were analysed as in previous chapters using ANOVA.

A1.3 Results

Survival

The addition of dye to the food had no effect on the longevity in any of the food delivery systems tested ($F_{1,146}$=0.515, p=0.473). Females mating status did have a significant effect upon longevity ($F_{1,146}$=6.510, p=0.011), with virgin females living significantly longer than the once mated females. The food delivery system also had a significant effect on longevity ($F_{2,146}$=26.874, p<0.001) with those females held on media where the food was placed directly on the surface of the agar living significantly longer than those from the other food delivery systems. There was also a significant interaction between female mating status and the food delivery system ($F_{2, 146}$=3.883, p=0.021), with mated females fed by the disc or pipette methods having particularly short lifespans.

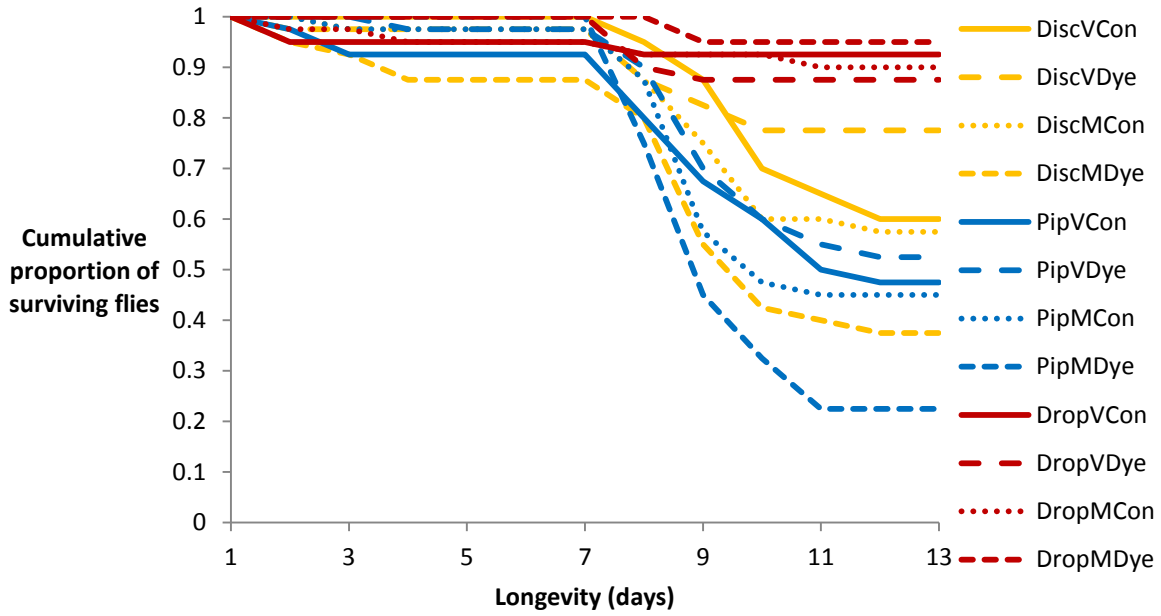

Fig A1.1 – Longevity of females maintained under different food delivery methods; disc fed single females on control food (DiscVCon), disc fed single females on control food with added dye (DiscVDye), disc fed mated females on control food (DiscMCon), disc fed mated females on control food with added dye (DiscMDye), pipette fed single females on control food (PipVCon), pipette fed single females on control food with added dye (PipVDye), pipette fed mated females on control food (PipMCon), pipette fed mated females on control food with added dye (PipMDye), Drop fed single females on control food (DropVCon), drop fed single females on control food with added dye (DropVDye), drop fed mated females on control food (DropMCon), drop fed mated females on control food with added dye (DropMDye).

Fecundity

There was a significant difference in the number of eggs laid by virgin in comparison to mated females ($F_{1,431}$=10.020, p=0.002), with virgin females laying fewer eggs. The feeding method also significantly affected the egg production, with females kept on the drop food method laying significantly more eggs than the other two treatment groups ($F_{2,431}$=57.091, p<0.001). Fecundity was not affected by the dye in the food in any of the food delivery systems ($F_{1,431}$=0.110, p=0.741), hence in fig A1.2 below I have integrated data from groups held with and without dye added. A significant interaction was seen between the feeding method and the female mating status ($F_{2,431}$=21.792, p<0.001), with mated females from the drop food method laying significantly more eggs.

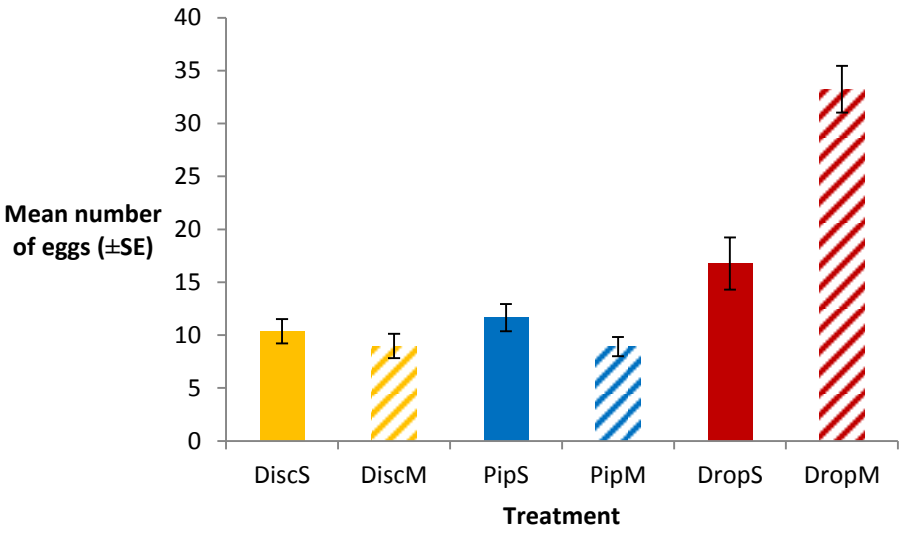

Fig A1.2 – Mean (±SE) number of eggs laid by the *Drosophila* females in the different feeding methods. Disc fed single females (DiscV), disc fed mated females (DiscM), pipette fed single females (PipV), pipette fed mated females (PipM), drop fed single females (DropV), drop fed mated females (DropM).

Offspring

The food delivery system had a significant effect on the number of offspring produced ($F_{2,223}$=6.593, p=0.002). Dye had no effect ($F_{1,223}$=0.024, p=0.877) hence the data on both standard food and food containing dye have been amalgamated.

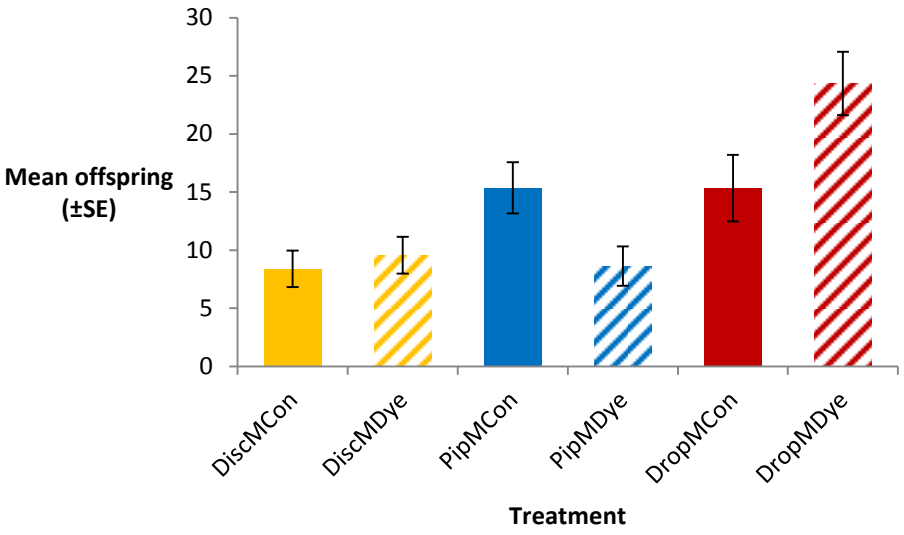

Fig A1.3 – Mean (±SE) number of offspring produced by the *Drosophila* females in the different feeding systems. Disc fed single females (DiscV), disc fed mated females (DiscM), pipette fed single females (PipV), pipette fed mated females (PipM), drop fed single females (DropV), drop fed mated females (DropM).

Qualitative observations on ingestion of food dye were observed, the flies in each group were scored independently by 4 separate researchers in the *Drosophila* field, on a scale of 1 to 5, for the intensity of blue dye in their abdomens. The scores were then analysed to compare uptake of dye in each treatment group. The mean uptake values were not identical but the rankings were found to be similar (mean average of range, disc fed 23.25, pipette fed 25.5, drop fed 24.5) suggesting that all groups took up similar amounts of dye in their food (see below). Therefore, there is little evidence that food uptake was dependent on the type of food delivery system.

Dye uptake

Fig A1.4 – Females from the disc food delivery system with added dye

Fig A1.5 – Females from the pipette food delivery system with added dye

Fig A1.6 – Females from the dropped food delivery system with added dye

A1.4 Conclusion

From the experiment above I concluded that the best food delivery system is where the food is dropped directly onto the agar plug at the bottom of the vial (the drop treatment). Female flies fed by the drop food delivery system lived longer, laid more eggs and had more viable offspring than the

other two methods. Therefore the drop food delivery system was used for the delivery of chemical activators of autophagy in my thesis work. Images of the females after eating showed that in all delivery systems there was a good uptake of food, using as a proxy the food dye intensity in the female's abdomens.

In all of the three delivery systems females became stuck in the food and in some groups this severely reduced the dataset. The Cafe food delivery system used by Ja et al. (2007) allowed flies to consume liquid food from a graduated glass microcapillary tube. Because of the sensitivity of the CAFE system Ja et al. (2007) were able to measure feeding patterns of individual flies (Ja, Carvalho et al. 2007). However, this method is not suited to the measurement of eggs as *D. melanogaster* females are selective in their choice of egg laying sites and will withhold eggs when there is no appropriate oviposition site available (Klionsky, Abeliovich et al. 2008). In the pipette food delivery system (comparable to the CAFE method above) there was no food media (i.e. agar only) in the bottom of the vial onto which eggs could be oviposited. This may have caused a lower number of eggs to be laid by the females maintained under this food delivery system.

It is widely known that dietary restriction extends lifespan in a wide range of species but it also reduces their reproductive output (Carey, Harshman et al. 2008). This experiment was set up to determine the best food delivery method but also the one that permitted the expression of wild type levels of reproductive output.

Many manipulations of *Drosophila* diet can alter lifespan, however, enhancing longevity by dietary restriction is caused almost exclusively by the level of yeast in the diet (Grandison, Piper et al. 2009). Thus, for work with food delivery in my thesis I used a yeast paste rather than liquid SYA food. It was thought this might boost fecundity still further and, because it has a thick consistency, might reduce the number of females becoming stuck on the food surface.

Appendix II Comparison of fecundity in females fed on two different mediums

A2.1 Introduction

This short experiment investigated whether females feeding upon a yeast drop placed onto the top of agar in a vial laid comparable numbers of eggs to females feeding upon normal SYA medium. Along with the data on food delivery methods shown in appendix 1, this could then validate whether the delivery of chemical activators of autophagy in yeast droplets was an appropriate and biologically relevant method by which to examine female life history.

A2.2 Materials and Methods

All flies were handled in the same manner as described in chapter 2. Once mated females (30 per treatment) were added to the experimental vials 24 hours following eclosion using CO_2 anaesthesia, and discarded on day 3. Two experiments with different yeast droplet preparations were done (experiments 1 and 2 as described below).

Statistical analysis was performed using SPSS v 18 (SPSS 2009). Fecundity data were analysed using ANOVA and were tested for normality using Kolmogorov-Smirnov tests and for homogeneity of variance using Levene's tests.

A2.3 Results

Experiment 1

In this experiment I compared the fecundity of females fed yeast paste with that of normal SYA medium, (n=30 per treatment group). A droplet of yeast paste containing blue food dye, as used in appendix 1 (50mg dye (E133 Brilliant Blue)/100ml food, http://www.fastcolours.com/e133-brilliant-blue-fcf-granular-food-blue-2-ci-42090-23-c.asp), was added to a vial containing an agar plug and to the normal SYA food. It was clear that both groups of females ate the yeast paste as seen by comparable intensities of blue colouration in their abdomens.

Over the course of each of the 3 days during the experiment, egg production was monitored. There were no significant differences in fecundity on any of the 3 days tested ($F_{1,169}$=1.471, p=0.227, table A2.1) or when counts for all 3 days were collated (statistics as above, p=0.227)

Table A2.1 – Mean egg production (±SE) over 3 days for once mated females maintained on agar versus SYA medium, each containing a droplet of yeast paste (2mm diameter) with added blue dye.

	Mean egg production			
Treatment	Day 1 (±SE)	Day 2 (±SE)	Day 3 (±SE)	All 3 days combined
Yeast drop on agar	44 (2.2)	55 (1.6)	66 (3.0)	55 (1.6)
Yeast drop on SYA	39 (2.0)	60 (2.2)	77 (4.1)	59 (2.3)

Experiment 2

In this second experiment I prepared the yeast paste solution in a more systematic manner. I added 10ml dried yeast granule and made up to 20ml with water, before adding the blue food dye as above. 20µl of this yeast solution was then added to each vial, either to vials containing SYA food or agar only, as above.

Female fecundity was then measured over the next 3 days. As above, there was no apparent difference in blue dye uptake. However, in this experiment egg production was significantly lower on the yeast paste + SYA in comparison to the yeast paste + agar medium ($F_{1,178}$=477.883, p<0.001). This was the same when all data from individual days was collated (analysis the same as above p<0.001).

Table A2.2 – Mean egg production (±SE) over 3 days for once mated females maintained on agar versus SYA medium each containing 20 µl of defined liquid yeast solution with added blue food dye.

	Mean egg production			
Treatment	Day 1 (±SE)	Day 2 (±SE)	Day 3 (±SE)	combined days
Defined yeast drop on agar	32 (2.7)	23 (2.2)	9 (1.2)	21 (1.6)
Defined yeast drop on SYA	59 (1.7)	76 (1.9)	79 (3.2)	71 (1.6)

A2.4 Conclusions

I concluded from these experiments that the defined yeast paste placed onto an agar base was suitable for use in the experiments. I could therefore place defined amounts of autophagy activators into the yeast solution to be placed onto the agar. This method ensured that the females would maximise their intake of the chemicals to be delivered whilst expressing near normal fecundity. Lower egg numbers were also preferable as the numbers of eggs to count was logistically difficult. The fact that the chemicals could be added to the defined yeast liquid also meant that far lower amounts of these chemicals could be used than if they were added to the base medium itself reducing the overall costs of subsequent experiments. The one downside was that the yeast liquid on agar medium appeared to compromise larval development. I therefore determined to score fertility in the main experiments in the thesis by counting the number of hatched and unhatched eggs, rather than by scoring emerging offspring numbers.

Appendix III DMSO toxicity determination

A3.1 Introduction

The aim of this short experiment was to determine if DMSO was toxic to flies, as it was used in chapter 4 as the carrier control for Torin1. Torin1 does not dissolve well in water and is normally dissolved in DMSO. However, the toxicity of DMSO to flies is not known and was therefore tested here in order to know the maximum volume of DMSO that could be given to females without incurring toxic effects.

A3.2 Materials and Methods

All flies were handled as described in chapter 2. Once mated females were anaesthetized using CO_2 and randomly assigned to one of 6 treatment groups (n=30 per treatment) 24 hours following eclosion. Each vial contained an agar plug to which a droplet of yeast paste (2mm diameter) was added with DMSO carrier control in the following amounts: 0, 0.5µl , 1µl, 5µl, 10µl, 40µl. Flies were transferred between vials without using anaesthesia twice a week until death. Longevity was scored daily.

Statistical analysis was performed using SPSS v 18 (SPSS 2009). Longevity was analysed using Log rank tests.

A3.3 Results

The results of the experiment showed significant differences in lifespan across the six treatment groups (Log Rank X^2=23.218, df=5, p<0.001). Unexpectedly, the control containing 0µl DMSO was not the longest (or shortest) lived, but had an intermediate lifespan. Otherwise, there appeared to be a varied dose response, with increasing amounts of DMSO leading to shorter lifespans. Further

log rank analysis revealed that there was a significant extension in lifespan in the control, 0.5 and 1µl DMSO treatments compared to both the 10 and 40µl groups (all at p≤0.038). A significant lifespan extension was also seen in the 5µl treatment group compared to the 10µl group.

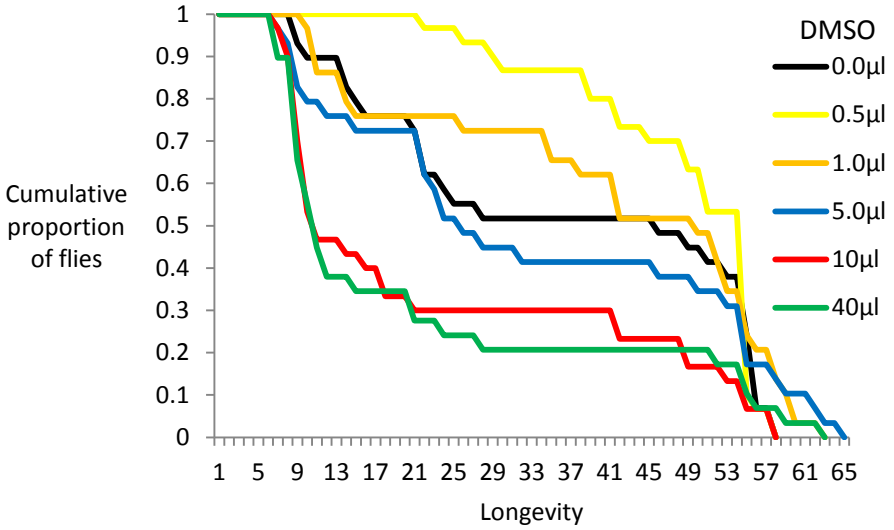

Fig A3.1 – Survivorship of once mated females exposed to 0, 0.5µl, 1µl, 5µl 10µ, 40µl DMSO in the diet.

A3.4 Conclusions

DMSO was toxic in increasing doses, causing a significant decrease in female lifespan at the highest doses tested. The reason for the extended lifespan at lower doses on DMSO was unclear and may arise because of feeding differences, perhaps females fed less at these doses extending lifespan via caloric restriction. It was clear therefore that the main experiments I needed to use one single dose of DMSO and to avoid exposing females to differing concentrations of DMSO, to avoid confounding effects on lifespan. I therefore decided to supply the Torin1 in the main experiments in 5µl of DMSO as this resembled most closely the control (0µl DMSO) conditions.