Acer, a homologue of the human angiotensin-1 converting enzyme, modulates the response of sleep, glycogen storage, lifespan, fecundity and stress resistance to diet in *Drosophila melanogaster*

By

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A thesis submitted to Lancaster University in fulfilment of the requirements for the degree of Doctor of Philosophy.

I declare that that this thesis is my own work and has not been submitted in substantially the same form for the award of a higher degree elsewhere.

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Abstract

Human angiotensin-1–converting-enzyme (ACE) plays a primary role in the regulation of blood pressure and electrolytes as part of the Renin-Angiotensin System (RAS) in humans. Renin cleaves angiotensinogen to angiotensin I and ACE regulates the vasoconstriction of blood vessels by converting angiotensin-1 to angiotensin-2 (a potent vasoconstrictor) and also by breaking down bradykinin (a potent vasodilator). Renin is regulated by a feedback loop mechanism and is inhibited by higher concentrations of angiotensin-2. A lack of or inhibition of ACE can lead to a reduction in blood pressure (BP) and may reduce the risk of diabetic nephropathy as high BP and high fluid retention can cause swelling in the kidneys. ACE inhibitors are used as treatments for these conditions as well as treating congestive heart failure (Stanley & Samson, 2002). ACE expression has been found in human adipose cells (Jonsson, et al., 1994) and ACE expression in this tissue was reduced when rats were treated with the ACE inhibitor Enalapril (Santos, et al., 2009). Currently ACE's role in this tissue is unknown and therefore the study of the *Drosophila* homolog ACER, which is expressed within the fly fat body which is similar in structure to human adipose cells, may highlight a role for ACE in adipose tissue.

To investigate the role of ACE-like enzymes in dietary effects on ageing-related and circadian health, function and metabolism we are studying ACER, a homologue of human ACE, in the fruit fly *Drosophila melanogaster.* Previous studies (Taylor, et al., 1996; Carhan, et al., 2010) have shown that *Acer* is expressed in the embryonic heart, adult head and adult fat body of the fly. The expression in the fat body is particularly interesting as the fly fat body acts like the human liver and adipose cells where ACE in humans is expressed. *Acer's* expression in the head shows a circadian cycle and appears to be regulated by the circadian gene *Clock*. $Acer^{\Delta}$ mutant flies exhibit normal circadian locomotor rhythms but show defects in the regulation of sleep, and the ACE inhibitor, Fosinopril, fed to flies disrupts night time sleep in the same way. This suggests a role for ACER in a circadian phenotypes in *Drosophila* and therefore a potential circadian role for ACE in humans (Carhan, et al., 2010).

The present study has found the effect of the loss of *Acer* expression in the *Acer* deletion mutant ($Acer^{\Delta}$) was complex and was often dependent on genetic background and sex. $Acer^{\Delta}$ mutants responded normally to dietary restriction (DR) for both sex and background therefore, *Acer* was not required for the DR response to

lifespan. Lipid storage in the *Acer*$^{\Delta}$ mutants was unaffected by the loss of *Acer* expression but glycogen storage was reduced on high food levels and did not show the normal increase in storage with increasing food compared to controls. Thus, indicating a role for *Acer* in the modulation of glycogen storage. The genetic backgrounds analysed in this study were the outbred white^{Dahomey} (*w*^{Dah}) and inbred white^{1118} (*w*^{1118}) backgrounds which did show a difference in the effect of the loss of *Acer* for certain phenotypes. Fecundity in *Acer*$^{\Delta}$ females was lower than in controls in the more fecund *w*^{Dah} background but not in the less fecund *w*^{1118} background. Sleep also showed an altered response between the backgrounds and sexes to changing diet in *Acer*$^{\Delta}$ mutants. *Acer*$^{\Delta}$ mutants were starvation and oxidative stress resistant but only in the *w*^{Dah} background and showed sensitivity when compared to controls in the *w*^{1118} background.

To investigate *Acer*'s role in the response to nutrition the effect of the loss of *Acer* on *drosophila*-insulin-like peptides (*dilps*) was investigated. Altered transcript levels of *dilps* in the head and body of the fly in *Acer*$^{\Delta}$ mutants indicated a possible link between *Acer* and the IIS (insulin/IGF-like signalling) nutrient-sensing pathway.

In this study a role for *Acer* in the modulation of nutrient responsive phenotypes was established.

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Abbreviations

ACE	-	Angiotensin-I Converting Enzyme
ACER	-	Angiotensin Converting Enzyme Related
ANCE	-	Angiotensin Converting Enzyme
Ang I	-	Angiotensin I
Ang II	-	Angiotensin II
BP	-	Blood Pressure
CAFE	-	Capillary Assay Feeder Experiment
CHF	-	Congestive Heart Failure
chREBP	-	Carbohydrate Response Element Binding Protein
CLK	-	CLOCK
CREB	-	cAMP-responsive Transcription Factor
CNS	-	Central Nervous System
CYC	-	CYCLE
CRY	-	CRYPTOCHROME
DILPs	-	*Drosophila* Insulin-Like Peptides
dInR	-	*Drosophila* Insulin Receptor
dnCLK	-	Dominant Negative CLOCK
dnCYC	-	Dominant Negative CYCLE
DR	-	Dietary Restriction
DTS – 3	-	Dominant Temperature Sensitive 3
Hrs	-	Hours
IGF	-	Insulin-like Growth Factor)
IIS	-	Insulin/IGF–like Signalling
IR	-	Insulin Receptor
IRS	-	Insulin Receptor Substrate
JNK	-	c-Jun N-terminal Kinase
LK	-	Leucokinin
Mins	-	Minutes
mNSCs	-	Median Neurosecretory Cells
MTH	-	Methuselah
PER	-	PERIOD
PCR	-	Polymerase Chain Reaction

RAS	-	Renin Angiotensin System
SOD	-	Superoxide Dismutase
TIM	-	TIMELESS
TOR	-	Target Of Rapamycin
w^{1118}	-	white ^{1118}
w^{Dah}	-	white ^{Dahomey}

Chapter 1: Introduction

1.1: Ageing – An Overview

Ageing is a natural process that occurs in most living creatures including humans and is described as an intrinsic decline in function during adulthood, which results in a decrease in fecundity and increases the probability of death (Finch, 1990). Ageing can be seen over time in many organisms including birds, insects, mammals and humans whose development is completed before reproduction begins (Vaupel, et al., 2004). In the case of humans this is evident in the development from baby, through puberty, reproductive stages of life and the elderly stage of life. This occurs over many decades. Historically the elderly (aged over 65) have always been outnumbered by children under the age of 5 worldwide. However, the elderly now outnumber the children and this is a trend seen all around the world. The elderly account for 8% of the world population and their numbers are growing (National Institute on Aging, n.d.).

Improvements in healthcare, sanitation and general living conditions, as well as the introduction of antibiotics, have enabled the human race to live longer now than in the early 20th century (Partridge, 2010). However, living longer has increased the number of occurrences of age-related diseases that are linked with age-related decline which previously were less prevalent due to shorter lifespans. These diseases include Alzheimer's disease, dementia, some age-related cancers and heart disease which can be devastating to the patients and their families alike (Partridge, 2010). This presents a medical paradox, as better living conditions and better treatment of disease have resulted in a longer lifespan, which has increased the number of people living long enough to suffer age-related diseases. This has led to a higher financial cost in caring for the elderly while those retired no longer contribute to the taxes that pay for healthcare. In the US the cost of treating dementia alone rose by 35.4% between 2010 and 2015 (Wimo, et al., 2017).

As humans age, it is not only the external changes such as wrinkling of the skin and lower muscle mass that occur. The internal organs of the body age as well, including the brain and the rest of the nervous system. Neurodegenerative diseases and cognitive decline are becoming more common and the largest risk factor in developing these diseases is from ageing itself (Bishop, Lu and Yankner 2010).

Therefore, it is crucial in our ageing society to study and understand the underlying mechanisms of ageing to find ways of improving human health at older ages.

A number of genetic and environmental interventions have been identified that can extend lifespan of model organisms, resulting in the identification of signalling pathways and cellular processes that modulate ageing. In the nematode worm *Caenorhabditis elegans* it was found that a single gene mutation could double the lifespan of the worms and keep the worms healthy and more youthful for longer with the discovery of *age1* (Friedman & Johnson, 1988) and subsequent genes such as *daf-2* (Kenyon, et al., 2003). Several other single mutations were also identified and these genes were discovered to be part of the insulin/IGF (insulin-like growth factor) – like signalling (IIS) nutrient sensing pathway (Kimura, et al., 1997; Lin, et al., 1997). In *Drosophila* a single gene mutation in *methuselah* was found to increase lifespan (Lin, et al., 1998) and in dwarf-mice a mutation in a transcription gene encoding for the pituitary gland resulted in long-lived dwarf mice. Male dwarf mice lived on average 350 days longer than controls while female dwarf-mice lived on average 470 days longer than controls (Brown-Borg, et al., 1996). The link to ageing to IIS was confirmed in *Drosophila* as mutations in the *Drosophila* insulin receptor and the receptor substrate *chico* resulted in lifespan extension (Tatar, et al., 2001; Clancy, et al., 2001). Furthermore, in the mouse model mutations in genes coding for insulin and the Igf-1 receptor also resulted in extended lifespan (Bluher, et al., 2003; Holzenberger, et al., 2003). Further research has found that the IIS pathway is evolutionarily conserved between animal species (Piper, et al., 2008; Partridge, 2010).

The IIS (Broughton & Partridge, 2009) and the Target of Rapamycin (TOR) nutrient sensitive pathways (Bishop, et al., 2010) as well as Dietary Restriction (DR) (Pletcher, et al., 2000), have been found to modulate lifespan in model organisms (worms, flies and mice). Flies with lower levels of insulin-like signalling have been found to be long-lived (Clancy, et al., 2001; Libert, et al., 2008) and have higher resistance to stress and starvation on low levels of food (Broughton, et al., 2010). A reduction in TOR signalling has been found to extend lifespan (Kapahi, et al., 2004; Kapahi, et al., 2010) as has dietary restriction (Katewa & Kapahi, 2011). Despite advances in the understanding of the genetics and potential mechanisms of ageing and lifespan, the mechanisms remain unclear therefore the relationship that exists between lifespan and healthspan is at the moment also unclear. Not all measures of health are improved in long-lived organisms. Walking behaviour in IIS reduced

mutants was found to be unaffected by IIS reduction but negative geotaxis behaviour was found to be positively affected when insulin levels were reduced throughout the fly but not when the IIS reduction was confined to the neurons specifically (Ismail, et al., 2015). This suggests that specific age-related health effects of reduced IIS may be linked to different types of tissue and that different tissues may age differently.

In this project the focus will be on the novel role for the *Acer* gene in ageing and health in response to nutrition. The aims of this project are to understand the role of ACER in nutrient sensing and its potential benefits to lifespan and possibly healthspan.

1.2: Diet, nutrient-sensing and ageing

Diet in *Drosophila melanogaster* consists of feeding on rotting and fermenting fruit in the wild but in the lab diet is defined using sugar and yeast (Skorupa, et al., 2008). Sugar provides a carbohydrate source while yeast provides a source of protein. Autolysed brewer's yeast and live yeast can be used to separately or combined in the diet (Skorupa, et al., 2008). In the lab the concentrations of sugar and yeast can be easily changed to manipulate diet and record the effects of different concentrations of carbohydrate and protein on lifespan and behaviour (Pulver, et al., 2011). An example of the diets used in this study can be found in Chapter 2.9, Table 3.

Dietary Restriction (DR) is an environmental intervention that has been shown to increase longevity in many organisms including *C*. *elegans*, *Drosophila melanogaster* and mice where DR is optimal for longevity (Pletcher, et al., 2000) but not for reproduction which continues to increase with increasing diet (Fontana, et al., 2010) (Figure 1). DR is characterised by the restriction of food without causing malnutrition in the subject (Kerr, et al., 2011), and, in the case of *D. melanogaster*, has been found to be reliant on the dilution of yeast (Mair, et al., 2005) in the food rather than the total calorific content (Bass, et al., 2007). Fecundity has been found to be reduced in flies on a DR diet (Burger, et al., 2007). Fecundity is affected by yeast concentration in the diet with higher yeast levels in the diet leading to higher fecundity levels. Higher yeast levels also lead to shorter lifespan, and therefore the protein content of the diet requires a balance to promote longevity but not reduced fecundity too much (Skorupa, et al., 2008). Fecundity shows an age-related decline of egg-laying but longevity achieved without DR has been found to not to be traded-off with fecundity (Marden, et al., 2003; Wit, et al., 2013), therefore DR could be extending

lifespan by a different mechanism and the 'trade-off' with fecundity maybe be for somatic maintenance.

DR has been shown to delay ageing in many organisms including *Drosophila* (Pletcher, et al., 2000), however, the mechanism that protects the subject from age-related decline, has yet to be found (Kerr, et al., 2011). It has been found that although DR showed an extension in lifespan in *Drosophila*, negative geotaxis behaviour (innate response against gravity) showed no delay in the onset of behavioural decline (Kerr, et al., 2011). Therefore DR does not appear to protect the decline of all behaviours alongside extended lifespan.

Nutrient sensing signalling pathways, such as the insulin/IGF (insulin-like growth factor) –like signalling (IIS) and the target of rapamycin (TOR) pathways, have been found to have a role in ageing, with a reduction of TOR/IIS signalling extending lifespan in model organisms (Bishop, et al., 2010). These nutrient sensing pathways are thought to mediate (Figure 2), at least in part, the effect of dietary restriction on lifespan and have been conserved throughout evolution through yeast, worms, flies and mammals (Fontana, et al., 2010).

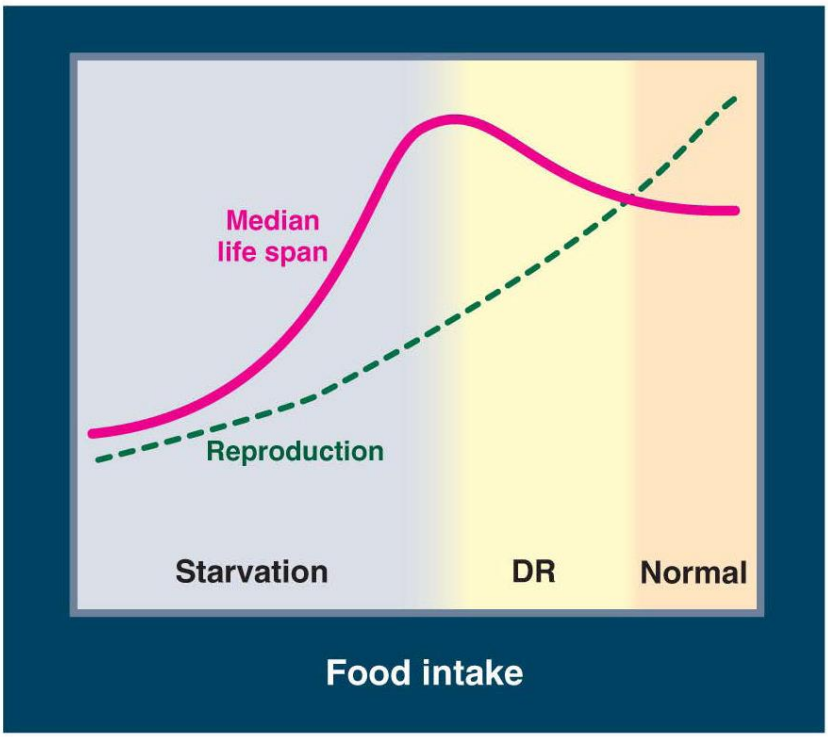

Figure 1: Food level, fecundity and longevity. Median lifespan and fecundity are negatively affected by a very low nutrient concentration in higher eukaryotes. However, lifespan but not fecundity is optimized by DR. (Fontana, et al., 2010).

The TOR signalling pathway is a regulator of protein synthesis, by detecting amino acids, and is expressed in most human cells. It has roles within growth and metabolism and the expression within the fly fat body has led to a possible role within ageing and lifespan being suggested (Katewa & Kapahi, 2011). Rapamycin is a TOR pathway inhibitor and has been shown to extend lifespan in both mice and flies (Marino, et al., 2008). Experiments using rapamycin on insulin-like signalling mutants have shown a further increase in longevity, suggesting that on occasion the pathways may work side-by-side to slow the process and the onset of ageing (Katewa & Kapahi, 2011). In conjunction with DR reduced TOR signalling has also been found to increase lifespan (Katewa & Kapahi, 2011).

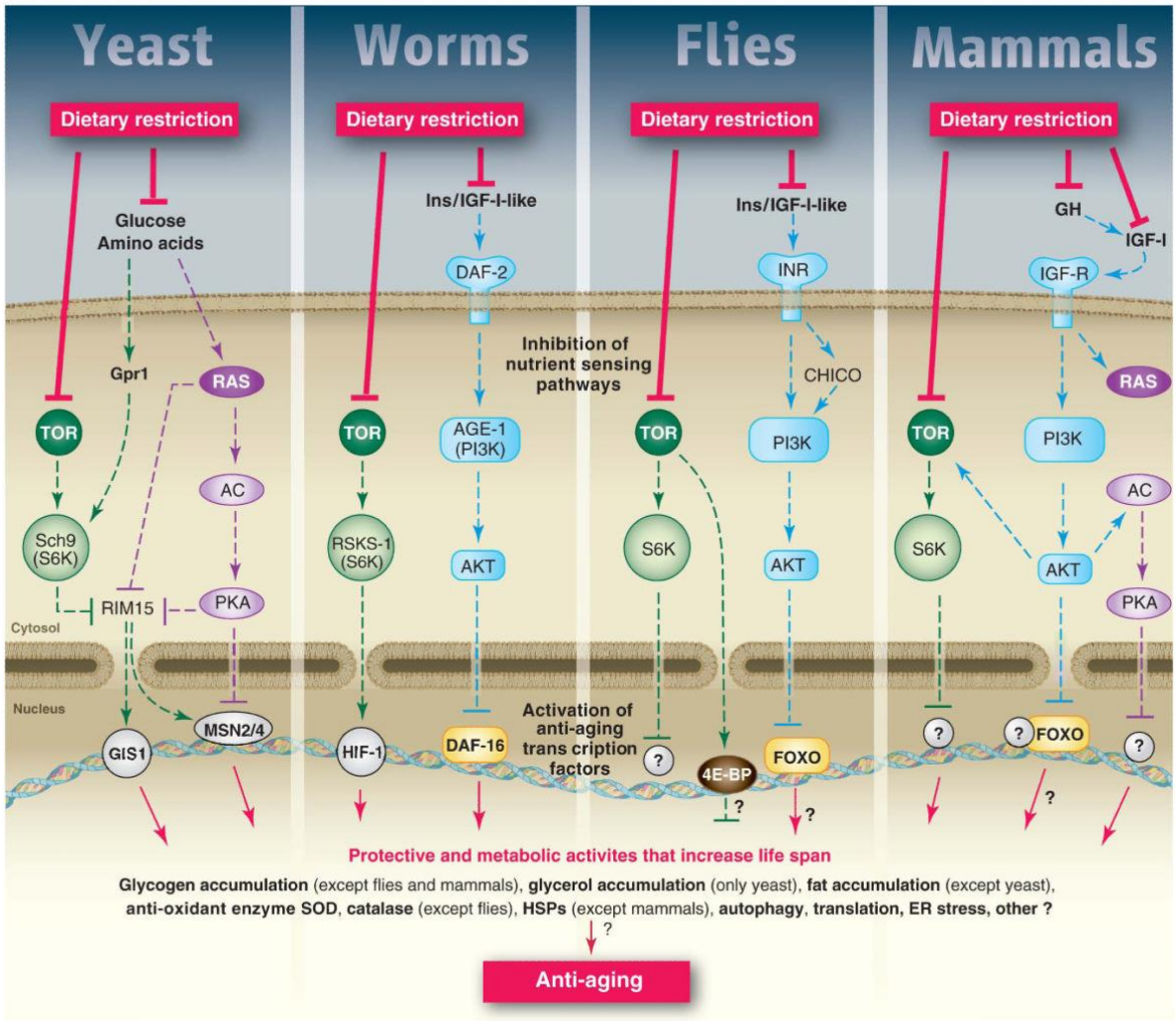

Figure 2: The evolutionary conservation of nutrient-sensing signalling pathways and their relationship to dietary restriction through yeast to mammals. (Fontana, et al., 2010).

Like the TOR signalling pathway, the IIS pathway has been found to be evolutionarily conserved through worms to mammals with the complexity of the pathway increasing with the complexity of the organism (Figure 2.) Manipulations of IIS have resulted in the extension of lifespan in model organisms (Fontana, et al., 2010). A reduction in IIS signalling has been found to extend lifespan in worms (Murakami, et al., 2005), flies (Clancy, et al., 2001) and mice (Selman, et al., 2008). The IIS pathway in worms and flies consists of a single insulin receptor (IR) and multiple substrate ligands to induce the signalling of the pathway but in mammals this is more complex with one insulin ligand which regulates the activity of the IR and two IGF ligands which regulate the IGF-1 R (IGF-1 receptor) (Broughton & Partridge, 2009).

IIS has been associated with the central nervous system (CNS) but other tissues have been found to secrete insulin ligands implying that insulin signalling is not only important for the nervous system but blood and other tissues as well (Broughton & Partridge, 2009). IIS is required to protect and sustain nerves and neural development, however, in model organisms, including *Drosophila melanogaster*, an increase in lifespan has been seen with reduced levels of IIS (Broughton & Partridge, 2009). Originally the brain was thought to be insensitive to IIS however, it has been suggested that the central nervous system (CNS) is able to respond to IIS from peripheral sources in the body in the development and maintenance (Broughton & Partridge, 2009).

It is possible the IIS may have a role in cognitive functions as Alzheimer's disease patients have been found to have a high resistance to IIS signalling. IIS is associated with the control of β-amyloid metabolism which is key in the pathogenesis of Alzheimer's disease (Carro & Torres-Aleman, 2004). This connection has also been found with multiple sclerosis, dementia and also schizophrenia (Broughton & Partridge, 2009).

Drosophila-insulin-like peptides (DILPs) are homologs of insulin-like ligands in humans and are expressed during insulin signalling in the fly (Broughton & Partridge, 2009). There are eight DILPs altogether expressed in the fly and Figure 3 shows the location of seven of the eight DILPs (Nässel, et al., 2015). DILPs 1 and 4 are thought to be primarily expressed in the larval stages of development but little is known about them (Brogiolo, et al., 2001; Ikeya, et al., 2002). DILPs 2, 3 and 5 are expressed in the

median neurosecretory cells of the fly brain (mNSCs) and DILP6 is expressed within the fly fat body (Grönke, et al., 2010). A reduction in specific DILP expression is compensated by the up-regulation of other DILPs (Grönke, et al., 2010) but DILP5 is the only DILP that has been found to respond to dietary changes in terms of expression (Broughton, et al., 2010).

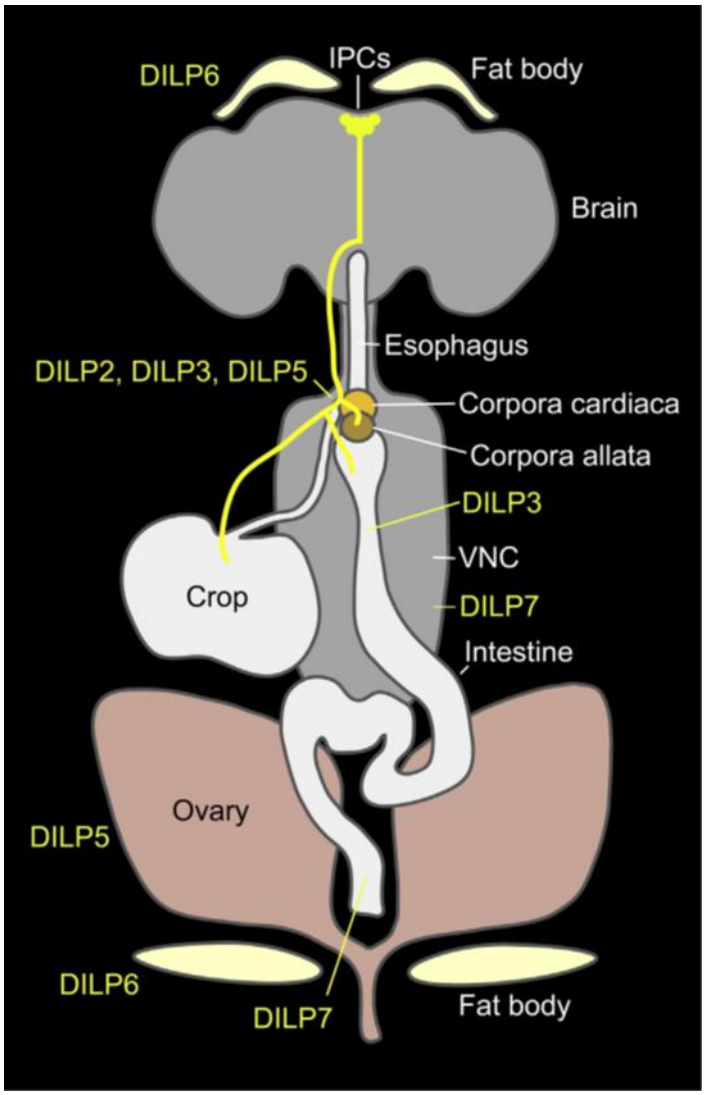

Figure 3: Overview of production and release sites of DILPs in the CNS and other organs of Drosophila. Three DILPs are produced in the insulin producing cells (IPCs) of the brain, shown in yellow: DILP2, 3 and 5. These DILPs are released from axon terminations in the corpora cardiaca (CC), corpora allata (CA), crop and anterior intestine. DILP 5 is additionally produced in the ovaries and Malpighian tubules (not shown). DILP6 is mainly produced by adipocytes of the fat body in the head and body of the fly. Finally DILP7 is produced by about 20 neurons of the abdominal neuromeres of the ventral nerve cord (VNC) and may be released onto the posterior intestine and

oviduct, as well as inside the CNS. This figure is redrawn and altered from an illustration by Toivonen and Partridge (2009) (Nässel, et al., 2015).

DILP2 is thought to regulate trehalose storage (Broughton, et al., 2008) while DILP6 expression is regulated by the *Drosophila* transcription factor FOXO and is induced in larvae when nutrient stores are low (Slaidina, et al., 2009). In the fat body over-expression of dFOXO has also been implicated in lifespan extension (Piper, et al., 2008). As well as increasing lifespan, reduced levels of insulin signalling have been shown to increase flies' ability to survive starvation and better resist the effects of oxidative stress (Broughton, et al., 2010). In response to high yeast diet, flies that have their mNSCs in the brain ablated to reduce IIS live longer than controls, suggesting that mNSCs are involved in the response of lifespan to increasing food (Broughton, et al., 2010). DILP7 expression occurs within the ventral nerve cord (VNC) of the fly and appears to be important in the female fly's analysis of suitable sites for egg-laying. Elevated levels of *dilp7* have been associated with increased fecundity in female flies (Yang, et al., 2008). DILP 8 is the most recently discovered DILP and is thought to be expressed in the adult ovaries and is thought to have roles in growth and development (Colombani, et al., 2012). Together these DILPs form part of the IIS pathway in *Drosophila melanogaste*r showing that IIS is complex and involved in many aspects of growth and development..

In mice it was found that lower levels of IIS achieved by deleting the insulin receptor substrate-1 (IRS-1) produced long-lived mice but caused insulin resistance at young ages, however, at older ages the mice were found to control glucose homeostasis better than controls. Other added benefits included improvements in movement and the immune system, and a lower risk of developing cataracts and osteoporosis (Selman, et al., 2008). A similar improvement was shown in *Drosophila* when mutation of the insulin receptor substrate (IRS) *chico* was found to increase lifespan and slow the decline of negative geotaxis behaviour as well as improving the functions of the immune system (Libert, et al., 2008). A study in *C. elegans* has also found increased longevity with reduced IIS as well as the inhibition of tumour growth, leading to a potential therapeutic pathway in targeting cancer (Pinkston-Gosse & Kenyon, 2007). These studies have shown that lifespan and some measures of healthspan can both be improved by manipulating the IIS pathway .As a result, the nutrient sensing IIS/TOR network is a major focus of research to identify targets of

potential therapy to delay the onset or slow the rate of age-related functional declines in humans (Broughton & Partridge, 2009). However, it is becoming clear that not all measures of health and function are improved in long-lived organisms and there are many unanswered questions about how manipulation of diet or the IIS/TOR network impacts on lifespan and healthspan.

1.3: *Drosophila* as a Model Organism to study ageing

The difficulties in identifying roles for different signalling pathways within ageing humans mean that model organisms with similar biological pathways and systems to human beings are very valuable in researching ageing, as phenotypes and effects can be seen much faster within model organisms that exhibit a shorter lifespan than humans.

Drosophila melanogaster, the nematode worm *C. elegans* and mice are often used as model organisms in scientific research because they have conserved genetic pathways with humans (Groteweil, et al., 2005).

Groteweil, et al. (2005) highlights the many different advantages of using *Drosophila* as a model organism. *Drosophila* are cheap to maintain with phenotypic differences which can be seen relatively easily and their lifespans are relatively short (between 50 and 80 days) allowing significant differences with age to be observed and analysed, with results seen within three months. *Drosophila* have two distinct sexes, male and female, and can be used to distinguish behaviour and molecular differences between the sexes and how single gene mutations can affect behaviour and appearance (Pulver, et al., 2011). Due to evolutionary conservation *Drosophila melanogaster* have many homologues of human genes that are similar in structure and function to their human counterparts, therefore linking the fly and human genomes and it is this link that makes *Drosophila melanogaster* a good and relevant model organism allowing research to be related to humans (Pulver, et al., 2011).

1.4: Genetic background influences phenotypes in *Drosophila melanogaster*

There are many different genetic backgrounds in *Drosophila melanogaster*, so when analysing genetic mutants the genetic background must be taken into consideration and the mutation tested within that particular genetic background.

Mutations in *Indy*, a transporter of intermediates of the Krebs cycle, extended lifespan in different genetic backgrounds but the percentage increase in lifespan was different between the backgrounds (Rogina & Helfand, 2013). A study into in human *superoxide dismutase* (*SOD*) using transgenically altered flies, also found that overexpression of *SOD* was affected by genetic background with varying effects of the overexpression (Spencer, et al., 2003). Svetec, et al. (2015) found that night time sleep responded differently in genetic backgrounds that were from different latitudes with equatorial populations sleeping twice as long as populations from higher latitudes and therefore a more temperate climate. In *Drosophila ananassae* it was found that response to cold stress was also affected by latitudinal genetic variation with equatorial populations recovering from cold chill coma more slowly than those of more temperate latitudes, suggesting that equatorial populations are sensitive to cold conditions (Sisodia & Singh, 2010). The expression of circadian clock genes and circadian rhythms have also been found to differ between latitudes and altitudes, indicating that genetic background could have an effect on the expression of different genes (Hut & Beersma, 2011).

In this study it was decided to conduct the investigations on *Acer* in two different genetic backgrounds: The inbred white ^{1118} (w^{1118}) and the outbred white $^{\text{Dahomey}}$ (w^{Dah}) backgrounds (Ziehm, et al., 2013). A partial deletion in the *white* (w) gene discovered by R. Levis led to the identification of a gene that controls eye colour in *Drosophila* (Bingham, 1980). The w^{1118} strain was developed from the Oregon R strain with mutated white eyes (Hazelrigg, et al., 1984) making it a temperate population. The w^{Dah} strain was developed in the Partridge lab (Broughton, et al., 2005) by crossing the mutated *white* gene in the w^{1118} strain with the out-bred Dahomey strain (Puijk & de Jong, 1972) which originates from West Africa making the w^{Dah} strain an equatorial population. Both the w^{1118} and w^{Dah} strains have white eyes instead of the usual red colour.

Flies with the mutated *white* gene have white eyes and are useful in developing transgenic strains where the insertion of the transgene, along with a red eye-colour marker, can be inserted into the genome. This results in the flies being red-eyed if they have taken up the transgene while those that have not remain white-eyed. This allows the selection of the progeny that contain the desired transgene by eye colour and without the need for Polymerase Chain Reaction (PCR) analysis (Klemenz, et al., 1987).

The *white* backgrounds were chosen for this study because these flies have been used in transgenic studies of ageing where transgenes use the w+ marker. Therefore in using these backgrounds, even though *Acer* is a deletion mutation and was not generated using transgenes, future studies may include the use of transgenes and comparison between the experiments will be applicable by using the same genetic background of fly. In these backgrounds *Acer* can be tested in an inbred background (w^{1118}) and an outbred background (w^{Dah}) to analyse the effect of the loss of *Acer* and how background can influence the effects.

1.5: ACE and the Renin-Angiotensin System (RAS)

The Renin-Angiotensin System (RAS) in humans regulates blood pressure and electrolyte balance in the blood and is regulated by a feedback loop mechanism (Corvol, et al., 2004). The pathway starts when low blood pressure or low renal blood flow is detected and Renin is activated. Renin cleaves Angiotensinogen to Angiotensin I (Ang I) (Santos, et al., 2009). ACE (angiotensin-1-converting enzyme) is then activated and cleaves Angiotensin I to Angiotensin II (Ang II). The presence of Ang II causes vasoconstriction to increase blood pressure and the reabsorption of sodium ions to combat dehydration. When the concentration of Ang II becomes too high it inhibits Renin and deactivates the pathway in a feedback loop manner (Corvol, et al., 2004) (Figure 3).

For this study the focus is on the role of ACE which exists as somatic ACE and testicular ACE (Guang, et al., 2012). ACER is a *Drosophila* homolog of human somatic ACE which is involved in the RAS. In humans, somatic ACE is a zinc-metallopeptidase and was the first of the M2 family to be characterised both biochemically and molecularly, (Corvol, et al., 2004).

Somatic ACE works by cleaving the C-terminal dipeptide or dipeptideamide from bioactive peptides or pre-cursors (Isaac, et al., 2007), when the substrate comes in contact with the endothelium of the blood vessel (Guang, et al., 2012). In the RAS the first target for ACE is Ang I and ACE removes the dipeptide at the C-terminal to form Ang II (Guang, et al., 2012). Ang II is a potent vasoconstrictor which works by narrowing the blood vessels and reducing volume of the lumen, therefore increasing the pressure of the blood passing through the vessel (Tom, et al., 2003).

Somatic ACE also breaks down bradykinin, which is a strong vasodilator, disabling it and therefore narrowing the blood vessel and raising blood pressure (Tom, et al., 2003). Inhibitors of ACE are currently being used to treat diseases of the cardiovascular system, including heart disease, Congestive Heart Failure (CHF) and high blood pressure (Danser, et al., 2007; Mancia, et al., 1997). Benefits of somatic ACE inhibitor treatment in humans have included increased function of the heart, reduction of symptoms including fatigue and reduction in visible damage after a myocardial infarction (Khalil, et al., 2001).

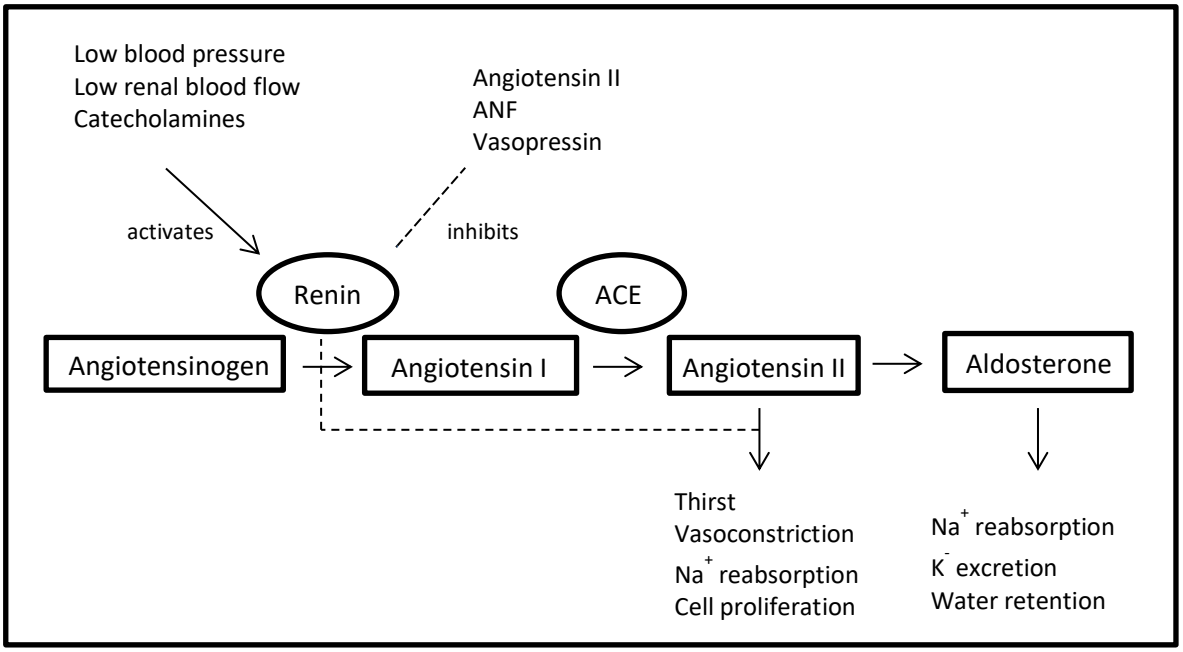

Figure 4: A diagram depicting the RAS and the roles of ACE, renin and AngII within the system (adapted from http://www.urology-textbook.com/kidney-renin-aldosterone.html).

Studies in rats have found that long-term treatment with Enalapril, a somatic ACE-inhibitor, reduced the body fat of rats, lowered their blood pressure and the rats showed a reduction in mortality compared to controls on a standard diet (Santos, et al., 2009). A combination treatment using the somatic ACE inhibitor Perindopril and bradykinin has also been found to improve the ventricular function in the myocardial infarction model in rats (Qu, et al., 2015).

Somatic ACE is also expressed in the adipose cells of humans (Jonsson, et al., 1994) but little is known about its role in this tissue.

1.6: ACE Homologues in *Drosophila melanogaster*

In *Drosophila melanogaster* there are two homologues of human somatic ACE, Angiotensin converting enzyme (ANCE) and Angiotensin converting enzyme related (ACER). However, there are structural differences between ACE and these homologues. Both ANCE and ACER are single domain proteins with only one active site each, whereas human somatic ACE has two domains (N and C) and two active sites (Siviter, et al., 2002). Both ANCE and ACER work as peptidyl-dipeptidases with likely roles in peptide metabolism cleaving different substrate targets (Siviter, et al., 2002).

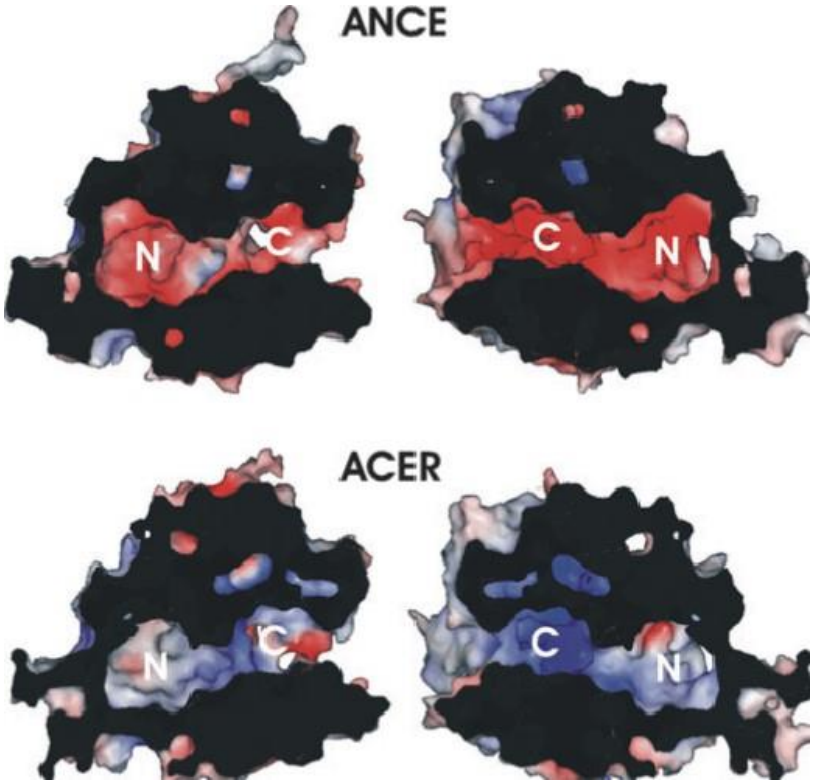

Figure 5: Surface representations of the electrostatic potential of ANCE and a homology model of ACER. The proteins have been sliced in half to show the internal substrate binding channel. The N-chamber and C-chamber (N and C) are postulated to bind up to 7 N-terminal residues and the C-terminal dipeptide of substrate, respectively. Molecular surfaces and electrostatic potential were calculated with the program SPOCK (http://quorum.tamu.edu). ANCE co-ordinates were obtained from the recently determined crystal structure (PDB accession code 1J36). The homology model of ACER was generated in SWISS-MODEL using the ANCE structure as a template. Positive and negative

charges are represented by shades of blue and red, respectively, with neutral areas coloured white. (Bingham, et al., 2006).

The somatic ACE binding sites of the N and C domains are positively charged with the N-domain binding channel appearing to be more positively charged than the C-domain channel (Tzakos, et al., 2003). Figure 5 shows the binding sites of ANCE and ACER which are similar in structure but the substrate-binding channels differ in that the ANCE channel is charged negatively and the ACER channel is charged positively, like the somatic ACE channels (Bingham, et al., 2006).

ACER, however, differs from ANCE and somatic ACE in one crucial way; ACER is unable to convert Ang I to Ang II or hydrolyse bradykinin which are the substrates of human somatic ACE (Bingham, et al., 2006; Siviter, et al., 2002). However despite these differences, ACER is still inhibited by some somatic ACE inhibitors, including Fosinopril (Isaac, et al., 2007). ACER is able to hydrolyse specific dipeptideamides much quicker than ANCE can hydrolyse Ang I, suggesting that ACER's role in peptide metabolism is mechanically different to ANCE's (Siviter, et al., 2002). ACER also has the ability to cleave amidated peptides such as Leucokinin-1 (Siviter, et al., 2002). Amidated peptides have the free carboxy group replaced with an amide group at the C-terminus which is often essential for the activity of neuropeptides and hormones. This suggests that ACER may have a wider range of substrates than ANCE which seems to be more substrate specific, but ACER only exhibited this ability at high concentrations of chloride ions (Siviter, et al., 2002).

1.7: Angiotensin converting enzyme (ANCE) in *Drosophila*

ANCE is expressed in the heart, midgut and the amnioserosa during *Drosophila* embryogenesis (Houard, et al., 1998; Kim, et al., 2003). ANCE was identified as a homologue of human ACE by its ability to convert Angiotensin-I to Angiotensin-II and the hydrolysis of the vasodilator bradykinin (Houard, et al., 1998). ANCE is also found in the tissues involved in *Drosophila* male reproduction, suggesting a role within the reproductive system and a potential link to testicular ACE (Siviter, et al., 2002).

ANCE is a single domain protein consisting of 615 amino acids and requires a zinc ion to catalyse reactions (Kim, et al., 2003). The crystalline structure of ANCE is

shown in Figure 6 and shows the essential zinc ion and the binding of Ang II in the active site as a competitive inhibitor.

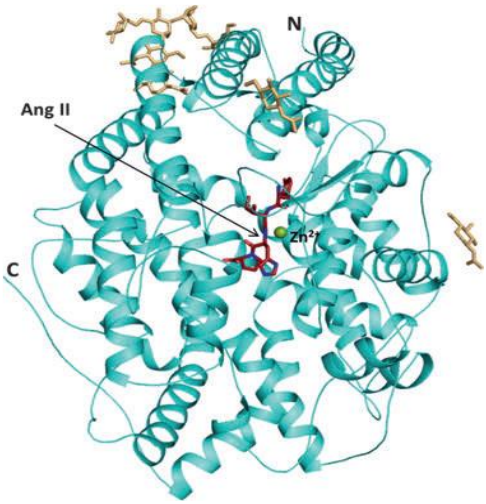

Figure 6: Substrate-bound *Drosophila melanogaster* AnCE crystal structure. AnCE (cyan) in cartoon representation, with Ang II as red sticks, glycosylation carbohydrates as yellow sticks. The catalytic zinc ion is shown as an olive green sphere. (Akif, et al., 2012)

The amino acids are structured in α-helices (21) and anti-parallel β-sheets (3) to form the ANCE protein structure (Kim, et al., 2003). The binding channel of ANCE is mainly negatively charged which suggests that positively charged substrates are preferred to those with a negative charge (Kim, et al., 2003). ANCE activity, like ACE activity, is inhibited by higher concentrations of Ang II. However, in the RAS it is Renin that is inhibited by Ang II and this has a knock-on effect of inhibiting ACE with reduced levels of Ang I. Somatic ACE itself is not inhibited by Ang II but Ang II directly inhibits ANCE as a competitive inhibitor (Akif, et al., 2012). Ang II cannot be broken down again by ANCE due to a proline amino acid placed in the penultimate position at the C-terminus of the molecule, allowing Ang II to competitively inhibit the active site of ANCE and stop any further cleavage of Ang I within the RAS (Akif, et al., 2012).

1.8: Angiotensin converting enzyme related (ACER) in *Drosophila*

ACER was identified in the embryonic heart of *Drosophila* and continues to be expressed there after development along with being expressed in the adult fat body and the head of the fly (Carhan, et al., 2010).

ACER expression has also been found in the testes and ovaries of flies which suggests a possible role within reproduction (Siviter, et al., 2002), however *Acer* null flies still develop as expected with no fertility problems indicating that ACER is not vital for development or reproduction within *Drosophila* (Carhan, et al., 2010).

ACER is expressed in the fly fat body, an organ which is important for storage of nutrients and the endocrine regulation of ageing, and is then secreted into the haemolymph, raising the possibility that ACER may be involved in the control of nutrient supply mechanisms to the brain (Carhan, et al., 2010).

Liao, et al. (2014) measured the effect of knocking down *Acer* specifically in the *Drosophila* heart and it was found that lifespan was decreased as well as heart function, suggesting that *Acer* was essential for heart function in *Drosophila*. However, this contradicts Carhan, et al. (2010) which found that *Acer* null flies, developed using P-element excision, were healthy and showed no obvious ill-effects of the loss of *Acer*.

Acer was found to be down-regulated when flies have been exposed to heat stress (36ºC) for one hour (Nielsen, et al., 2006) suggesting that *Acer* is not beneficial to heat stress resistance as genes involved in heat resistance were found to be up-regulated. *Acer* has also been found to be over-expressed in response to sleep deprivation and starvation (Thimgan, et al., 2015) suggesting potential roles in sleep deprivation resistance and starvation resistance.

The *Acer* null mutant flies also showed normal circadian locomotor rhythms and normal day-time sleep compared to control flies, however the *Acer* null mutants show a decrease in night-time sleep compared to wild type flies resulting in a decrease in total sleep of about 15% (Carhan, et al., 2010). Fosinopril, an inhibitor of ACE in mammals and ACER in *Drosophila* was also found to cause a similar disruption to night-time sleep as well as reducing the total amount of sleep (Carhan, et al., 2010). This is shown in Figure 7.

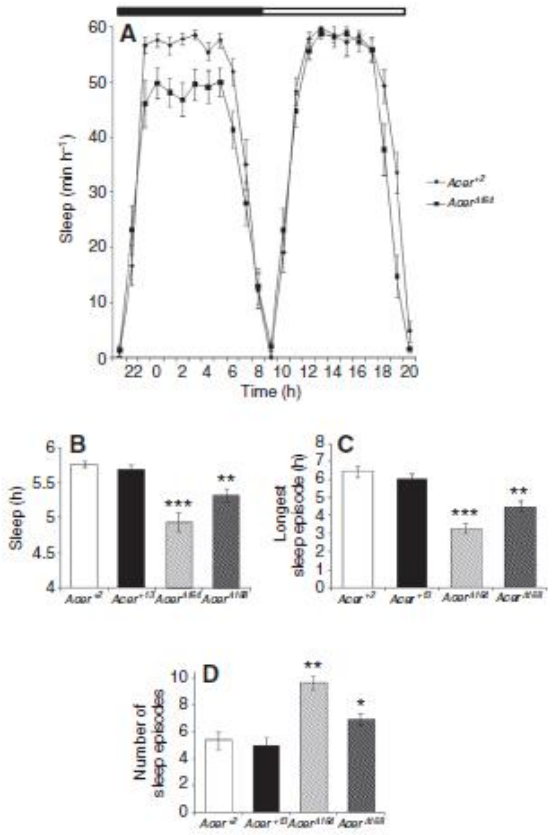

Figure 7: Lack of ACER results in disrupted night-time sleep. (A) Representative sleep profiles for Acer+2 (wild-type) and AcerD164 (Acer null) adult males. White and black horizontal bars indicate day-time and night-time, respectively. Sleep is expressed as minutes of sleep per hour. (B) Sleep between 00.00h and 06.00h for wild-type (Acer+2 and Acer+13) and ACER null (AcerD164 and AcerD168) adult male flies, expressed in hours. (C,D) Longest continuous period of sleep (C) and number of sleep episodes (D) of wildtype (Acer+2 and Acer+13) and ACER null (AcerD164 and AcerD168) adult male flies from lights-off (21.00h) to lights-on (09.00h). Values are means ± s.e.m., 60 flies of each genotype. ***P<0.0001 **P<0.001 and *P<0.05, statistical significance of the difference between wild-type and ACER null flies (Carhan, et al., 2010).

Acer has also been shown to be differentially expressed during night-time sleep as well as showing a range of expression in different genetic backgrounds at night (Svetec, et al., 2015). In addition, *Acer* has also been found to be over-expressed when flies were sleep deprived (Thimgan, et al., 2015) and together these data suggest a potential role for *Acer* in sleep regulation.

1.9: Sleep

Sleep in humans has been widely recognised as being essential to life, however, no singular function attributed to the need for sleep has been found (Kayser & Biron, 2016). It appears that sleep is required for multiple reasons including cognitive function (Gottlieb, et al., 2004) and metabolism (Tamura, et al., 2008).

Sleep, in humans, passes through five stages of sleep in a cyclic manner (Figure 8). Four of these stages are described as being in non-rapid eye-movement sleep (NREM) which consists of light and deep sleep and rapid eye-movement sleep (REM) where the eyes shift from side-to-side but remain closed throughout. (https://www.tuck.com/stages/). Through stages one and two a person can be awakened easily and often describes having not fallen asleep at all when woken. Brain movement and muscle movement slows in preparation for deep sleep. In stages three and four slow brain waves, called delta waves, are prominent and during stage three night terrors and sleep-walking are most likely to occur. Waking from stage four sleep often leaves the person feeling disorientated. REM sleep is where dreams are most likely to occur and brain waves are similar those seen when a person is awake (https://www.tuck.com/stages/).

In children, using sleep apnoea (pauses in breathing or shallowing breathing in sleep) as a measure of disrupted sleep, it was found that cognitive function and general intelligence was reduced in those suffering from disrupted sleep (Schechter, 2002). However, it was difficult to discern whether the reduced cognitive effects were due to disrupted sleep or reduced oxygen levels (Kayser & Biron, 2016). A further study investigating sleep fragmentation using snoring but without the oxygen levels being affected, showed that cognitive functions were negatively affected by the lack of sleep (Gottlieb, et al., 2004), therefore supporting the hypothesis that sleep is important for cognition (Kayser & Biron, 2016). In adults it was found that glucose metabolism was impaired in 60.5% of patients who suffered with sleep apnoea and that the prevalence increased with the severity of the sleep apnoea (Tamura, et al., 2008).

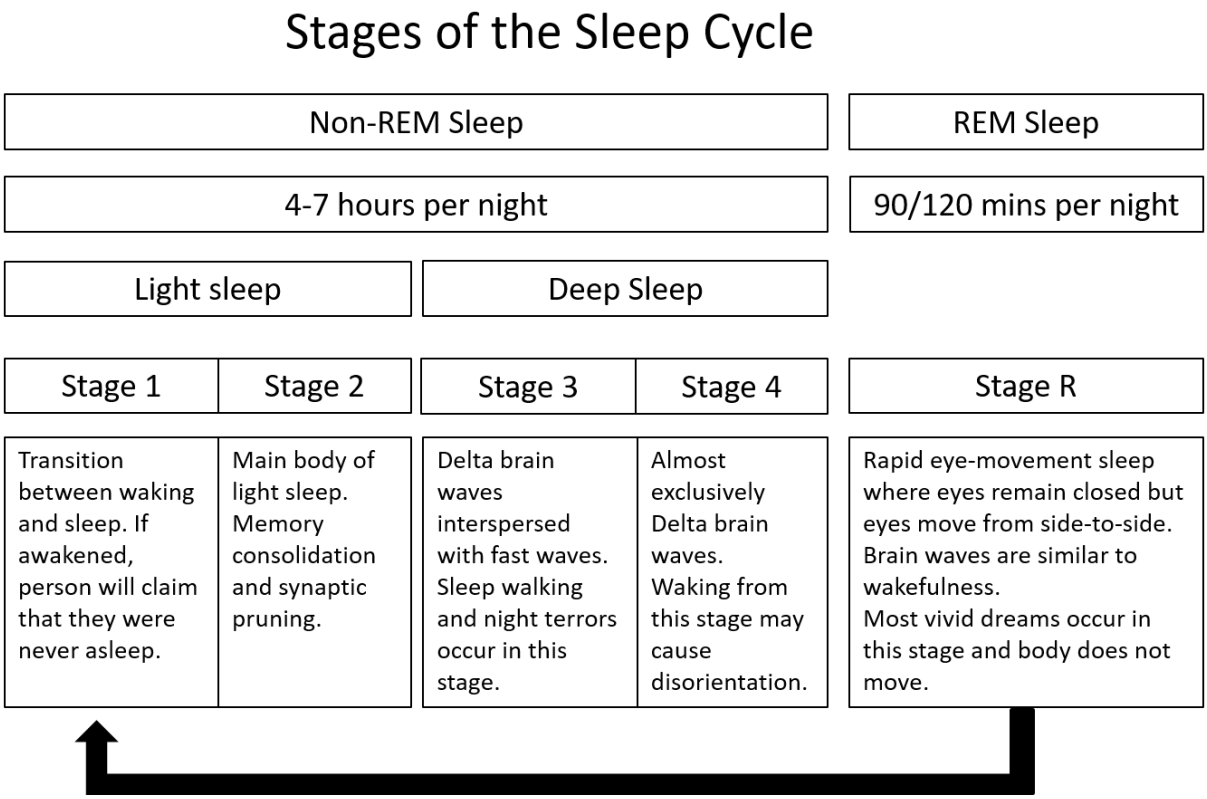

Figure 8: The Stages of the Human Sleep Cycle. Non-REM Sleep includes: Stages 1 and 2 are part of Light Sleep and Stages 3 and 4 are part of Deep Sleep. REM sleep only occurs in Stage R. Adapted from https://www.tuck.com/stages/.

In *Drosophila melanogaster*, like humans, sleep is controlled by circadian rhythms (Shaw, et al., 2000; Tataroglu & Emery, 2014). Circadian rhythms in the fly determine periods of activity and rest as well as the response to temperature change and the timing of feeding and courtship (Kaneko, et al., 2012; Tataroglu & Emery, 2014). Circadian rhythms in *Drosophila melanogaster* exhibit a feedback loop system over 24 hours (Figure 9). During the day the circadian transcription factors CLOCK (CLK) and CYCLE (CYC) combine to form a heterodimeric complex in the nucleus of the cell. This binding promotes the transcription of the *period* (per) and *timeless* (tim) genes (Allada, et al., 1998). At night, PER and TIM form a heterodimer of their own which enters the nucleus, resulting in the inactivation of CLK/CYC by phosphorylation (Menet, et al., 2009). During the day, gradual phosphorylation of PER/TIM results in the end of their repression of CLK/CYC, allowing CLK/CYC to restart the circadian cycle (Edery, et al., 1994). Environmental factors also affect the circadian cycle. Cryptochrome (CRY) is a blue light receptor which changes conformation in the

presence of blue light. This change allows it to bind to TIM and reset the clock (Hunter-Ensor, et al., 1996; Fang-Ju, et al., 2001).

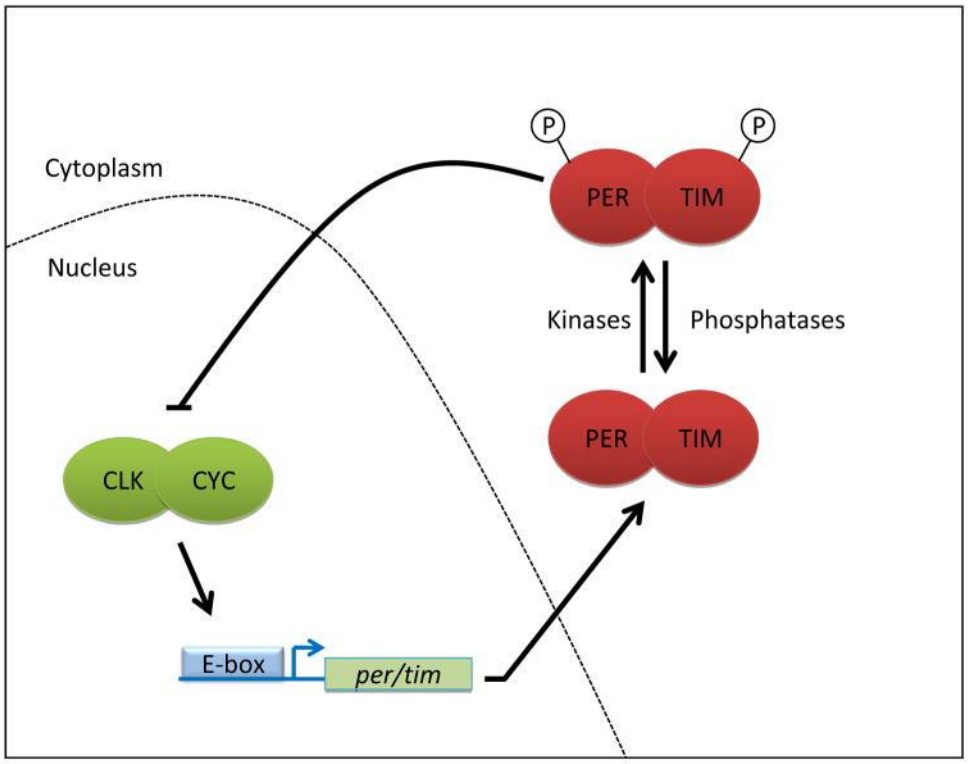

Figure 9: The circadian transcriptional feedback loop system in *Drosophila melanogaster*. CLOCK/CYCLE (CLK/CYC) drive the expression of their own repressors PERIOD (PER) and TIMELESS (TIM). PER/TIM go through various modifications during the day, until they are eventually turned over to release CLK/CYC from repression, starting the next cycle. (Tataroglu & Emery, 2014).

The expression of ACER in the head of the fly showed a daily cycle that appears to be regulated by the *Clock* gene (Carhan, et al., 2010). The daily cycle consists of activity at dawn and dusk separated by periods of relative inactivity and consolidated sleep during the night (Bushey, et al., 2010). Sleep and sleep fragmentation in flies increases with age, which is a phenotype often seen as humans age (Bushey, et al., 2010).

1.10: Sleep disruption and Nutrient sensing

Sleep in *Drosophila melanogaster* has been found to increase with age as well as the number of bouts increasing before reaching a threshold where sleep remains

constant (Bushey, et al., 2010). At night the number of bouts of sleep decreased while the number of bouts of sleep in the light increased but increased fragmentation was not seen (Bushey, et al., 2010). Selected *Hyperkinetic* mutants (Bushey, et al., 2010) have shown fragmentation in sleep as age increases and these mutants were found to have a shorter longevity than controls. Short-sleep mutants such as *fmn* (Yamazaki, et al., 2012) showed similar fragmentation as controls with fragmentation increasing with age and longer bouts of sleep being reduced at older ages. Ageing effects were more prominent on a higher calorie diet but the *fmn* mutants were found to be short-lived on higher calorific food compared to lower calorific food when compared to controls (Yamazaki, et al., 2012).

Sleep has also been found to be increased when flies were dosed with varying amounts of the active metabolite of the Ecdysone steroid hormone 20E (Ishimoto & Kitamoto, 2010). Ecdysone is important in larval moulting and metamorphosis. Increasing levels of 20E increased total sleep in the day and night with an increase in bout length and a decrease in wake bouts for female flies but walking behaviour was unaffected. In the heterozygous state the *dominant temperature sensitive – 3* (DTS-3) Ecdysone-synthesis mutant sleeps significantly less than controls with a large reduction in daytime sleep as well as a reduced night time sleep making it a short-sleep mutant (Ishimoto & Kitamoto, 2010). The flies also exhibit shortened sleep bout duration in both the night and day with these phenotypes being rescued when 20E levels were brought back to near the level of controls, suggesting that lower levels of 20E were responsible for the phenotypes observed. In sleep deprived flies the levels of 20E were higher than controls, this and the previous phenotypes, suggesting that adult Ecdysone plays a role in regulating sleep.

Genetic backgrounds have shown a variation in sleep patterns with flies originating from an equatorial climate sleeping twice as much as those from a temperate climate (Svetec, et al., 2015) as well as different circadian patterns observed in flies from different latitudes and altitudes (Hut & Beersma, 2011). This is why it is important to use flies of different genetic backgrounds to investigate whether *Acer*'s role, if any, in sleep is relatable to all genetic backgrounds.

Sleep in flies is also regulated by nutrition (Broughton, et al., 2010). Flies with the median Neurosecretory cells (mNSCs) in the brain ablated to reduced insulin-like signalling showed a reduction in night-time sleep under different nutritional conditions (Broughton, et al. 2010). The mNSC-ablated flies lived longer than controls on low

food with their activity was highest on the 0.1% sugar/yeast food and contrastingly their sleep was at its lowest on the same food. As the food concentration increased control activity decreased and sleep increased, suggesting a certain amount of nutrient dependency for sleep and activity (Broughton, et al., 2010).

Other nutrient sensing pathway mutants, such as c-Jun N-terminal Kinase (JNK) -knockdown mutants, have shown a decrease in lifespan and the subsequent effect of this on sleep was analysed (Takahama, et al., 2012). The controls showed a normal reduction of sleep associated with a high calorie diet but the JNK-knockdown flies showed no response to this diet. The number of sleep bouts was higher on low calorie food the knockdown flies which also showed a reduction in the number of long sleep bouts (Takahama, et al., 2012), suggesting a role for sleep regulation for the JNK pathway.

There are similarities with mammals where ACE is found in adipose tissue (Santos, et al. 2009) and may be part of a system which links metabolism and sleep. Enalapril, an inhibitor of ACE, was found to decrease levels of ACE activity within the adipose tissue, correlating with reduced body weight of the rats tested (Santos, et al. 2009). Ang II induces the growth of adipocytes and therefore a reduction in Ang II due to ACE inhibition may have caused the rats to lose body weight with a reduced number of adipocytes (Santos, et al. 2009).

The expression of ACER in the fat body of the fly is very interesting in terms of a role in nutrient sensing. Flies exhibit circadian clocks in different tissues throughout the *Drosophila* body including the fat body (Xu, et al., 2008). The expression of dominant negative CLOCK (dnCLK) to reduce CLOCK (CLK) signalling within the fat body, resulted in flies displaying characteristics of increased food consumption, a decreased level of stored glycogen and flies were more susceptible to starvation. The same results were seen using dominant negative CYCLE (dnCYC) (Xu, et al., 2008).The co-expression of *Clk* and *Cyc* and *Acer* in the fat body suggests a possible link between ACER and feeding and nutrition which has parallels with mammalian ACE research, where ACE expression has been found in adipocytes (Santos, et al., 2009).

Catterson, et al. (2010) experimented with sleep in conjunction with diet, specifically the levels of yeast in the food. It was found that dietary levels of yeast do cause a difference in sleep patterns in flies by fragmenting sleep-wake behaviour. Males were less likely to wake up quickly and reduced day and night time sleep while

females showed a lower level of locomotor activity on a 5% sugar and 2% yeast diet compared to a 5% sugar 0% yeast diet. Both sexes showed an increased number of bouts of sleep which were also shorter in length (Catterson, et al., 2010). This is similar to findings in Linford, et al. (2012), where an increase in dietary sugar and yeast increased the number of sleep bouts while reduced nutrients reduced the number of long sleep bouts and increased short and medium bouts, therefore increasing the fragmentation of sleep.

It is shown that, like humans, *Drosophila melanogaster* show an ultradian sleep-wake pattern from night-time sleep suggesting that the mechanisms that control sleep in flies may be similar to mechanisms that control sleep in mammals and the possible presence of an ultradian oscillator that specifically controls the action of waking up from sleep within the brain of the fly (Catterson, et al., 2010). However, this result was very varied and was only found to be significant when a large number of flies were tested.

Starvation resistance has been found to be linked to an increase in sleep in flies (Masek, et al., 2014) potentially suggesting that longer sleep favours starvation resistance.

1.11: Diet and Nutrient Storage

Diet can affect food intake, metabolism and the storage of nutrients. Unbalanced diets can cause states of health in *Drosophila* that are very similar to obesity and even diabetes in humans (Skorupa, et al., 2008). The diet for experimental flies consists of yeast to provide protein and sugar to provide carbohydrate. Flies that were fed on high sugar content food were found to maintain the same weight even when transferred to lower sugar diets. These flies also showed shorter longevity and reduced fecundity in female flies suggesting that too much sugar may be toxic to the flies (Skorupa, et al., 2008). Higher levels of yeast led to reduced longevity but increased fecundity, suggesting that although lifespan is reduced, the high level of yeast in the diet is not toxic to the flies, unlike high levels of sugar. Diet has proved important in the longevity and fecundity of flies with DR increasing longevity often, although not always, at the expense of fecundity in female flies (Burger, et al., 2007). In females it is possible that high yeast food requires a ‘trade-off’ between high levels of egg-laying and lifespan (Skorupa, et al., 2008). Physiologically flies show signs of

obesity when placed on a high carbohydrate diet at an early age, which was magnified by age, suggesting that diets that focus on one form of nutrient, either carbohydrate or yeast, can be detrimental to long term longevity and that a balanced diet is best. This may also be true for humans who exhibit similar responses to flies (Skorupa, et al., 2008).

Figure 10 shows that high levels of sugar promote strong adiposity with high related triglyceride levels whereas yeast levels appeared to counter-act the high levels of adiposity associated with high sugar. Protein storage has been found to be independent of carbohydrates and is determined by dietary yeast (Skorupa, et al., 2008).

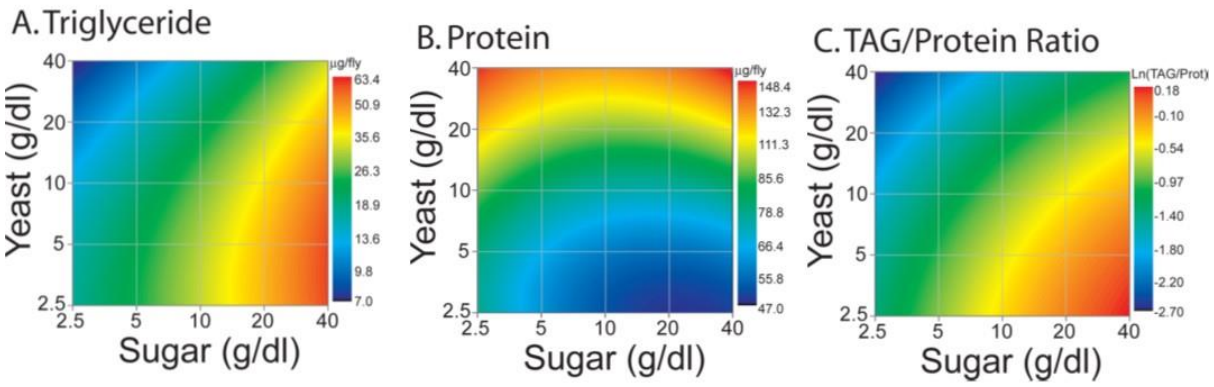

Figure 10: Diet Response Surfaces for metabolic phenotypes Levels (µg) of triglyceride (A) and protein (B). A summary measure of body composition, which is based on the relative ratio of TAG to total protein (C). Dietary sugar promotes TAG storage, while dietary protein suppresses it. Female flies of the yw strain are represented here. (Skorupa, et al., 2008).

Flies store carbohydrate and fat as glycogen, lipid and trehalose and Handel, (1965) discovered how to measure them separately. Glycogen storage, appears to be regulated by a clock gene located in the fly fat body (Xu, et al., 2008). When the clock gene in the fat body was disrupted glycogen levels were decreased whereas lipid levels only decreased slightly. Sensitivity to starvation was also increased and lipid levels have also been implicated in starvation resistance with higher levels contributing to resistance in *Drosophila simulans* (Ballard, et al., 2008). In mammals cAMP-responsive transcription factor (CREB) regulates glycogen and lipid and a *Drosophila* homolog, dCREB2 has been identified (Iijima, et al., 2009). Knockdown of dCREB2 in adult flies results in a reduction in glycogen and lipid storage while the blocking of

CREB only in neurons also resulted in a reduction in glycogen and lipid and increased sensitivity to starvation and oxidative stress. Blocking CREB specifically in the fat body resulted in reduced glycogen storage but increased lipid stores, with starvation resistance showing no difference to controls. These flies also had increased sensitivity to oxidative stress (Iijima, et al., 2009). Mio, a *Drosophila* homolog of human carbohydrate response element binding protein (chREBP), is a transcription factor which is thought to be responsible for the regulation of lipogenic enzymes and lipid storage as well as regulating food consumption (Sassu, et al., 2012). Reduced insulin signalling mutants, achieved by the ablation of the mNSCs in the brain, showed a higher than normal level of glycogen and lipid stores (Broughton, et al., 2005) but mutants with only DILP 2 knocked-down show no difference in glycogen and lip[id levels (Broughton, et al., 2008).

When studying nutrient storage a feeding assay is required to show whether any difference in nutrient storage is due to differences in the amount of food the fly has consumed or is due to the concentration of ingredients the food. One method is to feed flies an inert blue dye and then crush the flies and measure the absorption of blue colour in a photospectrometer. Using blue dye to determine consumption after six hours on food, Skorupa, et al. (2008) found that increased sugar in the diet increases consumption of food but the concentration of yeast does not increase the intake of food. It was also found that flies consumed the most calories on high/sugar/low yeast food and consumed the least calories on a medium protein/low carbohydrate diet. Conversely, Wong, et al. (2009) found no difference in feeding using blue dye between dietary restriction food (50g sugar and 100g yeast) and fully-fed food (50g sugar and 200g yeast) suggesting that DR does not affect food intake. Min & Tatar. (2006) also used blue food dye to assess feeding as well as dye marked faecal pellets and they found that females on higher food fed more than those on lower food and that younger flies ate more than older flies. They also found that females on higher food provided three times more excrement suggesting that these females ate more than those on the lower food. Males showed no significant difference between age and diet. Increased survival was seen for female flies on the DR diet but without any compensatory feeding so the DR flies consumed less yeast as well as fewer calories. Mair, et al. (2005) also saw a reduction in feeding on DR food using proboscis extension to identify feeding. This includes recording how many times the proboscis

is extended towards the food as a measure of feeding as this was found to be reduced in flies fed DR food.

Another alternative method to using blue food dye is the Capillary Feeder Assay (CAFE Assay) (Catterson, et al., 2010).This uses capillary tubes for flies to consume liquid food and the amount eaten can then be measured down the capillary tube as food is consumed. A blue food dye was also used to make the level of food in the capillary tubes clearer. It confirmed findings in (Skorupa, et al., 2008) that dietary yeast does not affect food intake (Catterson, et al., 2010).

Radioactivity has also been used to determine feeding behaviour (Zeng, et al., 2011) finding that DR flies increased their volume of food intake by twice as much as the full diet which contradicts Min & Tatar. (2006) who saw opposite phenotypes.

When circadian clock genes were disrupted specifically in the fly fat body, feeding behaviour was disrupted and flies subsequently increased feeding, suggesting that clock genes in the fly fat body regulate the feeding rhythm as well as the consumption of food (Xu, et al., 2008). Feeding was found to be reduced in starvation resistant mutants (Masek, et al., 2014), suggesting that flies resistant to starvation do not need to feed as much as flies that are not starvation resistant..

This study will analyse *Acer's* relationship to nutrient storage to determine if *Acer* is involved in the response of nutrient-responsive phenotypes, such as lipid and glycogen storage which are stored in the fat body where *Acer* is expressed.

1.12: Cold, heat, starvation and oxidative stress

Chill coma recovery has been used as a marker for stress in *Drosophila* for many years. Wit, et al., 2013 showed that lifespan extension was not related to susceptibility to chill coma stress in females but males did show a slight reduction in the ability to recover. Broughton, et al., 2005 found that long-lived mNSC-ablated flies were susceptible to cold shock and therefore took longer to recover from cold stress. Flies have also been shown to recover more slowly from cold chill coma at older ages (Burger & Promislow, 2006). Starvation before the cold stress was implemented was seen to increase resistance to cold stress with males showing a higher resistance than females, but starvation only increased resistance at young ages and not older ages (Le Bourg, 2013). These experiments were conducted analysing the lifespan of the flies after the cold stress had been enforced. Recovery time has also been measured

after a short period of cold stress (Morgan & Mackay, 2006) where flies were left for 3 hours at 0ºC and 50% of flies had recovered in 11 minutes. Cold resistance has been found to be heritable within populations, with cold resistance being transmitted to the next generation after the previous generation was exposed to cold stress. However, there was no correlation between cold stress resistance and resistance to other stresses such as heat, starvation and oxidative stress (Gerken, et al., 2016). Recovery time in comparison to diet has also been observed and it was found that there was no effect of food at young ages but at older ages those on lower food concentration recovered more slowly than those kept on higher concentration food (Burger, et al., 2007). Colinet & Renault, 2014 compared dietary effects on cold stress by using foods containing either no yeast, live yeast or autolysed brewer's yeast. When live yeast was added to a sugar and agar mix, female flies on the live yeast mix recovered quicker and lived longer after the stress was inflicted than those on the sugar and agar mixture alone. When the same live yeast mixture was compared to a mixture using autolysed brewer's yeast instead, it was found that the flies in both groups showed similar initial recovery times from the cold stress but that the flies on live yeast showed a reduced mortality after the cold stress. This suggests that live yeast levels may have a role in resistance to cold over time but yeast itself was important in initial recovery from the cold stress.

Dietary restriction was found to reduce survival post-cold shock in males and females (Le Rohellec & Le Bourg, 2009) but dietary restriction in this case meant the absence of live yeast on top of the inactivated brewer's yeast in the medium..

The ability of the fly to recover from cold temperatures has been shown to be affected by latitude in *Drosophila melanogaster* (Guerra, et al., 1997) and *Drosophila ananassae* (Sisodia & Singh, 2010). Flies from latitudes closer to the equator were found to have longer recovery times compared to those on latitudes further from the equator (Sisodia & Singh, 2010).

Like cold stress heat stress in *Drosophila* has been monitored in many different ways and at different temperatures. Morgan & Mackay, 2006 measured flies response to heat stress for different lengths of time at 38ºC before monitoring their survival after the stress. They found that the 50% survival threshold was 110 minutes at 38ºC. Wit, et al., 2013 also measured the time till death as a measure of resistance and found that long-lived flies were no more susceptible or resistant to heat than controls while it was found that long-lived, reduced insulin signalling mNSC-ablated flies showed

sensitivity to heat shock (Broughton, et al., 2005). Survival time has also been monitored at 37ºC at young age and old age and also in terms of diet (Le Rohellec & Le Bourg, 2009). They found in males that DR had no effect but survival time decreased with age. In females survival time also decreased with age but DR flies survived longer than controls but only at young ages. DR in this experiment consisted of no live yeast food on top of inactivated brewer's yeast medium. It has been found that genes that are beneficial to heat resistance, such as phototransduction genes, are up-regulated in response to heat stress. *Acer* was found to be down-regulated when flies were subjected to heat stress, suggesting that *Acer* is not beneficial to heat stress resistance (Nielsen, et al., 2006).

Starvation resistance has been monitored by using agar medium to provide water but no nutrients. Long-lived reduced insulin signalling mNSC-ablated flies were found to be starvation resistant (Broughton, et al., 2005) as well as DILP2 knockdown flies but to a lesser degree (Broughton, et al., 2008). Long-lived strains were also found to be resistant to starvation (Wit, et al., 2013) but although DR was found to initially increase resistance it later led to susceptibility (Burger, et al., 2007). Lipid and glycogen levels have also been linked with starvation resistance with higher levels of lipid associated with resistance and lower levels of glycogen associated with susceptibility (Ballard, et al., 2008; Xu, et al., 2008). Feeding was found to be decreased and sleep has been found to be increased in starvation resistant flies (Masek, et al., 2014). Thimgan, et al. (2015) found that *Acer* was one of many genes to be over-expressed in the response to starvation suggesting that *Acer* may play a role in response to starvation conditions.

Oxidative stress has been measured by feeding flies paraquat and hydrogen peroxide and then monitoring and recording lifespan until death. Long-lived flies were found to be resistant to paraquat oxidative stress compared to short-lived strains (Mockett, et al., 2001). Broughton, et al., 2005 found that mNSC-ablated flies, which were long-lived, were resistant to paraquat oxidative stress but Broughton, et al., 2008 found that DILP2 knockdown flies were not resistant or susceptible to hydrogen peroxide oxidative stress. Starvation before the stress was found to decrease resistance to hydrogen peroxide oxidative stress at all ages (Le Bourg, 2013) and DR was shown to have no effect at early ages but was found to decrease paraquat oxidative stress (Burger, et al., 2007).

The different stress responses have also been analysed to see if there is any connection between them. It was found that chill coma recovery was thought to be independent of the other stresses as flies resistant to chill coma were not resistant to other stresses (Gerken, et al., 2016).

1.13: Preliminary Data

Together, the published data indicate a possible role of ACER in metabolism, nutrient sensing and behavioural activity. Preliminary data by Dr. Susan Broughton and Dr. Alan Shirras raised the possibility that ACER plays a role in ageing. The effect of the loss of *Acer* on lifespan and fecundity were analysed. Fecundity in wild type flies shows a gradual reduction over time. At young ages more eggs are laid when the yeast content in food is high compared to lower yeast content (Skorupa, et al., 2008). Dietary Restriction (DR) is known to increase lifespan in model organisms, including in female flies but it is generally not as effective in male flies. The DR food concentration of 50g per litre of sugar and yeast was compared against the control of 50g of sugar and 200g of yeast per litre (FF).

Acer null flies were found to lay fewer eggs than wild type flies on both high (FF) and low yeast (DR) food suggesting a low egg-laying phenotype for *Acer* null females (Figure 11).

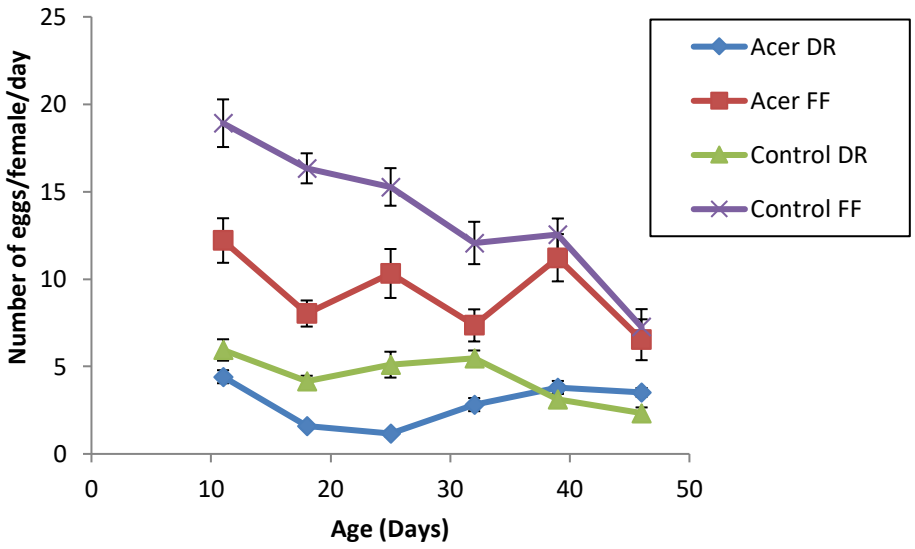

Figure 11: Fecundity of female flies comparing *Acer* nulls and w^{Dah} controls on DR and FF food.

The preliminary data showed that both *Acer* null males and females responded to DR with a lifespan extension and had a longer lifespan than controls on both of the foods tested (Figure 12).

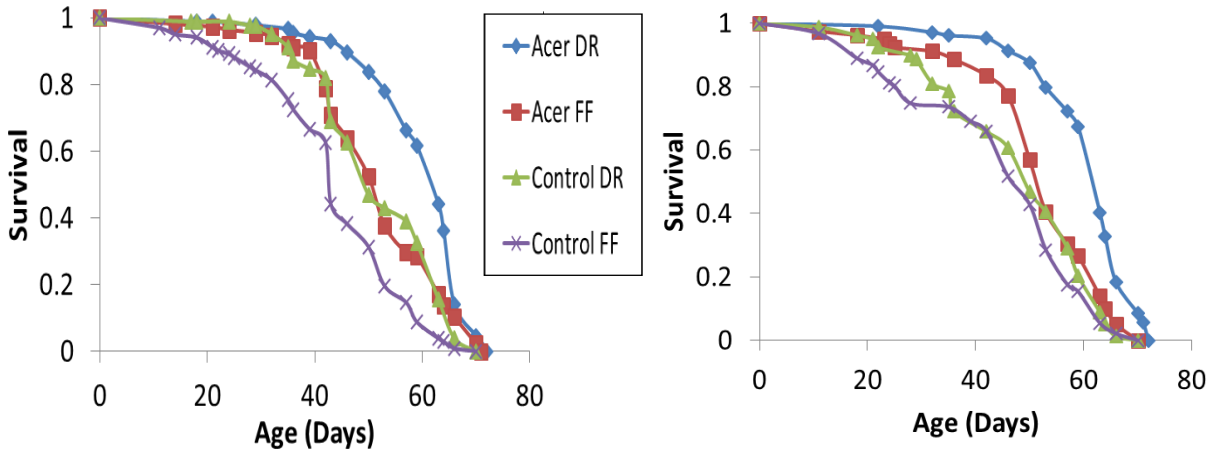

Figure 12: Lifespan of *Acer* null females and males compared to w^{Dah} controls on DR and FF food.

These data suggest that *Acer* nulls have a longer lifespan than controls regardless of the nutrient concentration of sugar and protein and therefore *Acer* may be a novel gene involved in the modulation ageing.

1.14: Aims and Objectives

The aim of this study is to investigate *Acer*'s potential role as a novel ageing gene and its involvement in modulating the nutrient response in *Drosophila melanogaster*. This will be done by monitoring phenotypes known to respond to nutritional changes.

1.14.1: Hypothesis

ACER modulates the dietary response, if any, of phenotypes to nutrition as well as the ageing response to nutrition.

1.14.2: Research Questions

The specific questions that will be addressed in the current study are: (1) Is *Acer* required in the ageing response to nutrition? (2) Does *Acer* modulate any response of phenotypes to nutritional changes? (3) Does *Acer* modulate the response of the IIS pathway to nutrition? (4) Do *Ance* deleted mutants, a different *Drosophila* homolog of human *Ace*, respond dietary changes in the same way as *Acer* deleted mutants?

1.14.3: Objective 1

To determine the role, if any, of *Acer* in the normal response of lifespan to nutrition.

This will address question 1 by monitoring *Acer* deleted males and females and their lifespan while feeding on different diets compared to controls of the same genetic background. These experiments will be conducted in the w^{Dah} and w^{1118} backgrounds (See Chapter 2.1) to confirm the effect of *Acer*. Preliminary results showed that *Acer* deleted males and females were long-lived on DR food (Chapter 1.13. and Chapter 2.9) compared to controls suggesting that *Acer* may modulate the response of lifespan to nutrition.

1.14.4: Objective 2

To determine *Acer's* role, if any, in phenotypes known to respond to dietary changes. The phenotypes studied are sleep, nutrient storage, fecundity and stress resistance.

This will address question 2 by monitoring *Acer* deleted males and females and their response to sleep, nutrient storage of lipid and glycogen, fecundity and stress resistance in the w^{Dah} and w^{1118} backgrounds. Preliminary results showed that *Acer* deleted females laid fewer eggs than controls but showed an extended lifespan on DR food, suggesting *Acer*'s involvement in the nutrient response. Sleep, glycogen and lipid storage as well as stress resistance have all been shown to respond differently to changes in nutrition. Therefore, these phenotypes will be tested to establish whether the loss of *Acer* affects their response to dietary change and *Acer*'s role. Flies will be analysed on the same diets as longevity. Feeding will also be analysed to ensure that any differences in glycogen and lipid storage are not the result of increased or reduced consumption of food. Due to *Acer's* expression in the ovaries and the testes of flies, female flies will be reciprocally mated to analyse if any difference in fecundity is due to the lack of *Acer* in either the testes of ovaries specifically (Chapter 2.15.1).

1.14.5: Objective 3

To analyse *Acer's* role in modulating the normal role of IIS to nutrition.

This will address question 3 by using real-time QPCR analysis of *Drosophila* insulin-like peptide (DILP) expression in the fly head and body comparing controls to *Acer* deleted males and females. Preliminary results showed an extension of lifespan for *Acer* deleted males and females which is similar to the extended lifespan observed in some insulin-signalling (IS) mutants (Figure 12).

1.14.6: Objective 4

To determine if *Ance* responds similarly to *Acer* in terms of longevity and fecundity.

This will address question 4 by comparing *Ance* deleted males and females in terms of longevity and in addition fecundity for females to different diets in the w^{Dah} genetic background. Preliminary results showed the loss of *Acer* extended lifespan and reduced fecundity, therefore lifespan and fecundity will be tested for *Ance* deleted males and females.

Chapter 2: Materials and Methods

2.1: Fly Stocks and Maintenance

The *Acer* deletion ($^{\Delta}$) (Carhan, et al., 2010) was analysed in the out-bred w^{Dah} (Broughton, et al., 2005) and the inbred w^{1118} genetic backgrounds. The *Acer* stock was initially backcrossed six times to each genetic background (see section 2.2) and once a year thereafter. The *Acer* deletion is a null mutation and the null flies are referred to as $Acer^{\Delta}$ throughout this study. The *Ance* deletion ($^{\Delta}$) was prepared in the same way in the w^{Dah} background ($Ance^{\Delta}$). The primary aim of this study was to investigate *Acer* therefore *Ance* was only tested in one genetic background.

The w^{Dah};$Acer^{\Delta}$, w^{1118};$Acer^{\Delta}$ and w^{Dah} ;$Ance^{\Delta}$ stocks, as well as the w^{Dah} and w^{1118} background stocks were maintained in separate *Drosophila* bottles, plugged with sponge bungs, under standard conditions of 25ºC with a 12 hour dark/light cycle. Each genotype was regularly maintained in 8 bottles of stock flies that were mixed and transferred every three weeks to four weeks allowing for overlapping generations of flies to mate.

2.2: Genetic backcrosses

Due to the *Acer* and *Ance* genes being located on the *Drosophila* autosomal chromosome 2 and the mutants being deletion mutants a strategy for backcrossing was devised. The $Acer^{\Delta}$ stock background was the w^{1118} background, however genetic backgrounds vary between laboratories. This was overcome by backcrossing the *Acer* deletion into the w^{Dah} background and the laboratory w^{1118} background so that comparisons could be made between the mutants and controls. The *Ance* deletion was only backcrossed into the w^{Dah} background.

Roughly 50 females carrying the *Acer* deletion in the homozygous state were crossed (1) with 30 males from either the w^{Dah} or w^{1118} backgrounds resulting in offspring which were heterozygous for the *Acer* deletion. The heterozygous *Acer* virgin female offspring (see section 2.3) were then crossed again (2) with males from the corresponding backgrounds, resulting in half of the flies being heterozygous for the deletion and the other half carrying the *Acer* gene (Figure 13).

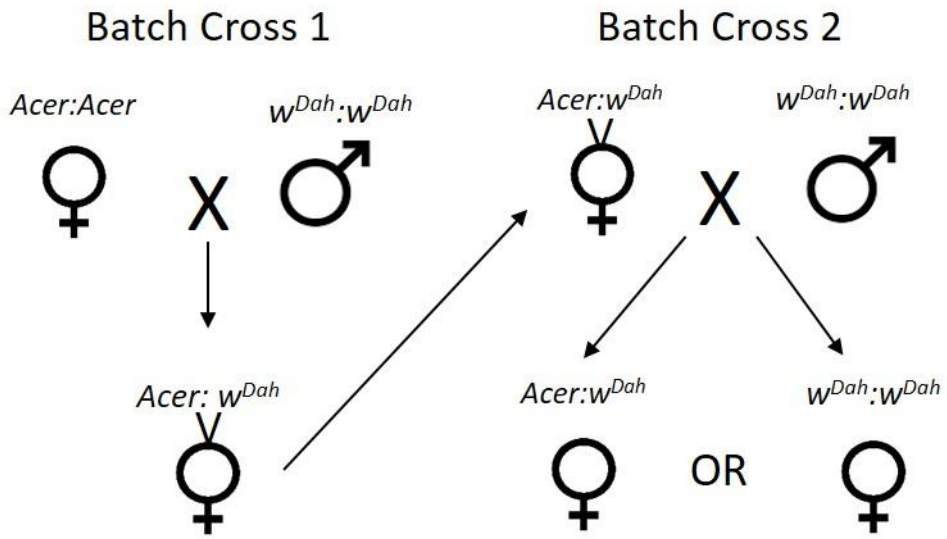

Figure 13: Batch Cross process for the *w^{Dah}* genetic background which was repeated in the *w^{1118}* background. Female *Acer* deletion mutants were crossed with *w^{Dah}* background males to give *$Acer^{\Delta}$* heterozygotes. These heterozygotes were crossed with *w^{Dah}* males to give either *$Acer^{\Delta}$* heterozygotes or *w^{Dah}* controls.

Single pair crosses (Figure 14) were set up using virgin females from cross 2 (Figure 9) and the appropriate background males. The flies were left to mate and the females laid eggs for 5-7 days. The females were then removed from the vial and their DNA was tested for the deletion using Polymerase Chain Reaction (PCR) (Chapter 2.4.1). The offspring of the single pair cross were kept if the female fly carried the *Acer* deletion and discarded if she did not. Single pair crosses were then set up again from the offspring of the female flies with the deletion (Figure 10). This was then repeated until 4 single pair crosses had been completed.

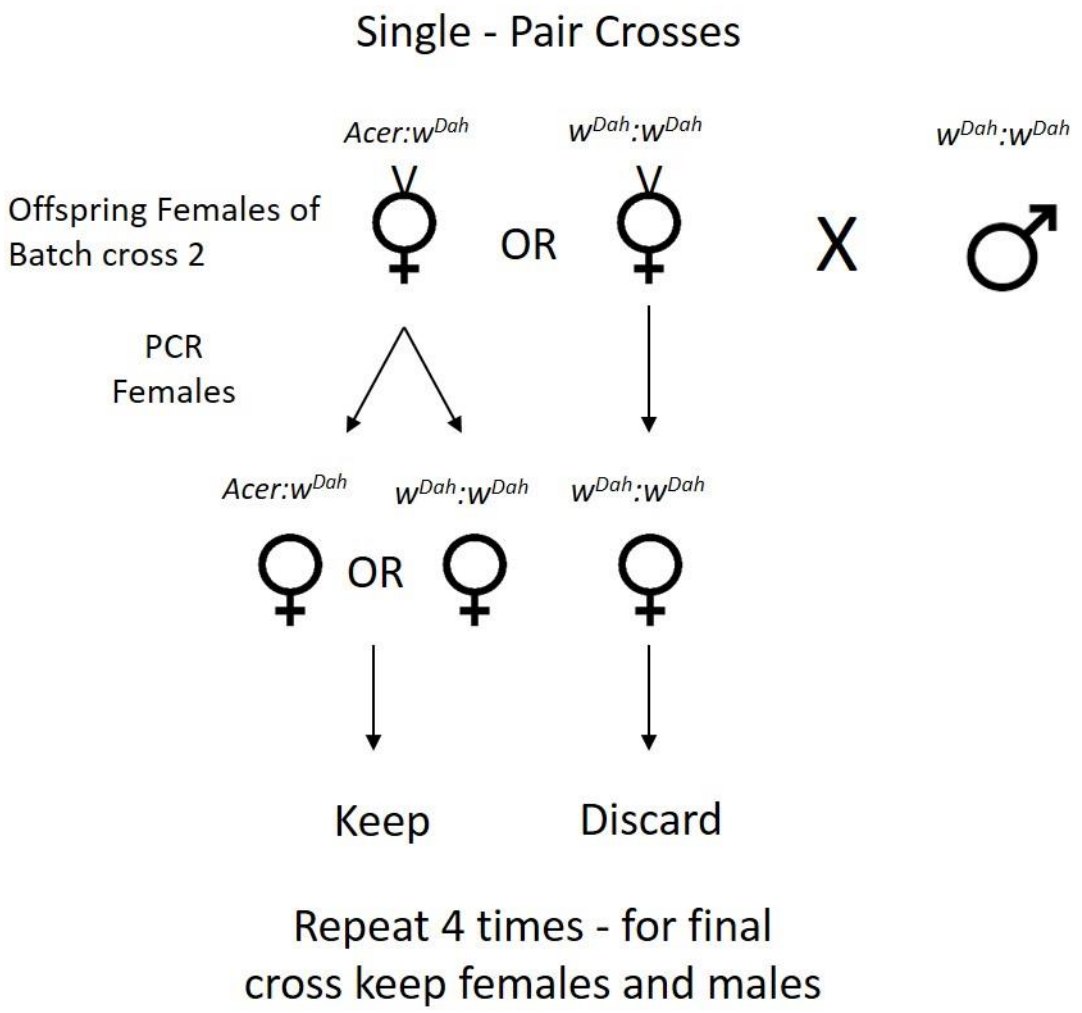

Figure 14: Single – Pair Cross process for the w^{Dah} genetic background. Female flies were tested for the *Acer* deletion by PCR and offspring of the flies with the deletion were kept while those without were discarded. This was repeated in the w^{1118} genetic background.

Once backcrossing was complete, a homozygous *Acer* deletion stock was generated using single pair crosses and PCR to confirm the genotype. (For agarose gels see Chapter 3.2.1 for $Acer^{\Delta}$ and Chapter 8.2.1 for $Ance^{\Delta}$.) 20 pairs of flies homozygous for the *Acer* deletion were used to establish the final stock to be used for experiments (Figure 15). This procedure was the same for the w^{1118} genetic background.

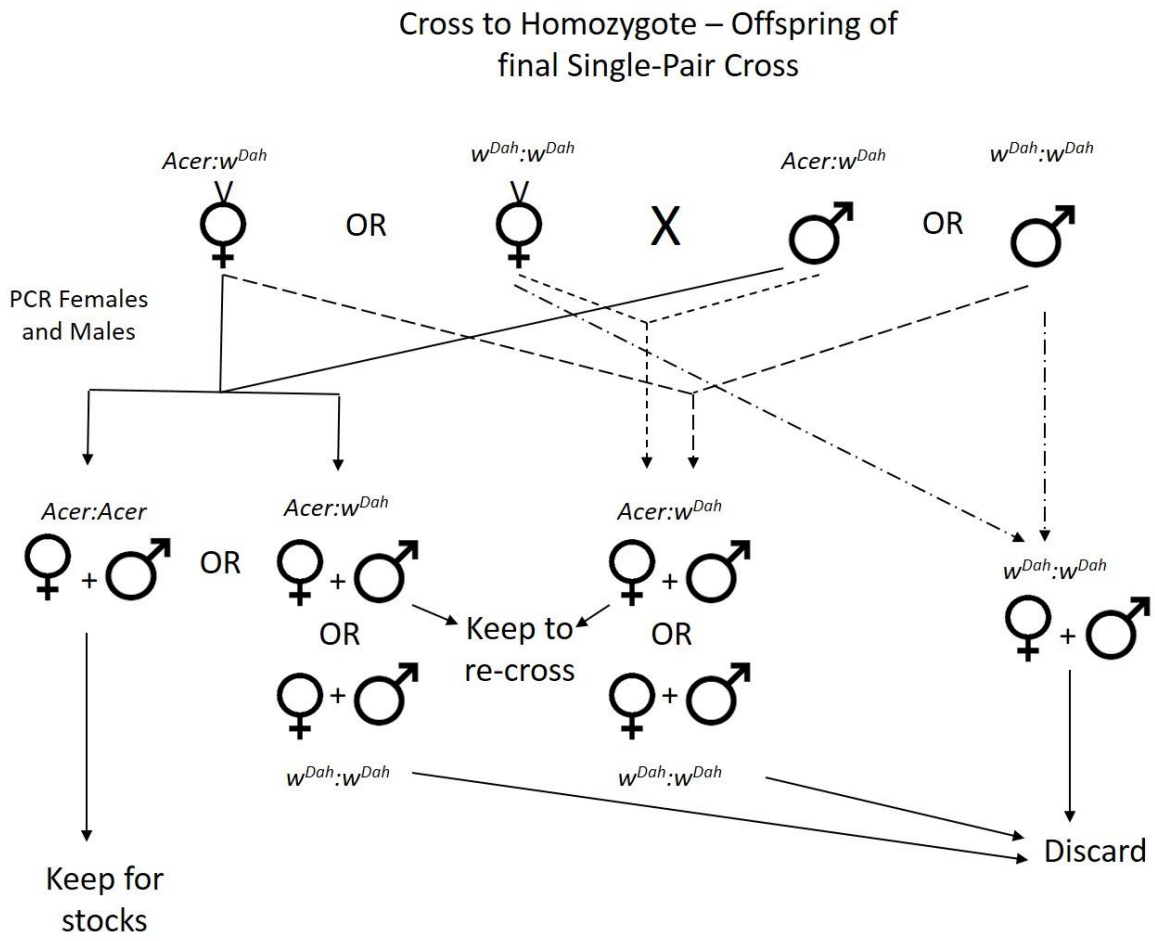

Figure 15: Single – Pair Cross to *Acer* homozygote. Offspring of (Females and Males) were crossed within themselves to create *Acer*$^{\Delta}$ homozygotes. Any flies that did not contain the *Acer* deletion were discarded. Potential heterozygotes were kept to be re-crossed while homozygotes were kept for stocks.

The same procedure was used to backcross the *Ance* deletion stock into the w^{Dah} background for future analysis.

2.3: Virgin Female Collection

To make sure that the offering generated from any genetic crosses are the offspring required, virgin female flies must be used in the genetic cross. This is because female flies are able to store sperm after copulation, therefore it cannot be guaranteed that the offspring produced will be the genotype wanted if the female fly has already mated. Virgin females are identified when they are less than six to eight hours old as they have little pigmentation and can be distinguished from older females

who are more pigmented. At this age they are unreceptive to male courtship and have not copulated. Females become receptive to male courtship after about six hours at 25°C. These virgin females were then used in the genetic crosses and experiments where required.

2.4: Fly genomic DNA preparation

A mixture of Proteinase K (PK) and 'squishing buffer'(10mM Tris-HCl (pH8.3), 1mM EDTA, 25mM NaCl), with the PK at a final concentration of 200µg/ml, was used. Single flies were homogenised in the solution using a pipette tip. The solution was placed in a heat block at 37ºC for half an hour and was then heated to 95ºC for 5 minutes to deactivate the PK. Afterwards the solution was either frozen at -20ºC or used immediately for PCR.

2.4.1: *Acer* Polymerase Chain Reaction (PCR) Primers

The primers below were used as a diagnostic tool to confirm the presence of the *Acer* deletion with the controls giving fragments of about 1,150 base pairs and the *Acer* deletion about 850 base pairs. A difference of about 300 base pairs. The primers were designed, validated and optimised by Dr. Matt Hodges.

Forward Primer (5'): TGTCCGGAATGCGGGTGTTCC
Reverse Primer (3'): TCGATCATGGCCTGGCGATTC

2.4.2: *Acer* genomic PCR Conditions

Step	Temperature (ºC)	Time (minutes)	Cycles
1	94	1	
2	94	0.5	Steps 2-4 cycled 30 times
3	60	1	
4	72	1.5	
5	72	10	
6	4	∞	

Table 1: *Acer* PCR temperature conditions and protocol.

A 20µl reaction required; 10µl of 2x BiomixRed Tag Mix (Bioline), 7µl distilled H_2O, 1µl of the *Acer* forward primer (10µM), 1µl of the *Acer* reverse primer (10µM) and 1µl of DNA.

2.4.3: *Ance* genomic PCR Primers

The primers below were used as a diagnostic tool to confirm the presence of the *Ance* deletion with controls giving fragments of about 1,650 base pairs and the *Ance* deletion about 400 base pairs. The primers were designed, validated and optimised by Dr. Matt Hodges.

Forward Primer (5'): ATAGCATCATGCAGAGCTGTTTC
Reverse Primer (3'): TTGTGACAAATTGCACTTTACG

2.4.4: PCR Conditions

Step	Temperature (ºC)	Time (minutes)	Cycles
1	94	5	
2	94	0.5	Steps 2-4 cycled 30 times
3	57	0.5	
4	72	3	
5	72	10	
6	4	∞	

Table 2: *Ance* PCR temperatures and protocol.

A 20µl reaction required; 10µl of 2x BiomixRed Tag Mix (Bioline), 7µl distilled H_2O, 1µl of the *Ance* forward primer (10µM), 1µl of the *Ance* reverse primer (10µM) and 1µl of DNA.

2.5: Agarose Gel Electrophoresis

A 1% agarose gel was prepared by mixing 1g of agarose per 100ml of 1X Tris Acetate EDTA (TAE) Buffer (Tris, Acetic Acid and EDTA) and then heating in a

microwave oven until the agarose had dissolved. The solution was allowed to cool until it could be held in the hand. Gel-Red (4µl per 100ml) was added to the gel and mixed. The gel was poured into a mould including a comb to form the wells and was allowed to set. Once the gel had set the comb was removed and the gel was placed in the electrophoresis machine and immersed in 1X TAE Buffer. The 1 Kb Plus DNA Ladder (Invitrogen) and the samples were then added to the wells and subjected to electrophoresis at 100V for 5 mins followed by two hours at 50V.
The gel was then imaged using a Bio Rad Gel Doc EZ Imager system and Image Lab software.

2.6: ACER Western Blot Analysis

The samples of whole flies were placed in liquid nitrogen (N_2) and once removed were quickly banged down on the desk to separate the fly heads from the bodies. The separate body parts were quickly poured into a clean weighing boat or petri dish to be sorted and collected, 20 heads or five bodies per sample. The bodies and heads were placed in separate tubes on ice containing 30µl of homogenisation buffer (25mM Tris-HCl pH7.5, 75mM NaCl, 1% Nonidet, 10mM MgCl2 and 15mM EGTA) and protease and phosphatase inhibitors (Sigma Aldrich). A pestle was used to homogenise the bodies or heads as quickly as possible after separation and the samples were kept on ice. After homogenisation, 30µl of 2xSDS loading buffer (62.5mM Tris HCl, pH 6.8, 10% Glycerol, 2% Sodium Dodecyl Sulfate (SDS), 0.005% Bromophenol Blue) in 9 parts to 1 part ratio with 5% 2-Mercaptoethanol (Sigma Aldrich) was added to each sample. The samples were centrifuged at 13,000 rpm for 10 minutes and the supernatant was transferred to a new tube and was centrifuged for a further three minutes. The supernatant (15µl for both heads and bodies) were then transferred onto a pre-cast get (10% Invitrogen) in 1x MOPS buffer (diluted 20% from 20x NuPAGE® MOPS SDS-Novex/Life Technologies). A Magic Marker protein ladder (MagicMark™ XP Western Protein Standard, Life Technologies) was added with a volume of 3.5µl to analyse the samples. The samples were run at 60V for 10-15 minutes to allow the proteins to stack in the gel before being run at 100V for 120-135 minutes. While the gel was running the nitrocellulose and blotting tissues were readied and the top left corner of the nitrocellulose membrane was cut to indicate the orientation of the samples. When the gel had finished running it was carefully removed

before the top left corner was cut to indicate orientation and the membranes and tissues were wetted in 1x Pierce semi-dry transfer buffer (PST) which was diluted from a 10x stock (Thermo Scientific). The transfer of the protein from the gel to the nitrocellulose membrane took place over 30 minutes using the semi-dry Trans-Blot® TurboTM Transfer System from BioRad with 1.0A current and a maximum voltage of 2.5V. After blotting was complete the membrane was transferred to a 1x TBS-T solution (one litre: 100 ml 10X TBS to 900 ml dH2O, mix, 0.1% Tween® 20(Sigma Aldrich)). The blocking solution contained 5% Marvel dried milk and 1% Bovine serum Albumin (BSA) in TBS-T. The membrane was blocked for four hours at room temperature or overnight at 4ºC on a shaker to ensure the membrane0000 was covered equally. The membrane was incubated in the anti-ACER primary antibody (1 in 2000 dilution) in the blocking solution for 4 hours at room temperature if originally blocked overnight, or overnight at 4ºC if blocking occurred at room temperature. Once incubated the membrane was washed in 1x TBS-T five times for 20 minutes each cycle and the antibody was kept at 4ºC for future use. The membrane was incubated with a secondary antibody (1 in 5000 dilution) in the blocking solution at room temperature for one hour. The membrane was washed using the same process used for the primary antibody but the secondary antibody wasn't kept. The ChemiDoc XRS system from BioRad, set to high chemisensitivity, was used to image the blot and was programmed to generate six images over a total time-span of four minutes. The signal was detected using an Enhanced Chemiluminescence (ECL) solution which consisted of Clarity™ Western ECL Substrate (Bio-Rad) in a 1:1 ratio with Clarity western peroxide reagent and Clarity western luminol/enhancer which was used to detect the conjugated Horseradish Peroxidase (HRP) signal attached to the secondary antibody for quantification during imaging. The membrane was coated in the ECL solution before being read and exposed on the ChemiDoc. The first image was captured at 10 seconds and the final image at 240 seconds.

2.7: Generation of flies for experiments

Flies of the appropriate genotype were put into cages (Figure 16) with grape juice agar plates used as a base, and with live yeast used as a food source. The flies were allowed to lay eggs for 24 hours.

Figure 16: *Drosophila* cage and narrow *Drosophila* vials from Dutscher Scientific. **http://www.dutscher-scientific.co.uk/frontoffice/browse_catalog?id=0H-19**

The grape juice agars were then swapped for new plates and yeast and the flies were left to lay eggs for another 24 hours. These eggs were then collected using a Phosphate Buffered Saline (PBS) wash and then using a widened pipette tip, squirted into a bottle of standard food. The same volume contains approximately the same amount of eggs so the flies developed and eclosed in similar environments. The bottles were incubated at 25°C for 10 days before eclosion occurred and adults were allowed to mate for 48 hours. The flies were then sorted at 3 days old into labelled vials with 10 flies per vial into K-resin plastic narrow Drosophila vials (Figure 12). Depending on the experiment this may have required a transfer to different types of food. This was done to ensure that all the flies tested in the experiment had all eclosed and matured in the same environment. To generate $Acer^{\Delta}$ heterozygotes virgin female w^{Dah} flies were crossed with $Acer^{\Delta}$ males in the cages to so that heterozygous eggs would be laid. They were then generated as above.

2.8: Recipe for grape juice agar plates

To generate large numbers of flies, cages were used with red grape juice agars and live yeast as a food source. The agars were made with 1L of red grape juice, 15g of agar, 15ml of nipagin (10% in 100% ethanol) and 3ml of propionic acid. The red grape juice was heated and the agar powder was added and mixed. The solution was brought to the boil and stirred until all of the agar powder had dissolved. The solution

was removed from the heat and was allowed to cool to 60°C. The nipagin and propionic acid were added and the solution was poured in to petri-dishes and allowed to set at room temperature. The plates were kept cool until use and live yeast paste was added for use.

2.9: Dietary manipulations

Flies were generated and maintained for 3 days post-eclosion on standard sugar/yeast (granulated sugar/brewer's yeast) food (SF) containing 50g/l sugar and 100g/l yeast (5% sugar and 10% yeast). The food also contained 30 ml per litre of nipagin (10% in 100% ethanol) and 3ml per litre of propionic acid (Table 3).

Diet	**Standard Food (0.5Sx1Y)**	**Starvation (Agar)**	**Low (0.1SY)**	**DR (0.5xSY)**	**FF (0.5Sx2Y)**
Water (ml)	700	1000	700	700	700
Agar (g)	15	15	15	15	15
Sugar (g)	50	0	10	50	50
Yeast (g)	100	0	10	50	200
Water at the end (ml)	170	0	217	196	118
Nipagin (ml)	30	30	30	30	30
Propionic Acid (ml)	3	3	3	3	3

Table 3: Dietary manipulations for experimentation.

Flies were then transferred to the appropriate food for each experiment at 3 days old. The Dietary Restriction food (DR) contained 50g/l sugar and yeast (5% sugar and yeast) and the Fully Fed food (FF) contained 50g/l sugar and 200g/l yeast (5% sugar and 20% yeast). The Low food contained 10g/l of sugar and yeast (1% sugar and yeast) and the starvation food contained agar and water. All diets contained the standard amount of agar, nipagin and propionic acid.

To make the food, the agar was added to 700ml of water and was heated and brought to the boil until all of the agar powder had dissolved. (The starvation food used

1000ml of water so no extra water was added at the end.) The sugar and yeast were added together and the mixture was brought to the boil again for 5 minutes with constant stirring. The mixture was removed from the heat and allowed to settle and once settled, extra water was added (SF: 170ml, Low: 217ml, DR: 196ml and FF: 118ml) to bring the volume to one litre. The mixture was left to cool to about 60°C and once cooled the nipagin and propionic acid (acting as preservatives) were added. The mixture was then poured into vials/bottles and allowed to set for 24 hours, while being covered by a breathable fabric. Once set and dry the vials or/bottles were plugged and were ready for use.

2.10: Sleep Analysis

Male and female *Acer*$^{\Delta}$ and control flies in the w^{Dah} background were generated as described in Chapter 2.7 for the first experiment and *Acer*$^{\Delta}$ and w^{1118} control males and females were generated separately for the second experiment. The same method was used for both experiments.

Figure 17: A single Trikinetics *Drosophila* Activity Monitor (DAM). **http://www.trikinetics.com/Downloads/DAMSystem%20User's%20Guide%203.0.pdf**

Following eclosion on day three the flies were transferred to medium containing Low, DR or FF diets (Chapter 2.9 – Table 3) and maintained at 25°C in a 12h light/dark cycle for 4 days. On day 5 individual flies (n=18 in the w^{Dah} background and n=24 in the w^{1118} background) were then placed in Trikinetics *Drosophila* Activity Monitors

(Figure 17) in tubes (5mm x 65 mm made of polycarbonate plastic) containing the same medium. Each DAM (Figure 13) contains 32 infra-red beams to detect activity. When the beam is broken by the fly moving across it the computer registers this as activity and five minutes onwards without the beam being broken is recorded as sleep. The DAMs also registered the difference between light and dark and therefore were able to distinguish between day and night sleep. Flies were assorted randomly with positions noted to reduce any differences between the DAMs or positioning in the controlled temperature room. Nine DAMs were used in both experiments with a total number of 216 flies monitored in the first (w^{Dah}) experiment and 288 flies monitored in the second experiment (w^{1118}). Activity was monitored in 1 minute bins for 5 days at 25°C in a 12h light/dark cycle. All the data gathered including light and dark sleep was recorded in the same experiment.

The sleep data was analysed for days 2-3 using the BeFLY! (Ed Green) Excel plug-in with sleep defined as 5 consecutive bins with no movement (Broughton, et al., 2005). Days two and three were analysed as maggot formation and movement could interfere with the detection of activity and not produce a true reading for female flies.

2.11: Lipid and Glycogen Separation

The lipid and glycogen separation was copied from (Handel, 1965) where lipid is extracted using chloroform:methanol and glycogen is removed by precipitation and adsorbed in sodium sulphate.

The flies were generated on standard food before being placed on the experimental foods; Starvation, Low, DR and FF. The flies were placed on the diets on day three for the four day experiment and frozen on day seven. For the two day experiment they were on SF until day five, when they were placed on the diets, before being frozen on day seven.

The weights of the flies were recorded in mg to three decimal places.

Each fly was then homogenised in 50µl of a solution of pre-saturated Na_2SO_4. Chloroform:methanol in a 1:1 ratio solution was added (1ml), vortexed and the mixture was centrifuged at 6000rpm for five minutes forming a glycogen pellet with lipid within in the organic phase. The organic phase containing the lipid was removed and placed in a separate tube. Both the lipid and glycogen were stored at -20°C.

2.12: Lipid assay

The colour change reagent for this assay was Vanillin which changes from yellow to pink in the presence of lipid. The Vanillin was freshly made for every lipid assay. This consists of 1.2% vanillin in 68% orthophosphoric acid. The lipid standard curve was formed using soybean oil. The highest concentration of soybean oil was 920µg/ml. This was then diluted using a 1 in 2 serial dilution using chloroform:methanol in a 1:1 ratio giving final concentrations of 920, 460, 230, 115, 57.5, 28.75 and 0µg/ml. The sample and standard curve solutions were placed in a heat block at 70ºC inside a fume cupboard until the cholorform:methanol had evaporated (approx. 1 hour), leaving a remaining residue of lipid. The lipid residue was re-suspended in 200µl of concentrated H_2SO_4 and heated at 90ºC for ten minutes. Once heated the tubes were cooled on ice. Once cool 1ml of the vanillin reagent mix was added to the tubes and mixed. The solutions containing the reagent were left for five minutes to allow the pink colour to develop and then 200µl of each sample was loaded into the wells of a 96-well microtitre plate. Absorbance of the pink colour was measured at 490nm by a Tecan Infinite M200Pro spectrophotometer. The higher the absorbance, the higher the concentration of lipid.

The DR and FF assays for the 2 day experiment in the w^{Dah} background were performed by Dr. Matt Hodges.

2.13: Glycogen assay

The glycogen pellets were air-dried in the fume cupboard for about two minutes to evaporate off any residual chloroform:methanol solution after separation.

The colour change reagent for this assay was Anthrone which changes from yellow to green in the presence of glycogen. The Anthrone solution was made up freshly for each set of assays. To make the solution 0.1125g of anthrone was dissolved in 70% H_2SO_4.

The glycogen standard curve was formed using porcine glycogen. Tube one contained 100µl of glycogen and was then diluted using a 1 in 2 serial dilution to give final concentration of 281, 140.5, 70.25, 35.13, 17.56, 8.78, 4.39 and 0µg/ml after the anthrone solution had been added. The glycogen pellets and the standard curve samples were then mixed with 1ml of anthrone and placed on a heat block at 90ºC for

10 minutes. During the heating gaseous pressure caused by the hot acid needed to be released by briefly opening the microtubes and replacing the tubes back into the heat block. While releasing the pressure the solution was mixed. Once the samples had been heated, 200µl of each sample was loaded into the wells of a 96-well microtitre plate. Absorbance of the green colour was measured at 620nm by a Tecan Infinite M200Pro spectrophotometer. The higher the absorbance, the higher the concentration of glycogen.

The DR and FF assays for the 2 day experiment in the w^{Dah} background were performed by Dr. Matt Hodges.

2.14: Feeding assay

The feeding experiment was adapted from (Wong, et al., 2009). Female and male flies were generated and sorted on to Low, DR and FF foods on day three after eclosion. On day seven, the flies were starved of food and water to encourage appetite for 1hr and 30mins and were then transferred on to the dietary foods containing 10g of blue food dye dissolved in 200ml of food (Dr. Oetker Gel Dye, Sky Blue) for 30mins and then frozen. The flies were then weighed and homogenised (5 per tube) in PBS (50µl/fly) using a pestle, centrifuged for 25 mins at 13,000rpm and the supernatant transferred to a fresh tube and spun again for 5 mins. The supernatant (200µl) was transferred to a 96-well microtitre plate and read at 625nm to read the absorbance of the blue dye.

2.15: Survival and Fecundity Analysis

100 female or male flies of each genotype on each food were maintained at 10 flies per vial throughout life. Deaths were scored on 5 out of 7 days each week and the data presented as proportion of surviving flies vs time. The fecundity of the female flies was measured by counting by eye the number of eggs laid over a two day period per vial at time points throughout life, and presented as mean number of eggs laid per female per day. The transfer to fresh food was carried out two times a week for male flies and in the first three weeks the vials containing female flies were changed three times a week due to maggot emergence and then subsequently twice a week as egg

laying was reduced, as is standard procedure for survival analyses (Broughton, et al., 2005; Broughton, et al., 2008).

2.15.1: Reciprocal Mating

The flies were generated as before except virgin female flies were collected as they eclosed instead of using mated females. Male flies were also collected alongside the virgin females and the flies were then mated three days after eclosion for 2 days.

The crosses were:
w^{Dah} virgin female X w^{Dah} male
w^{Dah};*Acer* virgin female X w^{Dah};*Acer* male
w^{Dah} virgin female X w^{Dah};*Acer* male
w^{Dah};*Acer* virgin female X w^{Dah} male

After mating, the female flies were placed into narrow *Drosophila* vials in groups of ten and their fecundity and lifespan were recorded over time, in the same way as a survival experiment.

2.16: Oxidative Stress Test - Hydrogen Peroxide Food (H_2O_2)

A stock solution of 30% H_2O_2 was diluted to 5% in 200ml of water. Sugar (10g) and agar (3g) were added to 160ml of water and brought to the boil until the agar had fully dissolved. The solution was allowed to cool until it was hand-hot. While cooling, 33.3ml of H_2O_2 was diluted in 6.7ml of water to give a volume of 40ml. Once cool the 40ml, including the H_2O_2, was added to the 160ml solution and mixed thoroughly. The solution was then poured into narrow *Drosophila* vials and kept at 4ºC until use. To prevent the degradation of the H_2O_2 this food was made 24 hours before the experiment.
Lifespan was recorded as a survival experiment and was taken from Broughton, et al., (2008).

2.17: Cold Coma Recovery

The flies of each genotype were generated and 3 days post-eclosion were sorted into males and females onto DR and FF foods with 10 flies per vial. On day 6 the flies were transferred to fresh food and sorted into groups of 5 flies per vial. The experiment was conducted at 9 days old for females (n=45 w^{Dah} and n=50 w^{1118}) and 10 days old for males (n=50 for both backgrounds). The flies were transferred to empty vials and placed on ice at 4ºC for 4 hours. The flies were then moved to a 25ºC constant temperature room, where they had been kept before the experiment, to recover and were constantly monitored until awake. Flies that stood up were counted as awake. This method was taken from Broughton, et al., (2005).

2.18: Heat Shock Resistance

The flies of each genotype were generated and 3 days post-eclosion were sorted into males and females onto DR and FF foods with 10 flies per vial. Due to the size of the experiment and the expected time till death, the heat experiment was conducted on day 9 for females (n=40) and day 10 for males (n=40) for both backgrounds. The flies were transferred to empty vials and placed into either a 37ºC or 39ºC water bath. The number of dead flies was counted every five minutes until all flies were dead. This method was taken from Broughton, et al., (2005).

2.19: Splitting Fly Heads from the Bodies for RNA Extraction

The flies were separated by sex before freezing 20 flies in a 1.5ml centrifuge tube at -80ºC. The centrifuge tubes containing the flies were placed in liquid nitrogen (N_2) for at least thirty seconds. Once frozen, the tubes were removed one at a time removed from the liquid N_2 using forceps and banged up and down on the bench to separate the fly heads from the bodies. The contents of the tube were quickly poured onto a clean petri-dish. The heads were counted and placed into 1ml of Trizol (in Ribolyzer tubes) using forceps. The same method was used for the bodies if they were

being used at the same time or the bodies were re turned to their original centrifuge tubes and placed into the liquid N_2 before being re-frozen at -80ºC.

2.20: Trizol RNA Extraction

The tubes containing either heads or bodies for the appropriate sex were chilled on ice and added to 1ml of Trizol (Gibco) in 1.7mm Zirconium Bead Ribolyser tubes (OPS DIAGNOSTICS). 20 fly heads, 10 fly bodies or 5 whole flies were used for various experiments. The samples were then homogenised in a ribolyser and left at room temperature for 5 minutes before adding 200µl of chloroform and shaken to mix for about 15 seconds. The tubes were incubated at room temperature for 3 minutes before being centrifuged at 12,000 rpm at 4ºC for 15 minutes. The upper aqueous layer was removed and transferred to a fresh tube and 500µl of Isopropyl alcohol was added and left overnight at -80ºC for RNA to precipitate. The tubes were then centrifuged at 12,000 rpm at 4ºC for 15 minutes, waste was removed and the pellet washed in 700µl of chilled 70% Ethanol diluted in DEPC (diethylpyrocarbonate) treated H_2O (Sigma-Aldrich). The tubes were spun at 10,000 rpm at 4ºC for 10 minutes and the washes were repeated twice more. The pellets were briefly air-dried and then re-suspended in 20µl of DEPC H_2O for bodies or 10µl for heads and frozen at -80ºC.

2.21: cDNA generation for QCPR analysis

For male and females heads cDNA was generated from 150ng of RNA in a volume of 11µl by dilution in DEPC H_2O, calculated from a nanodrop. For male and female bodies cDNA was generated from 500ng of RNA in a volume of 11µl by dilution in DEPC H_2O, calculated from a nanodrop. The RNA dilution was added to 1µl of Oligo $(dT)_{20}$, and 1 µl of 10mM dNTP mix to give a volume of 13µl and heated for 5 minutes at 65ºC and then placed on ice for 1 minute. The contents were collected by brief centrifugation and 4µl of 5x RT Buffer, 1µl of 0.1M DTT, 1µl of RNase Out Recombinant RNase Inhibitor, 0.5µl of SuperScript III and 0.5µl of DEPC H_2O were added to make a final volume of 20µl. All components were form Invitrogen.
The mixture was mixed by pipetting and incubated at 50ºC for 30-60 minutes and the reaction was terminated at 70ºC for 15 minutes and then chilled on ice. The samples were then diluted 1:2 by adding 20µl of PCR water (Sigma-Aldrich).

2.22: *Acer* Expression over 24hrs

Male and female w^{Dah} and w^{1118} flies were generated and sorted by sex and background onto DR and FF food on day 3. The flies were also grouped into 7 time-slots over 24 hours. On day six, the first flies were anesthetised using CO_2 and were frozen at 9am using a dry ice ethanol bath (n=30 per genotype and sex). The flies were then frozen at four hour intervals up to and including 9am the next day. For the night-time points, 9pm, 1am and 5am, a red light filter was used so as not to disturb the circadian rhythm by introducing light in the night cycle. Once frozen, the flies were analysed using QPCR to examine and compare *Acer* expression in w^{Dah} and w^{1118} flies. For QPCR Protocol see 2.23. The RNA extraction, cDNA synthesis and QPCR for this experiment was carried out by Dr. Matt Hodges.

2.23: Quantitative Polymerase /Chain Reaction (QPCR) – Plating and Reading

Separate master mixes were created using SYBR Green reagent (Sigma Aldrich) for each primer combination for QPCR analysis. The primers included β-actin for each reaction as an endogenous control. For fly heads *Dilps* 2, 3, 5 and 6 were tested for both male and female flies. For fly bodies *Dilps* 4, 5, 6, and 7 for both male and female flies. All primers were from Invitrogen.

β-Actin Primers

β-Actin Forward Primer: CACACCAAATCTTACAAAA
β-Actin Reverse Primer: AATCCGGCCTTGCACATG

DILP Primers:

The primers were supplied by Dr. Susan Broughton. (Broughton, et al., 2005)

Dilp 2 Forward Primer: ATGGTGTGCGAGGAGTATAATCC
Dilp 2 Reverse Primer: TCGGCACCGGGCATG

Dilp 3 Forward Primer: AGAGAACTTTGGACCCCGTGAA
Dilp 3 Reverse Primer: TGAACCGAACTATCACTCAACAGTCT

Dilp 4 Forward Primer: GCGGAGCAGTCGTCTAAGGA
Dilp 4 Reverse Primer: TCATCCGGCTGCTGTAGCTT

Dilp 5 Forward Primer: GAGGCACCTTGGGCCTATTC
Dilp 5 Reverse Primer: CATGTGGTGAGATTCGGAGCTA

Dilp 6 Forward Primer: CGATGTATTTCCCAACAGTTTCG
Dilp 6 Reverse Primer: AAATCGGTTACGTTCTGCAAGTC

Dilp 7 Forward Primer: CAAAAAGAGGACGGGCAATG
Dilp 7 Reverse Primer: GCCATCAGGTTCCGTGGTT

Acer Primers:

The *Acer* primers were designed, validated and optimised by Dr. Matt Hodges.

Acer Forward Primer: CAGTTGAATGGTCACCGCT
Acer Reverse Primer: GTAGCCGTGGAGCTGTCGG

For a 20µl reaction, 10µl of SYBR Green, 1µl of forward primer, 1µl of reverse primer, 7 µl of PCR water and 1µl of cDNA were added to a 96-well micro-titre plate. The plate was then run on a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler machine and analysed using Bio-Rad CFX Manager software Version 3.0.

DILP QPCR Protocol:

The protocol was supplied by Dr. Susan Broughton. (Broughton, et al., 2005)

1. 94ºC for 2 minutes
2. 94ºC for 1 minute
3. 55ºC for 30 seconds

4. 72ºC for 1 minute. Plate read.
5. Repeat steps 2-4 39 more times.
6. Melt curve 65ºC to 95ºC in increments of 0.5ºC for 5 seconds. Plate read. End.

Acer QPCR Protocol:

The RNA and cDNA were generated as above except that 1µl of Superscript III was used and therefore no DEPC-H_2O was used in the generation of the cDNA.

The protocol was designed by Dr. Matt Hodges.

1. 94ºC for 3 minutes
2. 94ºC for 30 seconds
3. 62ºC for 30 seconds
4. 72ºC for 1 minute
5. Repeat steps 2-4 39 more times
6. Melt curve 65ºC to 95ºC in increments of 0.5ºC for five seconds. Plate read. End.

2.24: Immunohistochemistry of Fly Brains

Fly brains were dissected in Phosphate Buffered Saline (PBS) and fixed in 4% paraformaldehyde in PBS for 45 mins at room temperature. The brains were washed in PBS for 3 x 20 minutes and then washes were repeated using Tris-NaCl-Tween (TNT: 0.1M Tris HCl/0.3M Na Cl (ph 7.4), 0.5% triton X-100) buffer. The brains were blocked in TNT containing 4% goat serum for 1.5 hours at room temperature with gentle shaking, followed by incubation in the same solution with a 1 in 50 dilution of DILP 5 rat antibody at 4ºC overnight. The brains were then washed for 6 x 20 minutes in TNT and then incubated at room temperature for 2 hours in a block solution containing 2% goat serum and a secondary rat antibody in a 1 in 200 dilution with a 488 tag.

The brains were then washed for 3 x 20 minutes in TNT before repeating in PBS and held in 2% n-propylgallate at 4ºC in the dark before being mounted in 200µl of 2% n-propylgallate. This method was adapted from (Broughton, et al., 2005).

The antibodies were provided by Dr. Susan Broughton.

Anti-DILP 5 #51: DFR GVV DSC CRK S / DML RVA CPN GFN SMF A

The polyclonal antibody was raised in rats against the peptides above and was immunised using the AS-DOUB-LX package (Eurogentec, Liege, Belgium) and then coupled to keyhole limpet haemocyanin and injected into rats (Broughton, et al., 2005).

The images were obtained using a confocal microscope at x20 magnification and z-stacks were imaged. The z-stacks were analysed in Image J at maximum intensity and analysed using integrated density, which analyses the maximum intensity divided by area. This ensured that the area of staining was taken into account with the maximum intensity of staining.

2.25: Statistics

All graphs were generated using Microsoft Excel 2013 using raw data. All statistical analyses were carried out in JMP Version 12.0 (SAS Institute, Cary, NC 27513, USA) statistical analysis software. Lifespan data were analysed in Excel by survival analysis and comparisons made by Log Rank tests. For other data, ANOVAs were performed and food and genotype found to be the main effects. Planned comparisons of means were made using Tukey-Kramer HSD test, with $p<0.05$.

Chapter 3: The role of *Acer* in sleep and response to nutrition.

3.1 Introduction

In order to analyse the role of *Acer* in *Drosophila melanogaster*, *Acer* null flies (*Acer*$^{\Delta}$) that had been created by imprecise P-element excision (Carhan, et al., 2010) were bred into the *w*^{Dah} and the *w*^{1118} genetic backgrounds. After backcrossing the lack of *Acer* in the *Acer*$^{\Delta}$ was confirmed by PCR analysis. In addition, Western Blot analysis was used to confirm the absence of ACER protein in the *Acer*$^{\Delta}$ flies in the *w*^{Dah} background.

The loss of *Acer* in *Acer*$^{\Delta}$ flies has previously been shown to result in increased activity, lower levels of sleep and night time sleep fragmentation (Carhan, et al., 2010). Fosinopril, an inhibitor of both ACE in mammals and ACER in *Drosophila*, has shown similar effects on sleep to the *Acer* deletion (Carhan, et al., 2010). *Acer* has also been found to be up-regulated in response to sleep deprivation, again suggesting that *Acer* may play a role in sleep regulation (Thimgan, et al., 2015).

Due to the suggestion of a role for *Acer* in sleep regulation and previous studies concluding a nutrient-responsiveness of sleep (Catterson, et al., 2010; Linford, et al., 2012) and genetic background variation of sleep (Svetec, et al., 2015), this study investigated the role of *Acer* in response to diet and sleep in different genetic backgrounds (*w*^{Dah} and the isogenic *w*^{1118} stock from the Vienna Drosophila Resource Centre, hereinafter referred to as the *w*^{1118} *background*).

3.1.2: Aims

To backcross the *Acer*$^{\Delta}$ (Carhan et al, 2010) deletion into the *w*^{Dah} and *w*^{1118} backgrounds and confirm the presence of the deletion following backcrossing.

To analyse the role of *Acer* in sleep and its response to diet by analysing the effect of loss of *Acer* on these phenotypes.

3.1.3: Research Design

To confirm the *Acer*$^{\Delta}$ flies did not contain the functioning *Acer* gene PCR was used to identify the deletion of about 300 base pairs compared to *w*^{Dah} and w^{1118} controls, using 10 female or male flies per genotype picked at random.

To confirm the lack of ACER protein production using western blot analysis 20 heads or 5 bodies for males and females were used for the protein extraction and analysed for presence of ACER protein in both *Acer*$^{\Delta}$ mutants and controls in the *w*^{Dah} background.

For sleep analysis *Acer*$^{\Delta}$ males and females in the *w*^{Dah} and *w*^{1118} backgrounds (n= 18 and 24 respectively) were analysed under Low, DR and FF conditions. The sleep experiment was adapted from Broughton, et al. (2010) and Total Activity, Total Sleep, Total Sleep in the Dark, Total Sleep in the Light, Number of Sleep Bouts in the Dark, Number of Sleep Bouts in the Light, Mean Bout Length in the Dark and Mean Bout Length in the Light were measured. Sleep was monitored in *Drosophila* Activity Monitors (DAMs) for four days and day 2 and day 3 were averaged and analysed using BeFLY! (Ed Green).

3.2: Results

3.2.1: Backcrossing and Validation of the *Acer*$^{\Delta}$ null genotype.

The *Acer* deletion was backcrossed through six generations into the either the w^{Dah} or w^{1118} background (Chapter 2.2). PCR analysis was used to validate and confirm the *Acer* deletion in the homozygote *Acer* deletion flies in both the w^{Dah} and w^{1118} backgrounds. As the *Acer*$^{\Delta}$ have part of the *Acer* gene deleted the DNA fragments will travel further along the gel representing a smaller number of base pairs and therefore a shorter band compared with controls.

Two lines containing the *Acer* deletion were originally established Δ164 and Δ168 by deleting the 5' end of the *Acer* gene. Using a reverse primer specific to an area within the second exon, PCR amplification results in a product of 1,137 bp for wild-type DNA and 800 bp for the deletion. A difference of 337 bp (Carhan, et al., 2010).

In the present study Δ168 was used and the diagnostic primers for the *Acer* deletion define a deletion of about 300 base pairs with the control band about 1,150 base pairs (bp) whereas the deletion band is about 850 bp (Figures 18 and 19). This confirms the *Acer* deletion seen in Carhan et al. (2010).

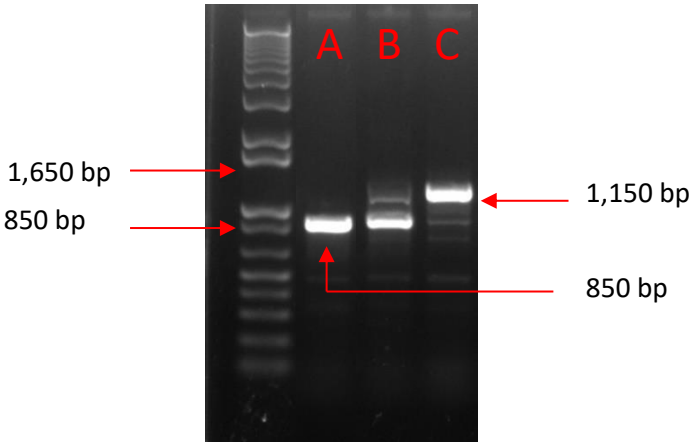

Figure 18: PCR analysis of the *Acer* deletion in the w^{Dah} background. A) The *Acer* deletion homozygote with a strong band at 850 bp. B) The *Acer* deletion heterozygote with a string band at 850 bp and a weaker band at 1,150 bp. C) The w^{Dah} control background with the *Acer* gene present with a strong band at 1,150 bp. (bp = base pairs).

The PCR gels for the w^{Dah} and w^{1118} backgrounds (Figures 18 and 19) clearly identify the homozygous *Acer* deletion ($Acer^{\Delta}$) with a strong signal of the shortest band and the controls show a strong signal with the longest band. The heterozygous *Acer* deletion identifies with a weaker band at the same length as the homozygous deletion as well as a much weaker band at the same length as the control.

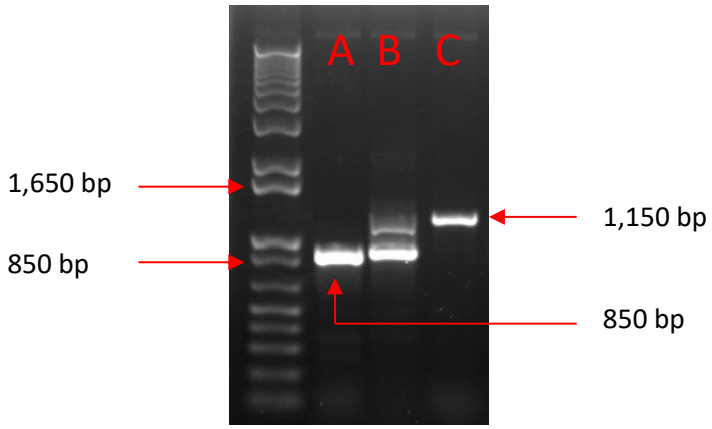

Figure 19: PCR analysis of the *Acer* deletion in the w^{1118} background. A) The *Acer* deletion homozygote with a strong band at 850 bp. B) The *Acer* deletion heterozygote with a strong band at 850 bp and a weaker band at 1,150 bp. C) The w^{1118} control background with *Acer* present with a strong band at 1,150 bp. (bp = base pairs).

Western Blot Analysis was used to validate and confirm the absence of the ACER protein in the w^{Dah} background compared to controls.

The western blots (Figure 20) for female and male heads and bodies clearly show the lack of ACER protein in the $Acer^{\Delta}$ flies. Bands corresponding to ACER protein can be identified in the controls but no bands can be seen for $Acer^{\Delta}$ confirming that no ACER protein is present. Therefore, the *Acer* deletion is a null mutation and the null flies are referred to throughout as $Acer^{\Delta}$.

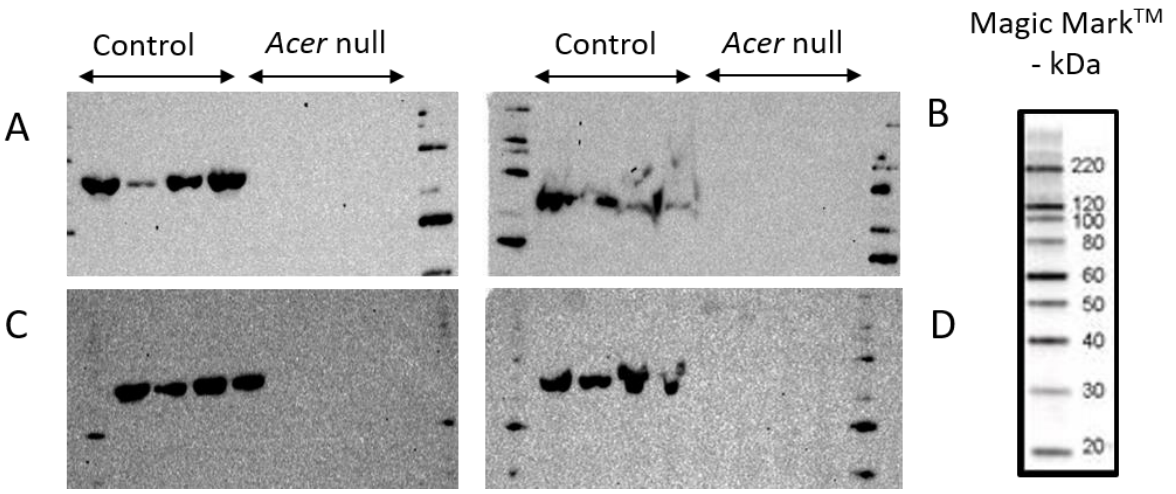

Figure 20: Western blot analysis of controls in the w^{Dah} background and *Acer* nulls. A) Female Heads (N=20). B) Female Bodies (N=5). C) Male heads (N=20). D) Male Bodies (N=5).

The PCR analysis confirmed the presence of the *Acer* deletion in the backcrossed flies and the Western blot analysis confirmed the lack of ACER protein in $Acer^{\Delta}$ flies. The backcrossed and validated $Acer^{\Delta}$ flies in the w^{Dah} and w^{1118} backgrounds were therefore used in all further experiments.

3.2.2: *Acer* regulates sleep and activity in response to diet in females but affects the response on high food in w^{1118} males only

Diet has been shown to play an important role in the response of sleep with male flies sleeping less when compared to a no yeast diet while female activity was reduced when yeast was added (Catterson, et al., 2010; Takahama, et al., 2012).

In this study, sleep was recorded over two days with flies aged seven days old at the start. Sleep was defined as five minutes without activity and one bin was equal to one minute (Chapter 210.). w^{Dah} control female flies showed a clear and significant decrease in total activity (Figure 21A and Table 4) as food levels increased as well as a significant increase in total sleep (Figure 21B and Table 4) as food levels increased.

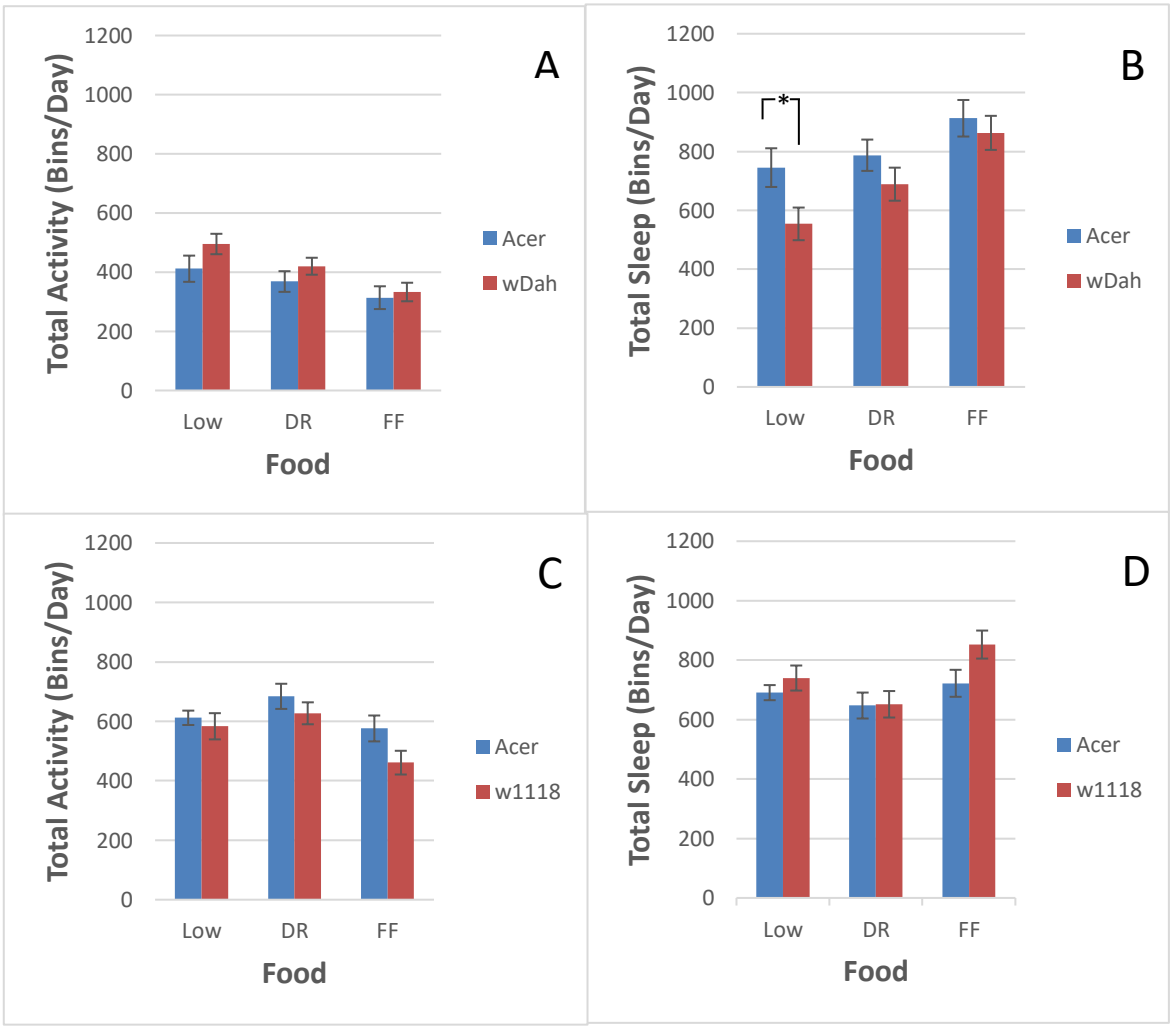

Figure 21: Female Total Activity (Bins/Day) and Total Sleep (Bins/ Day) on Low, DR and FF diets (Chapter 2.9. – Table 3). (1 Bin = 1 Minute). Total Activity (A) and Total Sleep (B) in the w^{Dah} background. Total Activity (C) and Total Sleep (D) in the w^{1118} background. (A) w^{Dah}: Low N = 15; DR

N = 17; FF N = 17. *Acer*$^{\Delta}$: Low N = 12; DR N = 17; FF N = 16. (B) *w*^{Dah}: Low N = 18; DR N = 18; FF N = 17. *Acer*$^{\Delta}$: Low N = 13; DR N = 18; FF N = 15. (C) *w*^{1118}: Low N = 23; DR N = 23; FF N = 24. *Acer*$^{\Delta}$: N =24 for all foods. (D) *w*^{1118}: Low N = 23; DR N = 23; FF N = 24. *Acer*$^{\Delta}$: N = 24 for all foods. Data was checked for normality and all data was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 4 and 5. *Indicates significant difference between genotypes (P=<0.05).

Control *w*^{1118} females showed a similar response with decreased activity on FF food and significantly increased sleep on FF food (Figures 17C and 17D and Table 4).

Genotype	Food Comparison	p-value for Total Activity	p-value for Total Sleep
w^{Dah}	Low-DR	0.2268	0.2132
	Low-FF	0.0021*	0.001*
	DR-FF	0.123	0.086
w^{Dah}:*Acer*$^{\Delta}$	Low-DR	0.7267	0.8743
	Low-FF	0.2144	0.1529
	DR-FF	0.5533	0.284
w^{1118}	Low-DR	0.7328	0.3542
	Low-FF	0.0863	0.1822
	DR-FF	0.0141*	0.0061*
w^{1118}:*Acer*$^{\Delta}$	Low-DR	0.3715	0.715
	Low-FF	0.7811	0.8383
	DR-FF	0.1142	0.3733

Table 4: Female Tukey-Kramer HSD comparisons of means for Total Activity and Total Sleep for controls and *Acer*$^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

Genotype	Food	Comparison to *Acer*$^{\Delta}$ for Total Activity	Comparison to *Acer*$^{\Delta}$ for Total Sleep
w^{Dah}	Low	0.143	0.0339*
	DR	0.2615	0.2122
	FF	0.7005	0.563
w^{1118}	Low	0.569	0.3186
	DR	0.3188	0.946
	FF	0.0577	0.0524

Table 5: Female ANOVA/Pooled t-test measure of variance of genetic effects on different diets for Total Activity and Total Sleep. *Indicates significant differences (P=<0.05).

Acer$^{\Delta}$ females in both genetic backgrounds did not show the normal response to changing diet in terms of total activity and total sleep (Figure 17 and Table 4) as

they showed no significant decrease in activity or increase in sleep between Low and FF food that was seen in controls. Although the lack of nutrient responsiveness in $Acer^{\Delta}$ females was seen irrespective of genetic background, differences in the specific effect of the loss of *Acer* were seen between the two backgrounds. When compared with controls $Acer^{\Delta}$ females slept significantly more on the Low food in the w^{Dah} background. This effect wasn't seen in the w^{1118} background where statistically no difference was observed between the genotypes. However, for both activity and sleep on FF food the differences between $Acer^{\Delta}$ females and w^{1118} controls are approaching significance, with $Acer^{\Delta}$ females appearing to show increased activity and decreased sleep on the FF food

The lack of response to increasing diet in $Acer^{\Delta}$ females suggests a role for *Acer* in regulating the response of sleep to diet in female flies, irrespective of genetic background. However, the direction of the effect is background dependent as the effects were seen on Low food in the w^{Dah} background and were close to significance in the w^{1118} background on FF food.

In contrast, male control flies in both backgrounds did not respond to dietary changes in terms of total activity and total sleep (Figure 22 and Table 6). Activity and sleep remained constant with no increase in sleep and decrease in activity as observed by the female controls between Low and FF foods.

Although $Acer^{\Delta}$ males in the w^{Dah} background behaved in the same way as controls with no response of sleep or activity to dietary change (Figures 22A and 22B and Tables 6 and 7), $Acer^{\Delta}$ males in the w^{1118} background showed a significant increase in total activity and a significant decrease in total sleep on FF food (Figures 22C and 22D and Tables 6 and 7). This is similar to the $Acer^{\Delta}$ females in the w^{1118} background, although unlike the males, the differences were approaching significance rather than significantly different (Table 5).

These data suggest that *Acer* is not involved in the response of activity and sleep in the w^{Dah} background for male flies, however, it appears that in the w^{1118} background *Acer* is potentially required to modulate sleep and activity levels on high yeast food.

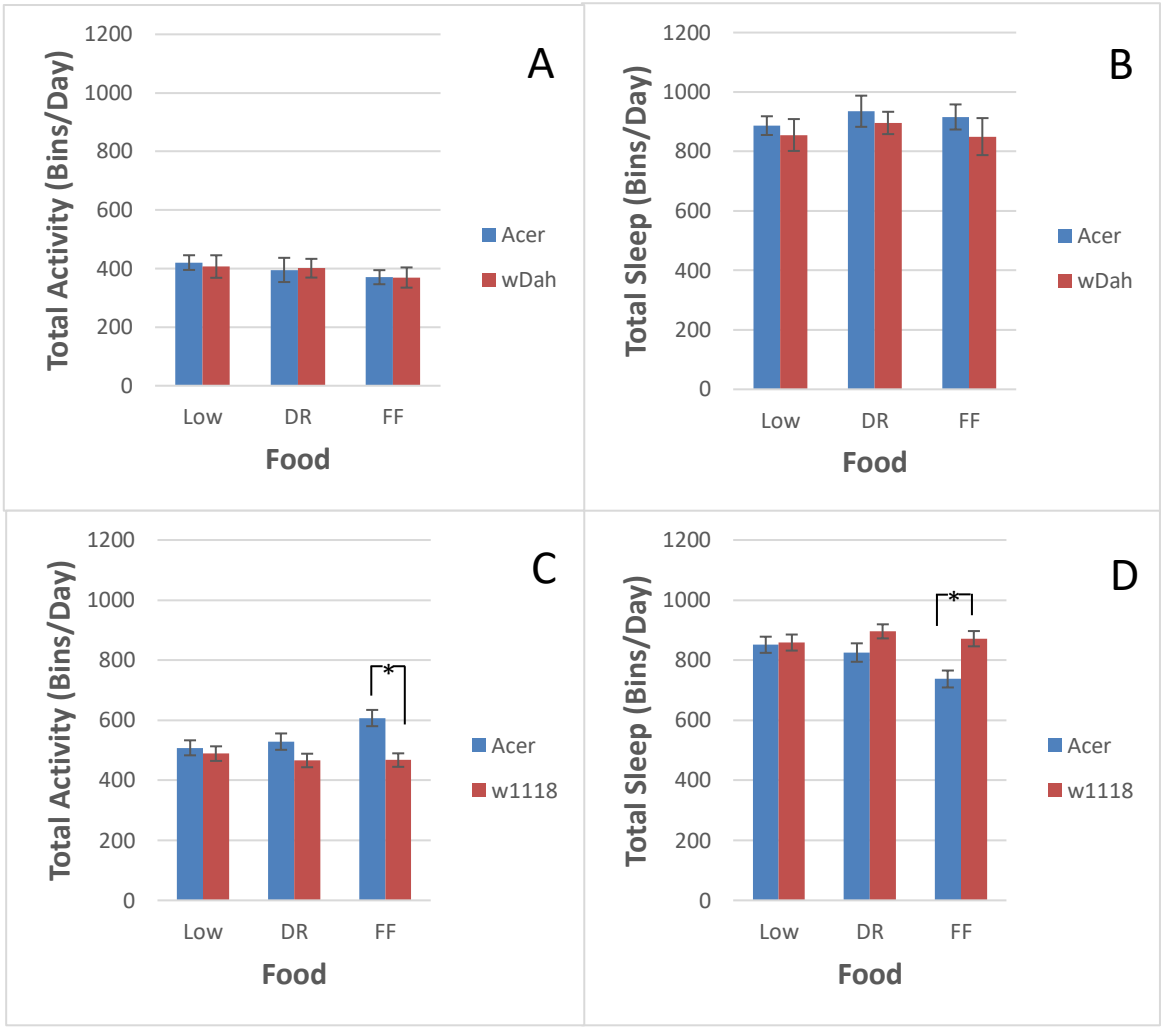

Figure 22: Male Total Activity (Bins/Day) and Total Sleep (Bins/Day) on Low, DR and FF diets. (1 Bin = 1 Minute). Total Activity (A) and Total Sleep (B) in the w^{Dah} background. Total Activity (C) and Total Sleep (D) in the w^{1118} background. (A) w^{Dah}: Low N = 18; DR N = 18; FF N = 14. $Acer^{\Delta}$: Low N = 14; DR N = 18; FF N = 17. (B) w^{Dah}: Low N = 18; DR N = 18; FF N = 16. $Acer^{\Delta}$: Low N = 14; DR N = 17; FF N = 18. (C) w^{1118}: Low N = 22; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 23; DR N = 20; FF N = 21. (D) w^{1118}: Low N = 22; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 23; DR N = 21; FF N = 22. Data was checked for normality and was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 6 and 7. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	p-value for Total Activity	p-value for Total Sleep
w^{Dah}	Low-DR	0.9922	0.837
	Low-FF	0.7474	0.997
	DR-FF	0.8111	0.8068
$w^{Dah};Acer^{\Delta}$	Low-DR	0.8545	0.7357
	Low-FF	0.5486	0.8919
	DR-FF	0.8427	0.9456
w^{1118}	Low-DR	0.769	0.5583
	Low-FF	0.7877	0.9316
	DR-FF	0.9991	0.7978
$w^{1118};Acer^{\Delta}$	Low-DR	0.8443	0.7654
	Low-FF	0.0242*	0.0159*
	DR-FF	0.1066	0.087

Table 6: Male Tukey-Kramer HSD comparisons of means for Total Activity and Total Sleep for controls and *Acer*$^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

Genotype	Food	Comparison to *Acer*$^{\Delta}$ for Total Activity	Comparison to *Acer*$^{\Delta}$ for Total Sleep
w^{Dah}	Low	0.7849	0.6408
	DR	0.9118	0.5422
	FF	0.9773	0.3773
w^{1118}	Low	0.5839	0.8468
	DR	0.0807	0.0719
	FF	0.0002*	0.001*

Table 7: Male ANOVA/Pooled t-test measure of variance of genetic effects on different diets for Total Activity and Total Sleep. *Indicates significant differences (P=<0.05).

Acer appears to modulate the normal response of total activity and total sleep to dietary changes in female flies irrespective of genetic background, however it appears to be involved in the response of sleep on low food in the w^{Dah} background and potentially high yeast food in the w^{1118} background. In males, *Acer* appears to affect the response of sleep and activity to high food within the w^{1118} background but has no effect in the w^{Dah} background.

3.2.3: *Acer* plays a role in regulating both dark and light sleep in male and female flies but its role is dependent on genetic background

Sleep was re-analysed from the same experiments as Chapter 3.2.2 by separating total sleep into sleep during dark hours and light hours.

Similarly to total sleep, both dark and light sleep in w^{Dah} control female flies was modified by diet, however in the w^{1118} background female controls only responded significantly to diet during light sleep (Figure 23).

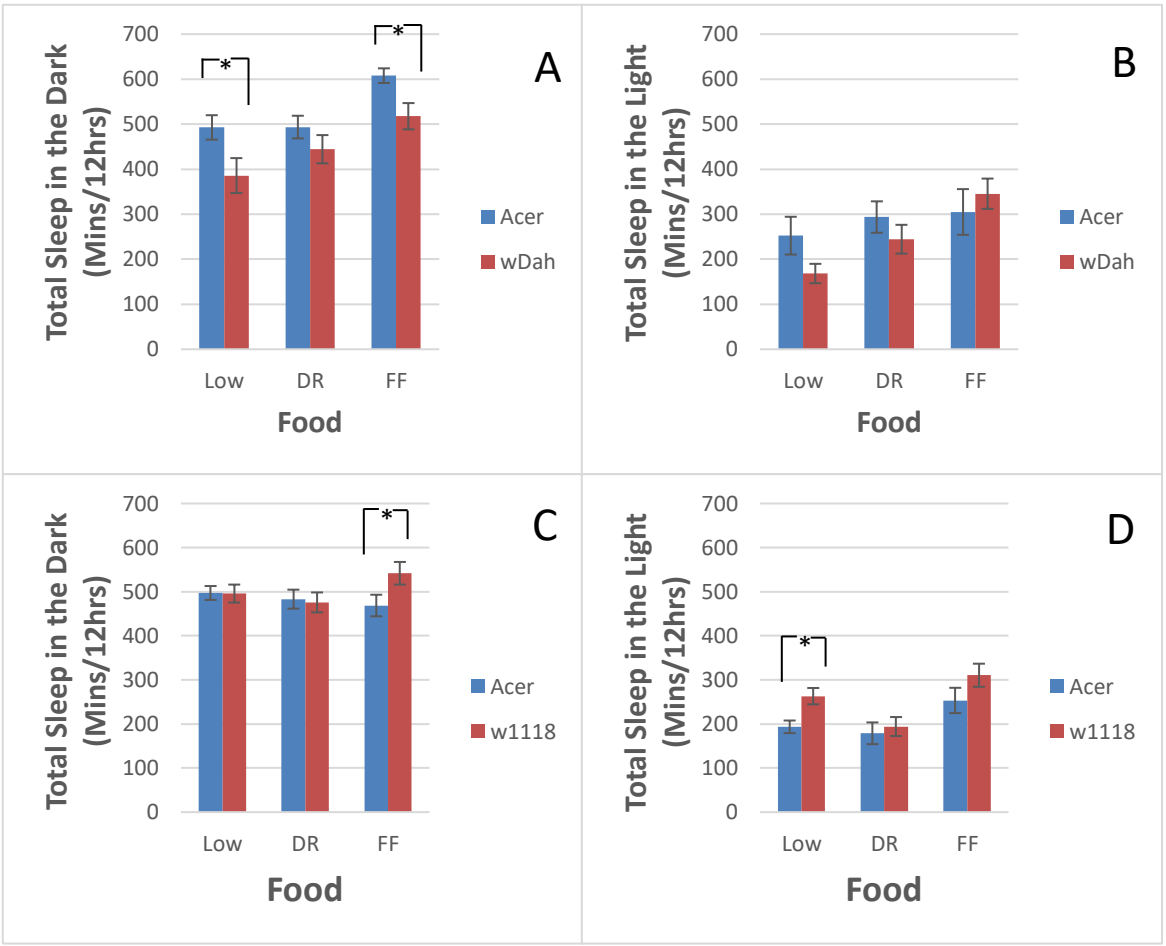

Figure 23: Female Total Sleep in the Dark (Min/12hrs) and Total Sleep in the Light (Min/12hrs) on Low, DR and FF diets. Total Sleep in the Dark (A) and Total Sleep in the Light (B) in the w^{Dah} background. Total Sleep in the Dark (C) and Total Sleep in the Light (D) in the w^{1118} background. (A) w^{Dah}: Low N = 18; DR N = 18; FF N = 17. $Acer^{\Delta}$: Low N = 13; DR N = 18; FF N = 15. (B) w^{Dah}: Low N = 18; DR N = 18; FF N = 17. $Acer^{\Delta}$: Low N = 13; DR N = 18; FF N = 15. (C) w^{1118}: Low N = 22; DR N = 22; FF N = 24. $Acer^{\Delta}$: Low N = 24; DR N = 23; FF N = 24. (D) w^{1118}: Low N = 23; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 24; DR N = 24; FF N = 24. Data was checked for normality and data was found to be normally

distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 8 and 9. *Indicates significant difference between genotypes (P=<0.05).

Interestingly, control females in the w^{Dah} background (Figures 23A and 23B and Table 8) showed the same phenotype of increasing sleep in both the light and the dark as food levels increased. However, in the w^{1118} background (Figures 23C and 23D and Table 8), control females showed a different pattern. Control w^{1118} females shoed no response to diet for sleep in the dark and appeared to sleep less in the light on the DR food compared to Low food, although the result is not significant it is approaching significance (Table 8), before showing an increase in sleep on FF food compared to DR food. Therefore, the normal response of dark and light sleep to nutrition is genetic background dependent.

Genotype	Food Comparison	p-value for Total Sleep in the Dark	p-value for Total Sleep in the Light
w^{Dah}	Low-DR	0.4309	0.1636
	Low-FF	0.0212*	0.0003*
	DR-FF	0.2823	0.0497*
$w^{Dah};Acer^{\Delta}$	Low-DR	0.9997	0.7747
	Low-FF	0.0054*	0.6831
	DR-FF	0.0026*	0.9793
w^{1118}	Low-DR	0.8197	0.0863
	Low-FF	0.34	0.2974
	DR-FF	0.1136	0.0013*
$w^{1118};Acer^{\Delta}$	Low-DR	0.8838	0.8976
	Low-FF	0.5984	0.1726
	DR-FF	0.8785	0.0689

Table 8: Female Tukey-Kramer HSD comparisons of means for Total Sleep in the Dark and Total Sleep in the Light for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

Similarly, the effect of loss of *Acer* on the response of dark and light sleep to nutrition is sensitive to genetic background. $Acer^{\Delta}$ females in the w^{Dah} background did not respond to dietary changes in the same way as controls and slept significantly longer on Low and FF food in the dark (Table 9). In the light $Acer^{\Delta}$ females did not respond to diet by increasing their sleep on the FF food compared to Low food and

DR food unlike controls. Despite this, there are no significant differences between the genotypes, however on Low food the difference between the genotypes is approaching significance (Table 9), with *Acer*$^{\Delta}$ females appearing to sleep longer on this food compared to controls.

In the *w*^{1118} background *Acer*$^{\Delta}$ females slept significantly less in the light on Low food compared to controls (Table 9) and although the increase in sleep on FF food wasn't significant, it was approaching significance and therefore a normal response. In the dark *Acer*$^{\Delta}$ females, like controls, showed no response to diet, however, *Acer*$^{\Delta}$ females slept significantly less on the FF food (Table 9).

Genotype	Food	Comparison to *Acer*$^{\Delta}$ for Total Sleep in the Dark	Comparison to *Acer*$^{\Delta}$ for Total Sleep in the Light
w^{Dah}	Low	0.046*	0.0636
	DR	0.23	0.3061
	FF	0.0142*	0.5021
w^{1118}	Low	0.9573	0.0045*
	DR	0.8152	0.6445
	FF	0.045*	0.148

Table 9: Female ANOVA/Pooled t-test measure of variance of genetic effects on different diets for Total Sleep in the Dark and Total Sleep in the Light. *Indicates significant differences (P=<0.05).

To analyse the light and dark sleep further the sleep data was analysed over 24 hours in a 12 hour light/dark cycle using the average sleep per hour of days two and three (Figure 24). The 24 hour cycle showed that all flies slept significantly more in the dark than the light (p=<.0001*) regardless of food or background. The least amount of sleep occurred as the lights turned on at 10am for control females in the *w*^{Dah} background but this was delayed for controls in the *w*^{1118} background until 11am, even though the lights turned on at 10am.

In the *w*^{Dah} background there was no significant difference of sleep between 10pm and 2am on all foods in the first four hours of the lights being switched off (Figure and Table 10). Sleep levels continued to plateau on all foods between 2am and 5am as sleep levels remained constant. Sleep significantly reduced between 5am and 9am in the final four hours of darkness for all foods (Table 10). *Acer*$^{\Delta}$ females in the *w*^{Dah} background followed the same pattern as controls, except significant increase in sleep was observed between 10pm and 2am on the FF food. In the light, controls showed no significant difference in sleep between 10am and 2pm on all foods in the first four

hours of the lights being switched on. Between 2pm and 5pm sleep plateaued on Low and DR food but increased significantly on FF food. A significant decrease in sleep was observed on Low food between 5pm and 9pm and, although not significant, a trend towards decreased sleep on DR and FF food was also observed. *Acer*$^{\Delta}$ females showed the same pattern of sleep in the light as controls but did not show a strong trend of decreased sleep on DR and FF food between 5pm and 9pm.

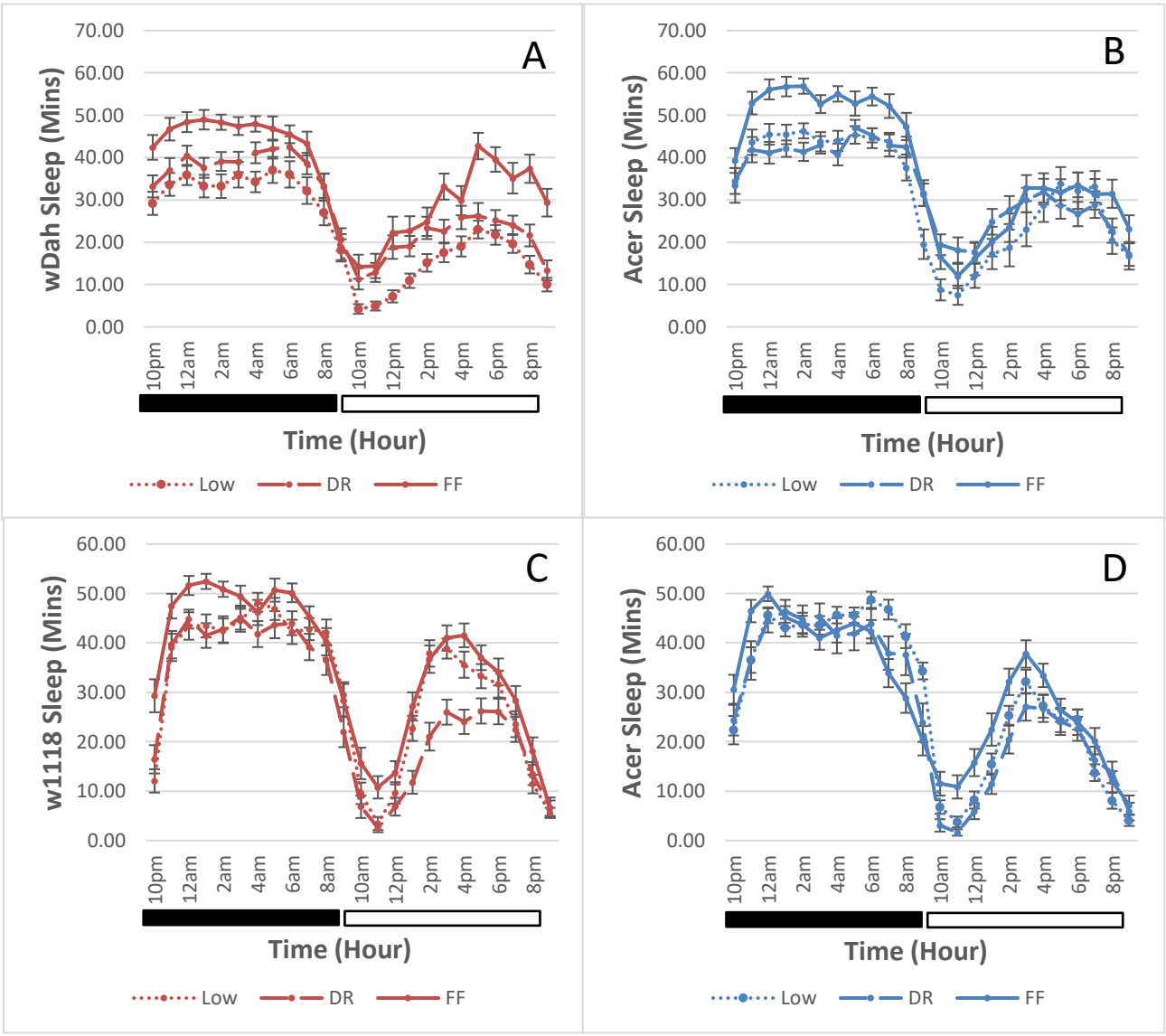

Figure 24: Female Sleep over a 24 hour period comparing the sleep response to Low, DR and FF foods for *w*^{Dah}, *w*^{Dah};*Acer*$^{\Delta}$, *w*^{1118} and *w*^{1118};*Acer*$^{\Delta}$. The data represents the average sleep (Mins) per hour over days 2 two and three. (A) Sleep for *w*^{Dah} controls on Low, DR and FF foods. Low: N = 18; DR N = 18; FF N = 17. (B) Sleep for *w*^{Dah};*Acer*$^{\Delta}$ on Low, DR and FF foods. Low N = 13; DR N = 18; FF N = 15. (C) Sleep for *w*^{1118} controls on Low, DR and FF foods. Low N = 23; DR N = 23; FF N = 24. (D) Sleep for *w*^{1118};*Acer*$^{\Delta}$ on Low, DR and FF foods. Low N = 24; DR N = 24; FF N = 24. Data was checked for

normality and data was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 10 and 11.

		w^{Dah}			$w^{Dah};Acer^{\Delta}$		
	Hourly Comparison	Low	DR	FF	Low	DR	FF
Dark	10pm-2am	1	0.9951	0.9993	0.3692	0.9809	0.0165*
	10pm-5am	0.8043	0.7183	1	0.5347	0.1311	0.2709
	10pm-9am	0.1578	0.0844	<.0001*	0.2276	1	0.9889
	2am-5am	1	1	1	1	0.9984	1
	2am-9am	0.0023*	0.0001*	<.0001*	<.0001*	0.5347	<.0001*
	5am-9am	<.0001*	<.0001*	<.0001*	<.0001*	0.0069*	0.0005*
Light	10am-2pm	0.1868	0.129	0.6306	0.8586	0.8864	0.9973
	10am-5pm	<.0001*	0.0096*	<.0001*	<.0001*	0.7128	0.0941
	10am-9pm	0.9925	1	0.0513	0.9824	1	0.9987
	2pm-5pm	0.8093	1	0.0044*	0.1051	1	0.9716
	2pm-9pm	0.9984	0.4522	1	1	0.4533	1
	5pm-9pm	0.0282*	0.0682	0.1779	0.0278*	0.2557	0.9565
		w^{1118}			$w^{1118};Acer^{\Delta}$		
	Hourly Comparison	Low	DR	FF	Low	DR	FF
Dark	10pm-2am	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	0.0467*
	10pm-5am	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	0.0347*
	10pm-9am	<.0001*	0.9969	1	0.0032*	1	0.3466
	2am-5am	0.9998	1	1	1	1	1
	2am-9am	0.0208*	<.0001*	<.0001*	0.0968	<.0001*	<.0001*
	5am-9am	<.0001*	<.0001*	<.0001*	0.0092*	<.0001*	<.0001*
Light	10am-2pm	<.0001*	0.0129*	<.0001*	<.0001*	<.0001*	<.0001*
	10am-5pm	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	0.0071*
	10am-9pm	1	1	0.6327	1	1	1
	2pm-5pm	0.9996	0.9989	1	1	1	0.9961
	2pm-9pm	<.0001*	0.0112*	<.0001*	<.0001*	0.0026*	<.0001*
	5pm-9pm	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*

Table 10: Female Tukey-Kramer HSD comparisons of means for hourly sleep within a 24 hour cycle for controls and $Acer^{\Delta}$ on Low, DR and FF foods. *Indicates significant differences (P=<0.05).

Overall the data showed that $Acer^{\Delta}$ females in the w^{Dah} background showed a similar pattern of sleep to controls over 24 hours, however, there were differences

between the genotypes but only on certain diets. This suggests that *Acer* is not involved in the circadian pattern in the w^{Dah} background and the differences observed were diet related rather than an alteration in the circadian pattern, which would have been observed in $Acer^{\Delta}$ females independently of diet.

In the w^{1118} background, unlike w^{Dah} controls, sleep significantly increased between 10pm and 2am on all foods in the first four hours of darkness. This was followed by a plateau of sleep levels between 2am and 5am before sleep levels significantly dropped between 5am and 9am (Table 10). For the hours of darkness, the same pattern was seen for $Acer^{\Delta}$ females (Table 10). In the light w^{1118} control females significantly increased sleep between 10am and 2pm before sleep plateaued between 2pm and 5pm on all foods. A significant decrease in sleep was observed between 5pm and 9pm on all foods (Table 10). $Acer^{\Delta}$ females in the w^{1118} background showed the same pattern as controls indicating that *Acer* is not involved in the circadian pattern.

The 24 hour data indicated that control females in the w^{Dah} background were more responsive to diet in their sleep patterns than control females in the w^{1118} background (Figure 24 and Table 10).

In the w^{Dah} background, control females at 10pm (when the lights switched off) showed a significant increase of sleep on FF food compared to Low food with the increase between DR and FF food nearing significance. The increase in sleep between Low and FF food was maintained through 2am and 5am with a significant increase in sleep also observed between DR and FF food at 2am but not 5am. Levels of sleep between all foods showed no significant difference at 9am. These data suggest that in the w^{Dah} background female sleep in the dark is affected by diet. $Acer^{\Delta}$ females in the w^{Dah} background showed a different response to diet. At 10pm $Acer^{\Delta}$ females, unlike controls, showed no response to diet. They did show an increase in sleep between Low and FF and DR and FF foods at 2am as well as a significant increase in sleep between Low and FF foods at 5am. At 9am sleep on the Low food was significantly reduced compared to both the DR and FF foods (Table 11).

In the light, w^{Dah} control females showed a significant increase in sleep between Low and FF foods throughout the light time points, with significantly increased sleep also observed between DR and FF food at 5pm and 9pm (Table 11). These data suggest that in the w^{Dah} background female sleep is highly responsive to diet in the light as well as the dark. $Acer^{\Delta}$ females showed no significant difference of sleep levels

between Low and FF food at any of the light time points. A significant increase in sleep was seen between Low and DR food at 10am but no other differences were observed. This confirms the observations of total sleep in the dark and the light (Figure 23A and Table 8).

		Genotype and Food Comparison					
	Hour	w^{Dah}			$w^{Dah};Acer^{\Delta}$		
		Low-DR	Low-FF	DR-FF	Low-DR	Low-FF	DR-FF
Dark	10pm	0.5547	0.0029*	0.0525	0.9759	0.4973	0.574
	2am	0.1718	<.0001*	0.0165*	0.1242	0.0003*	<.0001*
	5am	0.4118	0.037*	0.4316	0.788	0.0223*	0.0695
	9am	0.7901	0.9794	0.8941	0.0305*	0.0322*	0.9958
Light	10am	0.0681	0.0068*	0.6444	0.0368*	0.1929	0.7552
	2pm	0.0817	0.0365*	0.9239	0.2012	0.6513	0.6808
	5pm	0.6995	<.0001*	0.0002*	0.5907	0.9234	0.8155
	9pm	0.5977	<.0001*	<.0001*	0.9981	0.4044	0.3786
	Hour	w^{1118}			$w^{1118};Acer^{\Delta}$		
		Low-DR	Low-FF	DR-FF	Low-DR	Low-FF	DR-FF
Dark	10pm	0.5404	0.0001*	0.0056*	0.88	0.1228	0.2985
	2am	0.9951	0.0253*	0.0325*	0.9439	0.9998	0.9366
	5am	0.6291	0.5134	0.1083	0.4165	0.8462	0.7548
	9am	0.1976	0.9598	0.3056	0.0114*	0.0004*	0.5687
Light	10am	0.744	0.2271	0.047*	0.2968	0.1409	0.0025*
	2pm	<.0001*	0.9503	0.0003*	0.3499	0.1392	0.0035*
	5pm	0.1227	0.5414	0.0076*	0.9988	0.8208	0.7946
	9pm	0.8752	0.9039	0.9974	0.7228	0.3752	0.8352

Table 11: Female Tukey-Kramer HSD comparisons of means for sleep at specific time points within a 24 hour cycle for controls and *Acer*$^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

In the w^{1118} background, control females showed a significant increase in sleep in the dark at 10pm and 2am between Low and FF food and DR and FF food (Table 11). No difference in sleep levels was seen at either 5am or 9am between any of the foods. *Acer*$^{\Delta}$ females in the w^{1118} background showed no difference between diets at 10pm, 2am or 5am. At 9am a significant reduction in sleep was observed between Low and DR food and Low and FF food. In the light, w^{1118} controls showed a significant

increase in sleep between DR and FF foods at 10am. At 2pm, controls showed a significant decrease in sleep between Low and DR food and then a significant increase between DR and FF, with no difference between Low and FF food. A significant increase in sleep was observed at 5pm between DR and FF food while at 9pm there was no difference between the foods. *Acer*$^{\Delta}$ females showed a significant increase in sleep between DR and FF food at 10am and 2pm. No other differences were observed.

Diet appears to be an important factor for sleep in both the light and in the dark in both genetic backgrounds, however the effect of diet at time points within the circadian pattern is dependent on background. Sleep in the *w*^{Dah} background is more responsive to changing diet than sleep in the *w*^{1118} background, especially in the light (Table 10). In both backgrounds, sleep in *Acer*$^{\Delta}$ females did not respond in the same way as controls to differing levels of food, suggesting that *Acer* modulates the response of sleep to changing diet. Many of the differences between *Acer*$^{\Delta}$ females and *w*^{Dah} controls were seen in the hours of transition between dark and light (10pm, 9am, 10am and 9am). *Acer*$^{\Delta}$ females showed no response to diet at these times, except at 9am when a decrease in sleep was observed on Low food compared to DR and FF food and controls had shown no response to diet at that time. In the *w*^{1118} background differences were also seen at the transition points between dark and light but only at 10pm and 9am with *Acer*$^{\Delta}$ females responding differently to controls, suggesting a potential role for *Acer* in the transition between light and dark activity and sleep in response to diet.

Comparison of the genotypes at selected time points on each food revealed many differences between controls and *Acer*$^{\Delta}$ females in both backgrounds (Figure 25 and Table 12). At 10pm, as the light's switched off, there was no difference between *w*^{Dah} control females and *Acer*$^{\Delta}$ females on any of the foods but at 2am *Acer*$^{\Delta}$ females slept significantly more on Low and FF foods. At 5am *Acer*$^{\Delta}$ females continued to sleep significantly more on Low food with increased sleep levels nearing significance for both DR and FF food. At 9am *Acer*$^{\Delta}$ females slept significantly more on DR and FF foods but showed no difference in sleep levels on Low food. At 10am, as the lights switched on, *Acer*$^{\Delta}$ females slept significantly more on DR food than controls with increased sleep levels nearing significance on Low food but no difference was seen on FF food. No difference between the genotypes was observed at 2pm on all foods. At 5pm and a significant increase in sleep for *Acer*$^{\Delta}$ females was observed on Low food but a significant reduction in sleep was seen on FF food for *Acer*$^{\Delta}$ females. As

the light cycle came to the end at 9pm a significant increase in sleep was seen on Low food for *Acer*$^{\Delta}$ females.

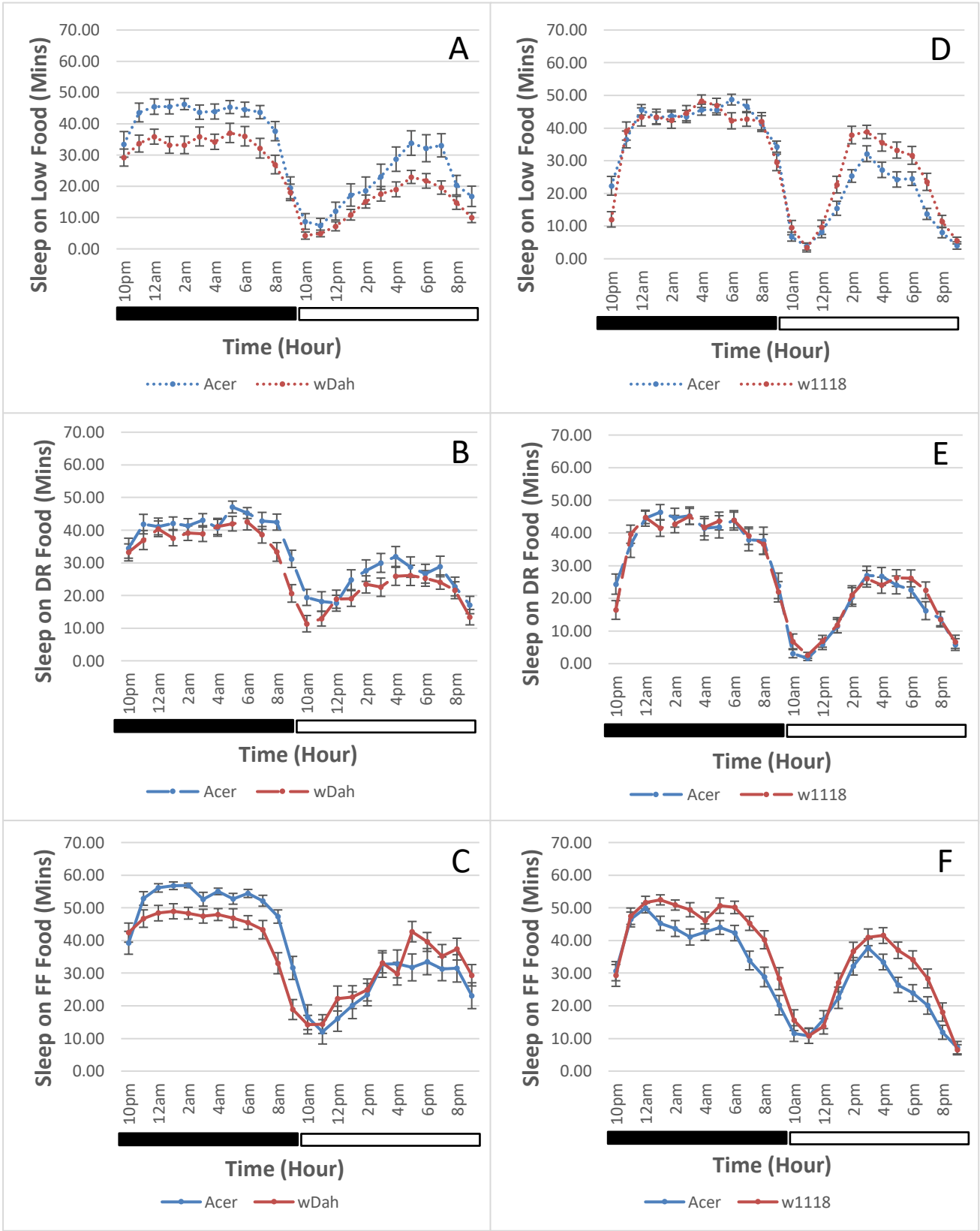

Figure 25: Female Sleep over a 24 hour period comparing controls to *Acer*$^{\Delta}$ flies in both backgrounds

on each food separately. The data represents the average sleep (Mins) per hour over days 2 two and three. (A-C) w^{Dah} background. (D-F) w^{1118} background. (A) Low food. w^{Dah} N = 18. $Acer^{\Delta}$ N = 13. (B) DR food. w^{Dah} N = 18. $Acer^{\Delta}$ N = 18. (C) FF food. w^{Dah} N = 17. $Acer^{\Delta}$ N = 15. (D) Low food. w^{1118} N = 23. $Acer^{\Delta}$ N = 24. (E) DR food. w^{1118} N = 23. $Acer^{\Delta}$ N = 24. (F) FF food. w^{1118} N = 24. $Acer^{\Delta}$ N = 24. Data was checked for normality and data was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Table 12.

		Comparison to $Acer^{\Delta}$ at specific times in the Dark and the Light					
Genotype		w^{Dah}			w^{1118}		
Food		Low	DR	FF	Low	DR	FF
Dark	10pm	0.3571	0.7515	0.4953	0.0071*	0.0641	0.7681
	2am	0.0006*	0.4659	<.0001*	0.6697	0.5601	0.0144*
	5am	0.0452*	0.0829	0.089	0.6361	0.6257	0.0364*
	9am	0.735	0.0064*	0.008*	0.1158	0.6638	0.0725
Light	10am	0.0773	0.0247*	0.6293	0.2805	0.1451	0.3057
	2pm	0.4303	0.3203	0.769	0.0002*	0.8799	0.2368
	5pm	0.0109*	0.5682	0.0385*	0.0094*	0.5752	0.0026*
	9pm	0.0463*	0.299	0.2247	0.3192	0.7554	0.7938

Table 12: Female ANOVA/Pooled t-test measure of variance of genetic effects on different diets for sleep at specific time points within a 24 hour cycle. *Indicates significant differences (P=<0.05).

These data confirm that $Acer^{\Delta}$ females in the w^{Dah} background slept more on Low and FF food in the dark and although no difference in sleep was seen when total sleep in the light was analysed, the 24 hour data showed that at the end of the light sleep cycle $Acer^{\Delta}$ females slept more than controls on Low food which contributed to the trend towards significantly increased sleep for $Acer^{\Delta}$ females on Low food in the light (Table 12).

In the w^{1118} background there were also differences between controls and $Acer^{\Delta}$ females (Figure and Table 12). At 10pm, when the lights were switched off, $Acer^{\Delta}$ females slept significantly more than controls on Low food with an increase in sleep for $Acer^{\Delta}$ females nearing significance on DR food. At 2am and 5am $Acer^{\Delta}$ females slept significantly less on FF food than controls with no difference observed on either Low or DR food. At 9am no difference was observed between the genotypes on any of the foods, however on FF food $Acer^{\Delta}$ females showed a decreased level of sleep that was nearing significance (Table 12). At 10am, when the lights turned on, there was no difference in sleep between the genotypes. At 2pm $Acer^{\Delta}$ females slept

significantly less on Low food compared to controls and this continued at 5pm with *Acer*$^{\Delta}$ females also sleeping less on FF food. At 9pm there was no difference between the genotypes on any of the foods.

These data confirm the reduction in total sleep in the dark on FF food and the reduction in sleep in the light on Low food for *Acer*$^{\Delta}$ females in the w^{1118} background.

In female flies in both genetic backgrounds *Acer* is not involved in maintaining the circadian pattern but it does appear to be involved in the response of the circadian pattern to dietary changes. These changes differ between the backgrounds and light and dark sleep making sleep a very complex behaviour to analyse. *Acer*'s role in sleep is linked to dietary response and requires further investigation.

Control males showed no response to nutrition, unlike females, for sleep in the light and dark (Figures 26 and Tables 13 and 14). This confirms the lack of nutritional response for total sleep in both backgrounds for control male flies (Chapter 3.2.2).

Acer$^{\Delta}$ males in the w^{Dah} background (Figure 26A and 26B) showed no response to diet in terms of dark and light sleep, however, *Acer*$^{\Delta}$ males slept significantly longer on DR food in the dark with an increase in sleep also nearing significance on FF food, suggesting that *Acer* may be involved in modulating sleep regulation to higher food levels. However, this difference was not enough to affect total sleep in the w^{Dah} background (Chapter 3.2.2.)

In the w^{1118} background *Acer*$^{\Delta}$ males showed a strong response to nutrition with a significant reduction in sleep on high food in the light (Figures 26C and 26D and Tables 13 and 14). The control males in the w^{1118} background showed no response to changing diet, therefore this reduction suggests that *Acer* modulates the response of sleep to high yeast content, as the mutants are unable to keep their sleep levels at the same level on high food. This reduction in sleep for *Acer*$^{\Delta}$ males in the light accounts for the reduced sleep level seen in total sleep for male *Acer*$^{\Delta}$ flies on FF food in the w^{1118} background (Chapter 3.2.2.).

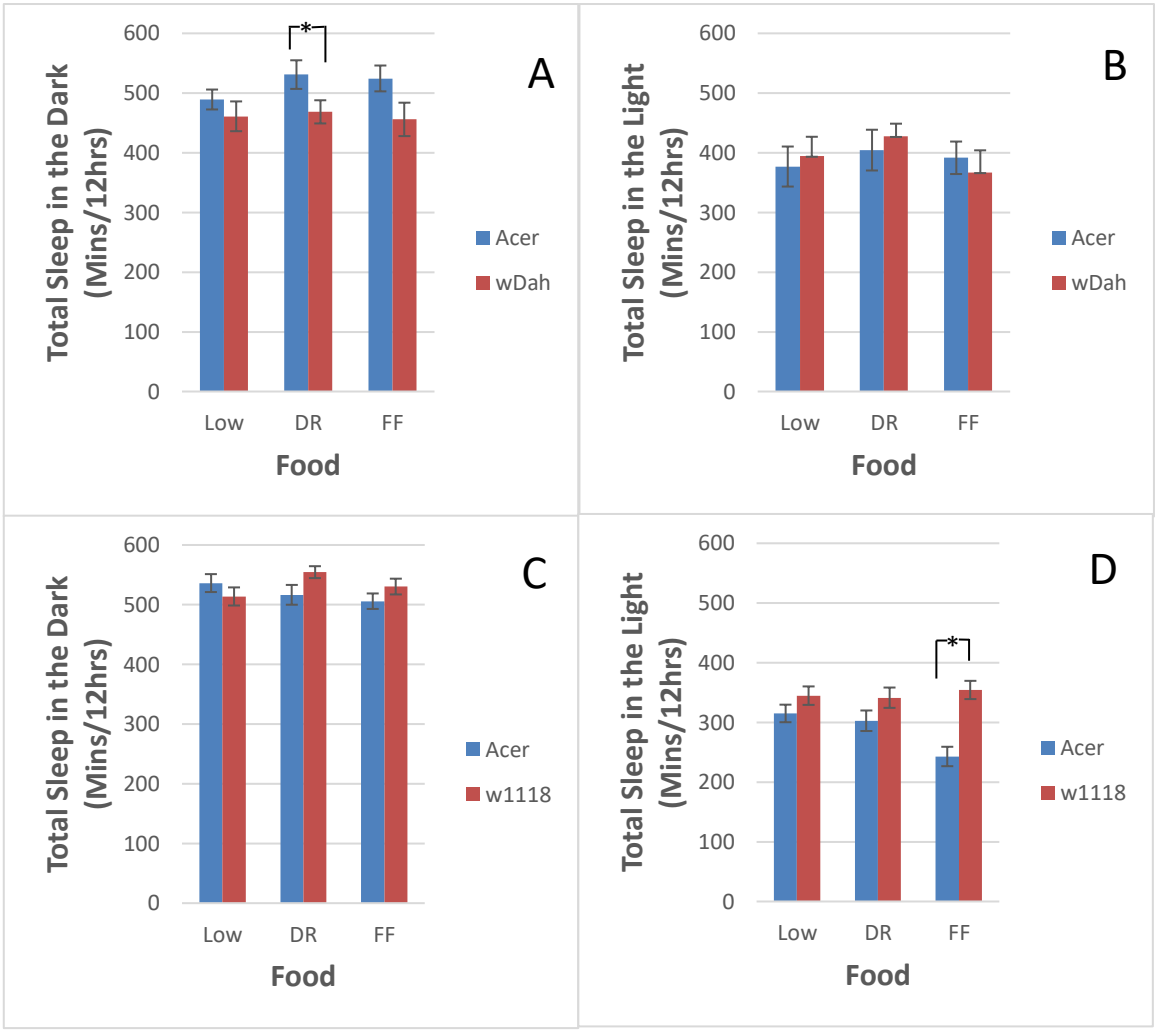

Figure 26: Male Total Sleep in the Dark (Mins/12hrs) and Total Sleep in the Light (Mins/12hrs) on Low, DR and FF diets. Total Sleep in the Dark (A) and Total Sleep in the Light (B) in the w^{Dah} background. Total Sleep in the Dark (C) and Total Sleep in the Light (D) in the w^{1118} background. (A) w^{Dah}: N = Low 18; DR N= 18; FF N= 16. . $Acer^{\Delta}$: Low N = 14; DR N = 17; FF N = 18. (B) w^{Dah}: Low N = 18; DR N = 18; FF N = 18. $Acer^{\Delta}$: Low N = 15; DR N= 17; FF N = 18. (C) w^{1118}: Low N = 22; DR N = 23; FF N = 23. $Acer^{\Delta}$: Low N = 23; DR N = 22; FF N = 21. (D) w^{1118}: Low N = 22; DR N = 23; FF N = 23. $Acer^{\Delta}$: Low N = 23; DR N = 21; FF N = 22. Data was checked for normality and data was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 13 and 14. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	p-value for Total Sleep in the Dark	p-value for Total Sleep in the Light
w^{Dah}	Low-DR	0.9731	0.7319
	Low-FF	0.9875	0.3584
	DR-FF	0.9285	0.8069
$w^{Dah};Acer^{\Delta}$	Low-DR	0.3887	0.8186
	Low-FF	0.4978	0.943
	DR-FF	0.9741	0.9531
w^{1118}	Low-DR	0.0781	0.9875
	Low-FF	0.6443	0.9072
	DR-FF	0.3884	0.8321
$w^{1118};Acer^{\Delta}$	Low-DR	0.618	0.8515
	Low-FF	0.3325	0.0057*
	DR-FF	0.8736	0.0299*

Table 13: Male Tukey-Kramer HSD comparisons of means for Total Sleep in the Dark and Total Sleep in the Light for controls and *Acer*$^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

Genotype	Food	Comparison to *Acer*$^{\Delta}$ for Total Sleep in the Dark	Comparison to *Acer*$^{\Delta}$ for Total Sleep in the Light
w^{Dah}	Low	0.3858	0.7126
	DR	0.05*	0.5656
	FF	0.0584	0.5955
w^{1118}	Low	0.3052	0.1695
	DR	0.0536	0.1187
	FF	0.1936	<.0001*

Table 14: Male ANOVA/Pooled t-test measure of variance of genetic effects on different diets for Total Sleep in the Dark and Total Sleep in the Light. *Indicates significant differences (P=<0.05).

To analyse the light and dark sleep further, like the females, the sleep data was analysed over 24 hours in a 12 hour light/dark cycle using the average sleep per hour of days two and three (Figure 27). Like the females, the 24 hour cycle showed that all flies slept significantly more in the dark than the light (p=<.0001*) regardless of food or background.

In the w^{Dah} background control males showed a significant increase in sleep between 10am and 2am in the first four hours of the lights having been switched off on all foods. Sleep levels plateaued between 2am and 5am on all foods before

significantly reducing between 5am and 9am. *Acer*$^{\Delta}$ males responded in the same way as controls (Table 15).

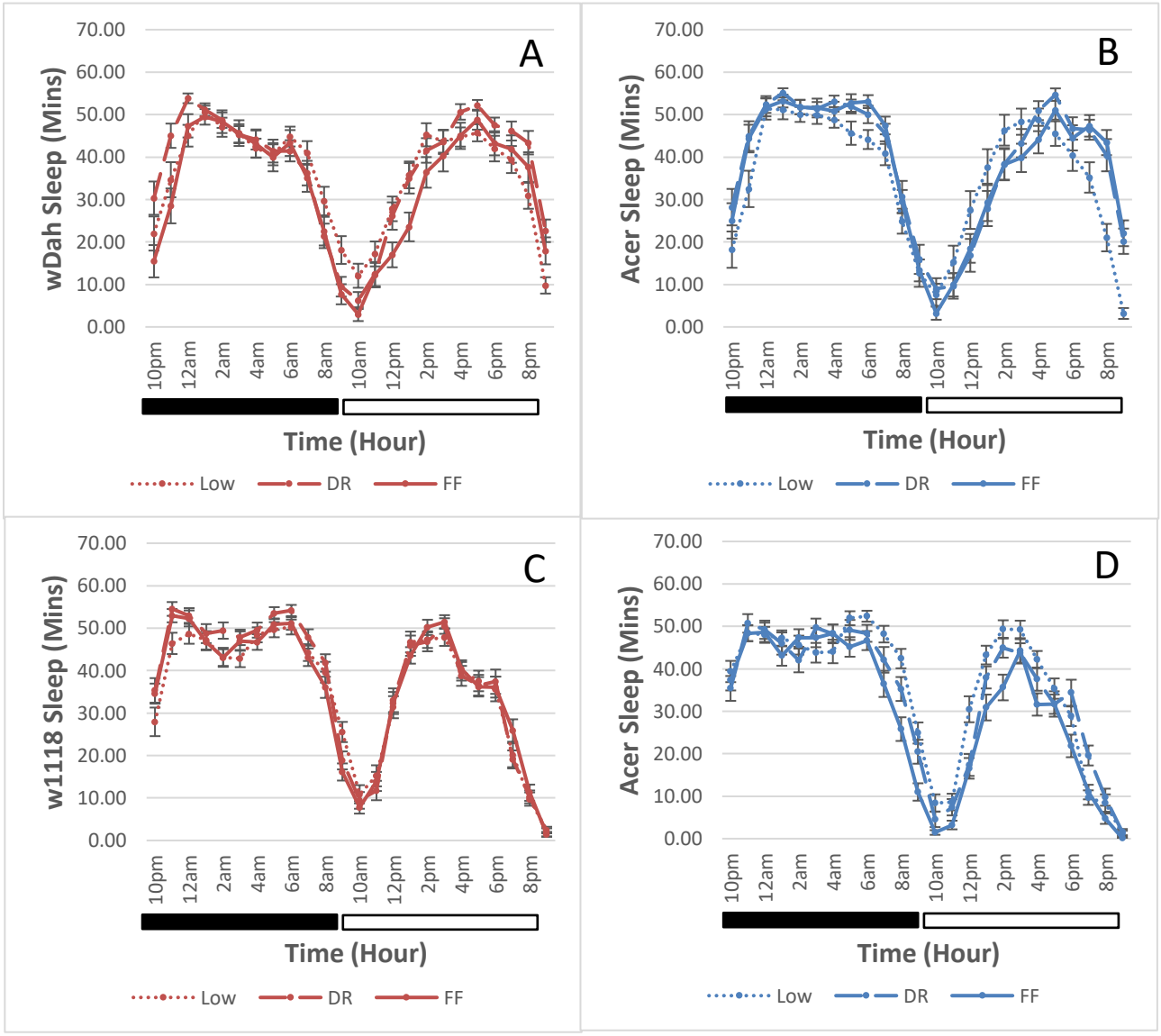

Figure 27: Male Sleep over a 24 hour period comparing the sleep response to Low, DR and FF foods for *w*^{Dah}, *w*^{Dah}*;Acer*$^{\Delta}$, *w*^{1118} and *w*^{1118}*;Acer*$^{\Delta}$. The data represents the average sleep (Mins) per hour over days 2 two and three. (A) Sleep for *w*^{Dah} controls on Low, DR and FF foods. Low: N = 18; DR N = 18; FF N = 18. (B) Sleep for *w*^{Dah}*;Acer*$^{\Delta}$ on Low, DR and FF foods. Low N = 15; DR N = 17; FF N = 18. (C) Sleep for *w*^{1118} controls on Low, DR and FF foods. Low N = 22; DR N = 23; FF N = 24. (D) Sleep for *w*^{1118}*;Acer*$^{\Delta}$ on Low, DR and FF foods. Low N = 22; DR N = 23; FF N = 22. Data was checked for normality and data was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 15 and 16.

		w^{Dah}			$w^{Dah};Acer^{\Delta}$		
	Hourly Comparison	Low	DR	FF	Low	DR	FF
Dark	10pm-2am	<.0001*	0.0001*	<.0001*	<.0001*	<.0001*	<.0001*
	10pm-5am	0.0009	0.2456	<.0001*	<.0001*	<.0001*	<.0001*
	10pm-9am	1	<.0001*	0.9857	0.999	0.2756	0.3186
	2am-5am	0.995	0.9387	0.9669	1	1	1
	2am-9am	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
	5am-9am	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
Light	10am-2pm	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
	10am-5pm	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
	10am-9pm	1	0.0014*	0.1058	1	0.4712	0.0005*
	2pm-5pm	1	0.3223	0.4111	1	0.0124*	0.1667
	2pm-9pm	<.0001*	<.0001*	0.0061*	<.0001*	0.0021*	0.009*
	5pm-9pm	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
		w^{1118}			$w^{1118};Acer^{\Delta}$		
	Hourly Comparison	Low	DR	FF	Low	DR	FF
Dark	10pm-2am	0.0006*	0.0005*	0.2994	0.9237	0.9633	0.2524
	10pm-5am	<.0001*	<.0001*	<.0001*	0.007*	0.0127*	0.7345
	10pm-9am	1	<.0001*	<.0001*	0.7503	0.0033*	<.0001*
	2am-5am	0.9289	0.9995	0.4858	0.9103	0.9129	1
	2am-9am	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
	5am-9am	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
Light	10am-2pm	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
	10am-5pm	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
	10am-9pm	0.5644	0.6281	0.8557	0.0004*	1	1
	2pm-5pm	0.2672	<.0001*	0.0003*	0.0008*	0.0237*	1
	2pm-9pm	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
	5pm-9pm	<.0001*	0.085	<.0001*	<.0001*	<.0001*	<.0001*

Table 15: Male Tukey-Kramer HSD comparisons of means for hourly sleep within a 24 hour cycle for controls and *Acer*$^{\Delta}$ on Low, DR and FF foods. *Indicates significant differences (P=<0.05).

In the light, control males showed a significant increase in sleep between 10am and 2pm before sleep plateaued between 2pm and 5pm on all foods. Between 5pm and 9pm control males showed a significant decrease in sleep on all foods. *Acer*$^{\Delta}$ males also showed the increase in sleep on all foods between 10am and 2pm as well

as the decrease in sleep between 5pm and 9pm on all foods. However, between 2pm and 5pm *Acer*$^{\Delta}$ males on DR showed a significant increase in sleep, unlike controls.

Overall the results show that *Acer* is not involved in maintaining the circadian pattern in males in the *w*^{Dah} background.

In the *w*^{1118} background control males showed a significant increase in sleep between 10pm and 2am on both Low and DR food but not FF food. Sleep levels plateaued between 2am and 5am before significantly reducing between 5am and 9am. *Acer*$^{\Delta}$ males in this background showed no increase in sleep levels on any food between 10pm and 2am. Sleep levels remained constant between 2am and 5am before significantly reducing between 5am and 9am. In the light *w*^{1118} control males significantly increased sleep between 10am and 2pm before significantly decreasing sleep between 2pm and 5pm on DR and FF foods but not Low food. A further reduction in sleep was seen between 5pm and 9pm on Low and FF food with the decrease on DR food nearing significance (Table 15). Between 10am and 2pm *Acer*$^{\Delta}$ males significantly increased sleep but, unlike controls, showed a reduction of sleep on Low and DR food but not FF food between 2pm and 5pm. Between 5pm and 9pm *Acer*$^{\Delta}$ males showed a significant decrease in sleep levels on all foods.

Although differences were seen in the circadian pattern, the differences were diet related and did not apply to all foods which would be expected if *Acer* was important in maintaining the circadian pattern. This suggests that *Acer* is not involved in maintaining the circadian pattern in *w*^{1118} males.

Total sleep and total sleep in the light and the dark showed that sleep in male flies in both backgrounds was unresponsive to diet. The 24 hour data confirms that sleep in male flies is less responsive to diet than female flies (Figure 26 and Tables 13 and 14).

In the *w*^{Dah} background male controls showed a decrease in sleep between DR and FF food at 10pm as the lights were turned off. No difference was seen between the foods at 2am or 5am but at 9am a significant reduction in sleep was seen between Low and FF foods (Table 16). *Acer*$^{\Delta}$ males showed no significant response of sleep to changing diet at any of the time points in the dark (Table 16). In the light *w*^{Dah} control males showed a significant reduction in sleep between Low and FF foods at 10am as the lights were switched on. No difference was seen between the foods at 2pm or 5pm, however at 5pm an increase in sleep between Low and DR food was nearing significance. At 9pm at significant increase in sleep between Low and DR food was

observed. *Acer*$^{\Delta}$ males showed no response to diet at 10am or 2pm, however a significant increase in sleep between Low and DR food was seen at 5pm. At 9pm a significant increase in sleep was seen between Low food and both the DR and FF foods.

		Genotype and Food Comparison					
	Hour	w^{Dah}			w^{Dah};*Acer*$^{\Delta}$		
		Low-DR	Low-FF	DR-FF	Low-DR	Low-FF	DR-FF
Dark	10pm	0.2923	0.4679	0.0227*	0.239	0.5065	0.8463
	2am	0.8628	0.9134	0.9931	0.7584	0.7568	1
	5am	0.9736	0.9896	0.9319	0.1124	0.0574	0.9569
	9am	0.064	0.0195*	0.8865	0.7467	0.9884	0.8131
Light	10am	0.153	0.0125*	0.5584	0.8951	0.3562	0.1456
	2pm	0.6766	0.1114	0.4675	0.2937	0.2804	1
	5pm	0.0505	0.4975	0.431	0.0141*	0.1962	0.4543
	9pm	0.0023*	0.0789	0.4174	<.0001*	<.0001*	0.8867
	Hour	w^{1118}			w^{1118};*Acer*$^{\Delta}$		
		Low-DR	Low-FF	DR-FF	Low-DR	Low-FF	DR-FF
Dark	10pm	0.1724	0.2349	0.9083	0.5318	0.8398	0.8631
	2am	0.0812	0.9998	0.0763	0.5328	0.87	0.2623
	5am	0.1708	0.8158	0.4362	0.6313	0.0631	0.3798
	9am	0.0818	0.0072*	0.6427	0.4185	0.0003*	0.0206*
Light	10am	0.849	0.4702	0.8034	0.2209	0.0075*	0.3657
	2pm	0.9825	0.5651	0.4461	0.4157	0.0004*	0.0261*
	5pm	0.9532	0.9115	0.9929	0.578	0.577	0.9999
	9pm	0.8772	0.625	0.8998	0.9029	0.3683	0.184

Table 16: Male Tukey-Kramer HSD comparisons of means for sleep at specific time points within a 24 hour cycle for controls and *Acer*$^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

In the w^{1118} background control males showed little response of sleep in the dark to diet except for a significant decrease in sleep between Low and FF food at 9am (Table 16). *Acer*$^{\Delta}$ males showed a similar response with no significant reaction to food at 10pm, 2am or 5am but showed a significant decrease in sleep between Low and FF food and DR and FF food at 9am. In the light w^{1118} controls showed no sleep response to diet at any of the time points. *Acer*$^{\Delta}$ males showed a significant reduction

in sleep between Low and FF food at 10am. At 2pm *Acer*$^{\Delta}$ males continued to show a significant reduction in sleep between Low and FF food as well as a reduction in sleep between DR and FF food. No difference in sleep was observed between the diets at 5pm or 9pm (Table 16).

Although in total no effect of diet was seen for sleep in the light and the dark for males, the 24 hour analysis showed that at certain time points diet did have an effect on sleep levels, especially in the w^{Dah} background. The effects were seen in the transition hours between light and dark in the 12 hour light/dark cycle but *Acer*$^{\Delta}$ males showed no response. In the w^{1118} background there was very little reaction to diet for male controls but *Acer*$^{\Delta}$ males showed a response to dietary change. This suggests that *Acer* is involved in modulating the sleep response to diet by either promoting a sleep response to changing diet or maintaining the level of sleep despite the change of diet.

The comparison of the genotypes for male flies revealed that in the w^{Dah} background sleep in the dark only differed at 5pm with *Acer*$^{\Delta}$ males sleeping more on both DR and FF food (Figure 28 and Table 17). In the light the only difference between the genotypes occurred at 9pm on Low food with *Acer*$^{\Delta}$ males sleeping less at that time (Table 17).

In the w^{1118} background (Figure 28 and Table 17) in the dark differences between the genotypes were seen at three of the four dark time points. At 10pm *Acer*$^{\Delta}$ males slept significantly more than controls on Low food but at 2am *Acer*$^{\Delta}$ males slept significantly less on DR food and less on FF food at 5am. In the light, *Acer*$^{\Delta}$ males slept significantly on FF food at 10am, 2pm and 9pm. This confirms the reduced level of sleep for *Acer*$^{\Delta}$ males on FF food in total sleep in the light in the w^{1118} background (Table 17).

Overall, the genetic differences confirm the results for total sleep in the dark and light in male flies. Though male flies are less responsive to dietary change it appears that *Acer*'s role in sleep is related to changing diet or to maintaining sleep levels regardless of dietary change.

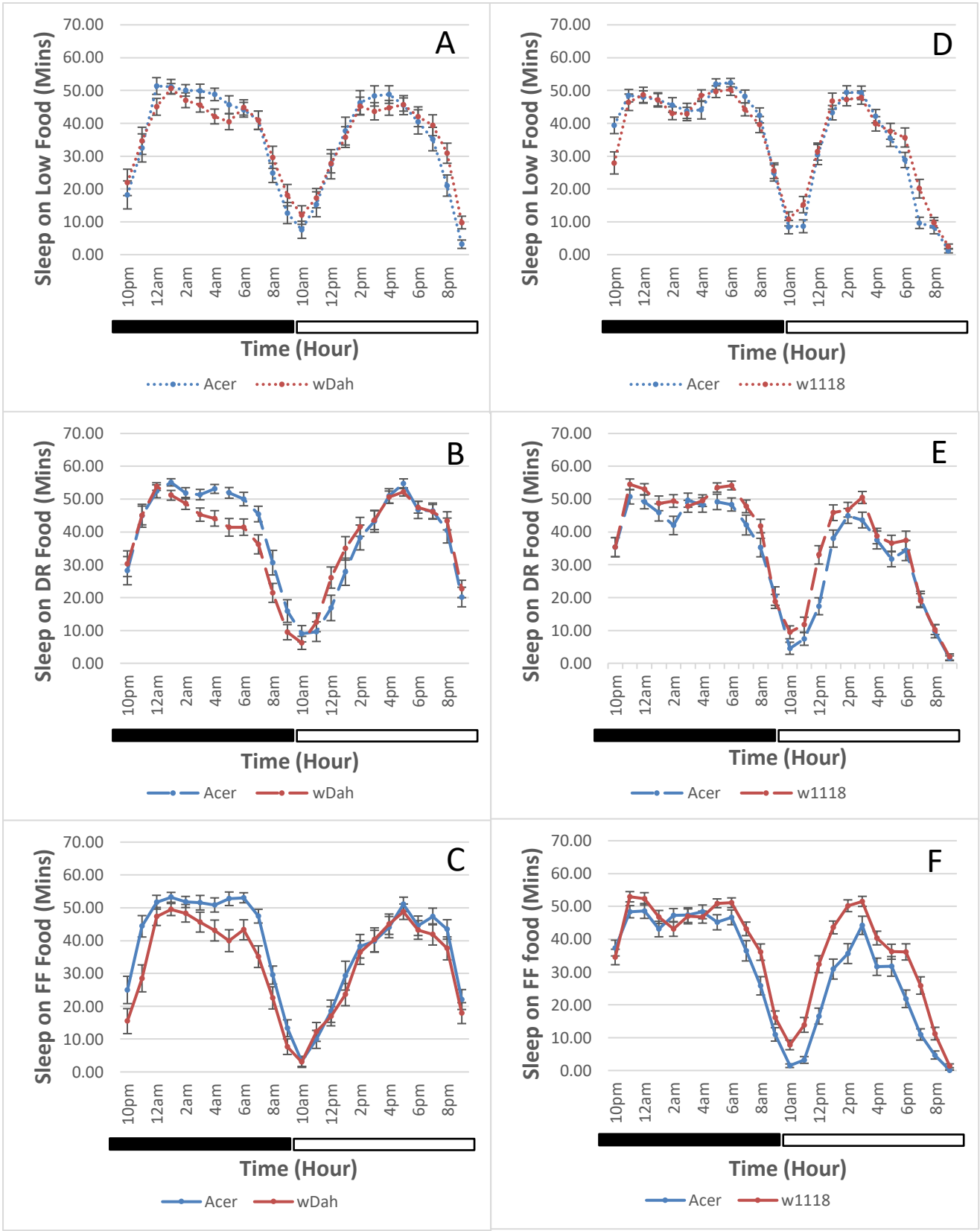

Figure 28: Male Sleep over a 24 hour period comparing controls to *Acer*$^{\Delta}$ flies in both backgrounds on each food separately. The data represents the average sleep (Mins) per hour over days 2 two and three. (A-C) *w*^{Dah} background. (D-F) *w*^{1118} background. (A) Low food. *w*^{Dah} N = 18. *Acer*$^{\Delta}$ N = 15. (B) DR

food. w^{Dah} N = 18. $Acer^{\Delta}$ N = 17. (C) FF food. w^{Dah} N = 18. $Acer^{\Delta}$ N = 18. (D) Low food. w^{1118} N = 22. $Acer^{\Delta}$ N = 23. (E) DR food. w^{1118} N = 23. $Acer^{\Delta}$ N = 22. (F) FF food. w^{1118} N = 24. $Acer^{\Delta}$ N = 23. Data was checked for normality and data was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Table 17.

		Comparison to $Acer^{\Delta}$ at specific times in the Dark and the Light					
Genotype		w^{Dah}			w^{1118}		
Food		Low	DR	FF	Low	DR	FF
Dark	10pm	0.5206	0.7151	0.0964	0.0074*	0.9925	0.4216
	2am	0.3113	0.2185	0.28	0.3994	0.0295*	0.1794
	5am	0.1709	0.0016*	0.0017*	0.3499	0.1094	0.031*
	9am	0.2443	0.1208	0.1133	0.85	0.643	0.078
Light	10am	0.2251	0.383	0.9211	0.4018	0.0745	0.0001*
	2pm	0.8087	0.4971	0.7116	0.4687	0.5519	<.0001*
	5pm	0.9564	0.1964	0.493	0.5164	0.1471	0.2352
	9pm	0.0079*	0.5382	0.3499	0.2192	0.7612	0.0303*

Table 17: Male ANOVA/Pooled t-test measure of variance of genetic effects on different diets for sleep at specific time points within a 24 hour cycle. *Indicates significant differences (P=<0.05).

In summary, the results showed that sleep in female controls in the w^{Dah} background was very responsive to dietary changes in both the light and the dark. $Acer^{\Delta}$ females showed a similar response to diet when sleep in the dark was isolated but slept significantly more on Low and FF food. $Acer^{\Delta}$ females showed no response to changing diet for sleep in the light. Control females in the w^{1118} background showed very little response of sleep to dietary changes in either the light or the dark. $Acer^{\Delta}$ females were found to sleep significantly less on FF food in the dark and significantly less on Low food in the light. Although light and dark sleep in the two genetic backgrounds behaved slightly differently in response to nutrition in females, a lack of *Acer* resulted in a reduced response to nutrition in the light irrespective of background. This indicates that *Acer* in females is required for the normal response of light sleep to nutrition.

The 24 hour analysis revealed that the differences between controls and $Acer^{\Delta}$ females in both backgrounds was diet related with the main differences in response to diet occurring at the transition times between the light and the dark. Genetic

differences between *Acer*$^{\Delta}$ females and their controls occurred throughout the 24 hour period suggesting that the differences in dietary response had a knock-on effect throughout the circadian pattern. This was clear in the w^{Dah} background with clearly increased sleep seen on both Low and FF food for *Acer*$^{\Delta}$ females while in the w^{1118} background reduced sleep for *Acer*$^{\Delta}$ females was seen on FF food in the dark.

Similarly to total sleep, w^{1118} control males showed no response of sleep in the dark and the light to changing diet. The lack of *Acer* had an effect on light sleep in the w^{1118} background with the *Acer*$^{\Delta}$ males seemingly unable to maintain the level of sleep on FF food. The 24 hour data further suggested that *Acer* is involved in the sleep response to nutrition with differences occurring in the transition hours between light and dark.

Circadian rhythms in both backgrounds and for both sexes were not affected by the lack of *Acer* as any observed difference would have been seen on all foods rather than on individual foods at particular time points.

The effect of the lack of *Acer* was predominately found to be either no response to diet when controls showed a dietary response or a dietary response when controls showed no reaction to changing diet. Many of the results showed a difference between the genotypes at low or high food levels in both backgrounds and for both sexes. This suggests that *Ace*r modulates the response of sleep to high and low diet by either promoting a dietary response or maintaining the level of sleep regardless of dietary change.

Sleep is complex and the data indicates that *Acer* is involved in the normal response of dark and light sleep to nutrition in a sex and genetic background dependent manner.

3.2.4: The effect of diet on sleep bout regulation is dependent on genetic background and sex. *Acer's* role in sleep bout regulation is sex and background dependent

The response of sleep bouts to diet varied with genetic background. The w^{Dah} background appeared to be more sensitive to dietary changes in terms of the number of bouts of sleep than the w^{1118} background. (Figure 29).

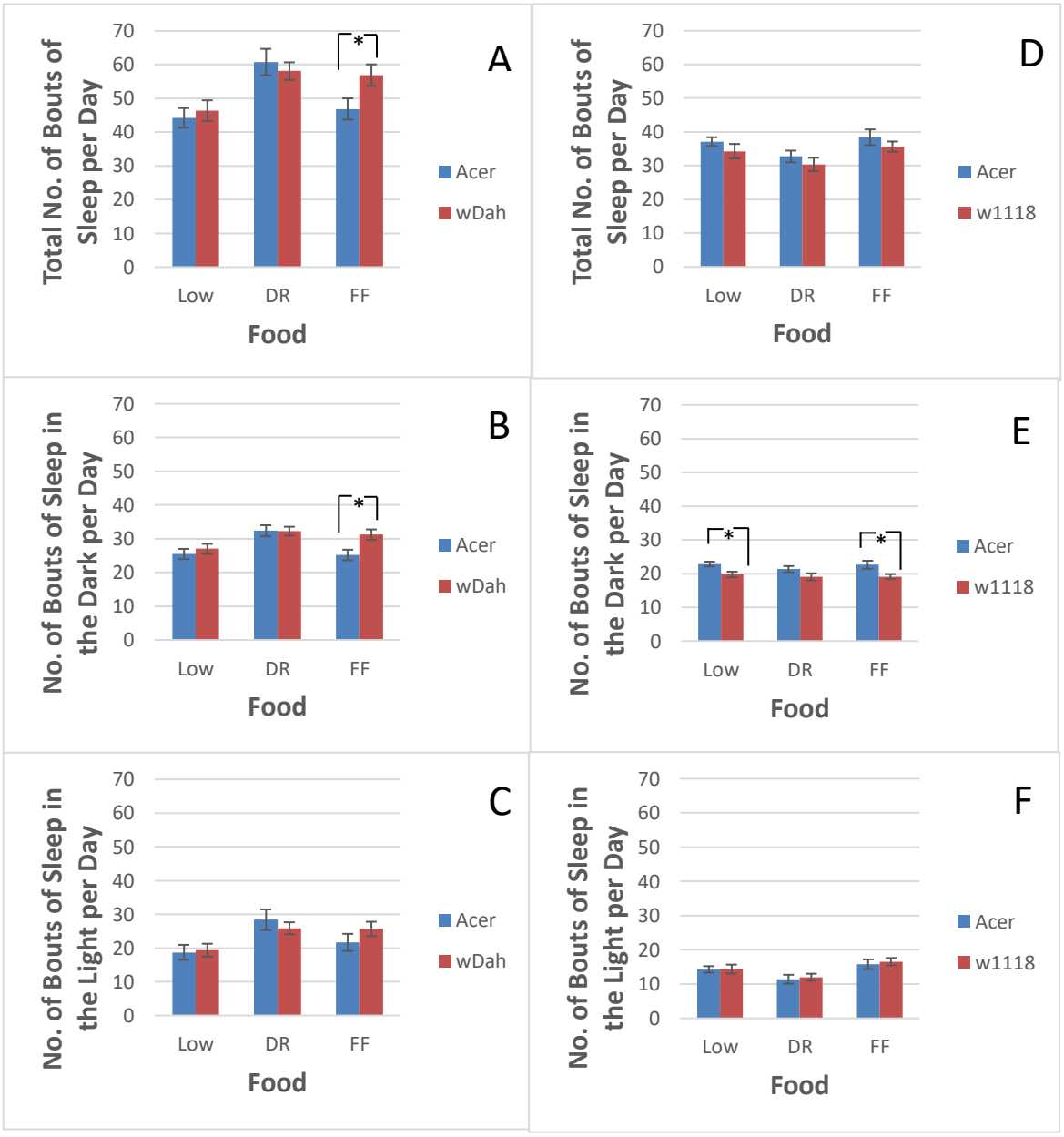

Figure 29: Female Total No. of Bouts of Sleep, No. of Bouts of Sleep in the Dark and the No. of Bouts of Sleep in the Light on Low, DR and FF diets. Total No. of Bouts per Day (A), No. of Bouts in the Dark per Day (B) and No. of Bouts in the Light per Day (C) in the w^{Dah} background. Total No. of Bouts per Day (D), No. of Bouts in the Dark per Day (E) No. of Bouts in the Light per Day (F) in the w^{1118}

background. (A) w^{Dah}: Low N = 18; DR N = 18; FF N = 17. $Acer^{\Delta}$: Low N = 13; DR N = 18; FF N = 15. (B) w^{Dah}: Low N = 18; DR N = 18; FF N = 17. $Acer^{\Delta}$: Low N = 13; DR N = 18; FF N = 15. (C) w^{Dah}: Low N = 18; DR N = 18; FF N = 17. $Acer^{\Delta}$: Low N = 13; DR N = 18; FF N = 15. (D) w^{1118}: Low N = 22; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 24; DR N = 24; FF N = 23. (D) w^{1118}: Low N = 23; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 24; DR N = 24; FF N = 24. (E) w^{1118}: Low N = 22; DR N = 22; FF N = 24. $Acer^{\Delta}$: Low N = 24; DR N = 24; FF N = 24. (F) w^{1118}: Low N = 22; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 24; DR N = 24; FF N = 24. Data was checked for normality and data was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 18 and 19. *Indicates significant difference between genotypes (P=<0.05).

Control females in the w^{Dah} background (Figures 29A, 29B and 29C and Table 18) showed a significant increase in total bouts of sleep between Low food and both the DR and FF foods. There was no difference in the number of bouts between DR and FF food. Bouts of sleep in the dark showed the same pattern as total bouts while in the light there was no difference between Low food and the DR and FF foods. However, the statistics showed a that the increase in bouts from Low food to the DR and FF foods was very close to being significant (Table 18), therefore suggesting that both light and dark sleep in the w^{Dah} background respond to increased food content by increasing the number of bouts of sleep, but that this is more prominent in the dark than the light.

Genotype	Food Comparison	p-value for Total No. of Bouts	p-value for No. of Bouts in the Dark	p-value for No. of Bouts in the Light
w^{Dah}	Low-DR	0.0174*	0.0338*	0.0534
	Low-FF	0.0395*	0.1112	0.0671
	DR-FF	0.9557	0.8744	0.9975
$w^{Dah};Acer^{\Delta}$	Low-DR	0.0053*	0.0114*	0.0469*
	Low-FF	0.8687	0.9912	0.7548
	DR-FF	0.016*	0.0056*	0.1885
w^{1118}	Low-DR	0.3195	0.8456	0.3164
	Low-FF	0.8672	0.872	0.3822
	DR-FF	0.1254	0.9988	0.0163*
$w^{1118};Acer^{\Delta}$	Low-DR	0.2164	0.5068	0.2377
	Low-FF	0.8732	0.9872	0.6712
	DR-FF	0.0807	0.6018	0.0399*

Table 18: Female Tukey-Kramer HSD comparisons of means for No. of Bouts of Sleep in the Dark and Light for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

In the w^{1118} background (Figure 29D, 29E and 29F and Table 18) female controls showed no significant change in the total number of bouts or the number of bouts in the dark with increasing food, however in the light a significant increase in bouts was observed between DR and FF food.

Genotype	Food	Comparison to $Acer^{\Delta}$ for Total No. of Bouts	Comparison to $Acer^{\Delta}$ for No. of Bouts in the Dark	Comparison to $Acer^{\Delta}$ for No. of Bouts in the Light
w^{Dah}	Low	0.6249	0.4843	0.8319
	DR	0.5748	0.9476	0.4818
	FF	0.0328*	0.0103*	0.2333
w^{1118}	Low	0.2615	0.0071*	0.9528
	DR	0.3713	0.1124	0.7296
	FF	0.3243	0.0161*	0.6777

Table 19: Female ANOVA/Pooled t-test measure of variance of genetic effects on different diets for No. of Bouts of Sleep in the Dark and No. of Bouts of Sleep in the Light. *Indicates significant differences (P=<0.05).

$Acer^{\Delta}$ females in the w^{Dah} background showed a similar increase in total bouts between Low and DR food as controls but showed a significant decrease in the number of bouts on FF food returning to a similar number of bouts observed on Low food. This was also the case in the dark. In the light, $Acer^{\Delta}$ females responded similarly to controls with a significant increase in bouts between Low and FF food and although a slight decrease in bouts was seen between DR and FF food, it was not significant. $Acer^{\Delta}$ females showed significantly fewer bouts of sleep compared to controls on only FF food for both total bouts and bouts in the dark (Table 19).These results suggest that the phenotype of an overall decrease in bouts for $Acer^{\Delta}$ females on FF food was mainly affected by the decrease in bouts seen on FF food in the dark.

In the w^{1118} background, $Acer^{\Delta}$ females, like controls, showed no response to diet in total bouts of sleep. In the dark, like controls they showed no response to dietary changes and in the light showed the same increase in the number of bouts between DR and FF food as controls. Despite this, $Acer^{\Delta}$ females showed more bouts of sleep on Low and FF food in the dark when compared to controls, however, this difference was not large enough to affect the total bouts.

These data suggest that *Acer* modulates sleep bouts in the dark in both backgrounds for female flies.

The male results showed that controls in the w^{Dah} background significantly decreased the number of bouts of sleep overall on FF food compared to Low and DR food (Figure 30A and Table 14). This pattern was observed in the dark with fewer bouts of FF food and in the light, controls showed a trend towards decreased bouts on FF food but it wasn't significant (Figures 30B, 30C and Table 20). In the w^{1118} background control males showed no significant response of the number of bouts of sleep in total or in the dark or the light with dietary changes (Figures 30D. 30E, 30F and Table 20).

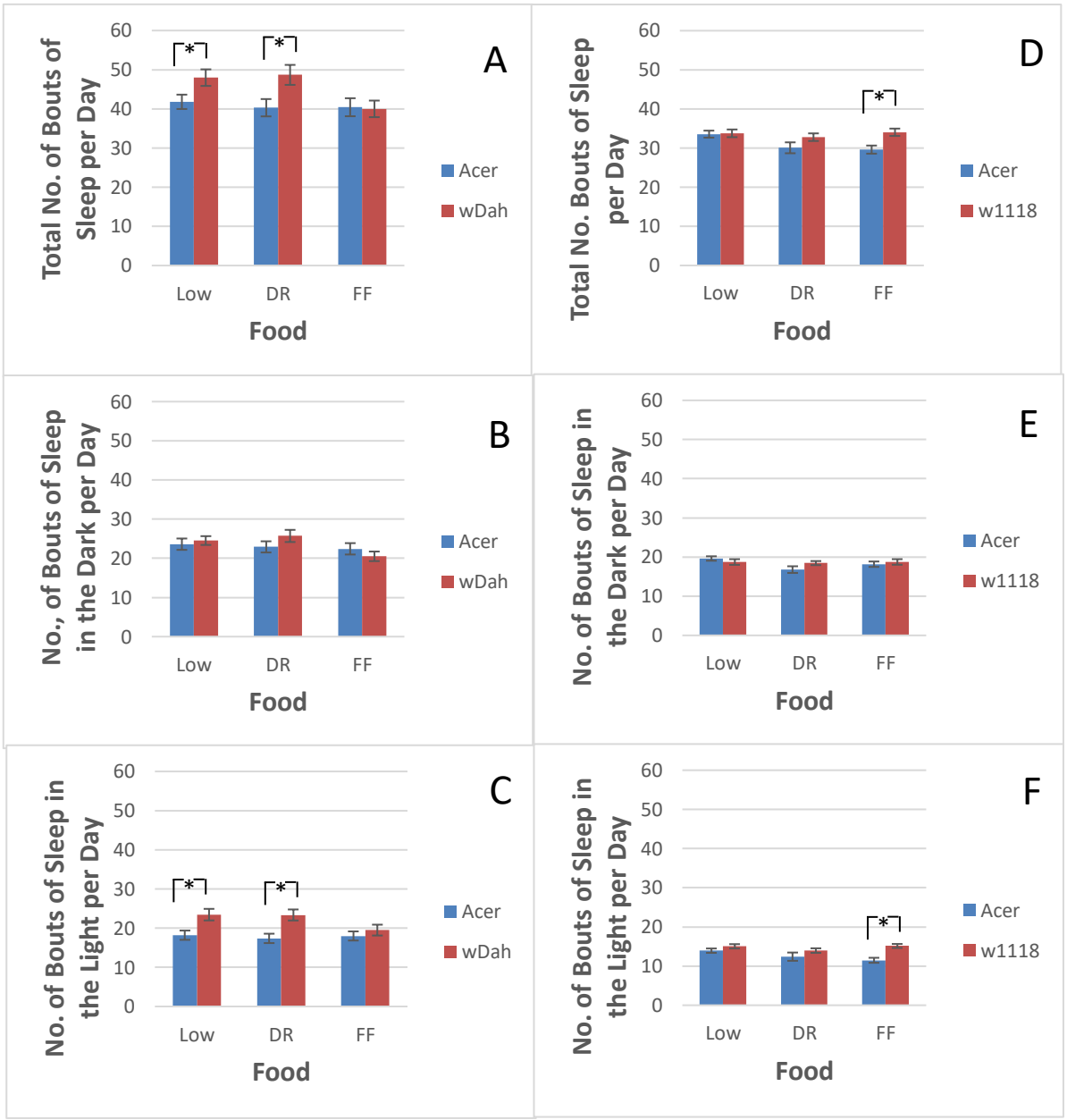

Figure 30: Male No. of Bouts of Sleep in the Dark and No. of Bouts of Sleep in the Light on Low, DR and FF diets. Total No. of Bouts per Day (A), No. of Bouts in the Dark per Day (B) and No. of Bouts in

the Light per Day (C) in the w^{Dah} background. Total No. of Bouts per Day (D), No. of Bouts in the Dark per Day (E) No. of Bouts in the Light per Day (F) in the w^{1118} background. (A) w^{Dah}: Low N = 18; DR N = 17; FF N = 18. $Acer^{\Delta}$: Low N = 15; DR N = 17; FF N = 18. (B) w^{Dah}: Low N = 18; DR N = 18; FF N = 18. $Acer^{\Delta}$: Low N = 15; DR N = 17; FF N = 18. (C) w^{Dah}: Low N = 18; DR N = 17; FF N = 18. $Acer^{\Delta}$: Low N = 15; DR N = 17; FF N = 18. (D) w^{1118}: Low N = 22; DR N = 23; FF N= = 23. $Acer^{\Delta}$: Low N = 23; DR N = 21; FF N = 22. (E) w^{1118}: Low N = 22; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 23; DR N = 22; FF N = 23. (F) w^{1118}: Low N = 22; DR N = 22; FF N = 22. $Acer^{\Delta}$: Low N = 23; DR N = 20; FF N = 21. Data was checked for normality and data was found to be normally distributed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 20 and 21. *Indicates significant difference between genotypes (P=<0.05).

$Acer^{\Delta}$ males in the w^{Dah} background, unlike controls, showed no significant response of the number of sleep bouts in total or in the dark and light to dietary changes (Figures 30A, 30B, 30C and Table 20). This led to significantly fewer sleep bouts on Low and DR food and similarly in the light (Figures 30A, 30C and Table 21). In the dark, like controls, $Acer^{\Delta}$ males showed no difference in the number bouts with changing diet and therefore no differences were seen between the genotypes (Figure 30B and Table 21).

Genotype	Food Comparison	p-value for Total No. of Bouts	p-value for No. of Bouts in the Dark	p-value for No. of Bouts in the Light
w^{Dah}	Low-DR	0.9738	0.8004	0.9991
	Low-FF	0.0392*	0.0793	0.1333
	DR-FF	0.025*	0.0187*	0.1445
$w^{Dah};Acer^{\Delta}$	Low-DR	0.8802	0.9421	0.882
	Low-FF	0.8953	0.8349	0.9923
	DR-FF	0.9991	0.9667	0.9243
w^{1118}	Low-DR	0.7513	0.9556	0.3461
	Low-FF	0.9787	0.9997	0.9924
	DR-FF	0.6234	0.9458	0.2848
$w^{1118};Acer^{\Delta}$	Low-DR	0.0756	0.0171*	0.2018
	Low-FF	0.0356*	0.3133	0.018*
	DR-FF	0.9554	0.3609	0.5673

Table 20: Male Tukey-Kramer HSD comparisons of means for No. of Bouts of Sleep in the Dark and No. of Bouts of Sleep in the Light for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

In the w^{1118} background, $Acer^{\Delta}$ males, unlike controls, showed a response of sleep bouts to dietary change (Figure 30D, 30E, 30F and Table 20). $Acer^{\Delta}$ males showed a significant decrease in bouts between Low and FF food for total bouts and bouts in the light. For bouts in the dark the decrease was seen between Low and DR food Table 20). This led to fewer sleep bouts for $Acer^{\Delta}$ males on FF food in total bouts and bouts in the light. There was no difference between the genotypes for sleep bouts in the dark (Table 21). This suggests that in the w^{1118} background *Acer* modulates the response of sleep bouts to high food levels.

Genotype	Food	Comparison to $Acer^{\Delta}$ for Total No. of Bouts	Comparison to $Acer^{\Delta}$ for No. of Bouts in the Dark	Comparison to $Acer^{\Delta}$ for No. of Bouts in the Light
w^{Dah}	Low	0.0388*	0.6108	0.0114*
	DR	0.0185*	0.1939	0.0029
	FF	0.8947	0.3193	0.4123
w^{1118}	Low	0.8763	0.3295	0.1641
	DR	0.1134	0.0997	0.09818
	FF	0.0028*	0.5644	<.0001*

Table 21: Male ANOVA/Pooled t-test measure of variance of genetic effects on different diets for No. of Bouts of Sleep in the Dark and No. of Bouts of Sleep in the Light. *Indicates significant differences (P=<0.05).

These data suggest that in males *Acer* is involved in the normal response of sleep bouts to nutrition in both backgrounds in light sleep specifically.

3.2.5: *Acer* is involved in the regulation of mean bout length in females but not in males

The results showed that female controls in the w^{Dah} background (Figures 31A and 31B and Table 22) did not respond to changing diet in terms of mean bout length in the dark but did show a response to diet in the light with bout length significantly increasing on FF food.

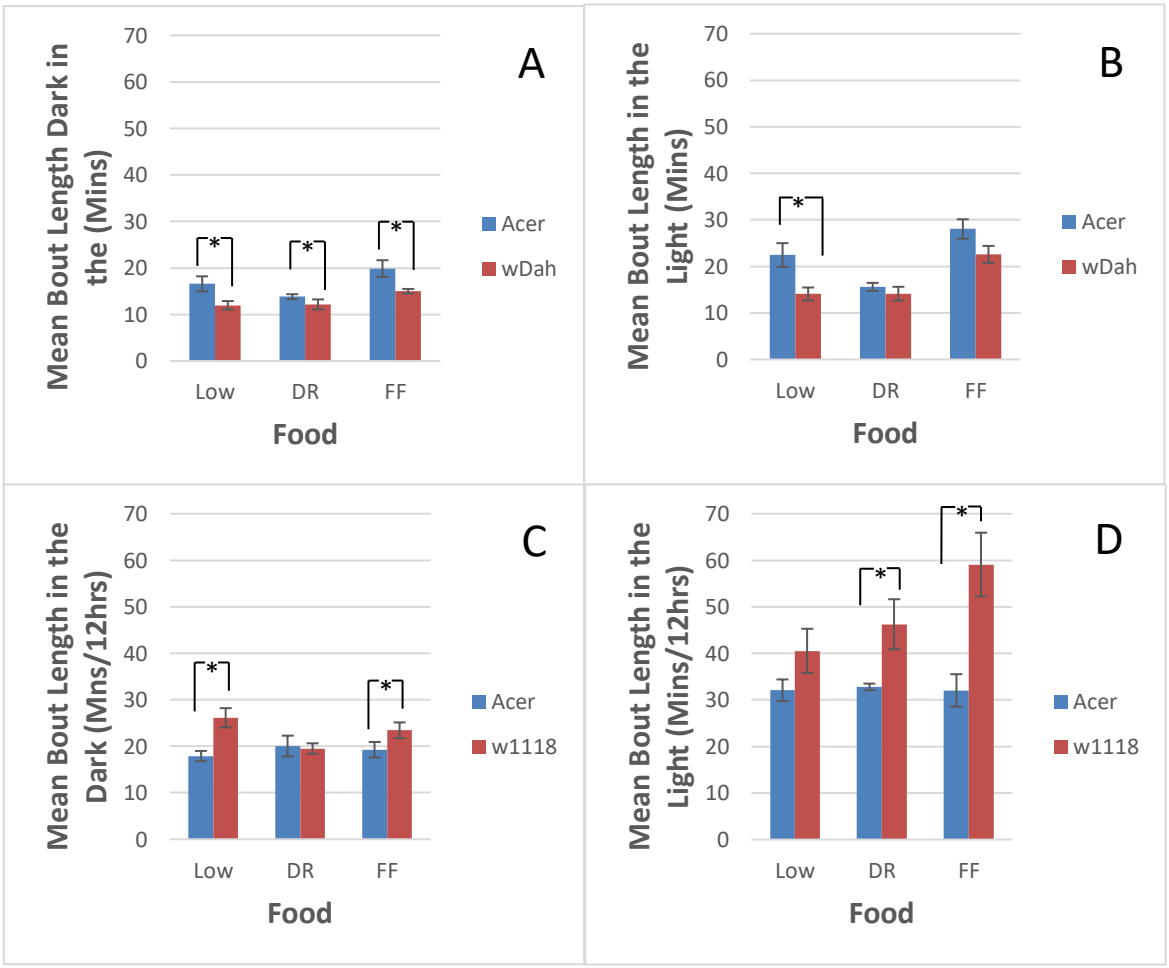

Figure 31: Female Mean Bout Length in the Dark (Mins/12hrs) and Mean Bout Length in the Light (Mins/12hrs) on Low, DR and FF diets. Mean Bout Length in the Dark (A) and Mean Bout Length in the Light (B) in the w^{Dah} background. Mean Bout Length in the Dark (C) and Mean Bout Length in the Light (D) in the w^{1118} background. (A) w^{Dah}: Low N = 17; DR N = 18; and FF N = 18. $Acer^{\Delta}$: Low N = 12; DR N = 18; FF N = 14. (B) w^{Dah}: N = 18 for all foods. $Acer^{\Delta}$: Low N = 12; DR N = 18; FF N = 13. (C) w^{1118}: Low N = 23; DR N = 22; FF N = 22. $Acer^{\Delta}$: Low N = 24; DR N = 23; FF N = 23. (D) w^{1118}: Low N = 21; DR N = 23; FF N = 23. . $Acer^{\Delta}$: Low N = 24; DR N = 23; FF N = 23. Data was checked for normality and data was found to be log distributed. Data is shown as raw data and analysed as log-transformed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and

variance statistics see Tables 22 and 23. *Indicates significant difference between genotypes (P=<0.05).

In the w^{1118} background (Figures 31C and 31D and Table 22), female controls showed a significant decrease in bout length in the dark between Low and DR food and increased bout length, though not significant, on FF food. In the light controls showed a trend of increased sleep with increasing diet which nears significance between the Low and FF foods.

Genotype	Food Comparison	p-value for Mean Bout Length in the Dark	p-value for Mean Bout Length in the Light
w^{Dah}	Low-DR	0.9912	0.9947
	Low-FF	0.114	0.0006*
	DR-FF	0.1386	0.0008*
$w^{Dah};Acer^{\Delta}$	Low-DR	0.3658	0.007*
	Low-FF	0.2686	0.0923
	DR-FF	0.008*	<.0001*
w^{1118}	Low-DR	0.0221*	0.7829
	Low-FF	0.5904	0.0994
	DR-FF	0.2036	0.3177
$w^{1118};Acer^{\Delta}$	Low-DR	0.9405	0.962
	Low-FF	0.9466	0.8861
	DR-FF	0.9998	0.9779

Table 22: Female Tukey-Kramer HSD comparisons of means for Mean Bout Length in the Dark and Mean Bout Length in the Light for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

Genotype	Food	Comparison to $Acer^{\Delta}$ for Mean Bout Length in the Dark	Comparison to $Acer^{\Delta}$ for Mean Bout Length in the Light
w^{Dah}	Low	0.0122*	0.0015*
	DR	0.0494*	0.1836
	FF	0.0304*	0.0506
w^{1118}	Low	0.0006*	0.1102
	DR	0.6641	0.032*
	FF	0.0447*	0.0007*

Table 23: Female comparison ANOVA/Pooled t-test measure of variance of genetic effects on different foods for Mean Bout Length in the Dark and Mean Bout Length in the Light. *Indicates significant differences (P=<0.05).

Acer$^{\Delta}$ females in the *w*^{Dah} background showed an increased mean bout length of sleep on all foods in the dark. As mean bout length in the dark appears not to be sensitive to nutrition it is possible that *Acer* is involved in mean bout length regulation in the dark independently of diet in *w*^{Dah} females. In the light, *Acer*$^{\Delta}$ females showed an initial increase in bout length on Low food, which was significantly longer than controls, before returning to the control phenotype for DR and FF food, suggesting that in the light *Acer* may play a role in regulating bout length on Low food. In the *w*^{1118} background, *Acer*$^{\Delta}$ females did not respond to changing diet in the dark or in the light resulting in a decrease in sleep compared to controls in the dark on Low and FF food and in the light on DR and FF food. These data suggest that *Acer* is involved in the normal response of sleep bout length to nutrition in the *w*^{1118} background (Table 23).

The male data showed that mean bout length in the dark and the light in both backgrounds in male flies is not responsive to nutrition (Figure 32 and Table 25).

Acer$^{\Delta}$ males in the *w*^{1118} background responded normally when compared with controls but in the *w*^{Dah} background *Acer*$^{\Delta}$ males showed a trend towards longer bouts in the light and the dark compared to controls although the result is not significant (Table 24).

Genotype	Food	Comparison to *Acer*$^{\Delta}$ for Mean Bout Length in the Dark	Comparison to *Acer*$^{\Delta}$ for Mean Bout Length in the Light
w^{Dah}	Low	0.0601	0.2497
	DR	0.1526	0.0585
	FF	0.0624	0.2512
w^{1118}	Low	0.6655	0.8789
	DR	0.935	0.2234
	FF	0.8664	0.7814

Table 24: Male comparison ANOVA/Pooled t-test measure of variance of genetic effects on different foods for Mean Bout Length in the Dark and Mean Bout Length in the Light. *Indicates significant differences (P=<0.05).

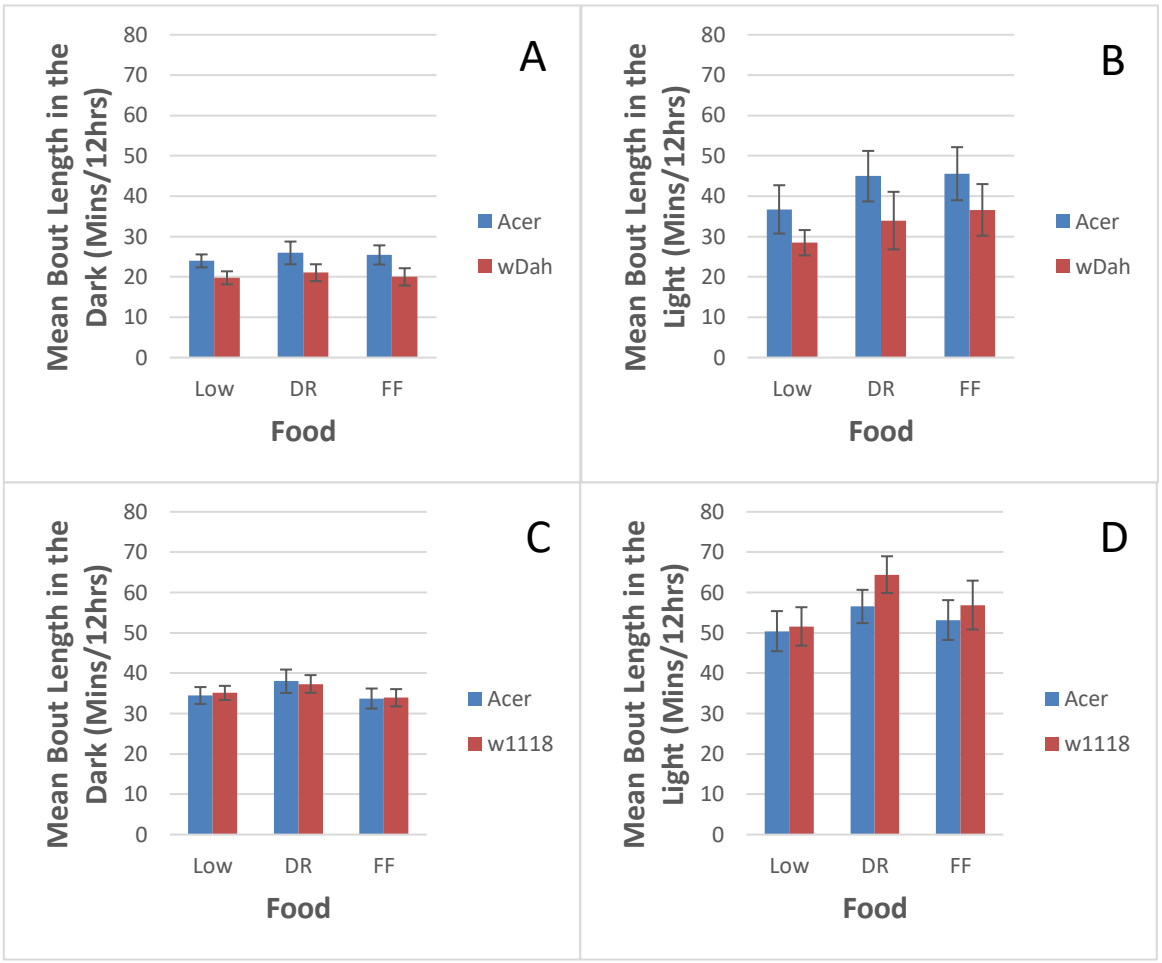

Figure 32: Male Mean Bout Length in the Dark (Mins/12hrs) and Mean Bout Length in the Light (Mins/12hrs) on Low, DR and FF diets. Mean Bout Length in the Dark (A) and Mean Bout Length in the Light (B) in the w^{Dah} background. Mean Bout Length in the Dark (C) and Mean Bout Length in the Light (D) in the w^{1118} background. (A) w^{Dah}: N = 18 for all foods. $Acer^{\Delta}$: Low N = 15; DR N = 18; FF N = 18. (B) w^{Dah}: Low N = 18; DR N = 18; FF N = 17. $Acer^{\Delta}$: Low N = 15; DR N = 17; FF N = 16. (C) w^{1118}: Low N = 21; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 22; DR N = 21; FF N = 22. (D) w^{1118}: Low N = 22; DR N = 23; FF N = 24. $Acer^{\Delta}$: Low N = 23; DR N = 19; FF N = 22. Data was checked for normality and data was found to be log distributed. Data is shown as raw data and analysed as log-transformed. Outliers and the data of dead flies were removed from the analysis. For mean comparison and variance statistics see Tables 24 and 25. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	p-value for Mean Bout Length in the Dark	p-value for Mean Bout Length in the Light
w^{Dah}	Low-DR	0.9428	0.9674
	Low-FF	0.9975	0.6986
	DR-FF	0.9181	0.8375
$w^{Dah};Acer^{\Delta}$	Low-DR	0.9825	0.5062
	Low-FF	0.9861	0.5701
	DR-FF	0.9998	0.9955
w^{1118}	Low-DR	0.8359	0.1081
	Low-FF	0.7733	0.8462
	DR-FF	0.4049	0.2795
$w^{1118};Acer^{\Delta}$	Low-DR	0.7554	0.4641
	Low-FF	0.9276	0.9043
	DR-FF	0.5297	0.7228

Table 25: Male Tukey-Kramer HSD comparisons of means for Mean Bout Length in the Dark and Mean Bout Length in the Light for controls and *Acer*$^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

These data suggest that *Acer* is involved in the regulation of bout length in females in both the dark and the light with the effect being dependent on genetic background. In males *Acer* is not involved in regulating bout length in the w^{1118} background however *Acer* is potentially involved in the regulation of bout length in the w^{Dah} background as *Acer*$^{\Delta}$ males showed a trend towards longer bouts in the light and dark independent of diet.

3.3: Discussion

The data presented here showed that sleep and its modulation by nutrition in *Drosophila* are dependent on genetic background with many differences being observed between the equatorial w^{Dah} background and the temperate w^{1118} background. This is in agreement with other data showing genetic and geographic variation in sleep (Svetec, et al., 2015). In this study, several sleep phenotypes were found to be responsive to dietary changes while some phenotypes did not respond to dietary change at all. The effect of phenotypes in the light and dark appears to be variable suggesting that day and night sleep are regulated separately, however *Acer* appears to be involved in both day and night time sleep. Insulin mutant females with the median-neurosecretory cells (mNSCs) of the brain ablated to reduce IIS showed reduced sleep on Low food but only at night also suggesting that day and night sleep are regulated separately and that the mNSCs may be involved in regulating night time sleep (Broughton, et al., 2010).

The circadian patterns of sleep were affected by the lack of *Acer* on specific foods only rather than across all foods, therefore *Acer*'s role in the circadian pattern was diet related rather than directly part of the circadian pattern itself. *Acer* is not involved in the regulation of the circadian pattern but is involved in the response of the circadian pattern to dietary changes. Many of the differences in the response to diet were found in the transition hours between light and dark, suggesting that *Acer* is involved in modulating the dietary response between light and dark hours in the 24 hour cycle. This study also showed a sex difference in sleep in mated flies with females responding to dietary changes for most phenotypes and little or no response to dietary changes for males in both backgrounds, suggesting that the sleep response to nutrition is regulated differently in male and female flies.

$Acer^{\Delta}$ females in both backgrounds showed a lack of response to dietary changes compared to controls in the sleep phenotypes in which controls responded to diet in both genetic backgrounds, namely total sleep and total sleep in the light. Sleep bout regulation also showed that the number of sleep bouts in females was disrupted in the dark with reduced bouts seen on FF food in the w^{Dah} background while increased bouts were seen on Low and FF food in the w^{1118} background. This suggests that *Acer* plays a role in the regulation of sleep bouts in the dark in female flies but the effect of the loss of *Acer* is background dependent. A potential role in the regulation of

mean bout length in females in the w^{Dah} background independent of diet was also observed. The lack of response to dietary changes in $Acer^{\Delta}$ females indicates that *Acer* is involved in the modulation of sleep in response to diet in female flies.

Male flies did not respond in terms of sleep and activity in the same way as females to dietary changes with only the number of sleep bouts responding to dietary change. $Acer^{\Delta}$ males showed no difference in response to sleep against w^{Dah} controls for total activity, total sleep and total sleep in the light and dark. However, $Acer^{\Delta}$ males showed a lack of response to diet in the number of total bouts and in the number of bouts in the light. $Acer^{\Delta}$ males, like females, also showed a trend of increased mean bout length independent of diet. In the w^{1118} background $Acer^{\Delta}$ males responded differently on FF food for total activity and total sleep as well as showing differences in total sleep in the dark and the light. $Acer^{\Delta}$ males showed reduced number of bouts overall as well as in the light on FF food but not in the dark. No difference was seen for mean bout length in the dark and light. These data highlight that although sleep in the genetic backgrounds responds differently to diet, *Acer* may still play a role in modulating male sleep but the loss of *Acer* has different effects in different genetic backgrounds. The loss of *Acer* affected the number of bouts of sleep in the light for male flies whereas in females this occurred in the dark, thus showing that sleep bouts are regulated differently in male and female flies.

Although the data in this study appears to contradict Carhan, et al. (2010) different food compositions means that a direct comparison between the experiments is difficult. Carhan et al. (2010) fed flies a 5% sugar medium which did not contain yeast and so is not comparable to Low, DR or FF food, which all contain yeast in addition to sugar. Our findings also contradict the findings of Catterson, et al. (2010) and Takahama, et al. (2012) who saw a reduction in sleep for controls on higher calorie food whereas we have found an increase in sleep and a decrease in activity for females with no significant difference for males. However, like Carhan et al. (2010), Catterson et al. (2010) used different diets with flies fed on a 5% sugar medium were compared to flies fed on a medium containing added 2% yeast. The phenotypes that were observed may be the difference between sleep on no yeast food compared to low yeast food whereas this experiment compared different levels of sugar and much higher concentrations of yeast.

The data presented here establish that *Acer* normally plays a role in the modulation of multiple sleep parameters in response to dietary changes. However, the

regulation of sleep by diet is complex, being sexually dimorphic and genetic background dependent. *Acer*'s role is also dependent on sex and background but the mechanism of its action is unknown. The experiment requires repetition to confirm these outcomes in both backgrounds and to produce a larger N.

Due to *Acer*'s likely involvement in dietary responses of sleep the following chapters will investigate: (1) the specificity of this role in the response to nutrition by analysing how the *Acer* deletion affects other phenotypes known to respond to changing diet (lipid and glycogen storage, ageing, fecundity and stress resistance); and (2) the mechanism by which *Acer* is involved in nutrient responses in a sex and background dependent manner by analysing how the *Acer* deletion potentially effects the nutrient sensing insulin/IGF-like signalling pathway and how *Acer* expression differs between the two genetic backgrounds.

Chapter 4: *Acer* modulates glycogen storage in response to high nutrient intake but is not required for lipid storage

4.1: Introduction

Like humans, *Drosophila melanogaster* store fat and carbohydrate as lipid and glycogen. Levels of sugar and yeast in the diet have been shown to be important in the storage of fats with higher levels of sugar being associated with higher lipid stores but with higher levels of yeast suppressing this phenotype, suggesting that protein suppresses triglyceride storage (Skorupa, et al., 2008).

These stores are found in the fly fat body (Xu, et al., 2008) and are regulated by the *Drosophila* homolog of human cAMP-responsive transcription factor (CREB) dCREB2 acting as an upstream control (Iijima, et al., 2009). Knocking down dCREB2 results in reduced glycogen and lipid stores and the blocking of CREB in the fly fat body resulted in reduced glycogen levels but increased lipid levels. Glycogen levels are thought to be regulated by a clock gene within the fly fat body. Clock genes maintain the cycles of gene expression within the tissue and glycogen levels have been found to be lower in flies with a disruption to the clock gene within the fly fat body but lipid levels were only slightly reduced (Xu, et al., 2008). Lipid levels are thought to be regulated by the transcription factor Mio which is required for the expression of lipogenic enzymes and lipid storage in the fat body and it is also thought to play a role in the consumption of food as a knockdown of Mio specifically in the fat body resulted in reduced feeding (Sassu, et al., 2012). Higher levels of lipid and glycogen have been found to be beneficial to starvation resistance (Ballard, et al., 2008; Xu, et al., 2008).

Flies have previously been found to increase feeding with increasing levels of yeast (Min & Tatar, 2006) and carbohydrates (Skorupa, et al., 2008). Flies subjected to dietary restriction were found not to compensate for the reduction of calories by increased feeding suggesting that increased lifespan was not due to increased feeding on DR food (Min & Tatar, 2006; Wong, et al., 2009).

Mutants with reduced levels of insulin/IGF-like signalling (IIS) due to the ablation of the DILP-producing mNSCs in the fly brain show higher levels of glycogen and lipid storage when compared to controls (Broughton, et al., 2010) but glycogen and lipid stores were not affected when solely DILP 2 was knocked-down (Broughton, et al., 2008).

Acer is expressed within the fat body of the fly (Carhan, et al., 2010), therefore *Acer* is potentially involved in the storage of nutrients in this tissue. In this study, levels of glycogen and lipid will be measured on different diets (Chapter 2.9 – Table 3) to assess their storage levels in $Acer^{\Delta}$ males and females compared to both w^{Dah} and w^{1118} controls.

4.1.1: Aims

Following the discovery of a role for *Acer* in the nutrient responsiveness of sleep, a potential role of *Acer* in glycogen and lipid metabolism in response to diet was investigated.

4.1.2: Research Design

The glycogen and lipid storage phenotypes of $Acer^{\Delta}$ males and females in the w^{Dah} and w^{1118} backgrounds were analysed under Starvation, Low, DR and FF conditions over two and four days feeding on the different foods. To determine if any effects on lipid and glycogen levels were due to differences in food consumption, feeding on these foods was additionally analysed.

The measurement of glycogen and lipid storage was adapted from Handel, (1965) with seven day old flies (n=10), which had been on the different foods for either 2 or 4 days prior to freezing, and their glycogen and lipid stores were measured as µg per mg of fly.

Wet weight was measured in mg before the flies were tested for glycogen and lipid levels (n=10).

Feeding was adapted from Wong, et al. (2009) and flies were fed on food stained with blue food dye (n=50) for 30 minutes after a starvation period of 90 minutes and flash-frozen before measuring the amount of blue dye ingested at 620nm.

4.2: Results

4.2.1: *Acer* is involved in the initial storage response of glycogen to high diet

The glycogen content of male and female $Acer^{\Delta}$ and control flies was measured after 2 or 4 days feeding on Starvation, Low. DR or FF foods.

Control females in both backgrounds (Figure 33, and Table 26) showed an increase in stored glycogen with increasing food concentration after two days feeding on the different foods. The decreased glycogen seen on the FF food for w^{Dah} females could be linked to an increase in egg-laying which occurs on the high yeast food (Chapter 5) (Skorupa, et al., 2008). However, the w^{1118} control females did not show the same decrease on FF food. After four days feeding controls in the w^{Dah} background showed a similar pattern to the two day storage on each food whereas controls in the w^{1118} background showed a plateau of glycogen storage on Low, DR and FF food.

$Acer^{\Delta}$ females in the w^{Dah} background showed an initial decrease in glycogen storage on DR and FF food after two days feeding but over four days this phenotype recovers to the level of the controls on both foods. However, after four days $Acer^{\Delta}$ females have higher glycogen stores on the Starvation food compared to controls (Figure 25B). In the w^{1118} background $Acer^{\Delta}$ females initially showed a decrease in glycogen storage on the FF food only and this phenotype continues after four days feeding. Unlike in the w^{Dah} background, $Acer^{\Delta}$ females in the w^{1118} background have the same amount of glycogen after Starvation as controls (Table 27).

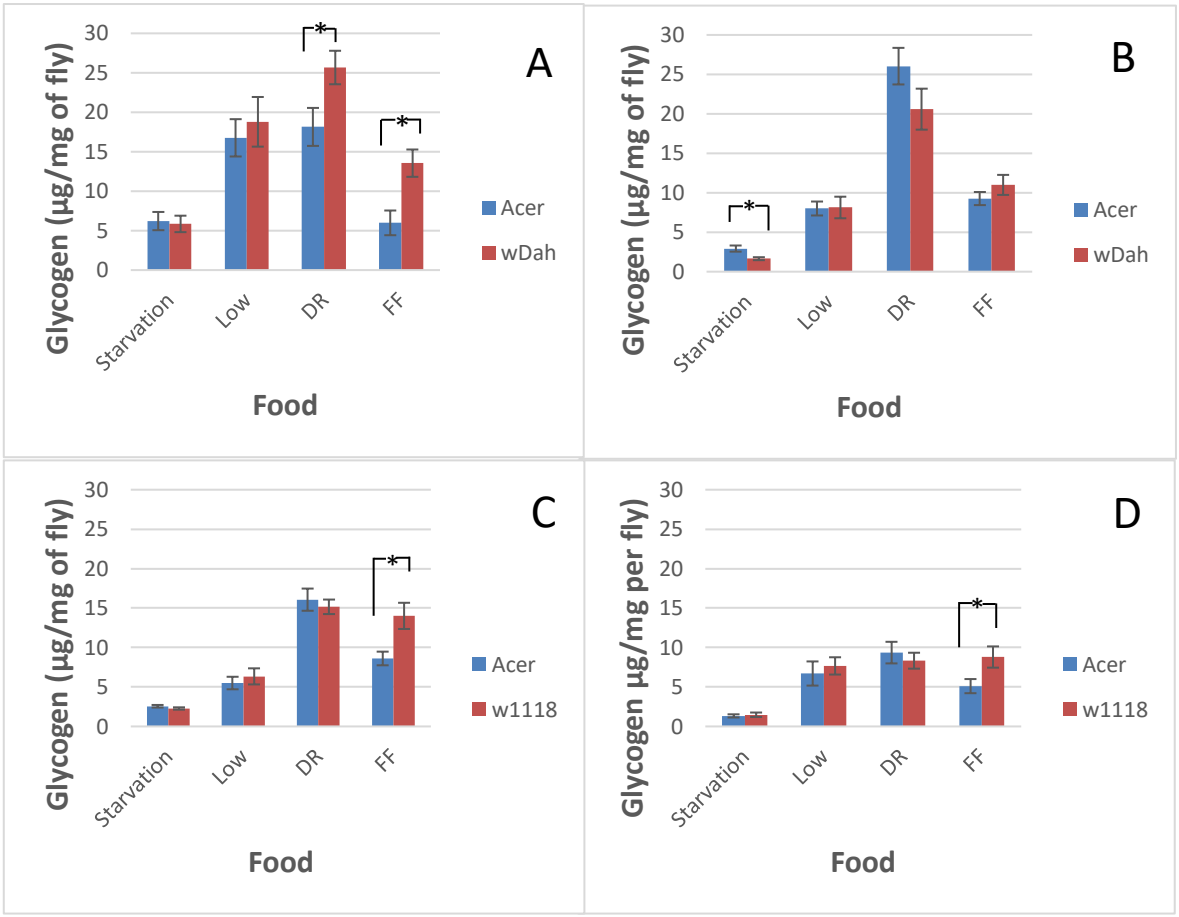

Figure 33: Female Glycogen Storage (µg/mg of fly) on Starvation, Low, DR and FF diets. (A and B) w^{Dah} background. (A) Glycogen levels after 2 days feeding. w^{Dah}: Starvation, Low and FF N = 10, DR N = 11. $Acer^{\Delta}$: DR N = 8; Starvation, Low and FF N = 10. (B) Glycogen levels after 4 days feeding. w^{Dah}: N = 10 for all foods. $Acer^{\Delta}$: FF N = 9; Starvation, Low and DR N = 10. (C and D) w^{1118} background. (C) Glycogen levels after 2 days feeding. w^{1118}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. (D) Glycogen levels after 4 days feeding. w^{1118}: Low N = 9; Starvation, DR and FF N = 10. $Acer^{\Delta}$: DR N = 9; FF N = 8; Starvation and Low N = 10. Data was checked for normality. (A, C and D) were found to be normally distributed. (B) was found to be log distributed. Data is shown as raw data and analysed as log-transformed. For mean comparison and variance statistics see Tables 26, 27 and 28. *Indicates significant difference between genotypes (P=<0.05).

Female controls in both backgrounds showed a difference in glycogen storage levels between two and four days of feeding on the different foods (Table 26). Control females in the w^{Dah} background showed a reduction in glycogen storage levels after four days feeding compared to two days feeding on the Starvation and Low foods, suggesting that glycogen stores were being used to combat the lack of food. Although a reduction in glycogen storage is seen on the DR and FF foods after four days feeding

the reduction is not significant. *Acer*$^{\Delta}$ females in the *w*^{Dah} background also showed a significant reduction in glycogen storage levels on Starvation and Low food but the reduction on Starvation food was not as strong as controls leading to the increased storage phenotype for *Acer*$^{\Delta}$ females on this food after four days feeding (Figure 33B). Conversely, on the DR and FF foods *Acer*$^{\Delta}$ females showed a significant increase in glycogen storage levels suggesting that the reduced phenotype seen on these foods after two days feeding may be due to a slower accumulation process caused by the lack of *Acer*.

Genotype	Food Comparison	p-value for Glycogen 2 Day Experiment	p-value for Glycogen 4 Day Experiment
w^{Dah}	Starvation-Low	0.0004*	<.0001*
	Starvation-DR	<.0001*	<.0001*
	Starvation-FF	0.0706	<.0001*
	Low-DR	0.1171	<.0001*
	Low-FF	0.3675	0.2557
	DR-FF	0.0007*	0.0162*
w^{Dah};*Acer*$^{\Delta}$	Starvation-Low	0.0002*	<.0001*
	Starvation-DR	<.0001*	<.0001*
	Starvation-FF	1	<.0001*
	Low-DR	0.9751	<.0001*
	Low-FF	0.0001*	0.7109
	DR-FF	<.0001	<.0001*
w^{1118}	Starvation-Low	0.0531	0.0008*
	Starvation-DR	<.0001*	0.0001*
	Starvation-FF	<.0001*	<.0001*
	Low-DR	<.0001*	0.9681
	Low-FF	<.0001*	0.8648
	DR-FF	0.8749	0.9875
w^{1118};*Acer*$^{\Delta}$	Starvation-Low	0.1263	0.0076*
	Starvation-DR	<.0001*	<.0001*
	Starvation-FF	0.0002*	0.1186
	Low-DR	<.0001*	0.3563
	Low-FF	0.0969	0.7645
	DR-FF	<.0001*	0.0738

Table 26: Female Tukey-Kramer HSD comparisons of means for glycogen storage levels after 2 or 4 days of feeding for controls and *Acer*$^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

Genotype	Food	p-value comparison to *Acer*$^{\Delta}$ for Glycogen 2 Day Experiment	p-value comparison to *Acer*$^{\Delta}$ for Glycogen 4 Day Experiment
w^{Dah}	Starvation	0.82	0.0042*
	Low	0.6124	0.8436
	DR	0.0118*	0.1333
	FF	<.0001*	0.3439
w^{1118}	Starvation	0.2154	0.6852
	Low	0.5228	0.6224
	DR	0.596	0.5488
	FF	0.0099*	0.0464*

Table 27: Female comparison ANOVA/Pooled t-test measure of variance of genetic effects on different foods for glycogen storage levels after 2 or 4 days feeding. *Indicates significant differences (P=<0.05).

In the *w*^{1118} background, control females showed a significant reduction in glycogen storage levels on all foods except the Low food (Table 28). *Acer*$^{\Delta}$ females in this background responded normally with the same decrease in glycogen storage for the same foods.

Genotype	Food	p-value comparing Glycogen storage levels between 2 and 4 days feeding
w^{Dah}	Starvation	0.0009*
	Low	0.0061*
	DR	0.1437
	FF	0.1064
w^{Dah};*Acer*$^{\Delta}$	Starvation	0.0149*
	Low	0.0027*
	DR	0.0118*
	FF	0.0051*
w^{1118}	Starvation	0.0227*
	Low	0.1679
	DR	<.0001*
	FF	0.0251*
w^{1118};*Acer*$^{\Delta}$	Starvation	0.0002*
	Low	0.6561
	DR	0.0034*
	FF	0.0132*

Table 28: Female comparison ANOVA/Pooled t-test measure of variance comparing glycogen storage levels after 2 and 4 days feeding on different diets for controls and *Acer*$^{\Delta}$. *Indicates significant differences (P=<0.05).

Therefore, in females *Acer* is involved in the normal response of glycogen metabolism to high yeast content in the diet as $Acer^{\Delta}$ females showed a reduced storage of glycogen on FF food in both genetic backgrounds after two days of feeding. A difference was also observed on the DR food on the w^{Dah} background with $Acer^{\Delta}$ females also showing reduced glycogen storage. The normal levels of glycogen after four days feeding on Low, DR and FF foods in $Acer^{\Delta}$ females in the w^{Dah} background only, suggests that *Acer* may be involved in an early response to altered diet, at least in this background.

Comparison of the genetic backgrounds revealed that w^{Dah} control females stored significantly more glycogen than w^{1118} control females after two days ($p=0.0004$) and four days ($p=0.0185$) feeding when the glycogen totals for all foods were averaged. Controls in the w^{Dah} background showed a significant reduction of glycogen between DR and FF foods but w^{1118} controls did not (Table 26). This was likely due to egg-laying as females have been found to lay more eggs on high yeast food but females in the w^{1118} background lay fewer eggs than females in the w^{Dah} background (Chapter 5.2.1).

Male controls in the w^{Dah} background (Figures 34A and 34B and Table 29) responded in the same way as females with increased glycogen stores as food levels increased over two days feeding. There was no decrease in storage on the FF food suggesting that the decrease seen in females is likely to be due to energy being used in egg-laying. After four days feeding the pattern continues with similar levels of glycogen being stored. In the w^{1118} background (Figures 34C and 34D), control males showed a steady increase of glycogen stores with increasing food which was also seen over four days. However, unlike males in the w^{Dah} background, levels of glycogen stores increased on the DR and FF food, suggesting a slower response of glycogen storage to diet.

Like females, $Acer^{\Delta}$ males in the w^{Dah} background (Figures 34A and 34B) showed an initial decrease in the storage of glycogen compared to controls on DR and FF food after two days feeding but levels were similar to the controls after four days feeding. In the w^{1118} background, $Acer^{\Delta}$ males responded normally to diet after two days feeding but after four days feeding $Acer^{\Delta}$ males did not increase glycogen stores as much as controls on the DR and FF foods (Figures 34C and 34D).

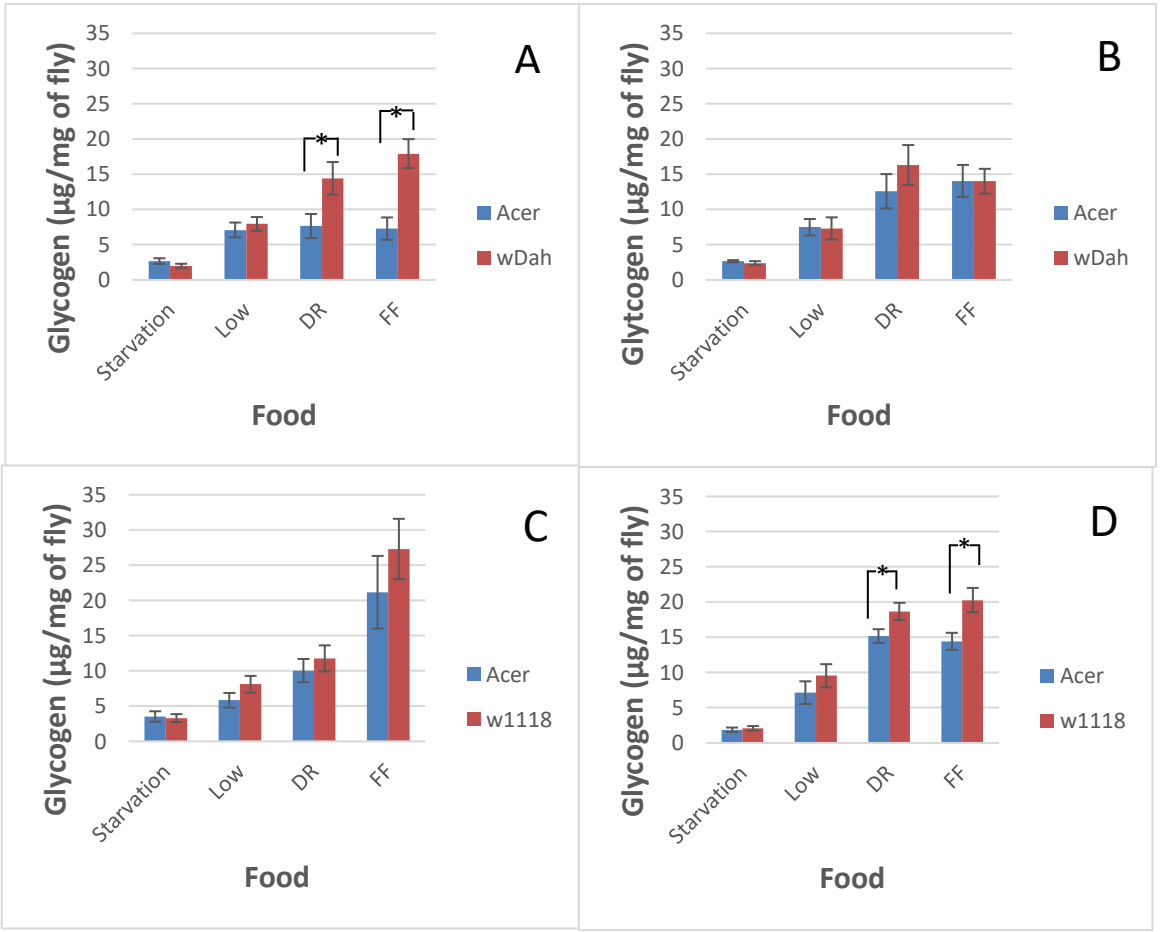

Figure 34: Male Glycogen Storage (µg/mg of fly) on Starvation, Low, DR and FF diets. (A and B) w^{Dah} background. (A) Glycogen levels after 2 days feeding. w^{Dah}: Starvation N = 12; Low N = 13; DR N = 9; FF N = 10. $Acer^{\Delta}$: Starvation N = 13; Low N = 13; DR N = 8; FF N = 7. (B) Glycogen levels after 4 days feeding. w^{Dah}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. (C and D) w^{1118} background. (C) Glycogen levels after 2 days feeding. w^{1118}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. (D) Glycogen levels after 4 days feeding. w^{1118}: DR N = 9; Starvation, Low and FF N = 10. $Acer^{\Delta}$: N = 10 for all foods. Data was checked for normality. (A and D) were found to be normally distributed. (B and C) were found to be log distributed. Data is shown as raw data and analysed as log-transformed. For mean comparison and variance statistics see Tables 29, 30 and 31. *Indicates significant difference between genotypes (P=<0.05).

Male controls in the w^{Dah} background showed no difference in glycogen storage levels between two days and four days feeding on the different foods. However, $Acer^{\Delta}$ males showed a significant increase in glycogen storage levels on FF food and an increase on DR food that was nearing significance (Table 31) similar to females.

Genotype	Food Comparison	p-value for Glycogen 2 Day Experiment	p-value for Glycogen 4 Day Experiment
w^{Dah}	Starvation-Low	0.0148*	0.0021*
	Starvation-DR	<.0001*	<.0001*
	Starvation-FF	<.0001*	<.0001*
	Low-DR	0.015*	0.0357*
	Low-FF	<.0001*	0.0525
	DR-FF	0.4517	0.9983
$w^{Dah};Acer^{\Delta}$	Starvation-Low	0.0019*	0.0004*
	Starvation-DR	0.0024*	<.0001*
	Starvation-FF	0.0085*	<.0001*
	Low-DR	0.9921	0.1842
	Low-FF	0.9999	0.0382*
	DR-FF	0.9992	0.8783
w^{1118}	Starvation-Low	0.0069*	<.0023*
	Starvation-DR	<.0001*	<.0001*
	Starvation-FF	<.0001*	<.0001*
	Low-DR	0.4154	0.0003*
	Low-FF	0.0002*	<.0001*
	DR-FF	0.0151*	0.8479
$w^{1118};Acer^{\Delta}$	Starvation-Low	0.1668	0.0111*
	Starvation-DR	0.0007*	<.0001*
	Starvation-FF	<.0001*	<.0001*
	Low-DR	0.1476	<.0001*
	Low-FF	0.0003*	0.0003*
	DR-FF	0.0997	0.9663

Table 29: Males Tukey-Kramer HSD comparisons of means for glycogen storage levels after 2 or 4 days of feeding for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

In the w^{1118} background, control males showed a reduction in glycogen storage on Starvation food that is nearing significance and a significant increase in glycogen stores on DR food (Table 31) after four days feeding. $Acer^{\Delta}$ males showed a similar pattern and responded normally.

Comparison of the genetic backgrounds showed no difference in glycogen storage levels between w^{Dah} and w^{1118} control males after two (p=0.209) and four days (p=0.2559) feeding when the glycogen totals for all foods were averaged. Males did not show the drop in glycogen storage between DR and FF food that was seen in females, suggesting that the reduction in glycogen on FF food in female flies was caused by egg-laying (Figure 25).

Genotype	Food	p-value comparison to *Acer*$^{\Delta}$ for Glycogen 2 Day Experiment	p-value comparison to *Acer*$^{\Delta}$ for Glycogen 4 Day Experiment
w^{Dah}	Starvation	0.207	0.2532
	Low	0.5571	0.8372
	DR	0.0125*	0.3886
	FF	0.0016*	0.8011
w^{1118}	Starvation	0.872	0.643
	Low	0.2467	0.3113
	DR	0.5111	0.0417*
	FF	0.2189	0.0127*

Table 30: Male comparison ANOVA/Pooled t-test measure of variance of genetic effects on different foods for glycogen storage levels after 2 or 4 days feeding. *Indicates significant differences (P=<0.05).

Genotype	Food	p-value comparing Glycogen storage levels between 2 and 4 days feeding
w^{Dah}	Starvation	0.419
	Low	0.7651
	DR	0.6047
	FF	0.1655
w^{Dah};*Acer*$^{\Delta}$	Starvation	0.9628
	Low	0.8087
	DR	0.0989
	FF	0.0394*
w^{1118}	Starvation	0.0751
	Low	0.4876
	DR	0.008*
	FF	0.1465
w^{1118};*Acer*$^{\Delta}$	Starvation	0.0538
	Low	0.5021
	DR	0.0152*
	FF	0.2195

Table 31: Male comparison ANOVA/Pooled t-test measure of variance comparing glycogen storage levels after 2 and 4 days feeding on different diets for controls and *Acer*$^{\Delta}$. *Indicates significant differences (P=<0.05).

Like females, the loss of *Acer* in male flies affects glycogen storage at higher food levels in both backgrounds. Interestingly, *Acer*$^{\Delta}$ males in the *w*^{Dah} background showed the same phenotype as *Acer*$^{\Delta}$ females in the same background with reduced glycogen storage on both DR and FF foods after two days of feeding. In the *w*^{1118} background, *Acer*$^{\Delta}$ males, unlike females, showed a reduced storage of glycogen on

both DR and FF food whereas females only showed the reduction on FF food. , *Acer*$^{\Delta}$ males also showed this response after four days feeding while females showed the response over both two and four days feeding, suggesting that *Acer*'s role in glycogen storage differs between the sexes.

Together, these data show that *Acer* is required to maximise glycogen storage and/or utilisation of glycogen, in response to high nutrient intake but the effect is dependent on genetic background.

4.2.2: *Acer* is not involved in lipid storage but potentially has a role in the utilisation of lipid in starvation conditions in w^{Dah} females.

The measurement of lipid storage was adapted from Van Handel, (1965) and was measured over two and four days on different diets.

Control females in the w^{Dah} background (Figures 35A and 35B and Table 32) did not significantly increase lipid levels after two days feeding on different foods but after four days feeding showed a significant increase in lipid storage between Starvation and DR food.

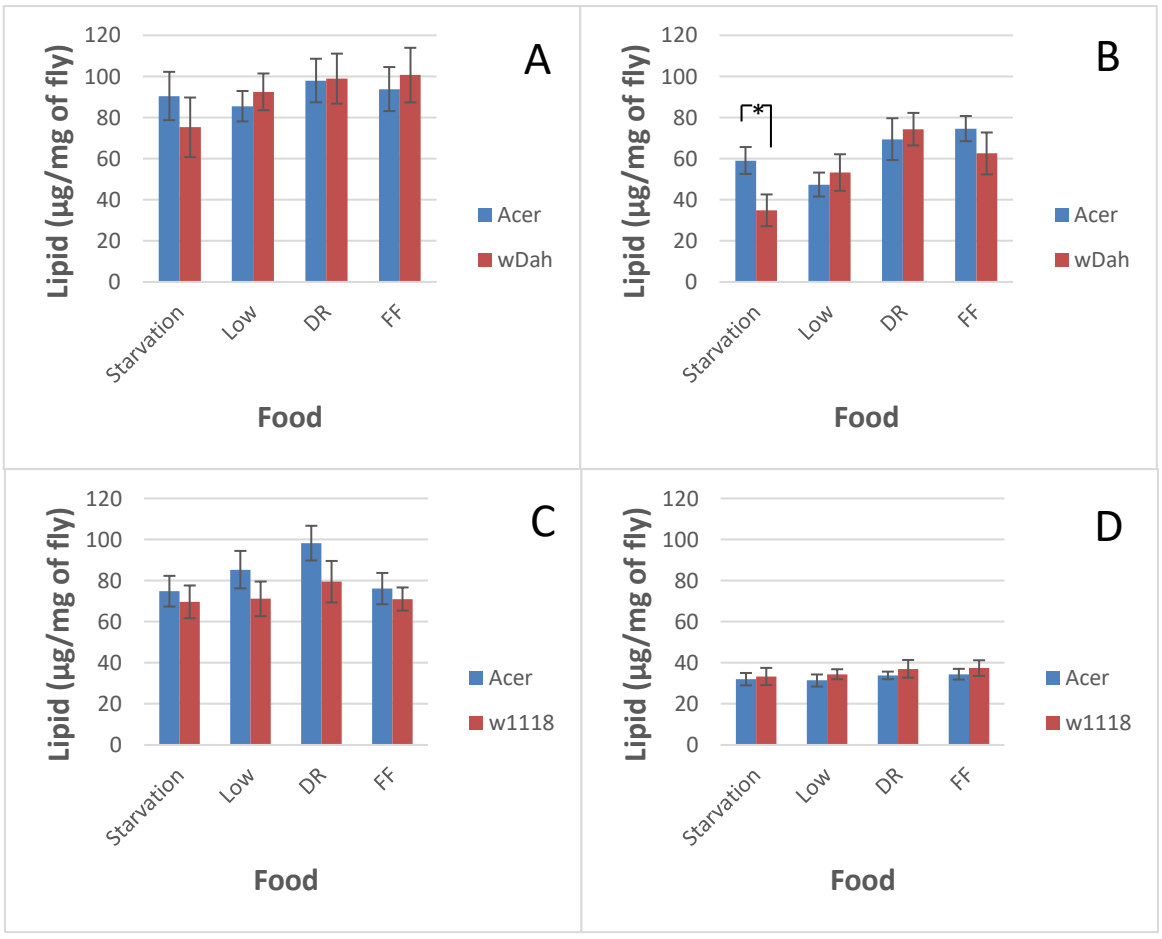

Figure 35: Female Lipid Storage (µg/mg of fly) on Starvation, Low, DR and FF diets. (A and B) Lipid levels w^{Dah} background. (A) Lipid levels after 2 days feeding. w^{Dah}: Starvation N = 9; Low N = 10; DR N = 9; FF N = 11. $Acer^{\Delta}$: FF N = 11; Starvation, Low and DR N = 10. (B) Lipid levels after 4 days feeding. w^{Dah}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. (C and D) Lipid levels in the w^{1118} background. (C) Lipid levels after 2 days feeding. w^{1118}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. (D) Lipid levels after and 4 days feeding. w^{1118}: DR N = 9; Starvation, Low and FF N = 10. $Acer^{\Delta}$: N = 10 for all foods. Data was checked for normality and was found to be normally distributed. For mean

comparison and variance statistics see Tables 32, 33 and 34. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	p-value for Lipid 2 Day Experiment	p-value for Lipid 4 Day Experiment
w^{Dah}	Starvation-Low	0.7724	0.4534
	Starvation-DR	0.533	0.0146*
	Starvation-FF	0.4158	0.1309
	Low-DR	0.9924	0.3344
	Low-FF	0.9778	0.8748
	DR-FF	1	0.7753
$w^{Dah};Acer^{\Delta}$	Starvation-Low	0.9977	0.9786
	Starvation-DR	0.9886	0.7532
	Starvation-FF	0.9995	0.4562
	Low-DR	0.9292	0.1671
	Low-FF	0.9819	0.0607
	DR-FF	0.9987	0.9606
w^{1118}	Starvation-Low	0.9993	0.9964
	Starvation-DR	0.8318	0.9967
	Starvation-FF	0.9994	0.8505
	Low-DR	0.888	1
	Low-FF	1	0.934
	DR-FF	0.8853	0.9385
$w^{1118};Acer^{\Delta}$	Starvation-Low	0.8019	0.9981
	Starvation-DR	0.2004	0.9602
	Starvation-FF	0.9995	0.9153
	Low-DR	0.6845	0.909
	Low-FF	0.8549	0.8435
	DR-FF	0.2419	0.9986

Table 32: Females Tukey-Kramer HSD comparisons of means for lipid storage levels after 2 and 4 days feeding for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant difference (p=<0.05).

In the w^{1118} background (Figures 35C and 35D and Table 32) controls, like w^{Dah} females, show no significant change of lipid levels with diet over two or four days.

$Acer^{\Delta}$ females (Figure 35) showed the normal response of lipid storage to diet in both backgrounds except $Acer^{\Delta}$ females stored higher amounts of lipid after four days feeding on Starvation food than w^{Dah} controls, suggesting an utilisation defect in response to starvation (Table 33).

Female controls in both backgrounds showed a significant decrease in lipid storage after four days feeding compared to two days feeding on all foods (Table 34).

Acer$^{\Delta}$ females also showed the same response in both backgrounds but not significantly on the DR and FF foods in the *w*^{Dah} background, however the reduction in lipid levels on these foods is approaching significance (Table 34).

Genotype	Food	p-value comparison to *Acer*$^{\Delta}$ for Lipid 2 Day Experiment	p-value comparison to *Acer*$^{\Delta}$ for Lipid 4 Day Experiment
w^{Dah}	Starvation	0.2771	0.0278*
	Low	0.5557	0.5868
	DR	0.9442	0.7108
	FF	0.6953	0.3238
w^{1118}	Starvation	0.6404	0.8015
	Low	0.267	0.4327
	DR	0.1713	0.8917
	FF	0.6001	0.5288

Table 33: Female ANOVA/Pooled t-test measure of variance of genetic effects on different foods for lipid storage levels after 2 or 4 days feeding. *Indicates significant differences (P=<0.05).

Genotype	Food	p-value comparing Lipid storage levels between 2 and 4 days feeding
w^{Dah}	Starvation	0.0008*
	Low	0.006*
	DR	0.0314*
	FF	0.0373*
w^{Dah}*;Acer*$^{\Delta}$	Starvation	0.0314*
	Low	0.0008*
	DR	0.068
	FF	0.1459
w^{1118}	Starvation	0.0008*
	Low	0.0006*
	DR	0.0009*
	FF	0.0001*
w^{1118}*;Acer*$^{\Delta}$	Starvation	<.0001*
	Low	<.0001*
	DR	<.0001*
	FF	<.0001*

Table 34: Female comparison ANOVA/Pooled t-test measure of variance comparing lipid storage levels after 2 and 4 days feeding on different diets for controls and *Acer*$^{\Delta}$. *Indicates significant differences (P=<0.05).

Comparison of the genetic backgrounds showed that w^{1118} control females stored on average less lipid after two (p=0.0029) and four days (p=<.0001) feeding compared to w^{Dah} control females.

In summary, lipid levels do not respond significantly to dietary changes over short-term feeding (2 to 4 days) for female flies but lipid levels are reduced after four days feeding in both genetic backgrounds, suggesting a utilisation effect after four days.

Unlike females, male controls in the w^{Dah} background (Figures 36A and 36B and Table 35) showed an increase in lipid storage with increasing diet after two days feeding on the different foods but the difference is negated after four days feeding as lipid levels showed no difference between the foods.

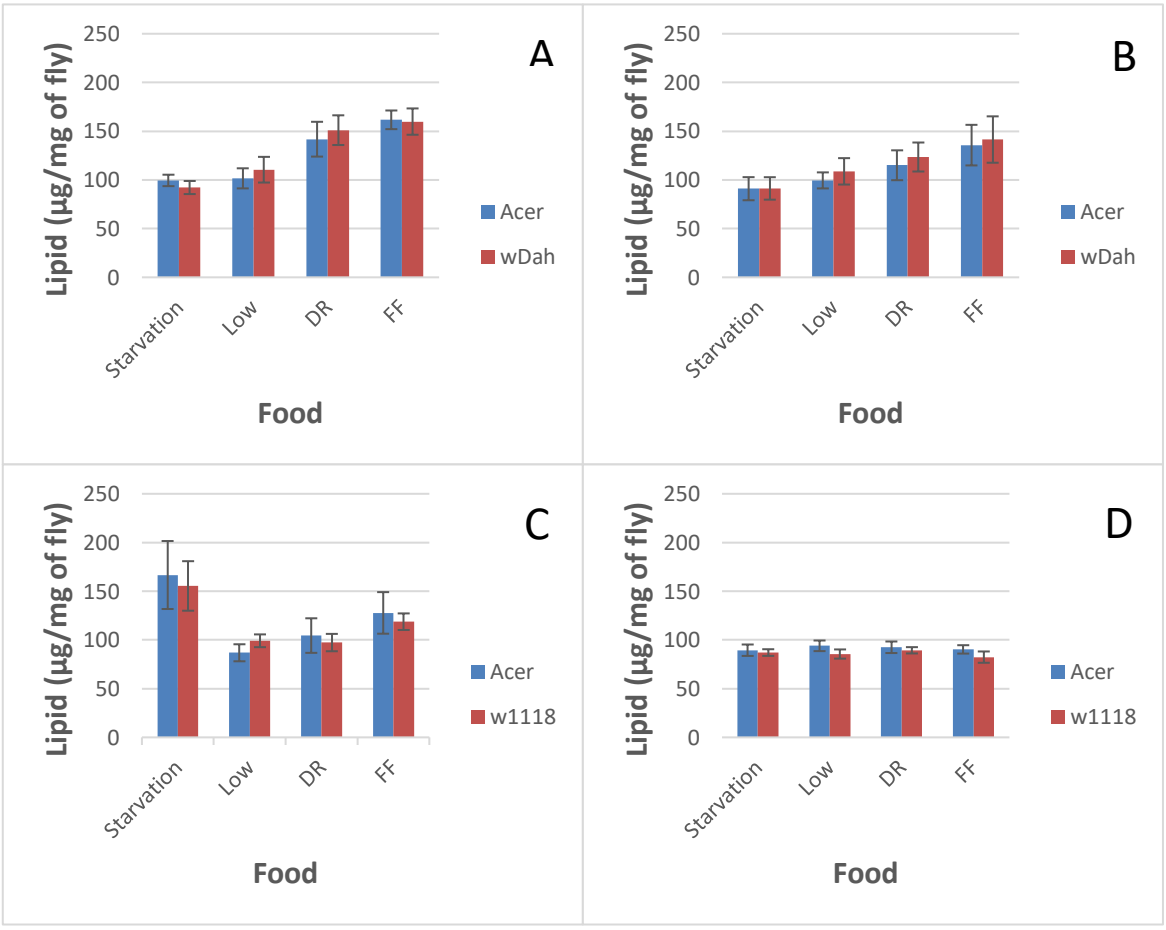

Figure 36: Male Lipid Storage (µg/mg of fly) on Starvation, Low, DR and FF diets. (A and B) Lipid levels in the w^{Dah} background. (A) Lipid level after 2 days feeding. w^{Dah}: Starvation N = 13; Low N = 12; DR N = 10; FF N = 10. $Acer^{\Delta}$: Starvation N = 13; Low N = 13; DR N = 9; FF N = 10. (B) Lipid levels after 4 days feeding. w^{Dah}: Starvation and Low N = 9; DR and FF N = 10. $Acer^{\Delta}$: DR N = 9; Starvation, Low and FF N = 10. (C and D) Lipid levels in the w^{1118} background. (C) Lipid level after 2 days feeding.

w^{1118}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. (D) Lipid levels after 4 days feeding. w^{1118}: N = 10 for all foods. $Acer^{\Delta}$: DR N = 9; Starvation, Low and FF N = 10. Data was checked for normality. (A and D) were found to be normally distributed. (B and C) were found to be log distributed. Data is shown as raw data and analysed as log-transformed. For mean comparison and variance statistics see Tables 35, 36 and 37. *Indicates significant difference between genotypes (P=<0.05).

In the w^{1118} background (Figures 36C and 36D and Table 35) control males did not respond to differing food levels in terms of lipid storage over two or four days but a reduction in storage levels was seen between two and four days.

Genotype	Food Comparison	p-value for Lipid 2 Day Experiment	p-value for Lipid 4 Day Experiment
w^{Dah}	Starvation-Low	0.6648	0.829
	Starvation-DR	0.0012*	0.4285
	Starvation-FF	0.0002*	0.2264
	Low-DR	0.0549	0.9112
	Low-FF	0.0111*	0.7058
	DR-FF	0.9776	0.9733
$w^{Dah};Acer^{\Delta}$	Starvation-Low	0.9999	0.8813
	Starvation-DR	0.0477*	0.5336
	Starvation-FF	0.0007*	0.1487
	Low-DR	0.0674	09184
	Low-FF	0.0011*	0.4821
	DR-FF	0.7156	0.8698
w^{1118}	Starvation-Low	0.131	0.9949
	Starvation-DR	0.0783	0.9821
	Starvation-FF	0.6937	0.8758
	Low-DR	0.9945	0.9267
	Low-FF	0.6637	0.9567
	DR-FF	0.5108	0.678
$w^{1118};Acer^{\Delta}$	Starvation-Low	0.1022	0.9342
	Starvation-DR	0.2956	0.9787
	Starvation-FF	0.776	0.9995
	Low-DR	0.938	0.9973
	Low-FF	0.5004	0.9624
	DR-FF	0.838	0.9916

Table 35: Male Tukey-Kramer HSD comparisons of means for lipid storage levels after 2 and 4 days feeding for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant difference (p=<0.05).

Genotype	Food	p-value comparison to *Acer*$^{\Delta}$ for Lipid 2 Day Experiment	p-value comparison to *Acer*$^{\Delta}$ for Lipid 4 Day Experiment
w^{Dah}	Starvation	0.4203	0.9559
	Low	0.5287	0.7301
	DR	0.6727	0.6913
	FF	0.9136	0.9324
w^{1118}	Starvation	0.9618	0.7277
	Low	0.2253	0.2559
	DR	0.962	0.6503
	FF	0.8876	0.2882

Table 36: Male ANOVA/Pooled t-test measure of variance of genetic effects on different foods for lipid storage levels after 2 or 4 days feeding. *Indicates significant differences (P=<0.05).

Lipid levels for *Acer*$^{\Delta}$ males in both backgrounds responded in the same manner as controls to dietary changes (Table 35).

Male controls in the *w*^{Dah} background showed no response of lipid levels to feeding after four days compared to feeding after two days (Table 37). *Acer*$^{\Delta}$ males in this background also showed no response between two and four days feeding and therefore responded normally.

Genotype	Food	p-value comparing Lipid storage levels between 2 and 4 days feeding
w^{Dah}	Starvation	0.942
	Low	0.916
	DR	0.2124
	FF	0.5095
w^{Dah};*Acer*$^{\Delta}$	Starvation	0.4978
	Low	0.8801
	DR	0.2319
	FF	0.272
w^{1118}	Starvation	0.0158*
	Low	0.1093
	DR	0.4141
	FF	0.0024*
w^{1118};*Acer*$^{\Delta}$	Starvation	0.0426*
	Low	0.495
	DR	0.5219
	FF	0.1024

Table 37: Male comparison ANOVA/Pooled t-test measure of variance comparing lipid storage levels after 2 and 4 days feeding on different diets for controls and *Acer*$^{\Delta}$. *Indicates significant differences (P=<0.05).

In the w^{1118} background male controls showed a reduction in lipid levels after four days feeding on the Starvation and FF foods (Table 37). $Acer^{\Delta}$ males in this background also showed a reduction in lipid levels on Starvation food but didn't show a reduction on FF food.

Comparison of the genetic backgrounds showed that there was no difference in average lipid storage levels after two days feeding on the different foods (p=0.4694), however after four days feeding w^{1118} control males stored lower levels of lipid compared to w^{Dah} controls (p=0.001).

Acer is not involved in lipid metabolism in male flies or females in the w^{1118} background, however it may play a role in the utilisation of lipid stores on Starvation food in response to starvation stress in females in the w^{Dah} background.

4.2.3: *Acer* is involved in the initial response of weight to changing diet in female flies but the direction is dependent on genetic background.

Wet weight was measured after two and four days feeding on the different diets. Wet weight in both genetic backgrounds for female flies increased significantly with changing diet (Figure 37 and Table 38) after two days and four days feeding on different diets.

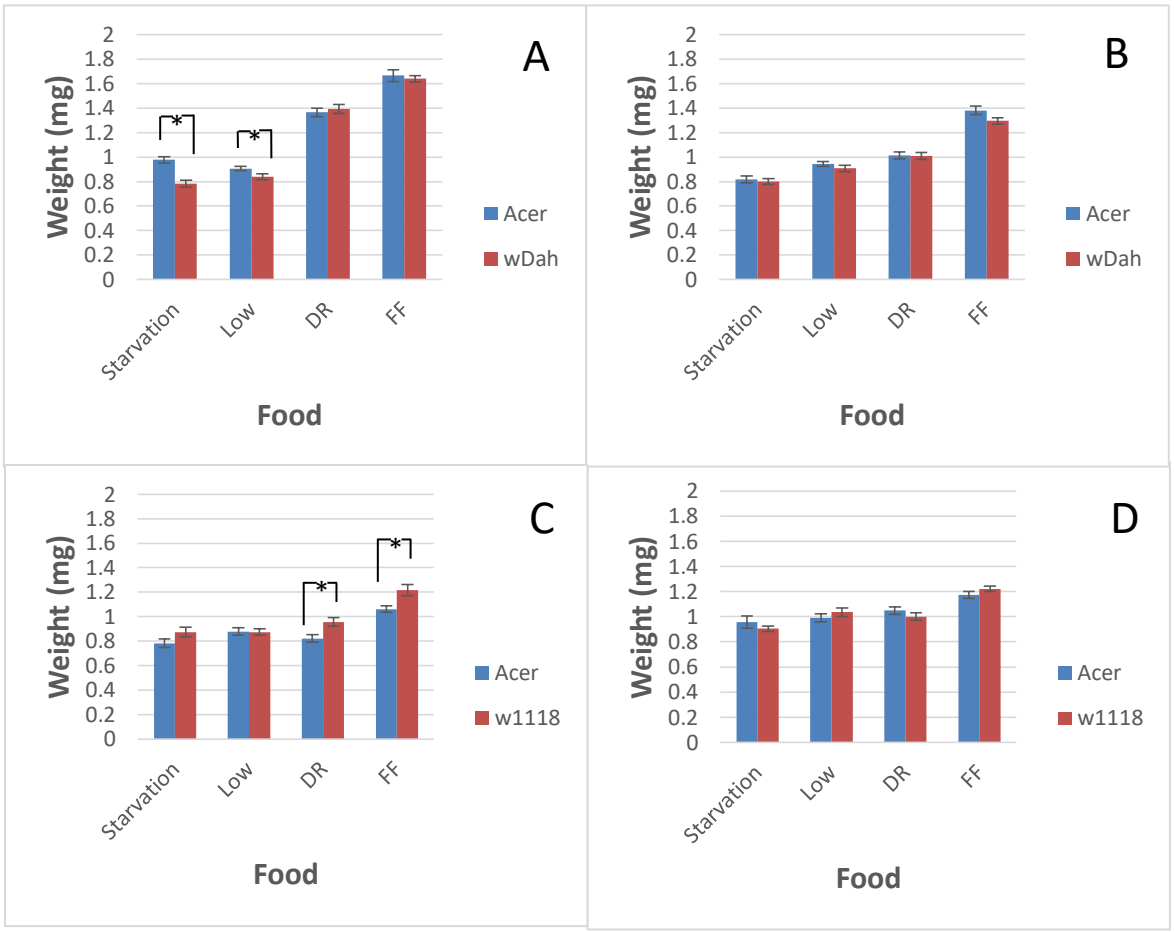

Figure 37: Female Wet Weight (mg) on Starvation, Low, DR and FF diets. (A and B) Weight in the w^{Dah} background. (A) Weight after 2 days feeding. w^{Dah}: N = 10 for all foods. $Acer^{\Delta}$: Starvation N = 9; Low N = 10; DR N = 10; FF N = 11. (B) Weight after and 4 days feeding. . w^{Dah}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. (C and D) Weight in the w^{1118} background. (C) Weight after 2 days feeding. w^{1118}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. (D) Weight after 4 days feeding, w^{1118}: DR N = 9; Starvation, Low and FF N = 10. $Acer^{\Delta}$: N = 10 for all foods. Data was checked for normality and found to be normally distributed. For mean comparison and variance statistics see Tables 38, 39 and 40. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	p-value for Weight 2 Day Experiment	p-value for Weight 4 Day Experiment
w^{Dah}	Starvation-Low	0.7522	0.041*
	Starvation-DR	<.0001*	<.0001*
	Starvation-FF	<.0001*	<.0001*
	Low-DR	<.0001*	0.0525
	Low-FF	<.0001*	<.0001*
	DR-FF	<.0001*	<.0001*
$w^{Dah};Acer^{\Delta}$	Starvation-Low	0.6281	0.0153*
	Starvation-DR	<.0001*	0.0001*
	Starvation-FF	<.0001*	<.0001*
	Low-DR	<.0001*	0.3421
	Low-FF	<.0001*	<.0001*
	DR-FF	<.0001*	<.0001*
w^{1118}	Starvation-Low	1	0.0108*
	Starvation-DR	0.403	0.0948
	Starvation-FF	<.0001*	<.0001*
	Low-DR	0.404	0.7959
	Low-FF	<.0001*	0.0003*
	DR-FF	<.0001*	<.0001*
$w^{1118};Acer^{\Delta}$	Starvation-Low	0.1559	0.9002
	Starvation-DR	0.8207	0.2575
	Starvation-FF	<.0001*	0.0006*
	Low-DR	0.613	0.6414
	Low-FF	0.0012*	0.0042*
	DR-FF	<.0001*	0.0804

Table 38: Female Tukey-Kramer HSD comparisons of means for Weight after 2 and 4 days feeding for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant difference (p=<0.05).

Genotype	Food	p-value comparison to $Acer^{\Delta}$ for Weight 2 Day Experiment	p-value comparison to $Acer^{\Delta}$ for Weight 4 Day Experiment
w^{Dah}	Starvation	<.0001*	0.6562
	Low	0.0425*	0.2731
	DR	0.5644	0.9558
	FF	0.6357	0.0671
w^{1118}	Starvation	0.0988	0.348
	Low	0.9207	0.3505
	DR	0.0123*	0.2538
	FF	0.0089*	0.2201

Table 39: Female ANOVA/Pooled t-test measure of variance of genetic effects on different foods for Weight after 2 or 4 days feeding. *Indicates significant differences (P=<0.05).

Acer$^{\Delta}$ females in the *w*^{Dah} background responded normally to changing diet over four days feeding but over two days feeding *Acer*$^{\Delta}$ females weighed significantly more than controls on Starvation and Low food (Table 38). In the *w*^{1118} background *Acer*$^{\Delta}$ females responded normally to changing diet over four days but after two days feeding they weighed significantly less on the DR and FF foods compared to controls (Table 38).

Genotype	Food	p-value comparing Weight between 2 and 4 days feeding
w^{Dah}	Starvation	0.662
	Low	0.0791
	DR	<.0001*
	FF	<.0001*
w^{Dah};*Acer*$^{\Delta}$	Starvation	0.0019*
	Low	0.1583
	DR	<.0001*
	FF	0.0004*
w^{1118}	Starvation	0.4867
	Low	0.0014*
	DR	0.351
	FF	0.9309
w^{1118};*Acer*$^{\Delta}$	Starvation	0.0091*
	Low	0.0235*
	DR	<.0001*
	FF	0.0101*

Table 40: Female comparison ANOVA/Pooled t-test measure of variance comparing weight after 2 and 4 days feeding on different diets for controls and *Acer*$^{\Delta}$. *Indicates significant differences (P=<0.05).

After four days feeding control females in the *w*^{Dah} background showed a decrease in weight on the DR and FF foods and no difference of weight on Starvation and Low food (Table 39). *Acer*$^{\Delta}$ females in this background showed a similar decrease in weight on the DR and FF foods but also showed a decrease in weight on the Starvation food (Table 39). In the *w*^{1118} background, control females showed little response of weight between two and four days feeding but did show an increase in weight on the Low food. In contrast, *Acer*$^{\Delta}$ females showed an increase in weight on all foods.

Average weight across all foods showed that after two days feeding w^{1118} control females were lighter than w^{Dah} control females (p= 0.0071) but after four days feeding there was no difference in weight between the two backgrounds (p=0.4128).

$Acer^{\Delta}$ females in both backgrounds respond normally to changes in diet in terms of weight over four days feeding but $Acer^{\Delta}$ females respond differently in the different genetic backgrounds with their initial response after two days feeding.

Male controls in the w^{Dah} background (Figures 38A and 38B and Table 41) showed a significant increase in weight as food levels increased for both two days and four days feeding on different foods. In the w^{1118} background (Figures 38C and 38D and Table 41), no weight difference was observed with changing diet after two days feeding but a significant increase in weight with increasing diet was seen after four days feeding on the different foods for control males.

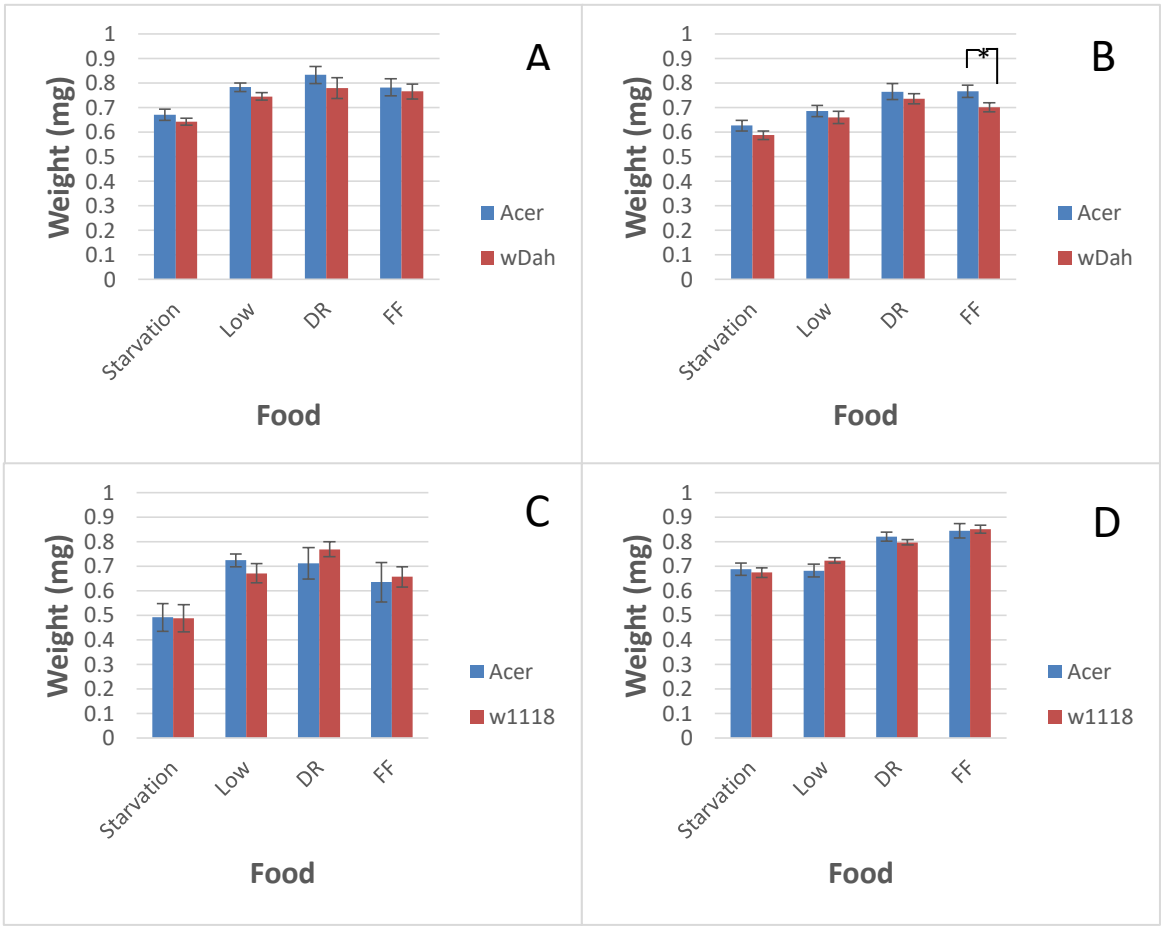

Figure 38: Male Wet Weight (mg) on Starvation, Low, DR and FF diets. (A and B) Weight in the w^{Dah} background. (A) Weight after 2 days feeding. w^{Dah}: Starvation N = 13; Low N = 12; DR N = 8; FF N = 9. $Acer^{\Delta}$: Starvation N = 13; Low N = 13; DR N = 9; FF N = 10. (B) Weight after 4 days feeding. w^{Dah}:

Starvation and Low N = 9; DR and FF N = 10. *Acer$^{\Delta}$*: DR N = 9; Starvation, Low and FF N = 10. (C and D) Weight in the *w^{1118}* background. (C) Weight after 2 days feeding. *w^{1118}*: N = 10 for all foods. *Acer$^{\Delta}$*: N = 10 for all foods. (D) Weight after 4 days feeding. *w^{1118}*: N = 10 for all foods. *Acer$^{\Delta}$*: DR N = 9; Starvation, Low and FF N = 10. Data was checked for normality. (A, B and D) were found to be normally distributed. (C) was found to be log-distributed. Data is shown as raw data and analysed as log-transformed. For mean comparison and variance statistics see Tables 41, 42 and 43. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	p-value for Weight 2 Day Experiment	p-value for Weight 4 Day Experiment
w^{Dah}	Starvation-Low	0.0002*	0.0785
	Starvation-DR	<.0001*	<.0001*
	Starvation-FF	<.0001*	0.0021*
	Low-DR	0.6569	0.0544
	Low-FF	0.9099	0.4968
	DR-FF	0.9875	0.6071
w^{Dah};Acer$^{\Delta}$	Starvation-Low	0.0071*	0.3883
	Starvation-DR	0.0002*	0.0033*
	Starvation-FF	0.0151*	0.0029*
	Low-DR	0.6223	0.1589
	Low-FF	1	0.1447
	DR-FF	0.6579	1
w^{1118}	Starvation-Low	0.131	0.1248
	Starvation-DR	0.0783	<.0001*
	Starvation-FF	0.6937	<.0001*
	Low-DR	0.9945	0.0079*
	Low-FF	0.6637	<.0001*
	DR-FF	0.5108	0.0812
w^{1118}:Acer$^{\Delta}$	Starvation-Low	0.1022	0.9989
	Starvation-DR	0.2956	0.0032*
	Starvation-FF	0.776	0.0005*
	Low-DR	0.938	0.0028*
	Low-FF	0.5004	0.0004*
	DR-FF	0.838	0.9062

Table 41: Male Tukey-Kramer HSD comparisons of means for Weight after 2 and 4 days feeding for controls and *Acer$^{\Delta}$* comparing dietary effects. *Indicates significant difference (p=<0.05).

Acer$^{\Delta}$ males responded normally in terms of weight to changing diet in both backgrounds except on the FF food in the *w^{Dah}* background where *Acer$^{\Delta}$* males were significantly heavier than controls.

Genotype	Food	p-value comparison to *Acer*$^{\Delta}$ for Weight 2 Day Experiment	p-value comparison to *Acer*$^{\Delta}$ for Weight 4 Day Experiment
w^{Dah}	Starvation	0.3015	0.175
	Low	0.0923	0.4521
	DR	0.262	0.4622
	FF	0.6881	0.0465*
w^{1118}	Starvation	0.9618	0.6725
	Low	0.2253	0.16
	DR	0.962	0.2921
	FF	0.8876	0.8509

Table 42: Male ANOVA/Pooled t-test measure of variance of genetic effects on different foods for Weight after 2 or 4 days feeding. *Indicates significant differences (P=<0.05).

Male controls in the *w*^{Dah} background showed a decrease in weight after four days feeding on all foods except DR food (Table 43) compared to two days feeding. *Acer*$^{\Delta}$ males only showed a decrease in weight on the Low food. In the *w*^{1118} background an increase in weight was observed on the Starvation and FF foods and *Acer*$^{\Delta}$ males responded normally (Table 43).

Genotype	Food	p-value comparing Weight between 2 and 4 days feeding
w^{Dah}	Starvation	0.0203*
	Low	0.0043*
	DR	0.2896
	FF	0.0158*
w^{Dah};*Acer*$^{\Delta}$	Starvation	0.1937
	Low	0.0026*
	DR	0.1439
	FF	0.7237
w^{1118}	Starvation	0.0059*
	Low	0.2161
	DR	0.3904
	FF	0.0004*
w^{1118};*Acer*$^{\Delta}$	Starvation	0.0054*
	Low	0.2854
	DR	0.1225
	FF	0.0252*

Table 43: Female comparison ANOVA/Pooled t-test measure of variance comparing weight after 2 and 4 days feeding on different diets for controls and *Acer*$^{\Delta}$. *Indicates significant differences (P=<0.05).

Average weight comparison over all of the foods between the two control backgrounds showed that w^{1118} control males weighed less than w^{Dah} control males after two days feeding on the different foods (p=0.0071) but after four days feeding w^{1118} control males weighed more than their w^{Dah} counterparts (p=<.0001).

Acer appears to be involved in the initial response of weight to changing diet in female flies but the direction is dependent on genetic background. *Acer* is not involved in the response of weight to changing diet in male flies.

4.2.4: *Acer* is not involved in the general feeding response to changing diet

The *Acer* deletion has little effect on lipid content and weight in response to nutrition which suggests that the effect on glycogen content is not due to a general feeding effect. The flies were placed on food containing blue dye for 30 minutes after a prior starvation period of 90 minutes before the amount of blue dye ingested was measured at 620nm.

Female controls in the w^{Dah} background (Figure 39A and Table 44) showed a significant increase in feeding with increasing food. In the w^{1118} background (Figure 39B and Table 44) control females show a significant increase in feeding from Low to DR food and Low to FF food but no difference between DR and FF food.

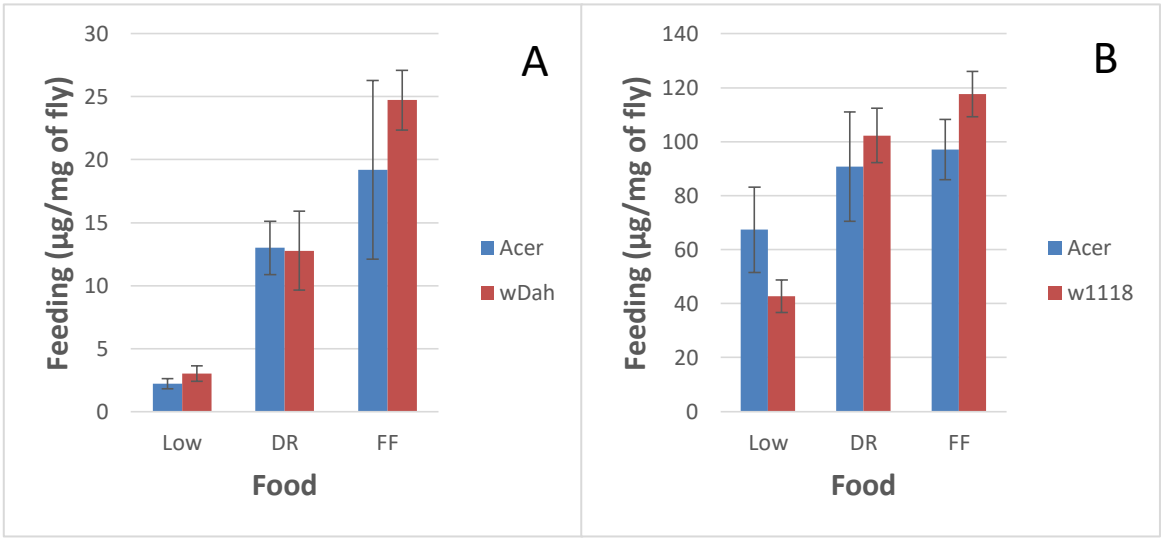

Figure 39: Female Feeding (µg/mg of fly) on Low, DR and FF diets. (A) Feeding in the w^{Dah} background on food containing 1% blue dye. w^{Dah}: Low N = 3; DR N = 4; FF N = 3. $Acer^{\Delta}$: Low N = 3; DR N = 6; FF N = 4. (B) Feeding in the w^{1118} background on food containing 5% blue dye. w^{1118}: Low N =10' DR N = 10; FF N= 9. $Acer^{\Delta}$: Low N = 9; DR N = 10; FF N = 8. Data was checked for normality and (B) was found to be normally distributed. (A) was found to be log distributed and data is shown as raw data and analysed as log-transformed. For mean comparison and variance statistics see Tables 44 and 45. *Indicates significant difference between genotypes (P=<0.05).

$Acer^{\Delta}$ females in the w^{Dah} background did not significantly increase their feeding with increasing food suggesting a lack of response to dietary changes. In the w^{1118} background $Acer^{\Delta}$ did not significantly alter their feeding in response to diet but did not significantly differ from controls.

Genotype	Food Comparison	p-value for Feeding
w^{Dah}	Low-DR	0.068
	Low-FF	0.0019*
	DR-FF	0.0295*
$w^{Dah};Acer^{\Delta}$	Low-DR	0.2273
	Low-FF	0.0641
	DR-FF	0.5239
w^{1118}	Low-DR	<.0001*
	Low-FF	<.0001*
	DR-FF	0.4156
$w^{1118};Acer^{\Delta}$	Low-DR	0.6029
	Low-FF	0.4842
	DR-FF	0.9646

Table 44: Female Tukey-Kramer HSD comparisons of means for Feeding for controls and *Acer*$^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

Genotype	Food	p-value comparison to *Acer*$^{\Delta}$ for Feeding
w^{Dah}	Low	0.3658
	DR	<.0001*
	FF	0.2805
w^{1118}	Low	0.1659
	DR	0.6158
	FF	0.184

Table 45: Female ANOVA/Pooled t-test measure of variance of genetic effects on different foods for Feeding. *Indicates significant differences (P=<0.05).

Male control feeding in the w^{Dah} background (Figure 40A and Table 46) significantly increased from Low to DR food and FF food but no difference was seen between DR and FF food.

In the w^{1118} background (Figure 40B Table 46) there was no significant difference of feeding between the foods however a trend of increased feeding with increasing yeast is observed. *Acer*$^{\Delta}$ males in both backgrounds fed normally with changing diet.

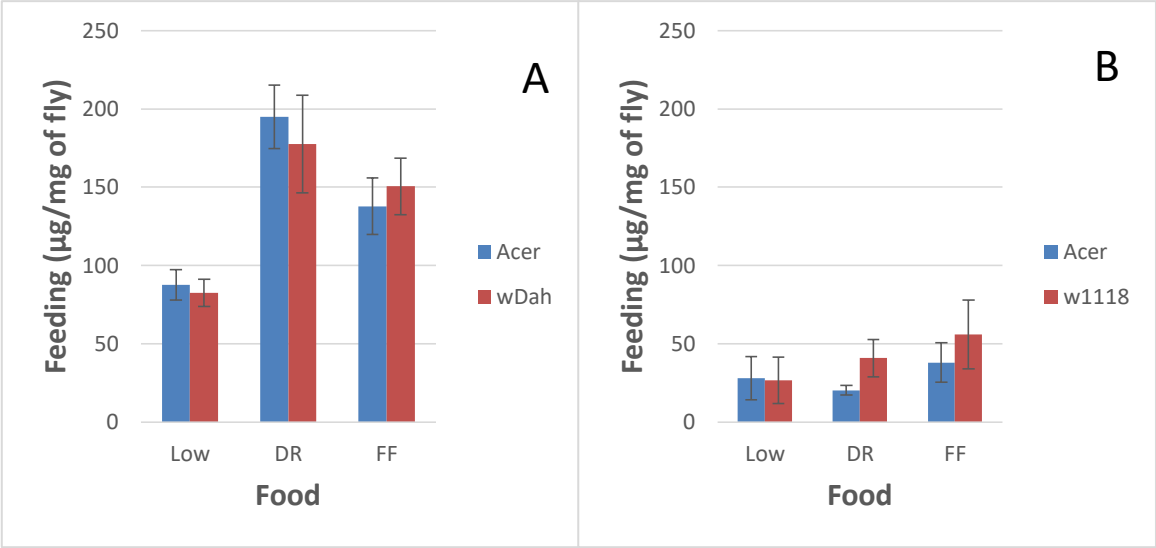

Figure 40: Male Feeding (µg/mg of fly) on Low, DR and FF diets. (A) Feeding in the w^{Dah} background on food containing 5% blue dye. w^{Dah}: N = 10 for all foods. $Acer^{\Delta}$: N = 10 for all foods. B) Feeding in the w^{1118} background on food containing 5% blue dye. w^{1118}: Low N = 5; DR N = 8; FF N = 10 $Acer^{\Delta}$: Low N = 6; DR N = 5; FF N = 4. Data was checked for normality and was found to be log-distributed. Data is shown as raw data and analysed as log-transformed. For mean comparison and variance statistics see Tables 40 and 41. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	p-value for Feeding
w^{Dah}	Low-DR	0.0047*
	Low-FF	0.014*
	DR-FF	0.8965
$w^{Dah};Acer^{\Delta}$	Low-DR	0.0001*
	Low-FF	0.0343
	DR-FF	0.0818
w^{1118}	Low-DR	0.3327
	Low-FF	0.3003
	DR-FF	0.9999
$w^{1118};Acer^{\Delta}$	Low-DR	0.9906
	Low-FF	0.4546
	DR-FF	0.5475

Table 46: Male Tukey-Kramer HSD comparisons of means for Feeding for controls and $Acer^{\Delta}$ comparing dietary effects. *Indicates significant differences (P=<0.05).

Genotype	Food	p-value comparison to *Acer*$^{\Delta}$ for Feeding
w^{Dah}	Low	0.7453
	DR	0.3704
	FF	0.5672
w^{1118}	Low	0.5956
	DR	0.2597
	FF	0.956

Table 47: Male ANOVA/Pooled t-test measure of variance of genetic effects on different foods for Feeding. *Indicates significant differences (P=<0.05).

The feeding phenotype has shown that after four days feeding on different foods *Acer*$^{\Delta}$ males and females did not feed differently to controls in the both genetic backgrounds. This suggests that the reduced glycogen phenotype is not due to a lack of feeding.

4.3: Discussion

Previously we found that there are differences between the w^{Dah} and w^{1118} genetic backgrounds when sleep was tested (Chapter 3) and this is also true of nutrient storage. On average across all foods female w^{Dah} controls stored more glycogen and lipid than w^{1118} female controls and were heavier after two days feeding on different foods but not after four days feeding. Male controls showed no difference in average glycogen levels or average lipid levels after two days feeding across all foods, but after four days feeding w^{1118} control males' average lipid levels were reduced compared to w^{Dah} controls. Weight was different between the two backgrounds with w^{Dah} males weighing more after two days feeding but w^{1118} males weighing more after four days feeding.

Acer's role in nutrient storage is affected by the different genetic backgrounds. In females, glycogen storage increases with increasing yeast with a reduction seen on the FF food in both backgrounds. This decrease is likely to be due to the energy used in egg-laying as female flies lay more eggs on high yeast food (Skorupa, et al., 2008). Initially, $Acer^{\Delta}$ females showed a lack of response to changing diet as glycogen levels are considerably reduced on DR and FF food in the w^{Dah} background and on FF food only in the w^{1118} background after two days feeding on different diets. After four days feeding, glycogen levels returned to normal for $Acer^{\Delta}$ females in the w^{Dah} background due to an increase in glycogen levels on these foods for $Acer^{\Delta}$ females and a decrease in glycogen levels for controls. On Starvation food $Acer^{\Delta}$ females in the w^{Dah} background stored higher levels of glycogen compared to controls but still showed a reduction in glycogen stores after four days on Starvation food. These data suggest that the lack of *Acer* is either slowing the rate of storage or increasing utilisation of glycogen in female flies in this background. There continued to be a reduction in glycogen storage compared to control females on FF food in the w^{1118} background with levels of glycogen reducing similarly to controls after four days feeding. These data show that *Acer* modulates glycogen levels in both backgrounds but the loss of *Acer* has slightly different effects.

In males, glycogen storage increased with increasing food for both backgrounds with $Acer^{\Delta}$ males in the w^{Dah} background, like females, showing an initial lack of response to changing diet on the DR and FF foods but glycogen levels returned to normal after four days feeding on different foods. After four days feeding glycogen

levels for *Acer*$^{\Delta}$ males increased on high food compared to two days feeding while control levels remained the same resulting in glycogen levels for *Acer*$^{\Delta}$ males mirroring control levels. These data, like the female data, suggests that *Acer* may be involved in the regulation of glycogen storage in the w^{Dah} background. In the w^{1118} background, *Acer*$^{\Delta}$ males initially responded normally to changing diet but then showed a reduction in storage levels after four days on food, suggesting a delayed reaction to changing diet.

Glycogen levels are known to be reduced when the clock gene, which maintains cycles of gene expression, in the fly fat body is disrupted with sensitivity to starvation related to low glycogen levels (Xu, et al., 2008) and *Acer* is known to be expressed in the fly fat body (Carhan, et al., 2010). The results showed an initial lack of response to changing diet in in the w^{Dah} background but a more delayed response in the w^{1118} background, suggesting that *Acer* is involved in glycogen storage but it may only be important at early stages of adaptation to changing diet in the w^{Dah} background but may be involved longer in the w^{1118} background. The increased stores of glycogen on the Starvation food for *Acer*$^{\Delta}$ females in the w^{Dah} background after four days feeding suggests a potential utilisation defect and a role for *Acer* modulating the usage of glycogen in response to starvation conditions.

Acer$^{\Delta}$ males and females regulate lipid metabolism normally in both genetic backgrounds but, like glycogen, *Acer*$^{\Delta}$ females have higher lipid stores on Starvation food after four days feeding, suggesting a potential utilisation defect similar to glycogen. Higher lipid and glycogen levels have been found to be associated with starvation resistance (Ballard, et al., 2008; Xu, et al., 2008) therefore with *Acer*$^{\Delta}$ females showing higher levels of lipid and glycogen after four days starvation it will be interesting to note whether *Acer*$^{\Delta}$ females are starvation resistant in the w^{Dah} background (Chapter 6.2.4).

Wet weight was measured and the results showed that after four days on different foods *Acer*$^{\Delta}$ females in both backgrounds responded normally to changing diet in terms of weight. However, initially they showed an increase in weight compared to controls in the w^{Dah} background on Starvation and Low food but in the w^{1118} background showed a reduction in weight on the DR and FF foods. The reduction in weight in the w^{1118} background correlates with reduced glycogen on the FF food but not the DR food, whereas the increase in weight does not correlate with our findings

for lipid and glycogen storage suggesting that other factors may affect weight such as protein levels.

Acer$^{\Delta}$ males and females in both backgrounds fed normally after four days feeding on different foods in response to changing diet suggesting that the reduction in glycogen storage after two days feeding in *Acer*$^{\Delta}$ flies was not due to a reduced feeding. However, analysing feeding after two days on the different foods would confirm if the reduction in glycogen storage is separate from feeding.

Chapter 5: The Role of *Acer* and diet in Lifespan and Fecundity

5.1: Introduction

Longevity and fecundity have been found to be responsive to dietary changes in *Drosophila melanogaster*. In the lab the diet of *Drosophila* consists of sugar for carbohydrate and yeast for protein (Skorupa, et al., 2008). Yeast is an important factor in regulating fecundity and is also important in the response to dietary restriction (DR) (Mair, et al., 2005). Therefore to test the response of longevity to DR, dietary changes of protein are required. Lifespan extension has being associated with DR which is characterised by a reduction in food without causing malnutrition (Kerr, et al., 2011) and in *Drosophila* it is achieved by the dilution of yeast concentration within *Drosophila* medium (Bass, et al., 2007). It is thought that the availability of nutrients is most important rather than the total calorific content being reduced (Bass, et al., 2007). Longevity has been found to peak under DR conditions but there is a trade-off with fecundity as flies lay fewer eggs under DR conditions and increase egg-laying with increasing food levels, but longevity decreases as food levels pass DR (Fontana, et al., 2010). DR is not optimal for fecundity and therefore a balance in diet is required to optimise the longevity and fecundity (Skorupa, et al., 2008).

Nutrient-sensing signalling pathways, such as the TOR and IIS, are thought to play roles in mediating the extension of lifespan by DR in *Drosophila* (Fontana, et al., 2010). Down-regulation, and therefore reduced signalling, of these pathways has led to extended lifespan in flies under DR conditions (Clancy, et al., 2001; Broughton, et al., 2010; Fontana, et al., 2010; Katewa & Kapahi, 2011). When reduced IIS was achieved by ablating the mNSCs in the fly brain, lifespan was extended but females showed a reduced level of fecundity. Lifespan extension by genetic manipulations, *such as Indy*, does not require reduced fecundity, suggesting that extended lifespan is not only extended by a traded-off with fecundity but there are other elements and mechanisms involved (Marden, et al., 2003).

We wanted to test if $Acer^{\Delta}$ flies respond to DR normally and if they are long-lived on DR or FF food compared to controls as well as testing fecundity on both these foods to see if egg-laying behaviour differs from controls. As we have already seen phenotypic difference due to genetic background, we tested the $Acer^{\Delta}$ males and females in both the w^{Dah} and w^{1118} backgrounds.

5.1.1: Aims

With *Acer's* apparent role in the response of nutrient responsive phenotypes to nutrition we wanted to identify if *Acer* plays a role in lifespan extension and whether $Acer^{\Delta}$ flies respond normally to DR.

With *Acer's* presence in the ovaries and testes we wanted to test whether egg-laying was affected by the lack of *Acer* secretion in $Acer^{\Delta}$ females and if any difference in egg-laying was due to female on male defects.

5.1.2: Research Design

The longevity and fecundity of $Acer^{\Delta}$ males and females in the w^{Dah} and w^{1118} backgrounds was analysed under DR and FF conditions.

For the measurement of longevity, 3 day old flies (N=100) were transferred to either DR or FF food for the duration of their life and their lifespan recorded.

For the measurement of fecundity eggs laid by female flies were recorded weekly until the amount of eggs laid was reduced to a minimal level on both foods.

5.2: Results

5.2.1: *Acer*$^{\Delta}$ mutants show a normal extension of lifespan in response to Dietary Restriction but females are less fecund in the *w*^{Dah} background.

A pilot study testing *Acer*$^{\Delta}$ female and male survival compared to controls in the *w*^{Dah} background on standard food (50g per litre of sugar and 100g per litre of yeast) showed that when fed standard food for the entirety of their lifespan *Acer*$^{\Delta}$ females were long-lived compared to controls (p=<.0001) whereas *Acer*$^{\Delta}$ males were short-lived compared to controls (p=<.0001) (Figure 41).

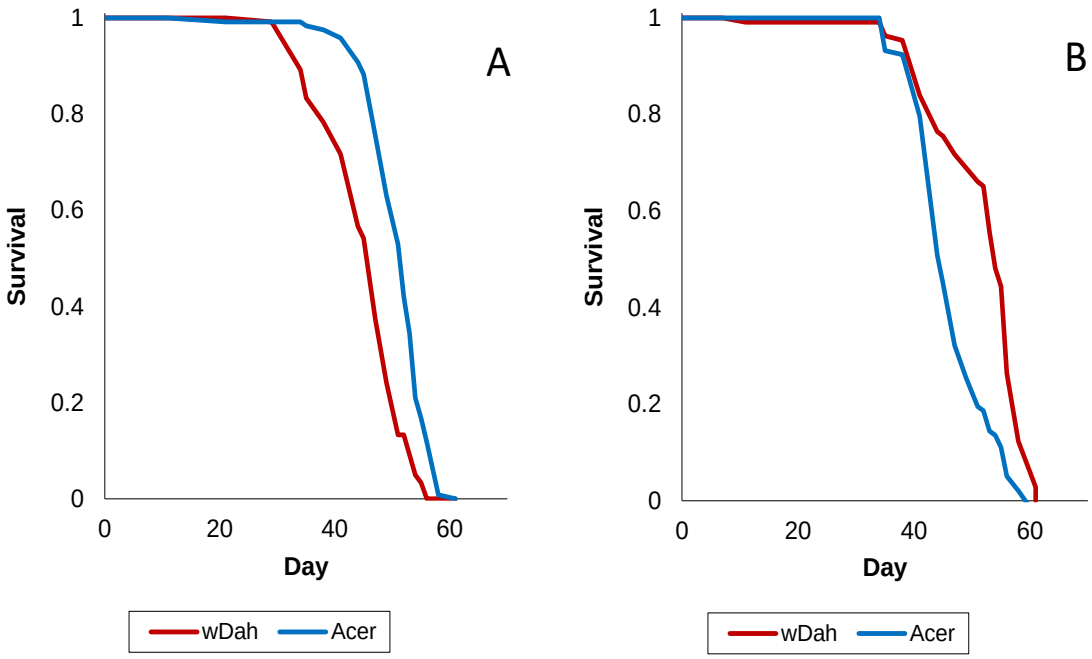

Figure 41: Female (A) and male survival (B) for *w*^{Dah} controls and *Acer*$^{\Delta}$ fed standard food (50g per litre of sugar and 100g per litre of yeast). (A) Median lifespan: *w*^{Dah} = 47 days, N = 120; *Acer*$^{\Delta}$ = 52 days, N = 120. p = <.0001. (B) Median Lifespan: *w*^{Dah} = 54 days, N = 120; *Acer*$^{\Delta}$ = 45 days, N = 123. P = <.0001. Survival analysis was performed in JMP using the log-rank test.

The lifespan experiments were conducted twice for *Acer*$^{\Delta}$ in each genetic background. In the *w*^{Dah} background (Figures 42A and 42B), control and *Acer*$^{\Delta}$ females responded to DR normally with a lifespan extension on the DR food compared to the FF food (Experiment A: *w*^{Dah}: p=0.0001; *Acer*$^{\Delta}$: p=<.0001. Experiment B: *w*^{Dah}: p=<.0001; *Acer*$^{\Delta}$: p=<.0001.). In the *w*^{1118} background (Figures 42C and 42D), control

and *Acer*$^{\Delta}$ females also responded normally to DR with a lifespan extension as expected (Experiment C: *w*^{1118}: p=<.0001; *Acer*$^{\Delta}$: p=<.0001. Experiment D: *w*^{1118}: p=<.0001; *Acer*$^{\Delta}$: p=0.0002). These data suggest that *Acer* is not required for the DR lifespan response.

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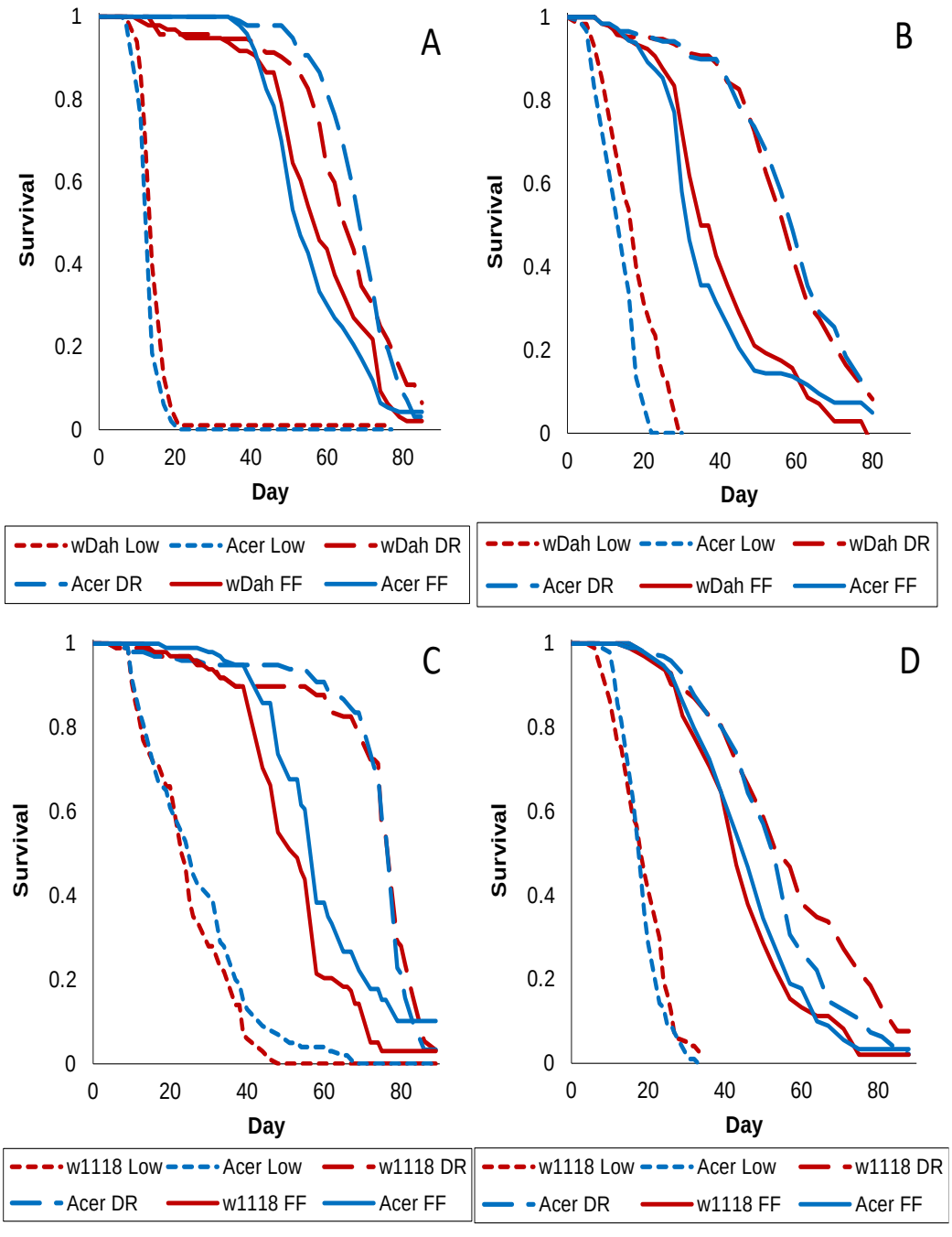

Figure 42: Female lifespan in response to feeding on Low, DR, and FF diets for controls and *Acer*$^{\Delta}$. (A and B) *w*^{Dah} background. (C and D) *w*^{1118} background. (A) Median Lifespan: *w*^{Dah}: Low = 14 days, N = 99; DR = 67 days, N = 98; FF = 58 days, N = 98. *Acer*$^{\Delta}$: Low = 14 days, N = 101; DR = 69 days, N = 98; FF = 53 days, N = 97. (B) *w*^{Dah}: Low = 18 days, N = 123; DR = 56 days, N = 189; FF = 39, N = 201. *Acer*$^{\Delta}$:

Low = 15 days, N = 124; DR = 59 days, N = 189; FF = 32 days, N = 198. (C) Median Lifespan: w^{1118}: Low = 24 days, N = 102; DR = 79 days, N = 85 days; FF = 51 days, N = 97. $Acer^{\Delta}$: Low = 25 days, N = 100; DR = 79 days, N = 94; FF = 58 days, N = 97. D) Median Lifespan: w^{1118}: Low = 18 days, N = 98; DR = 53 days, N = 95; FF = 43 days, N = 98. $Acer^{\Delta}$: Low = 18 days, N = 100; DR = 53 days, N = 99; FF = 46 days, N = 97. Survival analysis was performed in JMP using the log-rank test (Table 48).

Genotype Comparison	Food	Experiment	Log rank (p-value)
w^{Dah} *to* $Acer^{\Delta}$	Low	A	0.0081*
		B	<.0001*
	DR	A	0.7309
		B	0.4685
	FF	A	0.05
		B	0.031*
w^{1118} *to* $Acer^{\Delta}$	Low	C	0.2771
		D	0.4749
	DR	C	0.4398
		D	0.1898
	FF	C	0.0013*
		D	0.8451

Table 48: Female comparison of survival curves by genotype using the log rank test in JMP for each food for control and $Acer^{\Delta}$ females. *Indicates significant difference (P=<0.05).

In the w^{Dah} background in experiments A and B (Figure 42 and Table 48) $Acer^{\Delta}$ females were sensitive to low food, showed no difference in lifespan on DR food but were significantly short-lived on the FF food in experiment B, with a similarly shortened lifespan in experiment A approaching significance compared to control females.

$Acer^{\Delta}$ females in the w^{1118} background originally showed a lifespan extension on FF food compared to controls with no difference seen on the DR food (Figure 42C). However, in experiment D $Acer^{\Delta}$ females again showed no difference in lifespan on the DR food but also showed no difference in lifespan on the FF food (Table 48). In response to low food there was no difference in lifespan between $Acer^{\Delta}$ females and controls (Table 48).

The overall response of $Acer^{\Delta}$ females to changing diet compared to controls is mixed but we can confirm that $Acer^{\Delta}$ females respond normally to DR with an expected lifespan extension in both backgrounds, therefore *Acer* is not required for lifespan extension by DR. The response to high and low food is background dependent with $Acer^{\Delta}$ females in the w^{Dah} background sensitive to low and high food but in the

w^{1118} background $Acer^{\Delta}$ females show no sensitivity to low food and in experiment C were long-lived on FF food while in experiment D there was difference compared to controls. These suggest that *Acer* modulates the normal response of lifespan to high nutrition but the effect is background dependent and that *Acer* modulates the normal response of lifespan to low food in the w^{Dah} background.

The fecundity results (Figure 43) showed that in both backgrounds both genotypes showed an age-related decline in fecundity on both the DR and FF foods (p=<.0001) and laid significantly fewer eggs on the DR food than the FF food (p=<.0001).

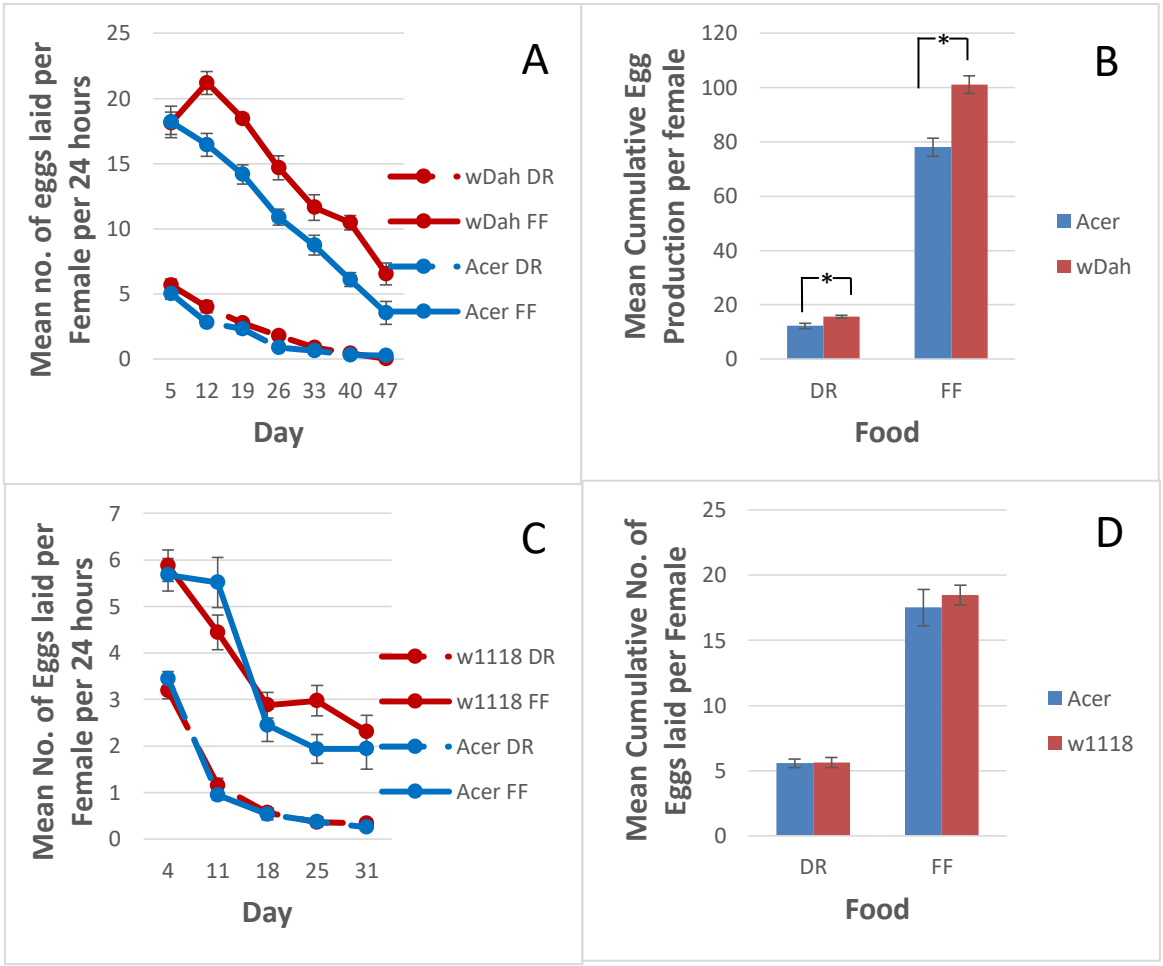

Figure 43: Fecundity for control and $Acer^{\Delta}$ females in response to feeding on DR and FF diets. (A) Mean no. of eggs laid per female per 24 hours in the w^{Dah} background. w^{Dah}: DR N =10; FF N = 10. $Acer^{\Delta}$: DR N =10; FF N = 10. (B) Mean cumulative no. of eggs laid per female in the w^{Dah} background. w^{Dah}: DR N =10; FF N = 10. $Acer^{\Delta}$: DR N =10; FF N = 10. (C) Mean no. eggs laid per female per 24 hours in the w^{1118} background. w^{1118}: DR N =10; FF N = 10. $Acer^{\Delta}$: DR N =10; FF N = 10. (D) Mean total no. of eggs laid in the w^{1118} background. w^{1118}: DR N =10; FF N = 10. $Acer^{\Delta}$: DR N =10; FF N = 10. Data was

checked for normality and data was found to be normally distributed. For mean comparison and variance statistics see Tables 49, 50, 51 and 52. * Indicates significant differences between genotypes. (P=<0.05)

		Day p-value						
Genetic Comparison	Food	5	12	19	26	33	40	47
w^{Dah} to $Acer^{\Delta}$	DR	0.586	0.0706	0.6213	0.0134*	0.23	0.6204	0.1272
	FF	0.998	0.0031*	0.0002*	0.0043*	0.045*	0.0006*	0.0558

Table 49: Tukey-Kramer HSD comparisons of means of eggs laid per female per 24 hours by age in the *w^{Dah}* background for controls and *$Acer^{\Delta}$* females. *Indicates significant differences (P=<0.05).

Genetic Comparison	Food	Mean cumulative no. of eggs laid per Female (p-value)
w^{Dah} to $Acer^{\Delta}$	DR	0.0118*
	FF	0.0002*

Table 50: ANOVA/Pooled t-test measure of variance of mean cumulative eggs laid per female in the *w^{Dah}* background for controls and *$Acer^{\Delta}$* females. *Indicates significant differences (P=<0.05).

In the *w^{Dah}* background (Figures 43A and 43B and Tables 49 and 50), *$Acer^{\Delta}$* females showed a significant reduction in egg-laying compared to controls on FF food at the majority of time-points but showed little difference on the DR food. The mean cumulative eggs laid per female over the course of the experiment shows that *$Acer^{\Delta}$* females laid significantly fewer eggs compared to controls on thee DR food, even though at specific time points the difference was not significant, and *$Acer^{\Delta}$* females laid significantly fewer eggs on the FF food compared to controls, suggesting that *Acer* modulates the response of fecundity to high food in this background. This is a reduced nutrient response in *$Acer^{\Delta}$* females which is similar to the reduced response observed previously in other nutrient responsive phenotypes, such as sleep and glycogen storage (Chapters 3 and 4).

In the *w^{1118}* background (Figure 43C and 43D and Tables 51 and 52) there was no significant difference between controls and *$Acer^{\Delta}$* females in the response of fecundity to dietary changes at any time points. This may be because the lower number of eggs laid by the *w^{1118}* females makes it more difficult to observe a difference in the number of eggs laid due to any difference being small.

		Day p-value				
Genetic Comparison	Food	4	11	18	25	31
w^{1118} to $Acer^{\Delta}$	DR	0.5173	0.5616	0.9746	0.9957	0.7672
	FF	0.9091	0.2513	0.5909	0.0779	0.7812

Table 51: Tukey-Kramer HSD comparisons of means of eggs laid per female per 24 hours by age in the *w^{1118}* background for controls and *$Acer^{\Delta}$* females. *Indicates significant differences (P=<0.05).

Genetic Comparison	Food	Mean cumulative no. of eggs laid per Female (p-value)
w^{1118} to $Acer^{\Delta}$	DR	0.993
	FF	0.7872

Table 52: ANOVA/Pooled t-test measure of variance of mean cumulative eggs laid per female in the *w^{1118}* background for controls and *$Acer^{\Delta}$* females. *Indicates significant differences (P=<0.05).

Fecundity in both backgrounds differed as *w^{Dah}* control females laid more eggs than *w^{1118}* control females on both DR (p=<.0001) and FF (p=<.0001) foods.. In the *w^{Dah}* background *Acer* appears to be involved the fecundity response to high food but this is not the case in the *w^{1118}* background.

The results for male lifespan showed that in both backgrounds control and *$Acer^{\Delta}$* males responded normally to DR (Experiment A: *w^{Dah}*: p=0.0059; *$Acer^{\Delta}$*: p=0.001. Experiment B: *w^{Dah}*: p=0.0042; *$Acer^{\Delta}$*: p=<.0001. Experiment C: *w^{1118}*: p=<.0001; *$Acer^{\Delta}$*: p=<.0001. Experiment D: *w^{1118}*: p=<.0001; *$Acer^{\Delta}$*: p=<.0001.).

In the *w^{Dah}* background (Figures 44A and 44B and Table 53) *$Acer^{\Delta}$* males are long-lived on both the DR and FF foods in experiment A. However in experiment B, *$Acer^{\Delta}$* males are not long-lived on FF food but are nearing significance on DR food. In both experiments *$Acer^{\Delta}$* males showed a sensitivity to low food (Table 47) which is the same phenotype seen in *$Acer^{\Delta}$* females in the *w^{Dah}* background

In the *w^{1118}* background (Figure 44C and 44D and Table 53) *$Acer^{\Delta}$* males were long-lived on DR food but not on FF food in experiment C, but in experiment D *$Acer^{\Delta}$* males were long-lived on FF food but not on DR food. In response to low food there is no difference between controls and *$Acer^{\Delta}$* males in experiment C but in experiment D *$Acer^{\Delta}$* males are sensitive to low food levels.

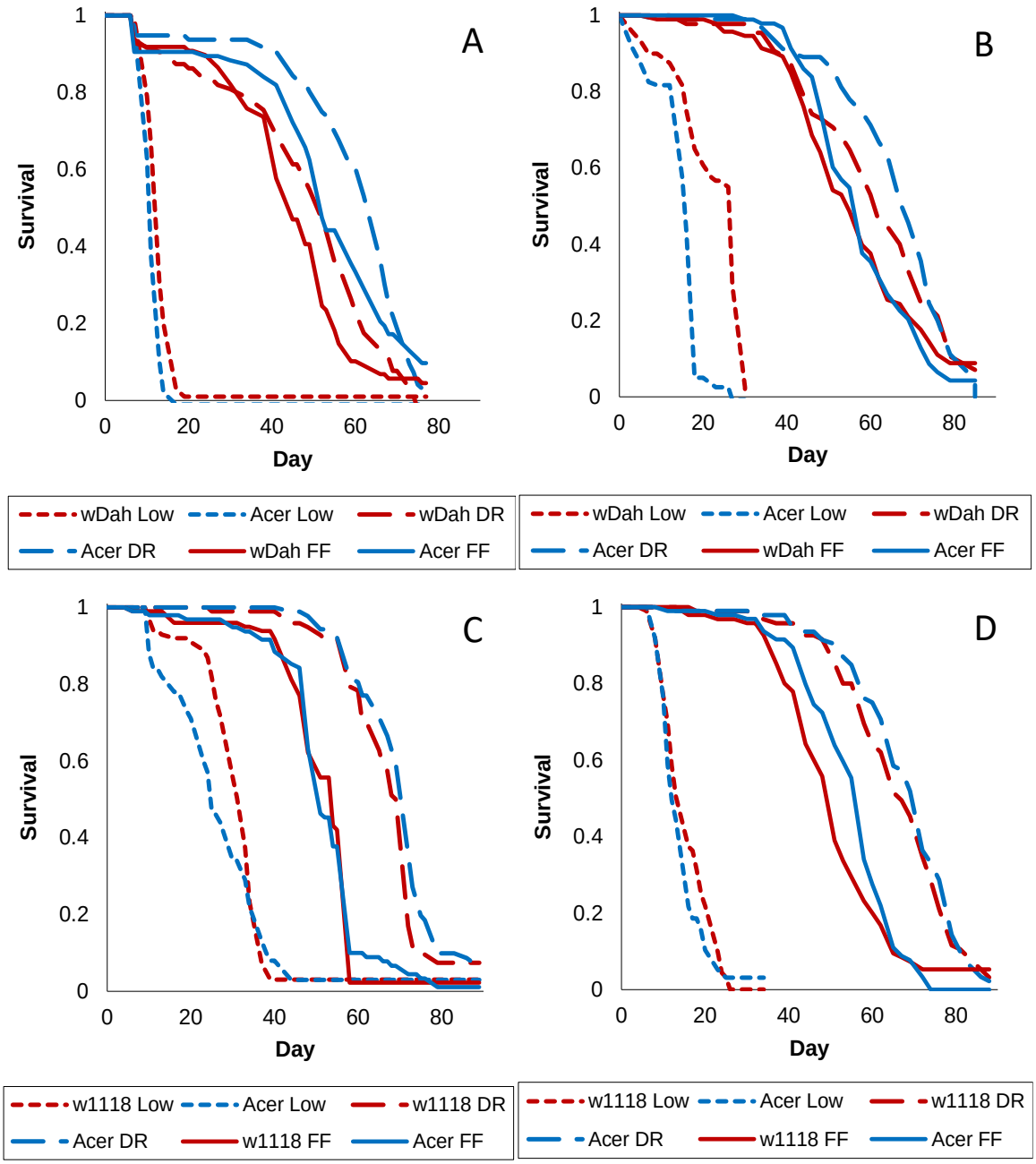

Figure 44: Male lifespan in response to feeding on Low, DR and FF diets for controls and *Acer*$^{\Delta}$. (A and B) *w*^{Dah} background. (C and D) *w*^{1118} background. (A) Median Lifespan: *w*^{Dah}: Low = 13 days, N = 99; DR = 52 days, N = 104; FF = 45 days, N = 98. *Acer*$^{\Delta}$: Low = 11 days, N = 101; DR = 66 days, N = 100; FF = 52 days, N = 92. (B) *w*^{Dah}: Low = 27 days, N = 120; DR = 62 days, N = 97; FF = 53 days, N = 94. *Acer*$^{\Delta}$: Low = 16 days, N = 121; DR = 69 days, N = 100; FF = 58 days, N = 97. (C) Median Lifespan: *w*^{1118}: Low = 31 days, N = 97; DR = 68 days, N = 93; FF = 54 days, N = 98. *Acer*$^{\Delta}$: Low = 25 days, N = 97; DR = 72 days, N = 92; FF = 51 days, N = 99. D) Median Lifespan: *w*^{1118}: Low = 16 days, N = 100; DR = 66 days, N = 97; FF = 51 days, N = 98. *Acer*$^{\Delta}$: Low = 12 days, N = 97; DR = 72 days, N = 98; FF = 58 days, N = 100. Survival analysis was performed in JMP using the log-rank test (Table 53).

Genotype Comparison	Food	Experiment	Log-rank (p-value)
w^{Dah} to Acer$^{\Delta}$	Low	A	<.0001*
		B	<.0001*
	DR	A	0.0002*
		B	0.078
	FF	A	0.0027*
		B	0.5776
w^{1118} to Acer$^{\Delta}$	Low	C	0.1476
		D	0.0067*
	DR	C	0.0036*
		D	0.3506
	FF	C	0.6669
		D	0.0134*

Table 53: Male comparison of survival curves by genotype using log rank test in JMP for each food for control and *Acer$^{\Delta}$* males. *Indicates significant difference (P=<0.05).

The response of *Acer*$^{\Delta}$ males to changing diet was mixed and requires repetition to establish whether *Acer*$^{\Delta}$ males are long-lived compared to controls on DR and FF foods.

5.2.2: Reduced fecundity in *Acer*$^{\Delta}$ females is likely to be caused by female flies alone

With the reduction in fecundity for *Acer*$^{\Delta}$ females and *Acer's* presence in the ovaries and testes we tested whether the reduction in egg-laying (Chapter 5.2.1.) was due to males or females by testing fecundity using reciprocal mating.

The results showed that all of the crosses showed a significant age-related decline in egg laying (p=<.0001*) for both genetic backgrounds (Figure 45 and Tables 54, 55, 56 and 57).

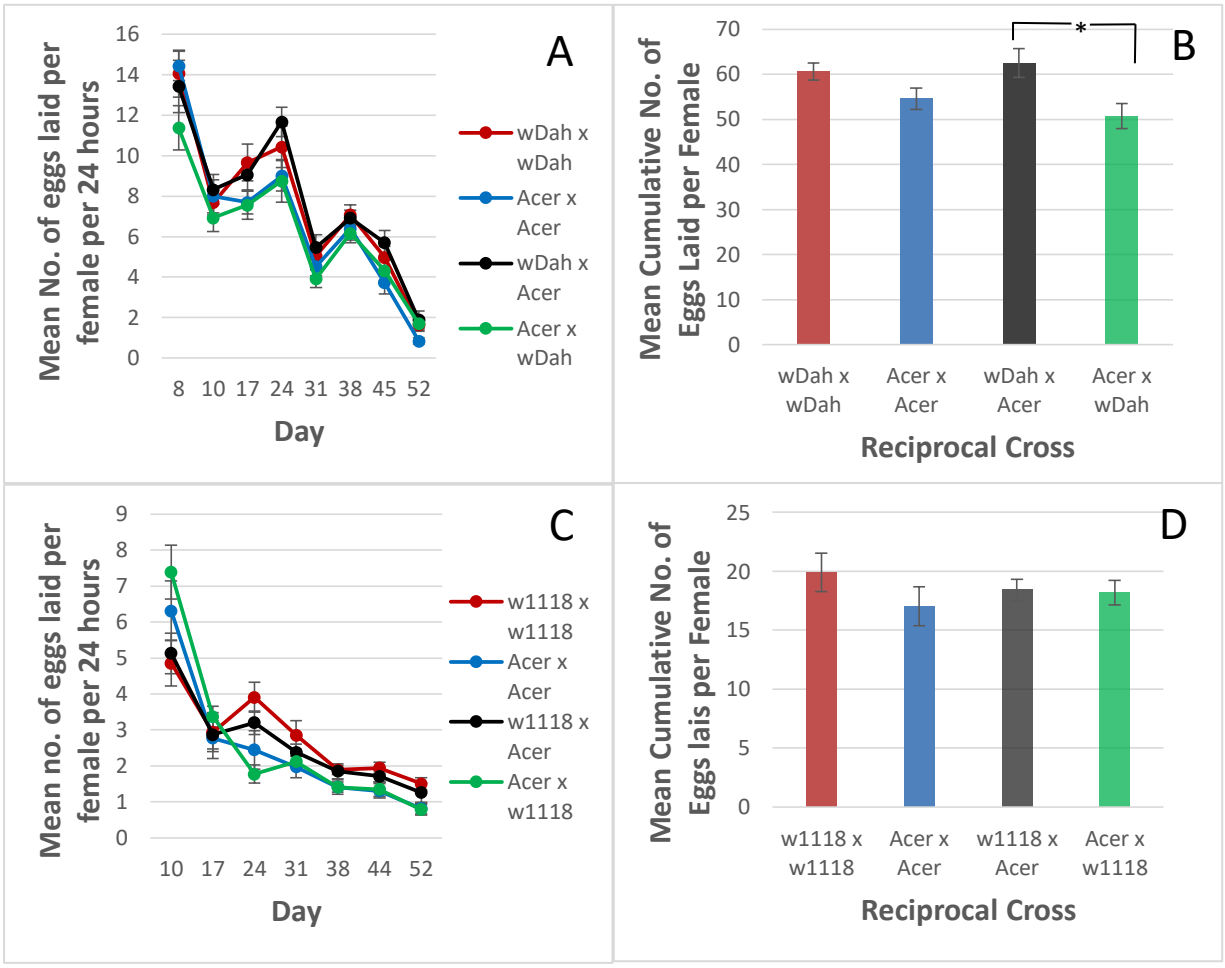

Figure 45: Reciprocal Mating Fecundity in response to feeding on DR and FF diets. (A) Mean no. of eggs laid per female per 24 hours in the *w*^{Dah} background. *w*^{Dah}: N = 10; *Acer*$^{\Delta}$: N = 10. (B) Mean cumulative no. of eggs laid in the *w*^{Dah} background. *w*^{Dah}: N = 10; *Acer*$^{\Delta}$: N = 10. (C) Mean no. eggs laid per female per 24 hours in the *w*^{1118} background. *w*^{1118}: N = 10; *Acer*$^{\Delta}$: N = 10. (D) Mean cumulative no. of eggs laid in the *w*^{1118} background. *w*^{1118}: N = 10; *Acer*$^{\Delta}$: N = 10. The first name of the cross indicates the genotype of the female while the second name indicates the genotype of the male. Data was checked for normality and data was found to be normally distributed. For mean

comparison and variance statistics see Tables 54, 55, 56 and 57. * Indicates significant differences between genotypes (P=<0.05).

In the w^{Dah} background reciprocal mating had no effect on fecundity but a trend towards reduced egg-laying for $Acer^{\Delta}$ females was seen throughout the experiment regardless of the genotype of the male that they were mated to. A significant difference in the mean cumulative no. of eggs laid per female was seen between reciprocally mated controls and reciprocally mated $Acer^{\Delta}$ females with the female controls laying significantly more eggs. Interestingly, control females reciprocally mated to $Acer^{\Delta}$ males responded normally, suggesting that any reduction in fecundity is due to $Acer^{\Delta}$ females.

	Day p-value							
Genetic Comparison	8	10	17	24	31	38	45	52
w♀ x w♂ to a♀ x a♂	0.9947	0.9893	0.2597	0.6803	0.891	0.7604	0.3769	0.3088
w♀ x w♂ to w♀ x a♂	0.9757	0.9187	0.9415	0.7704	0.9397	0.9953	0.781	0.9541
w♀ x w♂ to a♀ x w♂	0.3163	0.8782	0.2105	0.5571	0.4375	0.5484	0.8258	0.9997
a♀ x a♂ to w♀ x a♂	0.9116	0.9877	0.568	0.1779	0.5775	0.8776	0.0658	0.1221
a♀ x a♂ to a♀ x w♂	0.2106	0.7176	0.9993	0.9971	0.852	0.9849	0.8684	0.2647
w♀ x a♂ to a♀ x w♂	0.55	0.5173	0.9415	0.1209	0.1756	0.6934	0.2882	0.9743

Table 54: Tukey-Kramer HSD comparisons of means of eggs laid per female per 24 hours by age in the w^{Dah} background for normally and reciprocally mated females. w= w^{Dah} and a= $Acer^{\Delta}$. *Indicates significant difference (P=<0.05).

Genetic Comparison	Mean cumulative no. of eggs laid per Female (p-value)
w♀x w♂ to a♀x a♂	0.3795
w♀x w♂ to w♀x a♂	0.9561
w♀x w♂ to a♀x w♂	0.051
a♀x a♂ to w♀x a♂	0.1624
a♀x a♂ to a♀x w♂	0.7142
w♀x a♂ to a♀x w♂	0.0148*

Table 55: ANOVA/Pooled t-test measure of variance of mean cumulative eggs laid per female in the w^{Dah} background for normally and reciprocally mated females. w= w^{Dah} and a= $Acer^{\Delta}$. *Indicates significant difference (P=<0.05).

In the w^{1118} background (Figures 45C and 45D and Tables 56 and 57) reciprocal mating had no effect on fecundity with the mean cumulative no. of eggs per female showing no significant difference as well as little difference over the course of the experiment.

	Day (p-value)						
Genetic Comparison	10	17	24	31	38	45	52
w♀x w♂ to a♀x a♂	0.4492	0.94	0.0537	0.1311	0.1309	0.0887	0.025*
w♀x w♂ to w♀x a♂	0.948	1	0.8936	0.8349	0.9927	0.9355	0.6189
w♀x w♂ to a♀x w♂	0.0852	0.7805	0.0192*	0.5095	0.2503	0.1527	0.1332
a♀x a♂ to w♀x a♂	0.775	0.9502	0.224	0.5059	0.2181	0.2689	0.3046
a♀x a♂ to a♀x w♂	0.7758	0.4381	0.9732	0.832	0.9846	0.9931	0.9067
w♀x a♂ to a♀x w♂	0.2418	0.7598	0.0986	0.9442	0.3826	0.4036	0.7216

Table 56: Tukey-Kramer HSD comparisons of means of eggs laid per female per 24 hours by age in the w^{1118} background for normally and reciprocally mated females. w= w^{1118} and a= $Acer^{\Delta}$. *Indicates significant difference (P=<0.05).

However, a trend towards reduced egg-laying was seen for *Acer*$^{\Delta}$ females in the *w*^{Dah} background. Control females reciprocally mated to *Acer*$^{\Delta}$ males responded normally with egg-laying levels similar to normally mated controls, suggesting that any reduction in egg-laying was due to *Acer*$^{\Delta}$ females alone. Any differences in the *w*^{1118} background may have been difficult to see due to the lower number of eggs laid in this background. Further investigation with a higher N may also highlight any differences and confirm the differences seen in the *w*^{Dah} background.

Genetic Comparison			Mean cumulative no. of eggs laid per Female (p-value)
w♀x w♂	to	a♀x a♂	0.4498
w♀x w♂	to	w♀x a♂	0.8648
w♀x w♂	to	a♀x w♂	0.8103
a♀x a♂	to	w♀x a♂	0.8881
a♀x a♂	to	a♀x w♂	0.9291
w♀x a♂	to	a♀x w♂	0.9995

Table 57: ANOVA/Pooled t-test measure of variance of mean cumulative eggs laid per female in the *w*^{1118} background for normally and reciprocally mated females. w= *w*^{1118} and a= *Acer*$^{\Delta}$. *Indicates significant difference (P=<0.05).

The lifespan results for normally and reciprocally mated females showed that in both backgrounds there was no significant difference in lifespan for reciprocally mated females on standard food (5% sugar and 10% yeast) (Figure 46 and Table 58). Although in the *w*^{1118} background the log-rank statistics show a significant extension of lifespan for normally mated *Acer*$^{\Delta}$ females compared to normally mated control females, reciprocally mated *Acer*$^{\Delta}$ females to normally mated control females and reciprocally mated *Acer*$^{\Delta}$ females to reciprocally mated control females, the survival charts suggest no difference in lifespan. These data contradict the pilot data (Figure 41) which showed a lifespan extension for *Acer*$^{\Delta}$ females on standard food in the *w*^{Dah} background.

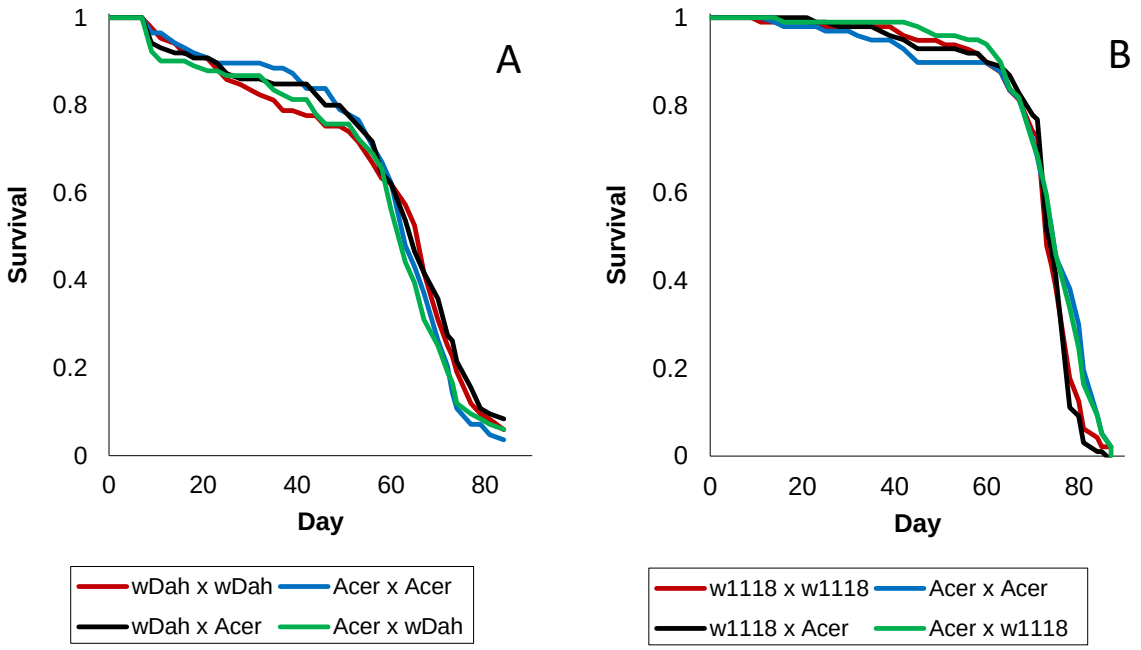

Figure 46: Lifespan for normally and reciprocally mated control and *Acer*$^{\Delta}$ females fed on standard food in the *w*^{Dah} background (A) and the *w*^{1118} background (B). (A) Median Lifespan: *w*^{Dah} x *w*^{Dah}: 67 days, N = 99; *Acer*$^{\Delta}$ x *Acer*$^{\Delta}$: 63 days, N = 98; *w*^{Dah} x *Acer*$^{\Delta}$: 65 days, N = 98; *Acer*$^{\Delta}$ x *w*^{Dah}: 63 days, N = 99. (B) Median Lifespan: *w*^{1118} x *w*^{1118}: 73 days, N = 98; *Acer*$^{\Delta}$ x *Acer*$^{\Delta}$: 75 days, N = 98; *w*^{1118} x *Acer*$^{\Delta}$: 74 days, N = 101; *Acer*$^{\Delta}$ x *w*^{1118}: 75 days, N = 100. Survival analysis was performed in JMP using the log-rank test (Table 58).

Genotype Comparison			Log-rank (p-value)
***w*^{Dah}**			
w♀x w♂	to	a♀x a♂	0.2036
w♀x w♂	to	w♀x a♂	0.8047
w♀x w♂	to	a♀x w♂	0.3328
a♀x a♂	to	w♀x a♂	0.1327
a♀x a♂	to	a♀x w♂	0.8916
w♀x a♂	to	a♀x w♂	0.2467
***w*^{1118}**			
w♀x w♂	to	a♀x a♂	0.0481*
w♀x w♂	to	w♀x a♂	0.8793
w♀x w♂	to	a♀x w♂	0.0368*
a♀x a♂	to	w♀x a♂	0.0507
a♀x a♂	to	a♀x w♂	0.8069
w♀x a♂	to	a♀x w♂	0.0378*

Table 58: Comparison of survival curves by genotype using non-parametric log rank tests with p values calculated for normally and reciprocally mated control and *Acer*$^{\Delta}$ females. *Indicates significant difference (P=<0.05).

In summary, *Acer*$^{\Delta}$ females showed the expected increase in egg-laying with an increased level of yeast in the diet in both backgrounds. However, in the *w*^{Dah} background *Acer*$^{\Delta}$ females laid significantly fewer eggs while feeding on both the DR and FF foods, suggesting *Acer*'s involvement in the egg-laying process. A decrease in egg-laying was not observed in the *w*^{1118} background but this may have been due to the lower levels of egg-laying observed in this background by control flies, therefore making it difficult to observe a change with low numbers of eggs being laid. The reciprocal mating experiment showed no difference in the *w*^{1118} background but in the *w*^{Dah} background a trend towards reduced fecundity was seen with the suggestion that this trend was caused by female *Acer*$^{\Delta}$ flies and not male *Acer*$^{\Delta}$ flies. The reciprocal mating experiment was conducted on standard food (Chapter 2.9 – Table 3) but the biggest difference in egg-laying in the *w*^{Dah} background was observed on FF food. Therefore, repetition on the FF food may give a stronger indication of the decreased fecundity of *Acer*$^{\Delta}$ females and whether the decrease is due to females, males or a mixture of both sexes.

5.3: Discussion

Once again we have seen differences between the w^{Dah} and w^{1118} backgrounds and $Acer^{\Delta}$ male and female response to dietary changes. The response of $Acer^{\Delta}$ flies to dietary changes was mixed throughout the backgrounds and the sexes for lifespan and fecundity.

$Acer^{\Delta}$ males and females in both backgrounds responded to DR normally, therefore *Acer* is not required for the normal lifespan extension by DR.

$Acer^{\Delta}$ females in the w^{Dah} background were significantly short-lived on FF food in experiment B and were also short-lived in experiment A with the result nearing significance (Figure 34 and Table 42). In the w^{1118} background, $Acer^{\Delta}$ females showed an extension of lifespan on the FF food in experiment C but showed no difference in experiment D (Figure 34 and Table 42), therefore repetition is required to establish the $Acer^{\Delta}$ phenotype in response to high food in this background. There was no difference for $Acer^{\Delta}$ females in either background on DR food. These data suggest that *Acer* modulates the response of lifespan to high nutrition in female flies but that the effect is background dependent.

The $Acer^{\Delta}$ male lifespan data results are variable. In the w^{Dah} background $Acer^{\Delta}$ males showed extended lifespan on DR food in experiment A with extension nearing significance in experiment B (Figure 36 and Table 47). This effect was the same in the w^{1118} background with extension of lifespan seen for $Acer^{\Delta}$ males in experiment C, however no difference was seen between the genotypes in experiment D, therefore lifespan needs to be repeated in this background. For both genetic backgrounds an extension in lifespan was seen on FF food (Figure 36A and D) but no difference between $Acer^{\Delta}$ males and control males was also seen (Figure 36B and C). These data suggest that in male flies *Acer* modulates the lifespan response to DR and high food but it is not required for the normal lifespan extension by DR.

The response to Low food did not differ between the sexes but did differ between the genetic backgrounds. $Acer^{\Delta}$ males and females are sensitive to low food in the w^{Dah} background but showed no difference in the w^{1118} background. These data suggest that *Acer* modulates the lifespan response to low food in the w^{Dah} background but has no effect in the w^{1118} background.

Fecundity differed between the two backgrounds with w^{Dah} female controls being significantly more fecund than w^{1118} females. Analysis of fecundity showed that

Acer$^{\Delta}$ females were significantly less fecund than controls in the w^{Dah} background on both DR and FF foods but showed no difference in fecundity compared to w^{1118} control females on either food (Figure 35 and Tables 43-46). This difference between the two backgrounds suggests that *Acer* modulates the response of fecundity to changing diet in the w^{Dah} background but not in the w^{1118} background.

The reciprocal mating experiment on standard food in the w^{Dah} background showed no difference in lifespan or fecundity for *Acer*$^{\Delta}$ females compared with controls however a trend towards decreased egg-laying for *Acer*$^{\Delta}$ females was observed with no effect of male mating. This suggests that any decrease in fecundity is solely due to females. There was no difference in lifespan or fecundity between the *Acer*$^{\Delta}$ females and controls in the w^{1118} background. With no lifespan or fecundity significance found on standard food and the reduction in total eggs seen in the w^{Dah} background on both DR and FF food (Figure 35), it is important to repeat this experiment in both genetic backgrounds on the DR and FF foods to assess whether the reduction in fecundity is solely due to female flies as thought.

Chapter 6: The role of *Acer* and diet in stress responses

6.1: Introduction.

Many different methods have been used to test the stress resistance of model organisms, including *Drosophila melanogaster*. These methods include, cold and heat tolerance, oxidative stress and starvation as ways of analysing stress on flies (Broughton, et al., 2005; Wit, et al., 2013).

Cold stress in *Drosophila* has been monitored in many different ways. Burger & Promislow., (2006) and Le Bourg., (2013), monitored the lifespan of flies after exposure to cold conditions. After the infliction of the cold stress, it was found that recovery time increased with age and that a prior cold stress at an early age aided recovery at older ages. It was also found that cold stress shortened lifespan, especially in male flies (Burger & Promislow, 2006). Starvation increased resistance to 16 hour cold stress and males fared better than females, with 24 hour starvation increasing resistance to cold stress in young flies but not at older ages (Le Bourg, 2013).

Recovery from cold stress, known as cold coma recovery, has also been used as a marker of stress resistance. In this assay, flies are placed on ice for a recorded number of hours and then moved to warmer conditions to recover. A fly was said to have recovered when it could stand (Gerken, et al., 2016). Diet was found to have little effect on cold coma recovery on young flies but older flies recovered quicker when they had been fed higher levels of food before the experiment (Burger, et al., 2007). Flies fed live yeast were found to recover more quickly from cold stress compared to flies that were fed sugar alone. When this was compared to autolysed brewer's yeast there was little effect on recovery suggesting that yeast is important to recovery from cold stress (Colinet & Renault, 2014). Cold resistance has been found to be heritable but does not correlate with resistance to other stress phenotypes (Gerken, et al., 2016). In *Drosophila ananassae* latitude was shown to be important in cold resistance as equatorial populations recovered significantly slower than populations from more temperate latitudes (Sisodia & Singh, 2010). This interesting as this study has tested cold resistance in the w^{Dah} background, which is an equatorial genetic background, and the w^{1118} background, which is a temperate background.

Heat stress has been monitored by analysing lifespan effects after a short exposure to heat (Morgan & Mackay, 2006) whereas (Le Rohellec & Le Bourg, 2009)

and (Broughton, et al., 2005) monitored fly resistance to heat until death. Starvation before heat shock was found to have no effect on heat resistance (Le Bourg, 2013) and insulin signalling reduced mutant mNSC-ablated flies were shown to be susceptible to heat shock (Broughton, et al., 2005). Longevity was found not to affect heat stress resistance (Wit, et al., 2013) and diet in some studies has been found to have little impact on heat stress resistance (Le Rohellec & Le Bourg, 2009). Nielsen, et al. (2006) found that *Acer* was down-regulated in response to heat stress, suggesting that *Acer* was not favourable to heat stress resistance.

Oxidative stress in *Drosophila* has been tested by adding different measures of paraquat (Broughton, et al., 2005, Mockett, et al., 2001) or hydrogen peroxide (Broughton, et al., 2008) to the flies' food and then monitoring lifespan until death. Broughton, et al. (2005) found that reduced insulin signalling mNSC-ablated flies were resistant to paraquat oxidative stress but Broughton, et al. (2008) found that the knockdown of DILP2 alone did not promote resistance to hydrogen peroxide stress. At older ages DR has been found to significantly decrease resistance to paraquat oxidative stress (Burger, et al., 2007) as well as prior starvation reducing resistance to hydrogen peroxide stress (Le Bourg, 2013).

Starvation stress has been tested by placing flies on agar medium for the duration of their life at a set time after eclosion (Broughton, et al., 2005, Wit, et al., 2013, Burger, et al., 2007). Starvation resistance has been observed in mNSC-ablated flies which have reduced levels of insulin signalling (Broughton, et al., 2005), flies with DILP2 knocked down (Broughton, et al., 2008) and long-lived strains (Wit, et al., 2013) among others. Burger, et al., (2007), found that DR initially increased resistance to starvation at young ages but this was reversed at middle and older ages. When flies previously fed on live yeast had the live yeast removed from their diet, survival decreased under starvation conditions in males, but interestingly a lack of live yeast increased starvation resistance in mated females (Le Rohellec & Le Bourg, 2009). Disruption to the fat body clock by expressing the dominant negative forms of *Clock* and *Cycle* (CLK and CYC) has been found to increase sensitivity to starvation through a reduction in glycogen levels (Xu, et al., 2008), while increased lipid levels have been found to be associated with starvation resistance (Ballard, et al., 2008). Feeding has been found to be reduced and sleep has been found to be increased in starvation resistant flies (Masek, et al., 2014).

In this study it has been shown that *Acer*$^{\Delta}$ females showed a reduction in glycogen levels on DR and FF food after two days feeding but a raised level of lipid and glycogen after four days on starvation food (see Chapter 4).Sleep was increased on Low food for female flies in the w^{Dah} background (see Chapter 3). It is possible that Acer modulates the response to starvation or is involved in the detection of the starvation threshold, therefore any change in response to starvation for $Acer^{\Delta}$ flies would be interesting.

It was decided to test whether *Acer* was potentially involved in stress responses and if diet played any part in the stress response itself.

6.1.1: Aims

With *Acer's* role in other nutritional responses we tested *Acer's* role, if any, in mediating stress responses, including chill coma recovery, heat stress and oxidative stress under different dietary conditions. Starvation will be analysed separately as flies will be removed from food completely.

6.1.2: Research Design

The stress resistance of $Acer^{\Delta}$ males and females in the w^{Dah} and w^{1118} backgrounds was analysed under DR and FF conditions.

For measurement of cold resistance, the chill coma recovery experiment was taken from Broughton, et al., (2005) and was employed. 9 day old females and 10 day old males (n=50) were placed in empty vials (5 flies/vial) at 4ºC for 4 hours and their time to recovery was recorded at 25ºC. Recovery was defined as the flies being able to stand.

Heat stress was measured as at 37ºC and 39ºC. 9 day old females and 10 day old males (n=~40) were placed in clean vials (10 flies/vial) and lifespan recorded until death.

Starvation resistance was taken from Broughton, et al. (2008) and flies (n=100) were placed onto 1% agar medium. This was done 3 days after eclosion to provide flies with water but no food with lifespan recorded until death. The oxidative stress

method was also taken from Broughton, et al. (2008) using 5% hydrogen peroxide in a 1.5% agar and 5% sugar mixture. Lifespan was recorded until death (n=100).

6.2: Results

6.2.1: *Acer* is not involved in the normal response to cold stress resistance

In a previous study diet had been shown to have no effect on cold resistance (Burger, et al., 2007) but with *Acer's* possible link to diet, $Acer^{\Delta}$ flies were compared to controls in the w^{Dah} and w^{1118} backgrounds. Flies were subjected to four hours at 4ºC after feeding on DR or FF food for a week prior to test their recovery at 25ºC. Recovery was defined as the flies being able to stand and recovery took place in the empty vials that were used to implement the cold stress.

The results showed that female controls in the w^{Dah} background (Figures 47A and 47B and Table 59 and 60) responded to the increase in yeast in experiment A by taking longer to recover on FF food but no response to diet was observed in experiment B with controls on both foods recovering at similar times to the flies on the DR food in experiment A.

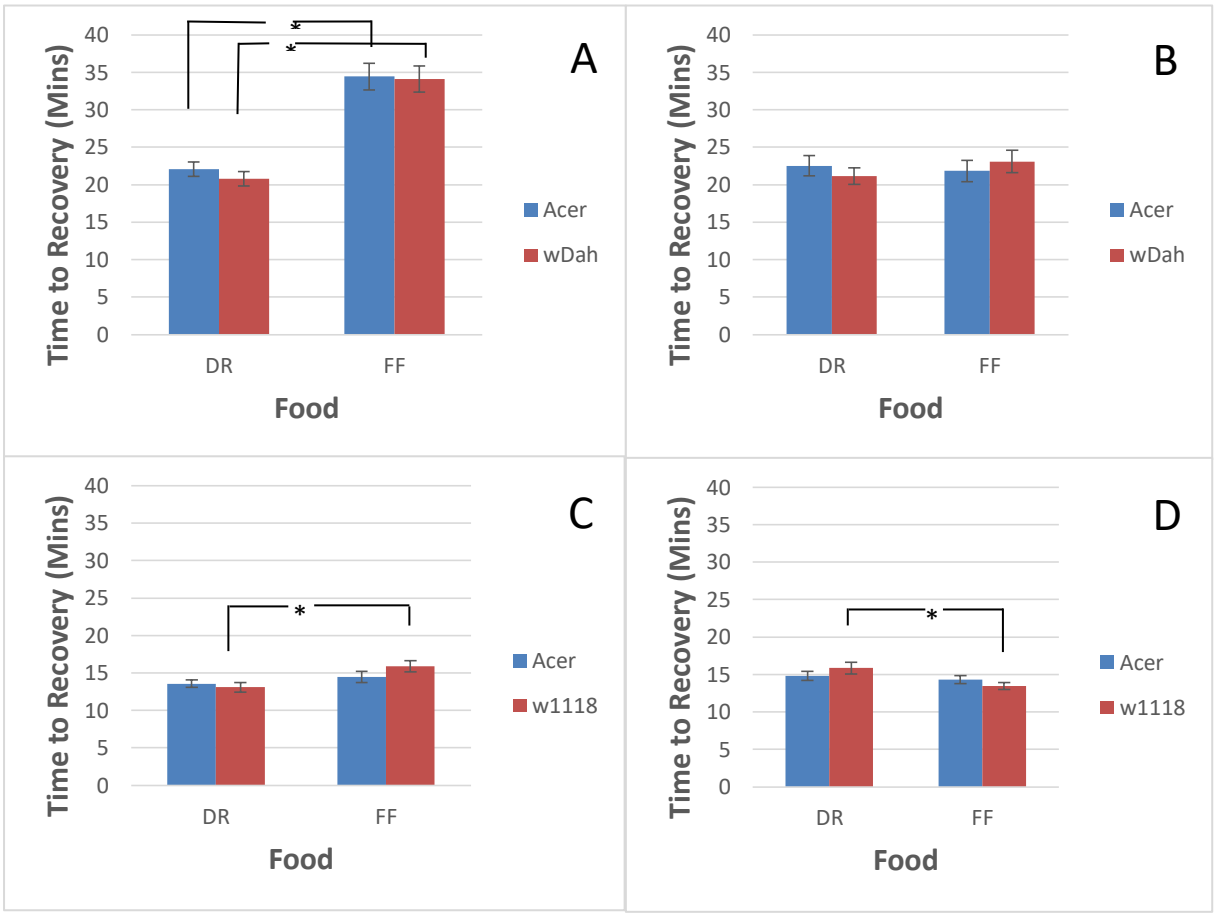

Figure 47: Female recovery response to cold stress (Mins) on DR and FF diets. (A and B) Recovery from cold stress in the w^{Dah} background. (A) w^{Dah}: DR N = 45; FF N = 45. $Acer^{\Delta}$: DR N = 45; FF N = 45.

(B) w^{Dah}: DR N = 42; FF N = 38. $Acer^{\Delta}$: DR N = 45; FF N = 42. (C and D) Recovery from cold stress in the w^{1118} background. (C) w^{1118}: DR N = 50; FF N = 49. $Acer^{\Delta}$: DR N = 50; FF N = 48. (D) w^{1118}: DR N = 48; FF N = 49. $Acer^{\Delta}$: DR N = 44; FF N = 44. Data was checked for normality. (A) was found to be normally distributed. (B, C and D) were found to be log distributed. Data is shown as raw data and analysed in log-transformation. For mean comparison and variance statistics see Tables 59 and 60. *Indicates significant difference between foods (P=<0.05).

Controls in the w^{1118} background (Figure 47C and 47D and Tables 59 and 60) initially showed a similar response to the w^{Dah} background with an increased recovery time on the FF food in experiment C but in experiment D controls showed a decrease in recovery time on the FF food compared to the DR food. The mean recovery times were similar for both experiments.

Genotype	Experiment	Food	p-value compared to $Acer^{\Delta}$
w^{Dah}	A	DR	0.3337
	B	DR	0.6713
	A	FF	0.8853
	B	FF	0.5005
w^{1118}	C	DR	0.3161
	D	DR	0.2906
	C	FF	0.1546
	D	FF	0.1879

Table 59: ANOVA/Pooled t-test measure of variance of mean recovery time in response to cold stress for females in the w^{Dah} and w^{1118} backgrounds comparing controls to $Acer^{\Delta}$ females on DR and FF foods. *Indicates significant difference (P=<0.05).

Genotype/Food	Experiment	p-value Comparing DR & FF
w^{Dah}	A	<.0001*
	B	0.4656
$w^{Dah};Acer^{\Delta}$	A	<.0001*
	B	0.6793
w^{1118}	C	0.0055*
	D	0.0029*
$w^{1118};Acer^{\Delta}$	C	0.609
	D	0.3953

Table 60: Tukey-Kramer HSD comparisons of means comparing the response of cold stress on DR and FF foods for control and $Acer^{\Delta}$ females. *Indicates significant difference (P=<0.05).

Acer$^{\Delta}$ females responded in the same way as control females in the w^{Dah} background for both experiments but *Acer*$^{\Delta}$ females in the w^{1118} background initially showed no response to diet compared to controls in experiment C but no genotype difference was observed. In experiment D, *Acer*$^{\Delta}$ females, like experiment C, showed no response to diet compared to the decrease in recovery time on FF food shown by control females but again no genotype difference was observed (Tables 59 and 60).

Comparison between the genetic backgrounds showed that w^{Dah} females are sensitive to cold, with longer recovery time, compared to w^{1118} females on the DR (p=<.0001) and FF (p=<.0001) foods.

The overall response of control females in both backgrounds to diet was mixed with the w^{Dah} background initially showing a response to diet (A) and then showing no response (B). In the w^{1118} background the initial response showed an increase in recovery time on FF food (C) but then showed a decrease in recovery time on FF food (D). *Acer*$^{\Delta}$ females in the w^{Dah} background reacted normally compared to controls however in the w^{1118} background *Acer*$^{\Delta}$ females showed no response to diet compared to controls but not enough for a significant difference on either food. There were no differences between the genotypes in either background when compared on the same foods, suggesting that *Acer* is not involved in the cold stress response in female flies.

Male controls in the w^{Dah} background (Figure 48A and 48B and Table 61) showed an initial increase in recovery time from cold stress in experiment A but in the experiment B no difference was seen. In the w^{1118} background (Figure 48C and 48D and Table 61) there was no difference between the diets for experiment C however control males in experiment D showed a decrease in recovery time on FF food compared to DR food.

In both genetic backgrounds *Acer*$^{\Delta}$ males showed no response of cold chill coma to changing diet, therefore *Acer*$^{\Delta}$ males showed a significant increase in recovery time on the DR food in the w^{Dah} background when the controls responded to diet (A) but not in the repeat experiment where controls did not respond to diet. In the w^{1118} background when the controls responded to changing diet (D) *Acer*$^{\Delta}$ males showed no response and recovered quicker on the DR food but when no response to diet was observed in controls *Acer*$^{\Delta}$ males responded normally.

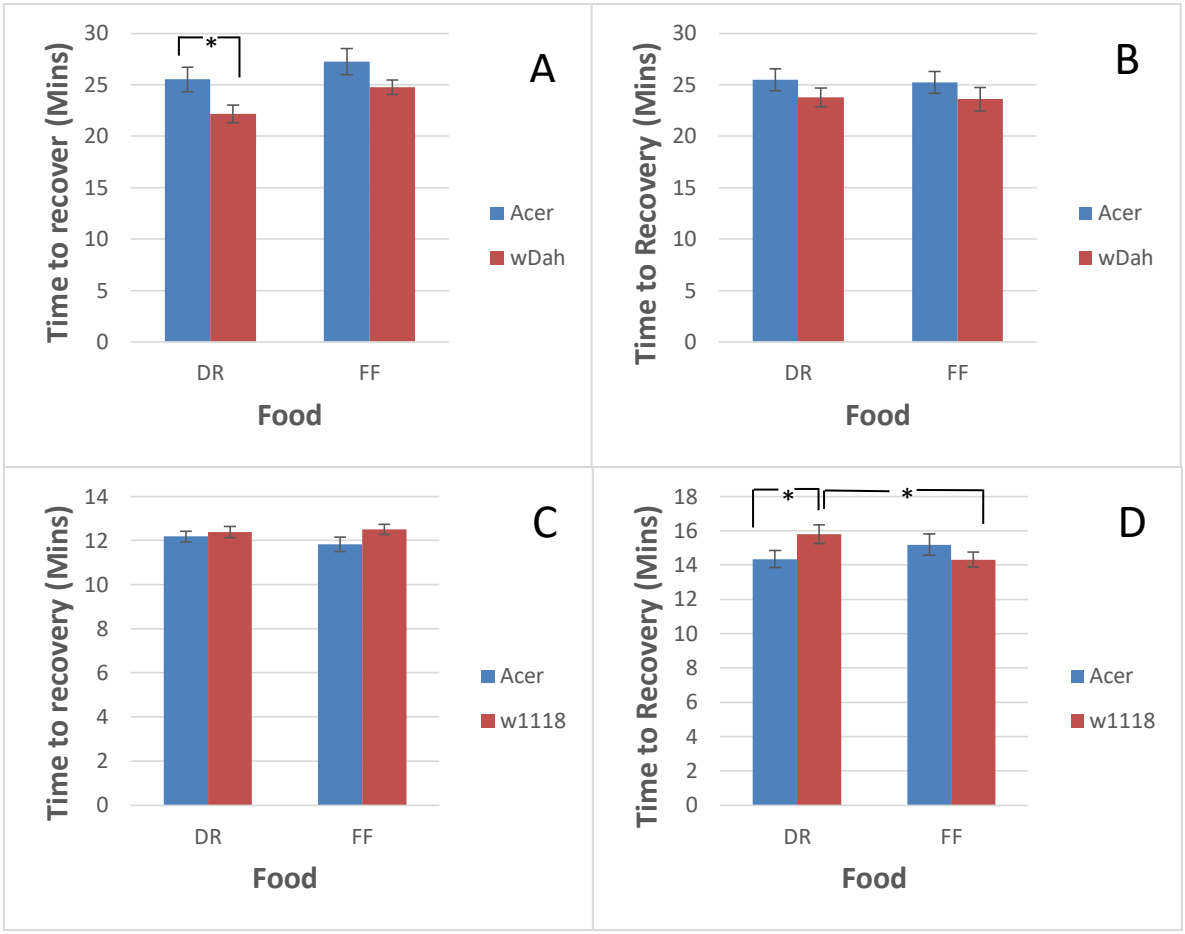

Figure 48: Male recovery response to cold stress (Mins) on DR and FF diets. (A and B) Recovery from cold stress in the w^{Dah} background. (A) w^{Dah}: DR N = 47; FF N = 47. $Acer^{\Delta}$: DR N = 46; FF N = 47 (B) w^{Dah}: DR N = 48; FF N = 48. $Acer^{\Delta}$: DR N = 48; FF N = 49. (C and D) Recovery from cold stress in the w^{1118} background. (C) w^{1118}: DR N = 48; FF N = 48. $Acer^{\Delta}$: DR N = 50; FF N = 47. (D) w^{1118}: DR N = 40; FF N = 4.4 $Acer^{\Delta}$: DR N = 47; FF N = 43. Data was checked for normality. (A and D) were found to be log distributed. Raw data is show and analysed as log-transformation. (B and C) were found to be normally distributed. For mean comparison and variance statistics see Tables 61 and 62. *Indicates significant difference (P=<0.05).

Comparison of genetic background showed that, like females, males in the w^{Dah} background took longer to recover on both DR (p=<.0001) and FF (p=<.0001) food than w^{1118} males and are therefore more sensitive to cold.

Genotype	Experiment	Food	p-value compared to $Acer^{\Delta}$
w^{Dah}	A	DR	0.0272*
	B	DR	0.2159
	A	FF	0.25
	B	FF	0.2968
w^{1118}	C	DR	0.5859
	D	DR	0.0475*
	C	FF	0.1032
	D	FF	0.3416

Table 61: ANOVA/Pooled t-test measure of variance of mean recovery time in response to cold stress for males in the w^{Dah} and w^{1118} backgrounds comparing controls to $Acer^{\Delta}$ males on DR and FF foods. *Indicates significant difference (P=<0.05).

Genotype/Food	Experiment	p-value comparing DR & FF
w^{Dah}	A	0.008*
	B	0.908
$w^{Dah};Acer^{\Delta}$	A	0.353
	B	0.861
w^{1118}	C	0.7275
	D	0.0491*
$w^{1118};Acer^{\Delta}$	C	0.3938
	D	0.3354

Table 62: Tukey-Kramer HSD comparisons of means comparing the response of cold stress on DR and FF foods for control and $Acer^{\Delta}$ males. *Indicates significant difference (P=<0.05).

In summary, male response to cold stress is inconclusive in both backgrounds however when a response to diet was shown by controls $Acer^{\Delta}$ males did not respond suggesting, as seen previously in nutrient responsive phenotypes, that $Acer^{\Delta}$ males showed a lack of response to changing diet.

In male and females flies it appears that *Acer* is not involved in the response to cold stress but the equatorial w^{Dah} background is sensitive to cold temperatures compared to the temperate w^{1118} background.

6.2.2: *Acer* modulates the response of high heat stress resistance to high diet in the w^{Dah} background

Diet has previously been shown to have little effect on heat stress resistance (Le Rohellec & Le Bourg, 2009) but with *Acer's* possible involvement in nutrition we tested the effect of food as well as genotype.

The results for females in the w^{Dah} background (Figures 49A and 49B and Table 63) showed that controls did not respond to diet regarding heat stress in experiment A at 37ºC but in experiment B at 39ºC controls on the FF food lived longer than controls on the DR food, suggesting that high food levels may be protective against high temperature heat stress in this background. In the w^{1118} background, the control females (Figures 49C and 49D and Table 63) did not respond to diet in terms of heat stress at either temperature.

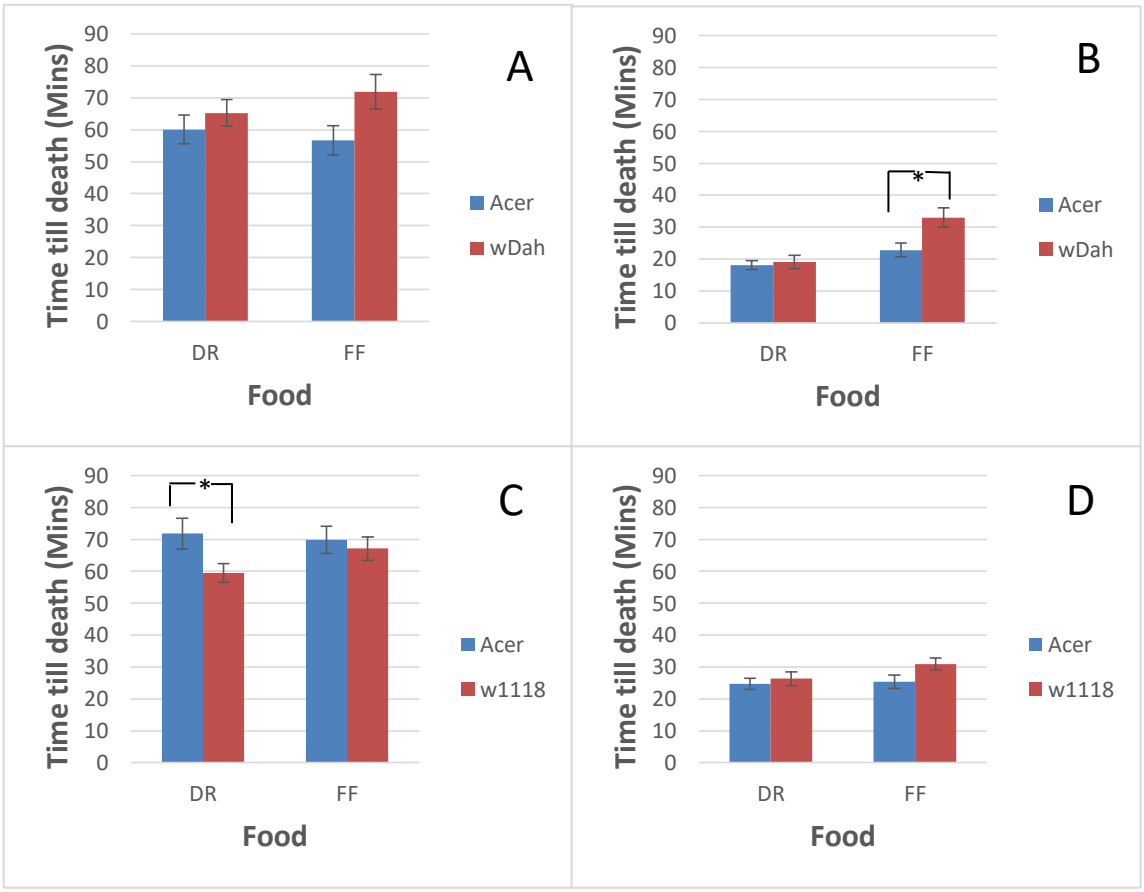

Figure 49: Female heat stress response until death (Mins) on DR and FF diets. (A) Response to 37ºC heat stress and (B) Response to 39ºC heat stress in the w^{Dah} background. (A) w^{Dah}: DR N = 48; FF N = 50. $Acer^{\Delta}$: DR N = 50; FF N = 50. (B) w^{Dah}: DR N = 43; FF N = 43. $Acer^{\Delta}$: DR N = 48; FF N = 46. (C)

Response to 37°C heat stress and (D) Response to 39°C heat stress in the w^{1118} background. (C) w^{1118}: DR N = 40; FF N = 40. $Acer^{\Delta}$: DR N = 38; FF N = 36. (D) w^{1118}: DR N = 44; FF N = 47. $Acer^{\Delta}$: DR N = 50; FF N = 49. Data was checked for normality. (A and B) were found to be log distributed. Data is shown as raw data and analysed as log-transformed. (C and D) were found to be normally distributed. For mean comparison and variance statistics see Tables 63 and 64. *Indicates significant difference (P=<0.05).

Acer$^{\Delta}$ females in the w^{Dah} background in both experiments did not respond to diet even when the controls did in experiment B, although the increase in time till death was nearing significance (Table 63). Therefore *Acer*$^{\Delta}$ females were sensitive to heat stress on the FF food compared to controls in this experiment. In the w^{1118} background *Acer*$^{\Delta}$ females, like controls, did not respond to diet but a significant resistance was seen on the DR food in the 37ºC experiment (C) but a trend towards sensitivity on the FF food was seen in 39ºC experiment (D) although it was not quite significant.

Genotype	Experiment	Temperature	Food	p-value compared to *Acer*$^{\Delta}$
w^{Dah}	A	37°C	DR	0.2829
	B	39°C	DR	0.9223
	A	37°C	FF	0.0673
	B	39°C	FF	0.0181*
w^{1118}	C	37°C	DR	0.0314*
	D	39°C	DR	0.551
	C	37°C	FF	0.6383
	D	39°C	FF	0.052

Table 63: ANOVA/Pooled t-test measure of variance of mean recovery time in response to heat stress for females in the w^{Dah} and w^{1118} backgrounds comparing controls to $Acer^{\Delta}$ females on DR and FF foods. *Indicates significant difference (P=<0.05).

Genotype/Food	Experiment	Temperature	p-value comparing DR & FF
w^{Dah}	A	37°C	0.582
	B	39°C	0.0003*
w^{Dah};*Acer*$^{\Delta}$	A	37°C	0.8569
	B	39°C	0.0738
w^{1118}	C	37°C	0.1101
	D	39°C	0.1086
w^{1118};*Acer*$^{\Delta}$	C	37°C	0.7675
	D	39°C	0.7982

Table 64: Tukey-Kramer HSD comparisons of means comparing the response of heat stress on DR and FF foods for control and $Acer^{\Delta}$ females. *Indicates significant difference (P=<0.05).

Temperature analysis showed that in both backgrounds and on both DR and FF foods control females died quicker at 39ºC than at 37ºC (p=<.0001), indicating that higher temperatures result in stronger heat stress. At 37ºC there was no difference in survival time between the control genotypes on either DR (p=0.2825) or FF food (p=0.4817), however at 39ºC w^{1118} females were resistant to heat stress on DR food compared to w^{Dah} females (p=0.0188) but not on FF food (p=0.5564).

The data presented suggests that *Acer* modulates the response of heat stress in female flies but the effect is background and temperature dependent.

Male controls in both backgrounds (Figure 50 and Table 65), like females, did not respond to dietary changes when heat stress was inflicted at either 37ºC (A and C) or 39ºC (B and D).

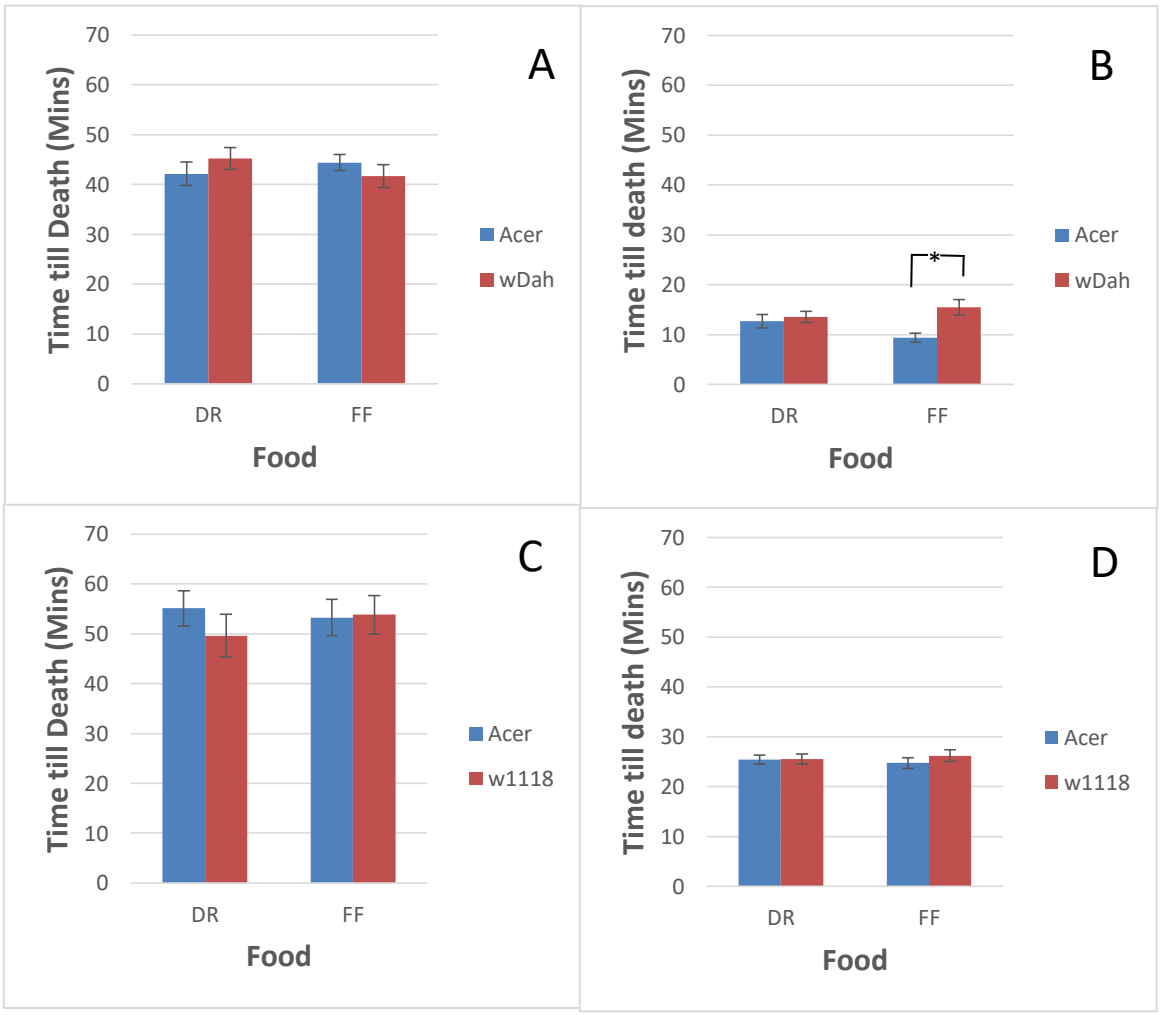

Figure 50: Male heat stress response until death (Mins) on DR and FF diets. (A) Response to 37°C heat stress and (B) Response to 39°C heat stress in the w^{Dah} background. (A) w^{Dah}: DR N = 50; FF N =

50. *Acer*$^{\Delta}$: DR N = 42; FF N = 50. (B) *w*^{Dah}: DR N = 34; FF N = 30. *Acer*$^{\Delta}$: DR N = 26; FF N = 33. (C) Response to 37^{o}C heat stress and (D) Response to 39^{o}C heat stress in the *w*^{1118} background. (C) *w*^{1118}: DR N = 40; FF N = 39. *Acer*$^{\Delta}$: DR N = 40; FF N = 40. (D) *w*^{1118}: DR N = 47; FF N = 45. *Acer*$^{\Delta}$: DR N = 47; FF N = 43. Data was checked for normality. (A and D) were found to be normally distributed. (B and C) were found to be log distributed. Data is shown as raw data and analysed as log transformed. For mean comparison and variance statistics see Tables 65 and 66. *Indicates significant difference (P=<0.05).

Acer$^{\Delta}$ males also showed no significant difference in resistance to heat stress between the different diets but did show a significant sensitivity to heat stress on the FF food in the *w*^{Dah} background at 39ºC similar to females (B).

Genotype	Experiment	Temperature	Food	p-value Compared to *Acer*$^{\Delta}$
w^{Dah}	A	37^{o}C	DR	0.349
	B	39^{o}C	DR	0.5971
	A	37^{o}C	FF	0.3373
	B	39^{o}C	FF	0.0015*
w^{1118}	C	37^{o}C	DR	0.1106
	D	39^{o}C	DR	0.9367
	C	37^{o}C	FF	0.9292
	D	39^{o}C	FF	0.332

Table 65: ANOVA/Pooled t-test measure of variance of mean recovery time in response to heat stress for males in the *w*^{Dah} and *w*^{1118} backgrounds comparing controls to *Acer*$^{\Delta}$ males on DR and FF foods. *Indicates significant difference (P=<0.05).

Genotype/Food	Experiment	Temperature	p-value Comparing DR & FF
w^{Dah}	A	37^{o}C	0.2781
	B	39^{o}C	0.4369
w^{Dah};*Acer*$^{\Delta}$	A	37^{o}C	0.4162
	B	39^{o}C	0.0703
w^{1118}	C	37^{o}C	0.3623
	D	39^{o}C	0.6416
w^{1118};*Acer*$^{\Delta}$	C	37^{o}C	0.5845
	D	39^{o}C	0.6241

Table 66: Tukey-Kramer HSD comparisons of means comparing the response of heat stress on DR and FF foods for control and *Acer*$^{\Delta}$ males. *Indicates significant difference (P=<0.05).

Temperature analysis showed that in both backgrounds and on both DR and FF foods, like females, control males died quicker at 39ºC than at 37ºC, indicating that

higher temperatures resulted in higher levels of heat stress. At 37ºC there was no difference in survival time between w^{Dah} and w^{1118} males on DR food (p=0.6045), however on FF food w^{1118} males survived for longer (p=0.0198). At 39ºC w^{1118} males were significantly longer lived than w^{Dah} males on both the DR (p=<.0001) and FF foods (p=<.0001).These data suggest that w^{1118} males are resistant to heat stress compared to w^{Dah} males.

In summary, control males in both backgrounds did not respond to diet in terms of heat stress at either temperature but w^{1118} males are resistant to heat stress at both temperatures compared to w^{Dah} males. w^{1118} control females did not respond to nutrition, however at 39ºC control females in the w^{Dah} background were resistant to heat stress on the FF food. $Acer^{\Delta}$ males and females in the w^{Dah} background are sensitive to high heat stress (39ºC) on FF food compared to controls but are resistant to lower levels of heat stress (37ºC) in the w^{1118} background on DR food.

6.2.3: *Acer* modulates oxidative stress resistance, the direction of which is dependent on genetic background

Oxidative stress resistance was measured by feeding male and female flies 5% hydrogen peroxide in an agar sugar mix and the flies were monitored until death.

In this experiment the control response of oxidative stress to diet in females in the w^{Dah} background (Figure 51B) was for DR to protect against oxidative stress with extended lifespan (p=<.0001) compared to FF food. The normal response of oxidative stress to diet for w^{1118} females (Figure 51D) was no effect of DR on longevity (p=0.0726).

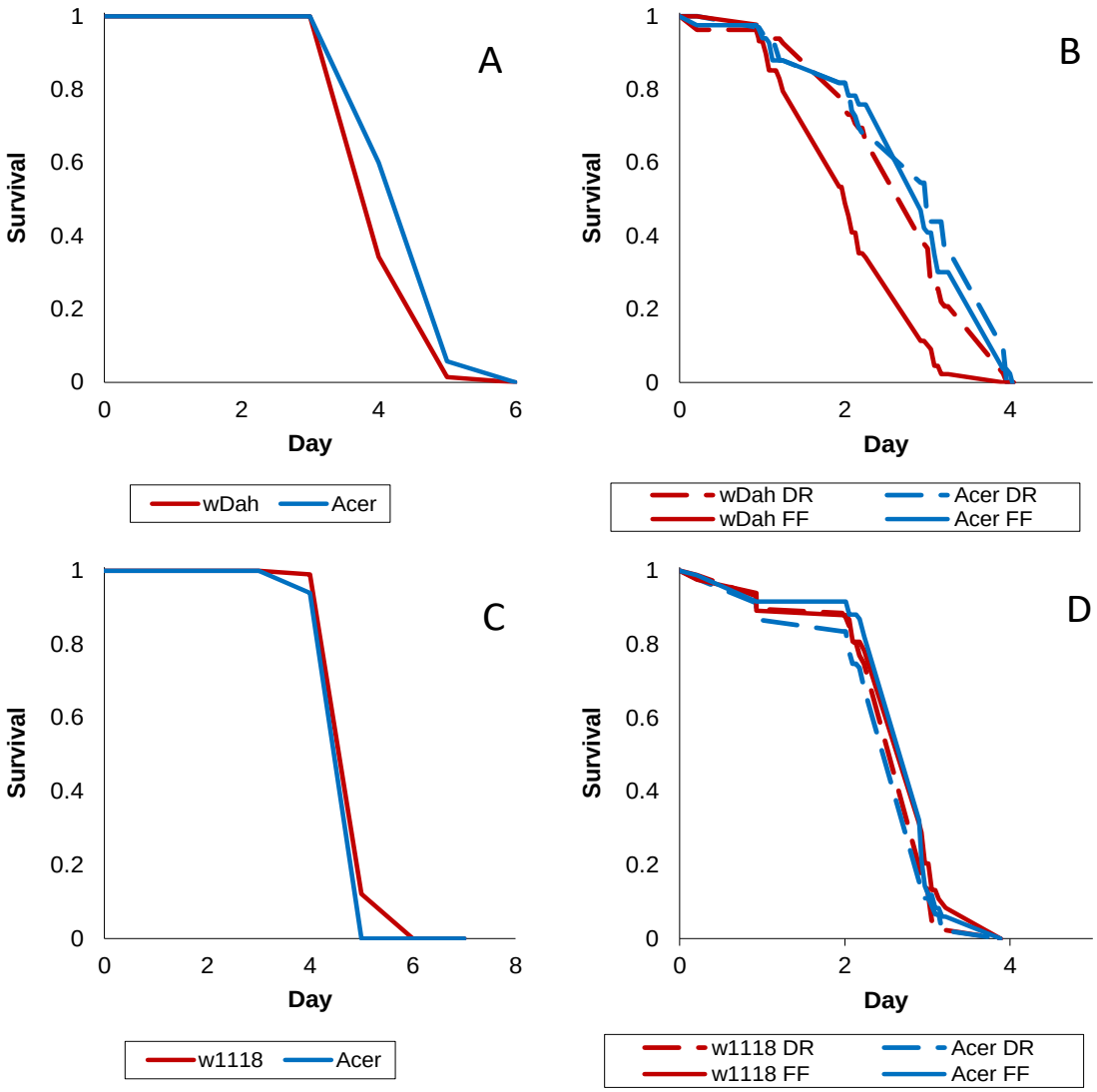

Figure 51: Female Lifespan in response to oxidative stress. (A and B) w^{Dah} background. (A) Median Lifespan: w^{Dah} = 4 days, N = 70; $Acer^{\Delta}$ = 5 days, N = 70. (B) Median lifespan after being placed on DR

and FF food for 2 days: w^{Dah}: DR = 2.92 days, N = 82; FF = 2 days, N = 89. $Acer^{\Delta}$: DR = 3 days, N = 66; FF = 2.92 days, N = 84. (C and D) w^{1118} background. (C) Median lifespan: w^{1118} = 5 days, N = 100; $Acer^{\Delta}$ = 5 days, N = 100. (D) Median lifespan after being placed on DR and FF food for 2 days: w^{1118}: DR = 2.9 days, N = 87; FF = 2.9 days, N = 83. $Acer^{\Delta}$: DR = 2.9 days, N = 91; FF = 2.9 days, N = 84. Survival analysis was performed in JMP using the log-rank test (Table 67).

Genotype Comparison	Experiment	Food	Log-rank (p-value)
w^{Dah} *to* $Acer^{\Delta}$	A		0.0018*
	B	DR	0.0442*
	B	FF	<.0001*
w^{1118} *to* $Acer^{\Delta}$	C		<.0001*
	D	DR	0.5612
	D	FF	0.6354

Table 67: Female comparison of survival curves by genotype for oxidative stress using the log rank test in JMP for each food comparing controls and $Acer^{\Delta}$ females. *Indicates significant difference (P=<0.05).

$Acer^{\Delta}$ females in the w^{Dah} background (Figures 51A and 51B and Table 67) showed resistance to oxidative stress in experiment A and also showed resistance on both the DR and FF foods compared to controls in experiment B. Unlike control females, $Acer^{\Delta}$ females did not respond to dietary changes with a lifespan extension on DR food (p=0.8066).

In the w^{1118} background (Figures 51C and 51D and Table 67), $Acer^{\Delta}$ females responded normally to oxidative stress in experiment C but in experiment D, $Acer^{\Delta}$ females responded to dietary change by an extended lifespan on FF food (p=0.0397), although the median lifespans were the same for both DR and FF foods (Figure 51D). Although a significant statistical difference is seen between genotypes in experiment C (Table 67), it is clear that the median lifespan was not significantly different for $Acer^{\Delta}$ females compared to control females.

Acer is involved in the normal response of oxidative stress resistance to DR in female flies in the w^{Dah} background but not the w^{1118} background.

The normal response of oxidative stress to diet in male controls in the w^{Dah} background (Figures 52A and 52B) was for DR to protect against oxidative stress (p=<.0001), the same as females in this background. In the w^{1118} background male

controls (Figures 52C and 52D), like females, showed that diet had no effect on oxidative stress resistance (p=0.394).

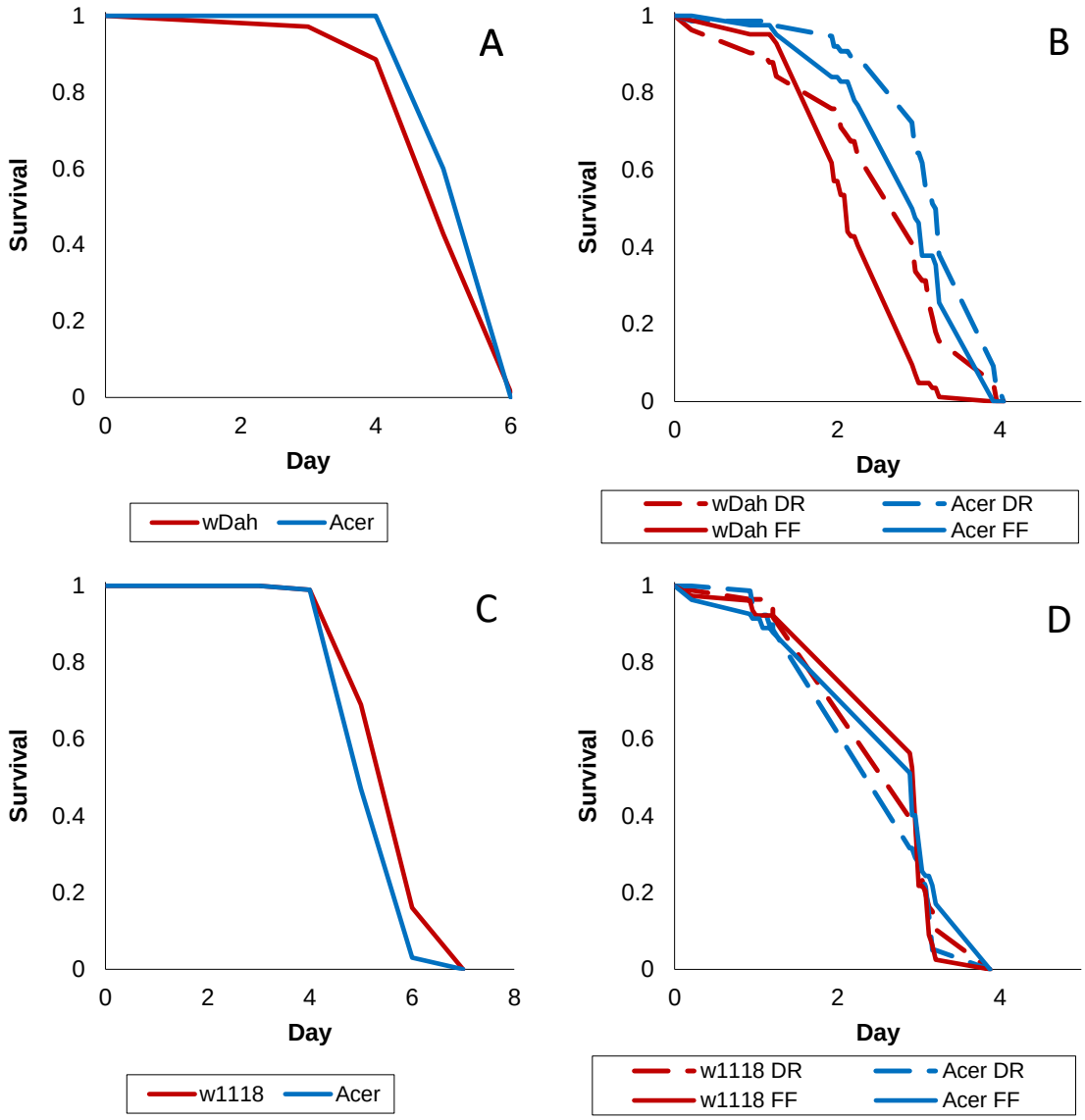

Figure 52: Male Lifespan in response to oxidative stress. (A and B) w^{Dah} background. (A) Median Lifespan: w^{Dah} = 5 days, N = 69; $Acer^{\Delta}$ = 6 days, N = 70. (B) Median Lifespan after being placed on DR and FF food for 2 days: w^{Dah}: DR = 2.92 days, N = 83; FF = 2.13 days, N = 84. $Acer^{\Delta}$: DR = 3.23 days, N = 76; FF = 2.94 days, N = 82 (C and D) w^{1118} background. (C) Median Lifespan: w^{1118} = 6 days, N = 100; $Acer^{\Delta}$ = 5 days, N = 100. (D) Median Lifespan after being placed on DR and FF food for 2 days: w^{1118}: DR = 2.89 days, N = 86; FF = 2.96 days, N = 78. $Acer^{\Delta}$: DR = 2.89 days, N = 79; FF = 2.92 days, N = 82. Survival analysis was performed in JMP using the log-rank test (Table 68).

Acer$^{\Delta}$ males in the *w*^{Dah} background (Figure 52), like controls, responded to DR with a lifespan extension on DR food compared to controls (p=0.0055). They also showed resistance to oxidative stress in experiment A and were resistant on both the DR and FF foods in experiment B compared to controls (Table 68). *Acer*$^{\Delta}$ males in the *w*^{1118} background did not show a response to diet however the statistics showed a reduction in lifespan on DR food compared to FF food that was nearing significance (p=0.0536). *Acer*$^{\Delta}$ males were sensitive to oxidative stress compared to controls in experiment C but no difference was observed when comparing DR and FF food to controls in experiment D (Table 68).

Genotype Comparison	Experiment	Food	Log-rank (p-value)
w^{Dah} *to Acer*$^{\Delta}$	A		0.0191*
	B	DR	<.0001*
	B	FF	<.0001*
w^{1118} *to Acer*$^{\Delta}$	C		0.0001*
	D	DR	0.276
	D	FF	0.1125

Table 68: Male comparison of survival curves by genotype for oxidative stress using the log rank test in JMP for each food comparing controls and *Acer*$^{\Delta}$ males. *Indicates significant difference (P=<0.05).

Oxidative stress in the *w*^{1118} background is not affected by diet in male and female flies. In this background *Acer*$^{\Delta}$ males and females show a slight sensitivity to oxidative stress.

In the *w*^{Dah} background DR protects against oxidative stress in control male and female flies and *Acer*$^{\Delta}$ males and females are resistant to oxidative stress independent of food showing an extended lifespan on both DR and FF food. *Acer*$^{\Delta}$ males, like controls, showed an extended lifespan on DR food compared to FF food, however *Acer*$^{\Delta}$ females did not show the normal response to DR, suggesting that *Acer* modulates the response of oxidative stress resistance to DR in female flies in this background.

Male and female flies in the *w*^{Dah} background in response to oxidative stress respond to dietary changes and *Acer* is involved in the dietary response. However, there is no response of oxidative stress to diet in male and female flies in the *w*^{1118} background and therefore there is no response of *Acer*. These data, along with

previous data involving nutrient-responsive phenotypes such as sleep, nutrient storage and fecundity, suggest that when a dietary response is observed in controls, *Acer* is often required for that response.

6.2.4: *Acer* plays a role in starvation resistance, the direction of which is dependent on genetic background

Starvation resistance was tested by placing flies onto 1% agar medium to provide water but no nutrients at three days of age and flies were monitored until death.

In the w^{Dah} background (Figures 53A and 53B and Table 69) $Acer^{\Delta}$ females were resistant to starvation when compared to controls. In the w^{1118} background (Figures 53C and 53D and Table 69) $Acer^{\Delta}$ females were sensitive to starvation compared to controls.

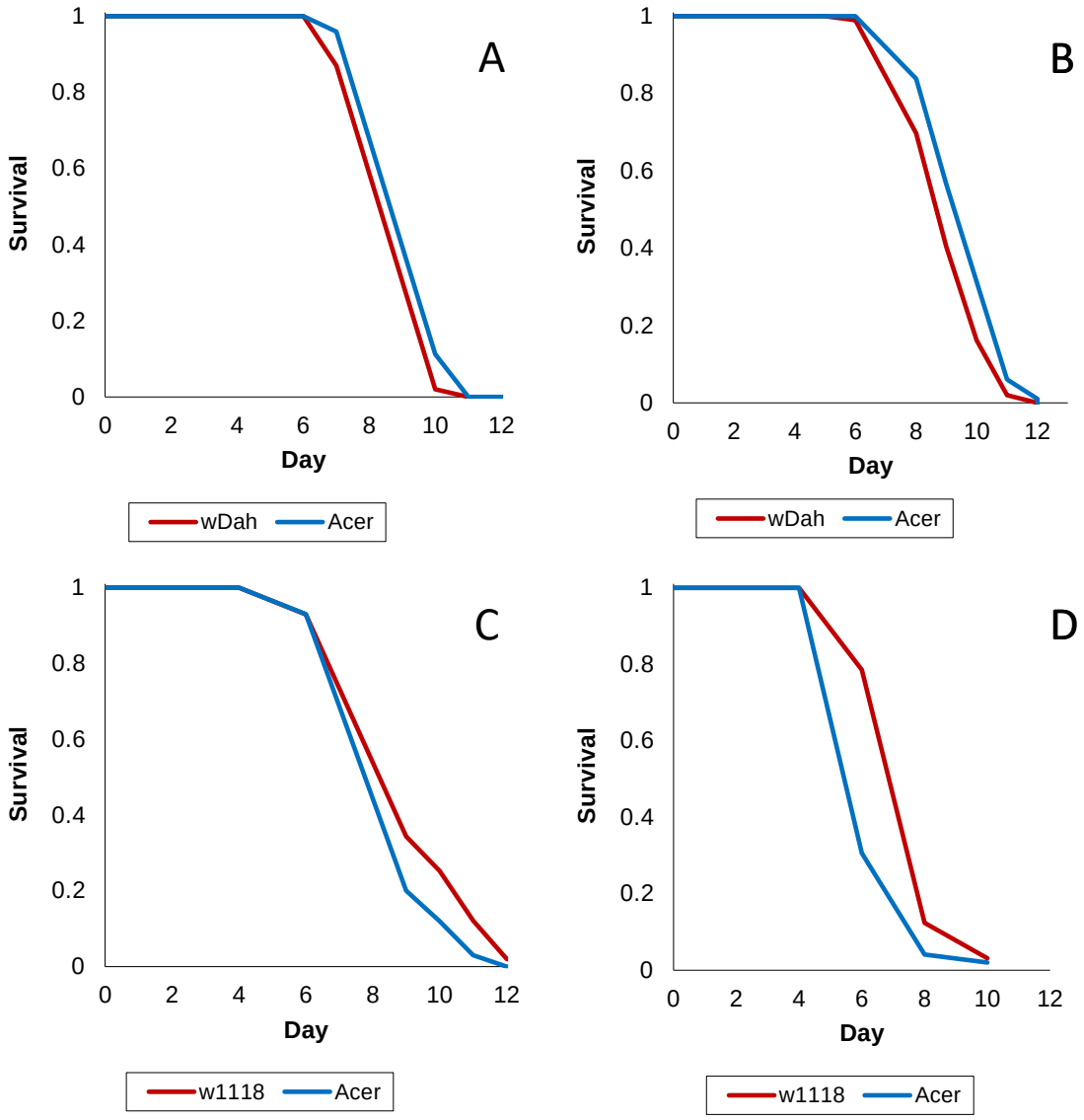

Figure 53: Female Lifespan in response to starvation. (A and B) w^{Dah} background. (A) Median Lifespan: w^{Dah} = 10 days, N = 100; $Acer^{\Delta}$ = 10 days, N = 100. (B) Median Lifespan (Days): w^{Dah} = 9 days,

N = 100; *Acer*$^{\Delta}$ = 10 days, N = 100. (C and D) *w*^{1118} background. (C) Median Lifespan: *w*^{1118} = 9 days, N = 100; *Acer*$^{\Delta}$ = 9 days, N = 100. (D) Median Lifespan: *w*^{1118} = 8 days, N = 98; *Acer*$^{\Delta}$ = 6 days, N = 97. Survival analysis was performed in JMP using the log-rank test (Table 69).

Genotype comparison	Experiment	Log-rank (p-value)
w^{Dah} *to Acer*$^{\Delta}$	A	0.001*
	B	0.0025*
w^{1118} *to Acer*$^{\Delta}$	C	0.0089*
	D	<.0001*

Table 69: Female comparison of survival curves by genotype for starvation using the log rank test in JMP comparing controls and *Acer*$^{\Delta}$ females. *Indicates significant difference (P=<0.05).

In the *w*^{Dah} background (Figure 54A and 54B and Table 70) *Acer*$^{\Delta}$ males were resistant to starvation compared to controls in both experiments.

In the *w*^{1118} background (Figure 54C and 54D and Table 70) in experiment C there was no difference between controls and *Acer*$^{\Delta}$ males but in experiment D *Acer*$^{\Delta}$ males were sensitive to starvation, the same as *w*^{1118} females.

Genotype comparison	Experiment	Log-rank (p-value)
w^{Dah} *to Acer*$^{\Delta}$	A	0.0001*
	B	0.0008*
w^{1118} *to Acer*$^{\Delta}$	C	0.4614
	D	<.0001*

Table 70: Male comparison of survival curves by genotype for starvation using the log rank test with in JMP comparing controls and *Acer*$^{\Delta}$ males. *Indicates significant difference (P=<.005).

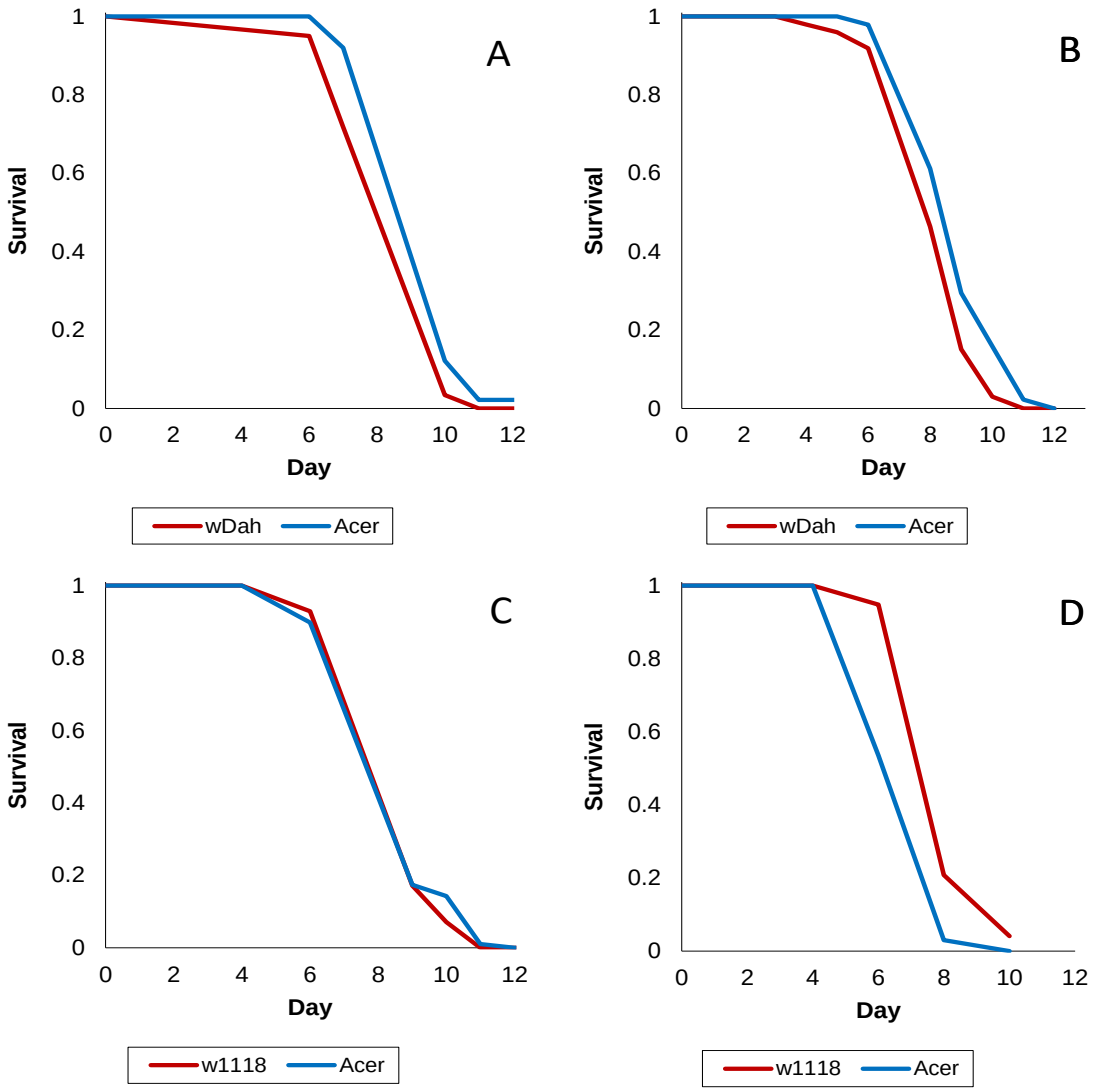

Figure 54: Male Lifespan in response to starvation. (A and B) w^{Dah} background. (A) Median Lifespan: w^{Dah} = 10 days, N = 100; $Acer^{\Delta}$ = 10 days, N = 98. (B) Median Lifespan: w^{Dah} = 8 days, N = 100; $Acer^{\Delta}$ = 9 days, N = 100. (C and D) w^{1118} background. (C) Median Lifespan: w^{1118} = 9 days, N = 100; $Acer^{\Delta}$ = 9 days, N = 100. (D) Median Lifespan: w^{1118} = 8 days, N = 96; $Acer^{\Delta}$ = 8 days, N = 100. Survival analysis was performed in JMP using the log-rank test (Table 64).

Like the oxidative stress phenotype there are differences in *Acer's* response to starvation in the w^{Dah} and w^{1118} backgrounds. $Acer^{\Delta}$ males and females are resistant to starvation in the w^{Dah} background whereas they are sensitive to starvation in the w^{1118} background, suggesting that *Acer's* role in starvation resistance is dependent on genetic background.

6.3: Discussion

As previously seen with previous phenotypes the w^{Dah} and w^{1118} backgrounds behave differently and the effect of the loss of *Acer* is dependent on genetic background. $Acer^{\Delta}$ males and females showed a lack of response to changing diet in sleep, nutrient storage and lifespan.

Cold stress appears to be potentially affected by diet but the results are inconclusive with some experiments showing a response to diet and others not. We have previously shown that *Acer* is involved in nutrient responsive phenotypes such as sleep, nutrient storage and lifespan but it does not appear to be involved in the normal cold stress response, however when a response to diet was observed in the controls $Acer^{\Delta}$ males showed no response as seen with previous phenotypes. The results confirm differences seen in the response of cold recovery in populations of *Drosophila melanogaster* (Guerra, et al., 1997) and *Drosophila ananassae* (Sisodia & Singh, 2010) where equatorial populations took longer to recover from cold stress compared to temperate populations. In this study the equatorial w^{Dah} background displayed sensitivity to cold by taking longer to recover from cold stress than the temperate w^{1118} background for both sexes.

The heat stress response showed that temperature is an important factor in the length of survival. Flies in both backgrounds and for both sexes were short-lived at the higher temperature of 39ºC than 37ºC regardless of food, suggesting a higher heat stress at the higher temperature. Males in the w^{1118} background were significantly resistant to heat stress compared to males in the w^{Dah} background at 39ºC and on FF food at 37ºC. Females in the w^{1118} background were resistant to heat stress at 39ºC on DR food compared to w^{Dah} females. This is surprising as the w^{Dah} background is an equatorial population and the w^{1118} background is a temperate population. The equatorial population would be expected to be heat resistant compared to the temperate population (Guerra, et al., 1997). However, temperate populations are more exposed to temperature changes and are therefore less sensitive to temperature change than equatorial populations (Kobey & Montooth, 2013). This suggests that the amplitude of the temperature change may constitute the main stress rather than the actual temperature itself. Control females in the w^{Dah} background showed an increase in survival time on FF food at 39ºC compared to DR food but $Acer^{\Delta}$ females showed no response to diet and were short-lived compared to controls. In the w^{1118} background

there was no response to diet at either temperature but *Acer*$^{\Delta}$ females were long-lived on the DR food at 37ºC. Male controls in both backgrounds did not respond to dietary change but *Acer*$^{\Delta}$ males were short-lived at 39ºC compared to *w*^{Dah} controls. These data suggest that *Acer* modulates the response of high heat stress to high food in the *w*^{Dah} background for male and female flies but in the *w*^{1118} background *Acer* has little or no effect.

Oxidative stress resistance is another phenotype that differed with genetic background. In the *w*^{Dah} background DR is protective against oxidative stress in male and female controls but in the *w*^{1118} background there is no difference between DR and FF food. *Acer*$^{\Delta}$ females in the *w*^{Dah} background showed resistance to oxidative stress but did not respond to diet normally. *Acer*$^{\Delta}$ females were resistant to oxidative stress on both the DR and FF foods with no difference of lifespan observed between the two diets. This lack of response to diet is similar to other nutrient responsive phenotypes, such as sleep (Chapter 3) and glycogen storage (Chapter 4). *Acer*$^{\Delta}$ males in the *w*^{Dah} background were, like females, resistant to oxidative stress on both foods but the DR food was still protective with an extended lifespan compared to FF food. This is similar to reduced insulin signalling mutants such as, mNSC-ablated mutants (Broughton, et al., 2005) and *dilp2-3,5* mutants (Grönke, et al., 2010), which are resistant to oxidative stress. Knockdown and overexpression of the G-coupled protein receptor methuselah, which is required for the normal release of neurotransmitters, has also been shown to result in resistance to oxidative stress (Gimenez, et al., 2013). In the *w*^{1118} background *Acer*$^{\Delta}$ males and females were initially found to be susceptible to oxidative stress but showed the normal response on DR and FF food.

Starvation resistance, like oxidative stress, differed between the two genetic backgrounds. *Acer*$^{\Delta}$ males and females in the *w*^{Dah} background showed resistance to starvation similar to long-lived flies reduced insulin signalling mutants (Broughton, et al., 2005) and long-lived flies in general (Wit, et al., 2013). However, in the *w*^{1118} background *Acer*$^{\Delta}$ males and females showed sensitivity to starvation. Starvation resistance has been linked with higher lipid and glycogen levels (Ballard, et al., 2008; Xu, et al., 2008) and more sleep (Masek, et al., 2014). Previously we have shown that *Acer*$^{\Delta}$ females in the *w*^{Dah} background slept longer on Low food and after four days and on starvation food have higher lipid and glycogen stores. These phenotypes are thought to aid in starvation resistance and *Acer*$^{\Delta}$ males and females were found to be starvation resistant in this background. Interestingly, these phenotypes are not seen

in w^{1118} females which are sensitive to starvation. The increased glycogen and lipid levels are also not seen in males in the w^{Dah} background which are starvation resistant, suggesting that the starvation resistance in the w^{Dah} background may have a different cause for male flies or that starvation resistance in $Acer^{\Delta}$ females is not a result of higher lipid and glycogen levels.

In conclusion, *Acer* modulates the response of diet to heat and oxidative stress but the response is background and sex dependent. *Acer* also modulates the response to starvation stress in a background dependent manner but is not involved in the response to cold stress.

Chapter 7: Expression Analysis

7.1: Introduction

Acer's involvement in nutrient responsive phenotypes (see previous chapters) suggested the possibility that *Acer* is linked to nutrient-sensing signalling pathways. The genetic background and sex dependent effects due to loss of *Acer* further suggested that *Acer* may be differentially expressed. Svetec, et al. (2015) has already shown that *Acer* expression is differential in different genetic backgrounds and at night time.

The reduction in insulin signalling in *Drosophila* has been found to extend lifespan and increase resistance to starvation (Broughton & Partridge, 2009). A mutation in the insulin receptor substrate *chico* was found to extend lifespan and improve the age-related decline of negative geotaxis behaviour (Libert, et al., 2008). Originally seven *Drosophila* insulin-like peptides were discovered as ligands for the *Drosophila* insulin receptor (dInR) (Brogiolo, et al., 2001) and DILP8 was discovered recently (Colombani, et al., 2012). The DILP producing median neurosecretory cells (mNSCs) of the fly brain are where *dilps 2*, *3* and *5* are readily expressed throughout development and in adult flies (Grönke, et al., 2010; Nässel, et al., 2015). *Dilp5* expression in adults responds to changing diet with an increase in transcription with increasing food levels, suggesting a role in the normal response to diet (Broughton, et al., 2010). When DILP5 levels alone were reduced a compensatory mechanism of increased DILP2 occurred suggesting that DILP expression is interlinked (Grönke, et al., 2010). Ablation of the mNSCs results in reduced levels of insulin signalling in the brain and these flies are long-lived and resistant to starvation and oxidative stress but are sensitive to cold and heat stress and show reduced fecundity (Broughton, et al., 2005). However, the overall knock-down of DILP2 only resulted in flies responding normally to longevity and fecundity and although a slight starvation resistance was seen there was no resistance to oxidative stress, but higher levels of the trehalose carbohydrate store was seen (Broughton, et al., 2008). *Dilp5* is also expressed in within the abdomen in the follicle cells of the ovary and the renal tubules of adult flies and *dilps 2*, *3* and *5* are all expressed in the embryonic mesoderm and the IPCs in larval stages of development (Nässel, et al., 2015).

Dilp6 is expressed within the fly fat bodies in the head and abdomen in adult flies and the *Drosophila* transcription factor (dFoxO) is required for *dilp6* expression (Slaidina, et al., 2009; Nässel, et al., 2015). *Dilp6* expression is induced under starvation conditions showing a high level of expression in comparison to fed conditions (Slaidina, et al., 2009). The expression of *dilp6* in the fly fat body is interesting because *Acer* is also expressed within the fat body of the fly (Carhan, et al., 2010), therefore suggesting a potential link to IIS.

Dilp7 is expressed within the midgut during embryonic development and the abdominal neuromeres of the ventral nerve cord (VNC) during larval and adult stages (Nässel, et al., 2015) and is thought to be involved in the decision process of egg-laying in female flies (Yang, et al., 2008).

Dilp 4 expression is required for midgut development in the embryonic and larval stages but so far has not been analysed in adults (Nässel, et al., 2015).

To test *Acer's* link to the IIS signalling pathway the expression of *DILPs* within the fly head and the fly body for male and female flies was tested. Due to the expression of *dilps 2*, *3* and *5* within the mNSC's of the fly brain of and *dilp6* expression within the head fat body of the fly, the expression of these *dilps* was tested in the fly head. The expression of *dilp5* within the ovaries and renal tubules, *dilp6* within the abdominal fat body and *dilp7* within the abdominal neuromeres led to testing the expression of these *dilps* within the fly body. *Dilp4* expression has been found within the midgut of fly embryos and larvae so the expression of this *dilp* was tested in the adult body.

As previously seen in Broughton, et al. (2010) DILP5 protein levels in the mNSCs increases with increasing diet so DILP5 protein in the fly head was tested to identify if DILP5 expression was effected by the loss of *Acer*.

As well as differential expression in different genetic background (Svetec, et al., 2015), *Acer* expression has also been found to be down-regulated when heat stress was applied (Nielsen, et al., 2006) and increased under sleep deprivation and starvation conditions (Thimgan, et al., 2015). In our investigations into *Acer* we have found differences between the w^{Dah} and w^{1118} genetic backgrounds. To investigate if *Acer* is differentially expressed in these backgrounds and whether there is a difference between the sexes, *Acer* expression on DR and FF food over 24 hours was tested in both genetic backgrounds and for both sexes.

7.1.1: Aims

With *Acer's* involvement in the reaction of nutrient responsive phenotypes to nutrition we wanted to test *Acer's* link, if any, to the nutrients-sensing signalling pathways such the IIS signalling pathway under different dietary conditions.

In our investigations into *Acer's* role in nutrition we have discovered that the w^{Dah} and w^{1118} genetic backgrounds respond differently to nutrition in a range of phenotypes and that *Acer's* role may differ between backgrounds. *Acer* had previously been shown to be differentially expressed at night (Svetec, et al., 2015) so it was investigated whether there was a difference in expression of *Acer* between the two genetic backgrounds in response to diet.

7.1.2: Research Design

The expression of *dilps* 2, 3, 5 and 6 were analysed in the fly head while the expression of *dilps* 4, 5, 6, and 7 were analysed in the fly body for $Acer^{\Delta}$ male and female flies in the w^{Dah} background. The expression was analysed under Starvation, DR and FF conditions by quantitative RT-PCR (QPCR) (N=3) after four days on Starvation, Low, DR and FF food.

Acer expression was monitored in males and females in the w^{Dah} and w^{1118} backgrounds under DR and FF conditions after four days feeding on these diets (N=6) for 24 hours at 4 hour intervals.

7.2: Results

7.2.1: *dilp* Expression

Transcript levels of *dilps* within the fly head and body of *Acer*$^{\Delta}$ females and males compared to controls in the *w*^{Dah} background were analysed.

The results confirmed that *dilp5* in controls (Figure 55C and Table 71) was the only *dilp* that responded to changing diet when analysed in the female head with increasing levels of transcription with increasing diet (Broughton, et al., 2010). *Acer*$^{\Delta}$ females also responded to increasing diet with increasing levels of *dilp5* transcription but levels of *dilp5* did not rise as much as controls on DR food.

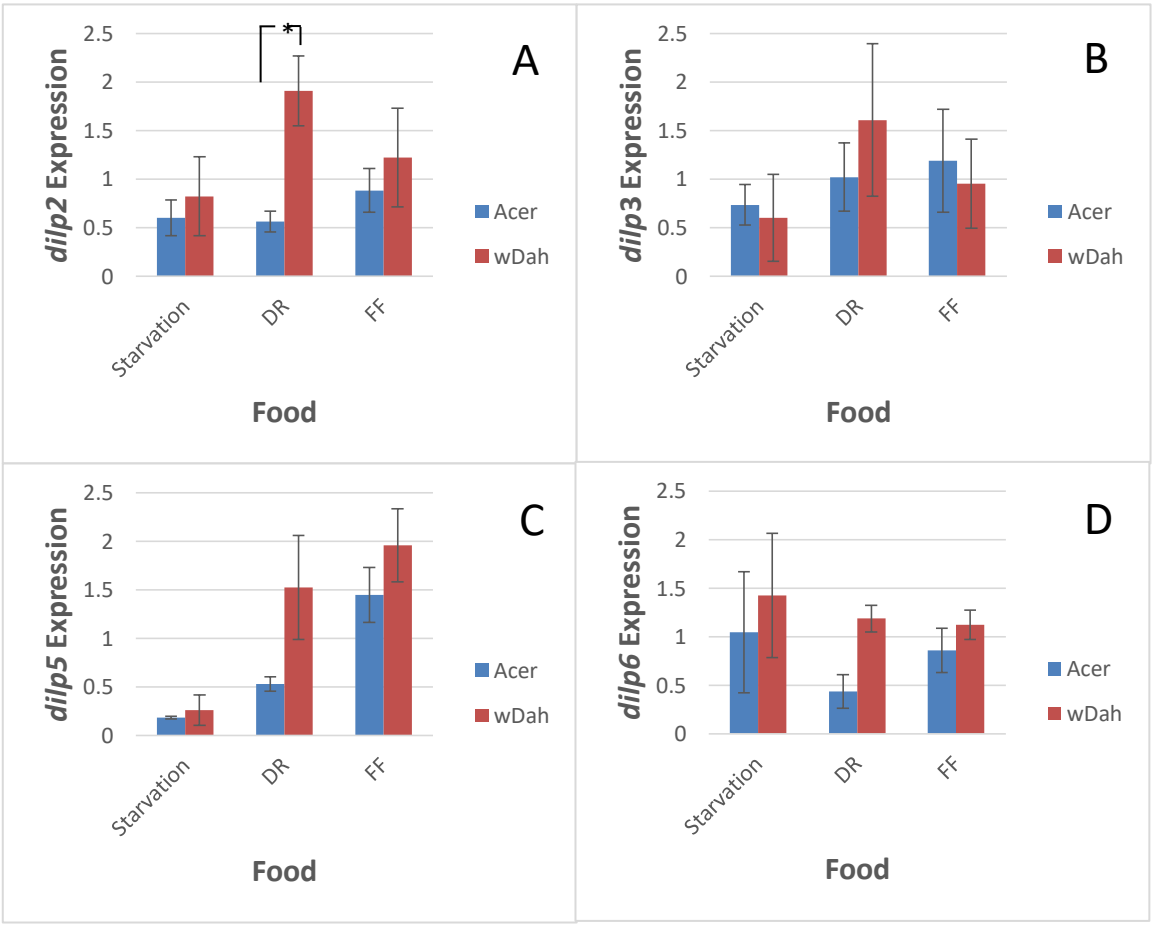

Figure 55: *dilp* Expression in Female Heads in the *w*^{Dah} background on Starvation, DR and FF diets. (A) *dilp2* (B) *dilp3* (C) *dilp5* (D) *dilp6*. (A) *w*^{Dah}: Starvation N = 3; DR N = 2; FF N = 3. *Acer*$^{\Delta}$: Starvation N = 2; DR N = 3; FF N = 3. (B) *w*^{Dah}: Starvation N = 3; DR N = 2; FF N = 3. *Acer*$^{\Delta}$: Starvation N = 2; DR N = 3; FF N = 3. (C) *w*^{Dah}: Starvation N = 3; DR N = 2; FF N = 3. *Acer*$^{\Delta}$: Starvation N = 2; DR N = 3; FF N = 3. (D) *w*^{Dah}: Starvation N = 3; DR N = 2; FF N = 3. *Acer*$^{\Delta}$: Starvation N = 2; DR N = 3; FF N = 3. Data was

checked for normality. (A, B and D) were found to be log-distributed and data is shown as raw data and analysed as log-transformed. (C) data was normally distributed. For mean comparison and variance statistics see Tables 71 and 72. *Indicates significant difference between genotypes (P=<0.05).

Genotype/DILP	Food Comparison	p-value
DILP2		
w^{Dah}	Starvation - DR	0.3001
	Starvation - FF	0.7045
	DR - FF	0.6416
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.9889
	Starvation - FF	0.6701
	DR - FF	0.5271
DILP3		
w^{Dah}	Starvation - DR	0.4628
	Starvation - FF	0.8741
	DR - FF	0.7006
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.8998
	Starvation - FF	0.774
	DR - FF	0.9557
DILP5		
w^{Dah}	Starvation - DR	0.0802
	Starvation - FF	0.0334*
	DR - FF	0.9044
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.0245*
	Starvation - FF	0.0014*
	DR - FF	0.0189*
DILP6		
w^{Dah}	Starvation - DR	0.9996
	Starvation - FF	0.9889
	DR - FF	0.9908
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.4739
	Starvation - FF	0.9939
	DR - FF	0.459

Table 71: Tukey-Kramer HSD comparisons of means comparing *dilp* expression in the female head with changing diet for controls and $Acer^{\Delta}$ females. *Indicates significant difference (P=<0.05).

The N of this experiment is low on Starvation food for $Acer^{\Delta}$ females (N= 2) for all *dilps* and w^{Dah} controls on DR food (N= 2) for all *dilps*, therefore a higher N is required to confirm our findings. Although not significant, there is a trend towards $Acer^{\Delta}$

females transcribing lower levels of all *dilps* on the DR and FF foods but not Starvation food in the female head.

DILPs	Food	Comparison to $Acer^{\Delta}$ (p-value)
DILP2		
w^{Dah}	Starvation	0.8568
	DR	0.0265*
	FF	0.654
DILP3		
w^{Dah}	Starvation	0.8383
	DR	0.4861
	FF	0.7548
DILP5		
w^{Dah}	Starvation	0.9374
	DR	0.0897
	FF	0.3293
DILP6		
w^{Dah}	Starvation	0.6998
	DR	0.1075
	FF	0.3805

Table 72: ANOVA/Pooled t-test measure of variance of mean *dilp* expression in female heads comparing $Acer^{\Delta}$ females and controls on Starvation, DR and FF foods. *Indicates significant difference (P=<0.05).

The results in the male head showed that, like females, *dilp5* in controls was the only *dilp* that significantly modulates transcription with dietary changes (Figure 56 and Table 73). Interestingly, $Acer^{\Delta}$ males showed a heightened response to diet with increased transcription levels of *dilp2* and *dilp5* with the $Acer^{\Delta}$ males showing a trend of higher transcription than controls on FF food. This is in contrast to the effect in females where there was a reduced response to diet in $Acer^{\Delta}$ females.

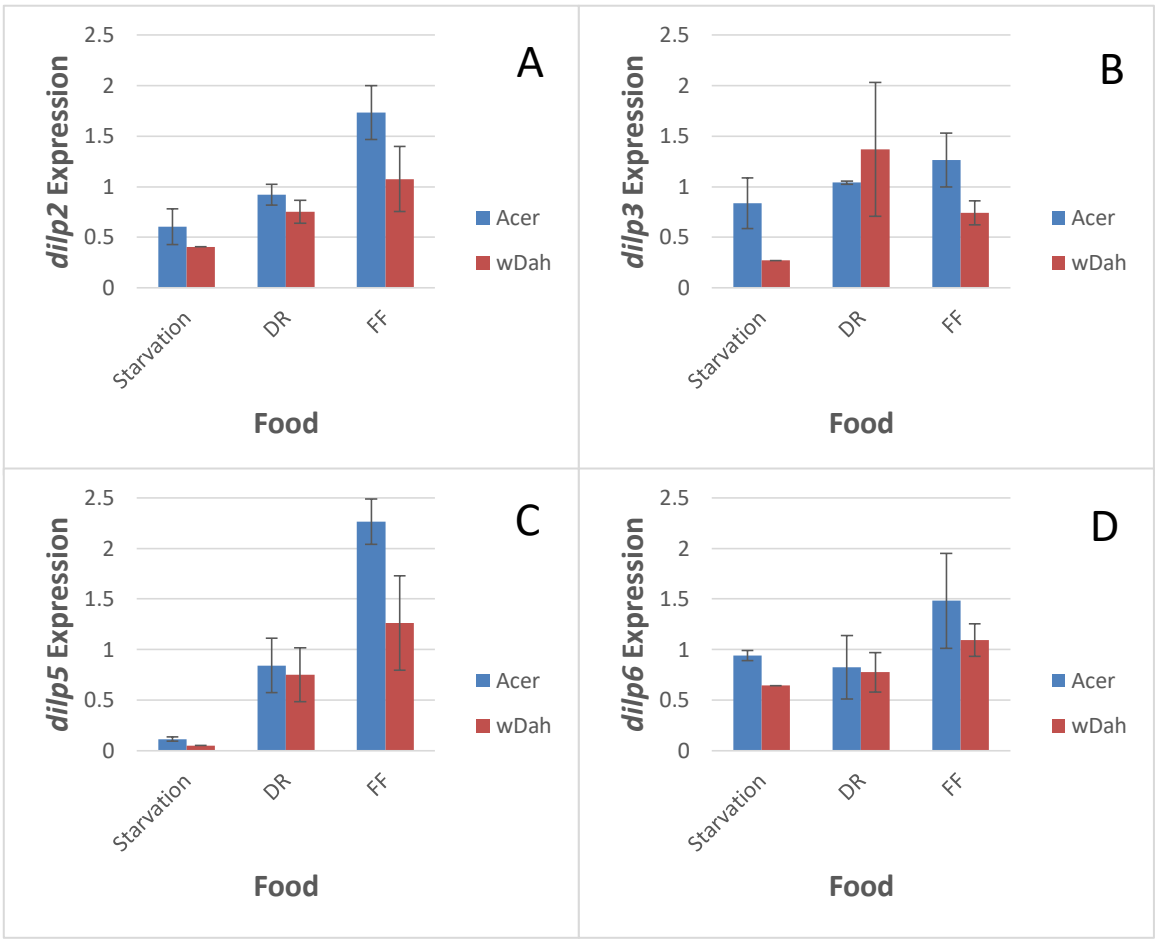

Figure 56: *dilp* Expression in Male Heads in the w^{Dah} background on Starvation, DR and FF diets. (A) *dilp2* (B) *dilp3* (C) *dilp5* (D) *dilp6*. (A) w^{Dah}: Starvation N = 1; DR N = 2; FF N = 3. $Acer^{\Delta}$: Starvation N = 3; DR N = 2; FF N = 3. (B) w^{Dah}: Starvation N = 1; DR N = 3; FF N = 3. $Acer^{\Delta}$: Starvation N = 3; DR N = 2; FF N = 3. (C) w^{Dah}: Starvation N = 1; DR N = 2; FF N = 3. $Acer^{\Delta}$: Starvation N = 3; DR N = 3; FF N = 3. (D) w^{Dah}: Starvation N = 1; DR N = 3; FF N = 3. $Acer^{\Delta}$: Starvation N = 3; DR N = 3; FF N = 3. Data was checked for normality and was found to be log distributed. Data shown as raw data and analysed as log-transformed. For mean comparison and variance statistics see Tables 73 and 74. *Indicates significant difference between genotypes (P=<0.05).

Like the female head experiment the N for many of the conditions is low and needs to be raised to confirm our findings. This includes the w^{Dah} control males on Starvation food (N= 1) for all *dilps* and on DR food (N= 2) for *dilp2* and *dilp5*. For $Acer^{\Delta}$ males this includes DR food (N= 2) for *dilp2* and *dilp3*.

Genotype/DILP	Food Comparison	p-value
DILP2		
w^{Dah}	Starvation - DR	0.5538
	Starvation - FF	0.3037
	DR - FF	0.7474
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.3601
	Starvation - FF	0.0293*
	DR - FF	0.2453
DILP3		
w^{Dah}	Starvation - DR	0.3714
	Starvation - FF	0.5463
	DR - FF	0.848
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.6934
	Starvation - FF	0.4038
	DR - FF	0.9109
DILP5		
w^{Dah}	Starvation - DR	0.0814
	Starvation - FF	0.0446*
	DR - FF	0.6754
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.0065*
	Starvation - FF	0.0006*
	DR - FF	0.0589
DILP6		
w^{Dah}	Starvation - DR	0.9484
	Starvation - FF	0.4889
	DR - FF	0.4537
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.8106
	Starvation - FF	0.7045
	DR - FF	0.3766

Table 73: Tukey-Kramer HSD comparisons of means comparing *dilp* expression in the male head with changing diet for controls and $Acer^{\Delta}$ males. *Indicates significant difference (P=<0.05).

DILPs	Food	Comparison to $Acer^{\Delta}$ (p-value)
DILP2		
w^{Dah}	Starvation	0.6409
	DR	0.4247
	FF	0.1658
DILP3		
w^{Dah}	Starvation	0.2174
	DR	0.9736
	FF	0.1289
DILP5		
w^{Dah}	Starvation	0.1649
	DR	0.9164
	FF	0.121
DILP6		
w^{Dah}	Starvation	0.0629
	DR	0.9828
	FF	0.6227

Table 74: ANOVA/Pooled t-test measure of variance of mean *dilp* expression in male heads comparing $Acer^{\Delta}$ males and controls on Starvation, DR and FF foods. *Indicates significant difference (P=<0.05).

In female bodies levels of *dilp4*, *dilp5*, *dilp6* and *dilp7* all respond to dietary change (Figure 57 and Table 75). Transcription levels of *dilp4*, *dilp5* and *dilp6* significantly decrease with increasing food with the lowest levels of transcription seen on the FF food, whereas *dilp7* transcription increases significantly with increasing diet. The decrease in *dilp6* expression confirms previous data in Slaidina, et al. (2009) which found that *dilp6* expression was increased in response to starvation compared to normally fed flies.

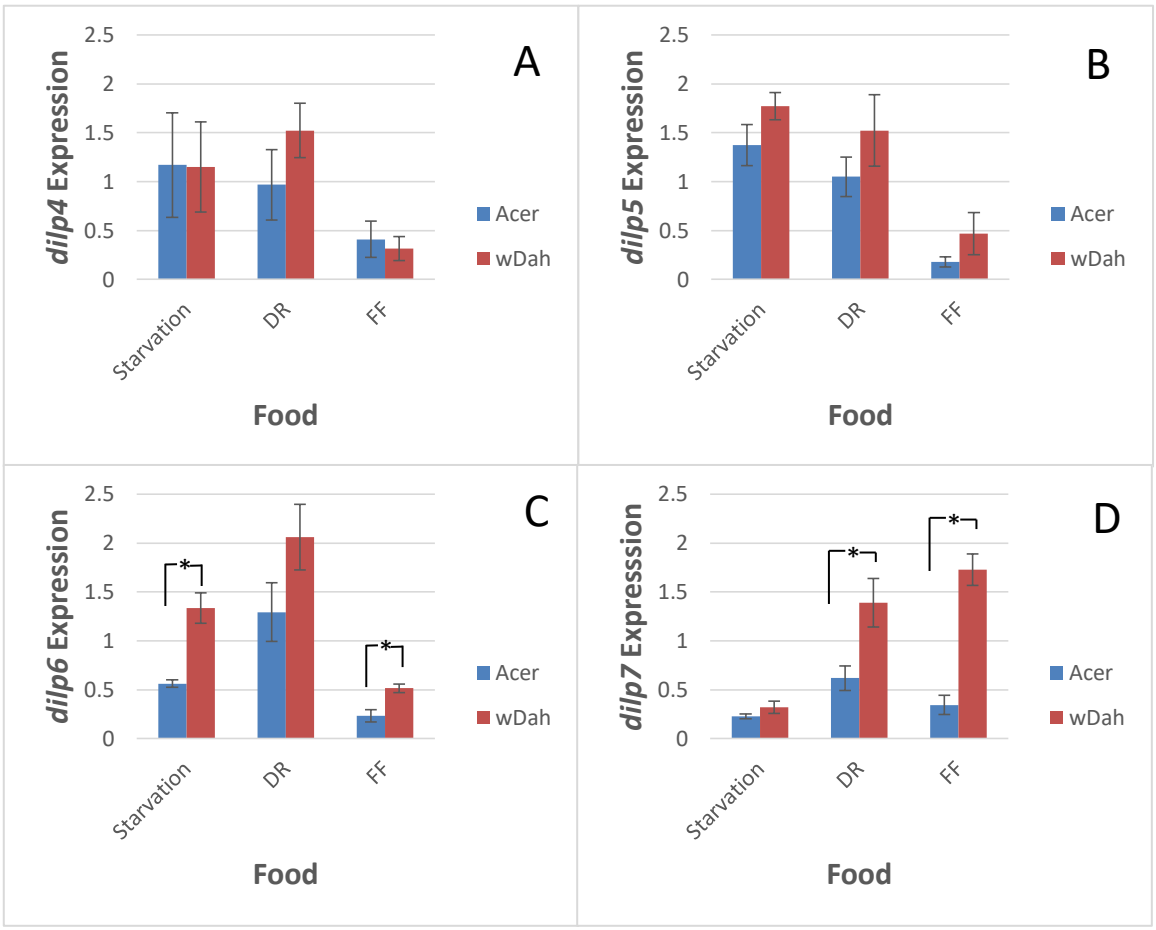

Figure 57: *dilp* Expression in Female Bodies in the w^{Dah} background Starvation, DR and FF diets. (A) *dilp4* (B) *dilp5* (C) *dilp6* (D) *dilp7*. (A) w^{Dah}: Starvation N = 3; DR N = 3; FF N = 3. $Acer^{\Delta}$: Starvation N = 3; DR N = 3; FF N = 3. (B) w^{Dah}: Starvation N = 3; DR N = 3; FF N = 3. $Acer^{\Delta}$: Starvation N = 3; DR N = 3; FF N = 3. (C) w^{Dah}: Starvation N = 3; DR N = 3; FF N = 3. $Acer^{\Delta}$: Starvation N = 3; DR N = 3; FF N = 3. (D) w^{Dah}: Starvation N = 3; DR N = 3; FF N = 3. $Acer^{\Delta}$: Starvation N = 3; DR N = 3; FF N = 3. Data was checked for normality. (A, B and D) were found to be log distributed. Data is shown as raw data and analysed as log-transformed. (C) data was found to be normally distributed. For mean comparison and variance statistics see Tables 75 and 76. *Indicates significant difference between genotypes (P=<0.05).

$Acer^{\Delta}$ females (Figure 49) showed a similar response to diet with *dilp4* and *dilp5* transcription levels decreasing with increasing diet in the fly body. Levels of *dilp6* transcription were found to be significantly lower than controls on Starvation and FF food and showed a trend towards decreased in transcription on the DR food, suggesting that *dilp6* transcription levels are lower in $Acer^{\Delta}$ females regardless of diet.

Genotype/DILP	Food Comparison	p-value
DILP4		
w^{Dah}	Starvation - DR	0.6798
	Starvation - FF	0.0771
	DR - FF	0.0268*
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.974
	Starvation - FF	0.7531
	DR - FF	0.6294
DILP5		
w^{Dah}	Starvation - DR	0.7819
	Starvation - FF	0.0275*
	DR - FF	0.0629
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.4246
	Starvation - FF	0.0061*
	DR - FF	0.0257*
DILP6		
w^{Dah}	Starvation - DR	0.1151
	Starvation - FF	0.0041*
	DR - FF	0.0006*
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.1133
	Starvation - FF	0.056
	DR - FF	0.004*
DILP7		
w^{Dah}	Starvation - DR	0.0012*
	Starvation - FF	0.0005*
	DR - FF	0.5525
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.0199*
	Starvation - FF	0.3245
	DR - FF	0.1368

Table 75: Tukey-Kramer HSD comparisons of means comparing *dilp* expression in the female bodies with changing diet for controls and $Acer^{\Delta}$ females. *Indicates significant difference (P=<0.05).

Although $Acer^{\Delta}$ females showed an increase in *dilp7* transcription levels between Starvation and DR food they showed an overall lack of response to changing diet with significantly reduced levels of transcription on the DR and FF foods compared to controls.

DILPs	Food	Comparison to *Acer*$^{\Delta}$ (p-value)
DILP4		
w^{Dah}	Starvation	0.7239
	DR	0.2924
	FF	0.7357
DILP5		
w^{Dah}	Starvation	0.1885
	DR	0.3216
	FF	0.2607
DILP6		
w^{Dah}	Starvation	0.0031*
	DR	0.1831
	FF	0.0392*
DILP7		
w^{Dah}	Starvation	0.2134
	DR	0.0428*
	FF	0.0012*

Table 76: ANOVA/Pooled t-test measure of variance of mean *dilp* expression in female bodies comparing *Acer*$^{\Delta}$ females and controls on Starvation, DR and FF foods. *Indicates significant difference (P=<0.05).

In summary, *Acer*$^{\Delta}$ females did not show the normal response of *dilp6* and *dilp7* transcription in the body to dietary changes suggesting that *Acer* normally plays a role in modulating the expression of these *dilps* in response to diet.

In control male bodies (Figure 58 and Table 77) none of the *dilps* significantly reacted to changing diet. However a trend of decreasing transcription with increasing food can be seen for *dilp7*. *Acer*$^{\Delta}$ males showed a trend of reduced transcription for all *dilps* on most foods with significantly lower levels of transcription of *dilp6* and *dilp7* on FF food compared to controls.

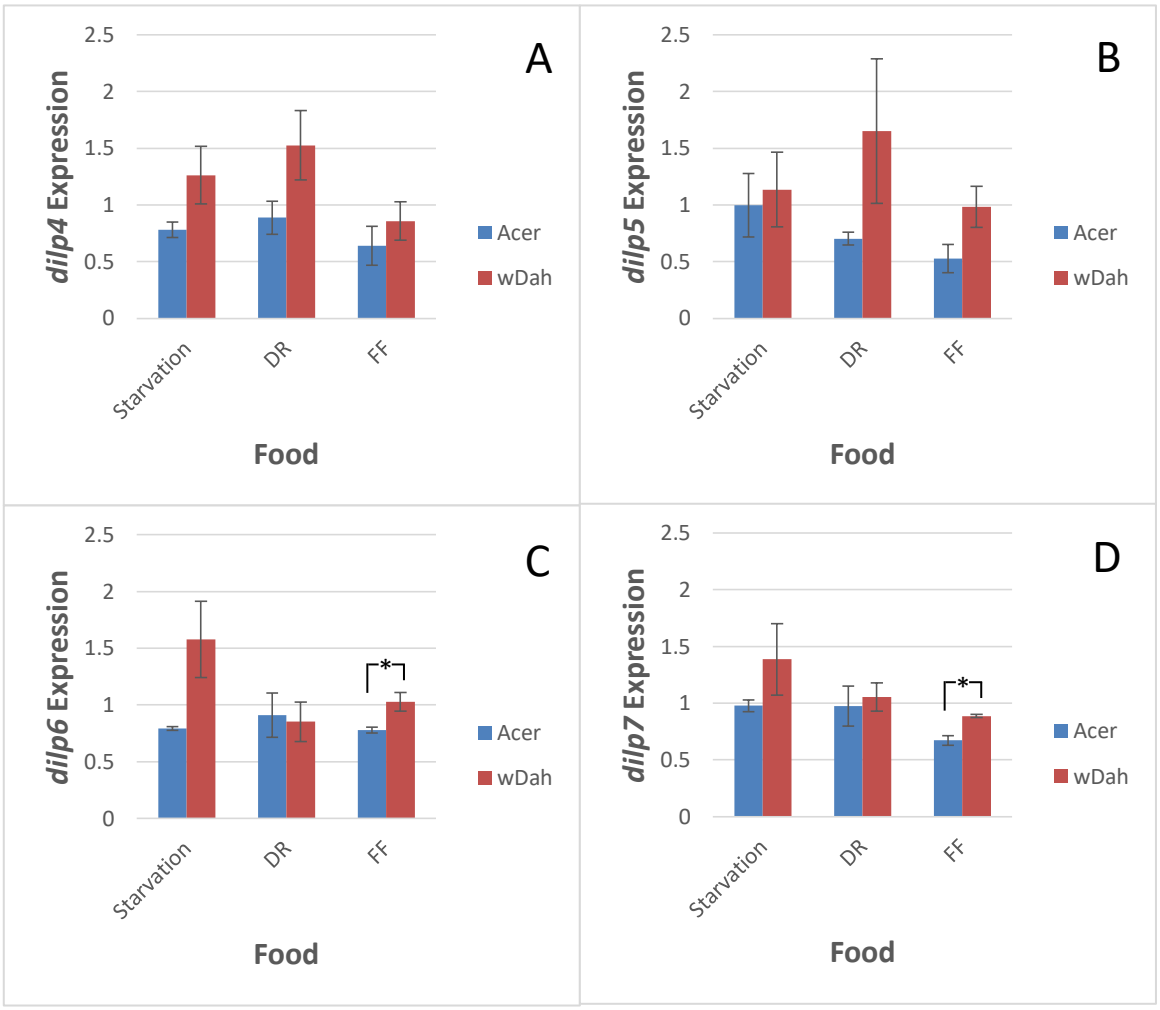

Figure 58: *dilp* Expression in Male Bodies in the w^{Dah} background on Starvation, DR and FF diets. (A) *dilp4* (B) *dilp5* (C) *dilp6* (D) *dilp7*. (A) w^{Dah}: Starvation N = 3; DR N = 3; FF N = 2. $Acer^{\Delta}$: Starvation N = 3; DR N = 3; FF N = 3. (B) w^{Dah}: Starvation N = 3; DR N = 3; FF N = 2. $Acer^{\Delta}$: Starvation N = 3; DR N = 3; FF N = 3. (C) w^{Dah}: Starvation N = 3; DR N = 3; FF N = 2. $Acer^{\Delta}$: Starvation N = 2; DR N = 3; FF N = 3. (D) w^{Dah}: Starvation N = 3; DR N = 3; FF N = 2. $Acer^{\Delta}$: Starvation N = 2; DR N = 3; FF N = 3. Data was checked for normality and found to be log distributed. Data is shown as raw data and analysed as log-transformed. For mean comparison and variance statistics see Tables 77 and 78. *Indicates significant difference between genotypes (P=<0.05).

Genotype/DILP	Food Comparison	p-value
DILP4		
w^{Dah}	Starvation - DR	0.7699
	Starvation - FF	0.4935
	DR-FF	0.2462
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.9271
	Starvation - FF	0.6204
	DR - FF	0.4249
DILP5		
w^{Dah}	Starvation - DR	0.7718
	Starvation - FF	0.9954
	DR - FF	0.7626
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.6249
	Starvation - FF	0.1687
	DR - FF	0.5246
DILP6		
w^{Dah}	Starvation - DR	0.1919
	Starvation - FF	0.5246
	DR - FF	0.7789
$w^{Dah};Acer^{\Delta}$	Starvation- DR	0.8842
	Starvation - FF	0.9947
	DR - FF	0.8032
DILP7		
w^{Dah}	Starvation - DR	0.6711
	Starvation - FF	0.4272
	DR - FF	0.8437
$w^{Dah};Acer^{\Delta}$	Starvation - DR	0.9845
	Starvation - FF	0.1997
	DR - FF	0.188

Table 77: Tukey-Kramer HSD comparisons of means comparing *dilp* expression in the male bodies with changing diet for controls and $Acer^{\Delta}$ males. *Indicates significant difference (P=<0.05).

DILPs	Food	Comparison to *Acer*$^{\Delta}$ (p-value)
DILP4		
w^{Dah}	Starvation	0.1087
	DR	0.1145
	FF	0.4313
DILP5		
w^{Dah}	Starvation	0.8632
	DR	0.1404
	FF	0.1467
DILP6		
w^{Dah}	Starvation	0.1455
	DR	0.8267
	FF	0.0333*
DILP7		
w^{Dah}	Starvation	0.4535
	DR	0.6663
	FF	0.0441*

Table 78: ANOVA/Pooled t-test measure of variance of mean *dilp* expression in male bodies comparing *Acer*$^{\Delta}$ males and controls on Starvation, DR and FF foods. *Indicates significant difference (P=<0.05).

In summary, *Acer*$^{\Delta}$ flies showed a reduced response of *dilp* transcription levels to dietary changes with the specific *dilp* affected differing between males and females. In the head of the fly only *dilp5* responds to dietary changes in both males and females with transcription levels increasing with increasing diet. *Acer*$^{\Delta}$ females showed a trend towards reduced transcription levels whereas in males the trend is towards higher levels of transcription on the FF food in the head. In the female body, all *dilps* showed a reduction in transcript levels with increasing diet especially on the FF food except for *dilp7* which clearly showed an increase in transcription with increasing diet. *Acer*$^{\Delta}$ females showed a significantly reduced transcript level for *dilp7* on the DR and FF foods while also showing reduced transcription on the Starvation and FF foods for *dilp6*. *Acer*$^{\Delta}$ females also showed a trend towards reduced *dilp* levels overall which could be confirmed with a higher N. In males, none of the *dilps* responded to dietary changes however a significant reduction in transcript is seen on the FF food for both *dilp6* and *dilp7* for *Acer*$^{\Delta}$ males.

7.2.2: DILP5 expression in the median neurosecretory cells (mNSCs) of the fly brain is altered in *Acer*$^{\Delta}$ flies

Previously, Broughton, et al. (2010) found that DILP5 protein expression in the mNSCs of the fly brain responded to changing diet with an increase in expression as food levels increased. In this study it was found that *dilp5* gene expression levels in the head of the fly were lower in *Acer*$^{\Delta}$ females on DR and FF foods while *Acer*$^{\Delta}$ males appeared to have higher levels of *dilp5* expression on FF food (Chapter 7.2.1).

DILP5 protein was analysed in the mNSCs of the fly brain using a DILP5 antibody (Broughton, et al., 2005) to compare *w*^{1118} controls and *Acer*$^{\Delta}$ males and females after two days feeding on either Low, DR or FF food (Figures 59 and 60).

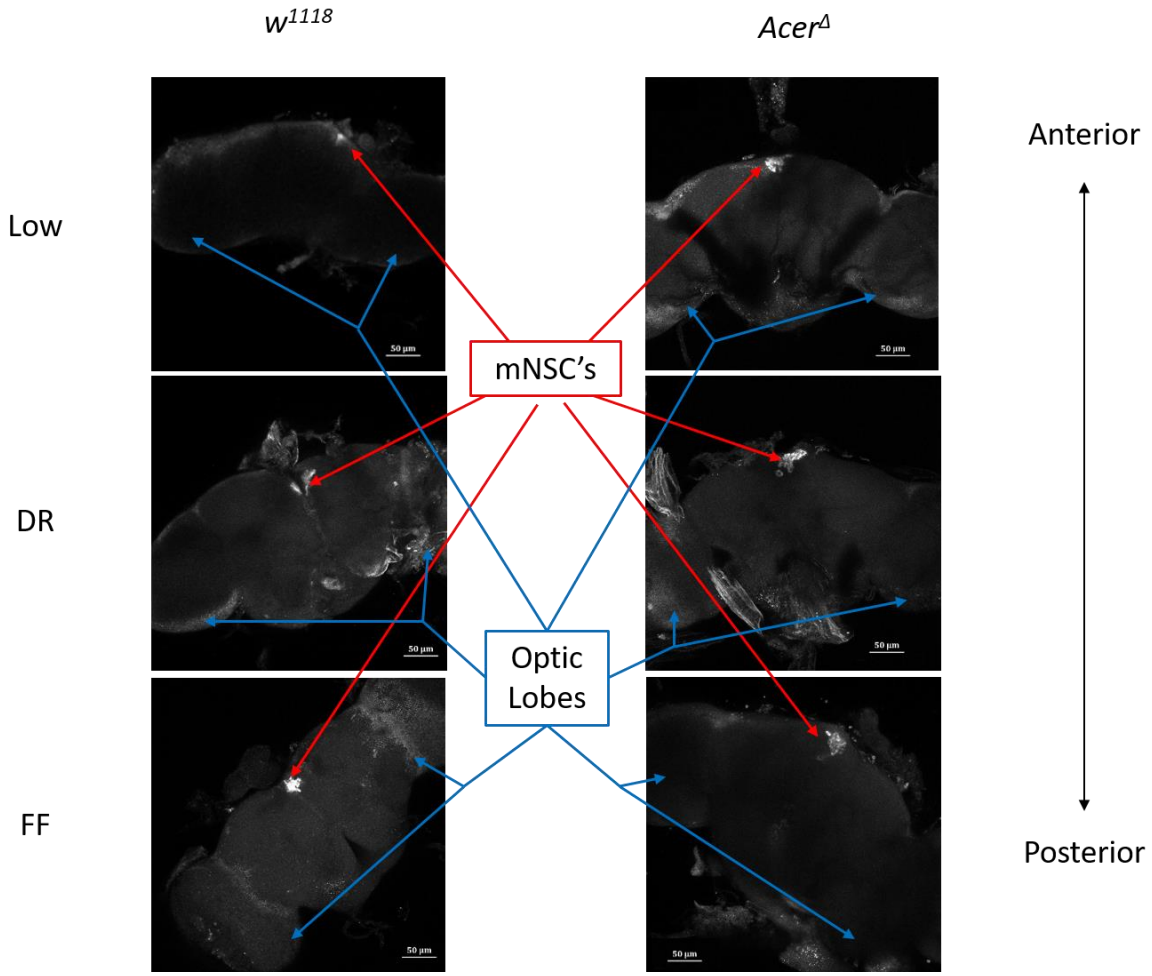

Figure 59: Immunohistochemical analysis of DILP5 protein in the mNSCs of the fly brain in *w*^{1118} controls and *Acer*$^{\Delta}$ females on Low, DR and FF diets. The images show the maximum intensity of staining from the z-stacks imaged using a confocal microscope. For all brains analysed, see Appendix 1.

The analysis of the DILP5 staining was carried out using the programme Image J. The program recorded the intensity of z-stack staining at maximum density and divided the result by the area stained.

The results showed that control females in the w^{1118} background (Figures 59 and 60A) showed a steady increase in DILP5 expression with increasing food, although not significant the increase in DILP5 between Low and FF food was nearing significance (Table 79). There was no significant difference in the number of cells expressing DILP5 protein between the different foods (Figure 60B and Table 79).

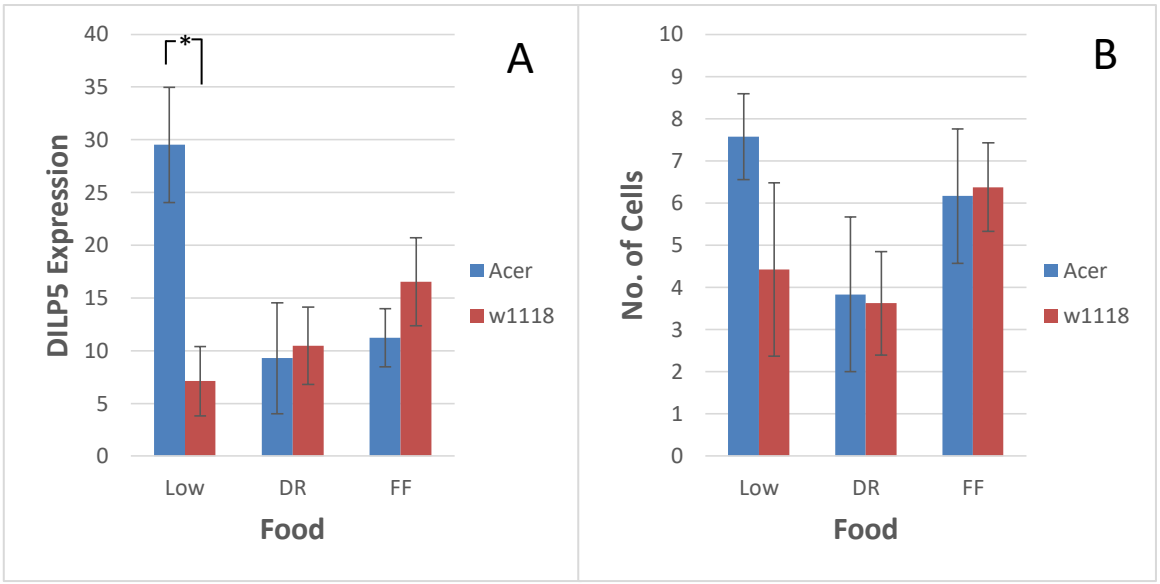

Figure 60: DILP5 Expression in the mNSCs of the fly brain for w^{1118} control and $Acer^{\Delta}$ females on Low DR and FF diets. Expression was quantified using Image J on the samples shown in Figure 51 and Appendix 1. (A) DILP5 expression in the mNSCs. w^{1118}: Low N = 7; DR N = 8; FF N = 8. $Acer^{\Delta}$: Low N = 7; DR N = 5; FF N = 6. (B) Number of cells stained. w^{1118}: Low N = 7; DR N = 8; FF N = 8. $Acer^{\Delta}$: Low N = 7; DR N = 6; FF N = 6. Data was checked for normality. (A) Data was found to be log-distributed and data is shown as raw data but was analysed as log-transformed. (B) Data was found to be normally distributed. For mean comparison and variance statistics see Tables 79 and 80. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	DILP5 Expression (p-value)	No. of Cells (p-value)
w^{1118}	Low - DR	07181	0.9214
	Low – FF	0.1499	0.625
	DR - FF	0.4596	0.3761
w^{1118};$Acer^{\Delta}$	Low - DR	0.0326*	0.2147
	Low – FF	0.1392	0.7888
	DR - FF	0.6712	0.5525

Table 79: Tukey-Kramer HSD comparisons of means comparing DILP5 expression in mNSCs of the fly brain and the number of cells stained with changing diet for controls in the w^{1118} background and $Acer^{\Delta}$ females. *Indicates significant difference (P=<0.05).

Genetic Comparison	Food	DILP5 Expression (p-value)	No. of Cells (p-value)
w^{1118} to $Acer^{\Delta}$	Low	0.0055*	0.1961
	DR	0.8803	0.9233
	FF	0.511	0.9151

Table 80: ANOVA/Pooled t-test measure of variance of mean DILP5 expression in the mNSCs of the fly brain and the number of cells stained comparing $Acer^{\Delta}$ females and controls on Low, DR and FF foods. *Indicates significant difference (P=<0.05).

$Acer^{\Delta}$ females showed a significantly higher level of DILP5 expression on Low food than DR food (Figure 60A and Table 80). This heightened expression for $Acer^{\Delta}$ females on Low food was significantly higher than controls on the same food (Table 80). There no difference between genotypes in DILP5 expression on the DR and FF foods. There was no difference in the number of cells stained between the foods for $Acer^{\Delta}$ females and no difference in the number of cells stained between the genotypes on any of the foods Table 80).

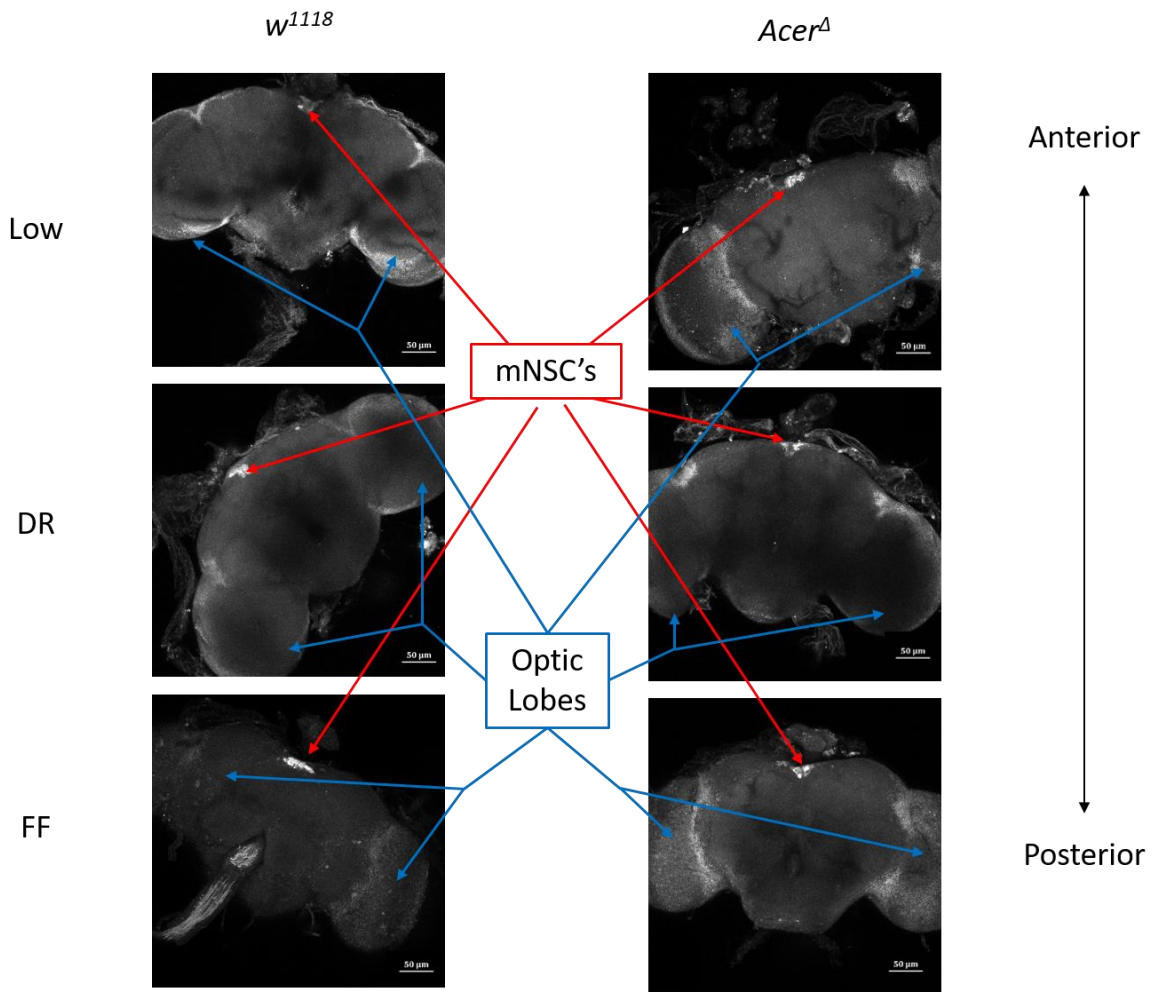

Figure 61: Immunohistochemical analysis of DILP5 protein in the mNSCs of the fly brain in *w*^{1118} controls and *Acer*$^{\Delta}$ males on Low, DR and FF diets. The images show the maximum intensity of staining from the z-stacks imaged using a confocal microscope. For all brains analysed, see Appendix 1.

Control males in the *w*^{1118} background (Figures 61 and 62A), like females, showed an increase in DILP5 protein as food increased, although not significant the increase in expression between Low and FF food was nearing significance (Table 81). There was no significant difference between the numbers of cells stained between the foods (Figure 62B) for control males however, the increase in the number of cells stained between DR and FF food was nearing significance (Table 81).

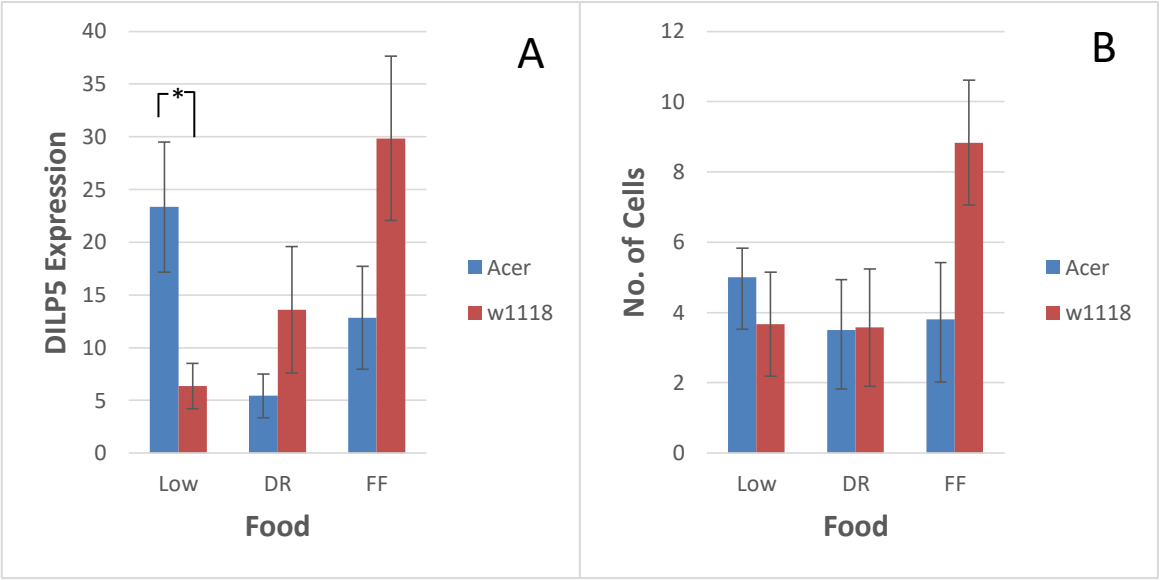

Figure 62: DILP5 Expression in the mNSCs of the fly brain for w^{1118} control and $Acer^{\Delta}$ males on Low DR and FF diets. Expression was quantified using Image J on the samples shown in Figure 53 and Appendix 1. (A) DILP5 expression in the mNSCs. w^{1118}: Low N = 6; DR N = 7; FF N = 6. $Acer^{\Delta}$: Low N = 5; DR N = 5; FF N = 5. (B) Number of cells stained. w^{1118}: Low N = 6; DR N = 7; FF N = 6. $Acer^{\Delta}$: Low N = 5; DR N = 6; FF N = 5. Data was checked for normality. (A) Data was found to be log-distributed and data is shown as raw data but was analysed as log-transformed. (B) Data was found to be normally distributed. For mean comparison and variance statistics see Tables 81 and 82. *Indicates significant difference between genotypes (P=<0.05).

Genotype	Food Comparison	DILP 5 Expression (p-value)	No. of Cells (p-value)
w^{1118}	Low - DR	0.9897	0.9991
	Low – FF	0.0774	0.1117
	DR - FF	0.0846	0.0896
$w^{1118};Acer^{\Delta}$	Low - DR	0.0592	0.7163
	Low – FF	0.2994	0.8205
	DR - FF	0.5838	0.9864

Table 81: Tukey-Kramer HSD comparisons of means comparing DILP5 expression in mNSCs of the fly brain and the number of cells stained with changing diet for controls in the w^{1118} background and $Acer^{\Delta}$ males. *Indicates significant difference (P=<0.05).

Genetic Comparison	Food	DILP5 Expression (p-value)	No. of Cells (p-value)
w^{1118} to $Acer^{\Delta}$	Low	0.0098*	0.4772
	DR	0.7208	0.9752
	FF	0.0928	0.0703

Table 82: ANOVA/Pooled t-test measure of variance of mean DILP5 expression in the mNSCs of the fly brain and the number of cells stained comparing *Acer*$^{\Delta}$ males and controls on Low, DR and FF foods. *Indicates significant difference (P=<0.05).

Acer$^{\Delta}$ males showed an increase in DILP5 expression on Low food compared to DR food that was nearing significance (Figure 62A and Table 81) and showed a significantly higher expression of DILP5 on Low food than controls (Table 82). *Acer*$^{\Delta}$ males also showed a lower level of DILP5 expression on FF food compared to controls that was nearing significance. No difference between the numbers of cells stained was observed between the foods for *Acer*$^{\Delta}$ males but a lower number of cells were stained on FF food compared to controls with the result nearing significance (Table 82).

In summary, DILP5 expression in *Acer*$^{\Delta}$ males and females was significantly higher than controls on Low food. These data suggest that *Acer* is involved in modulating the response of DILP5 in the mNSCs to low food. Interestingly, *dilp5* transcript was found to be reduced in the heads *Acer*$^{\Delta}$ males and females, although this result does require verification with a higher N, it appears that even though *Acer*$^{\Delta}$ flies showed reduced transcript levels the DILP5 protein was much higher in the mNSC's. This suggests that, although *dilp5* transcript levels are reduced, the mNSC's are unable to release the DILP5 protein and *Acer* therefore modulates the release of DILP5 protein from the mNSC's in response to low food.

7.2.3: The 24 hour expression of *Acer* does not significantly alter in the w^{Dah} background but in the w^{1118} background *Acer* expression increases at night

Some genes are known to be expressed differentially throughout a 24 hour cycle with latitudinal differentiation and *Acer* has been found to be expressed differentially through night time hours (Svetec, et al., 2015). *Acer's* expression in the w^{Dah} and w^{1118} genetic backgrounds over 24 hours in response to DR food was tested (Figure 63).

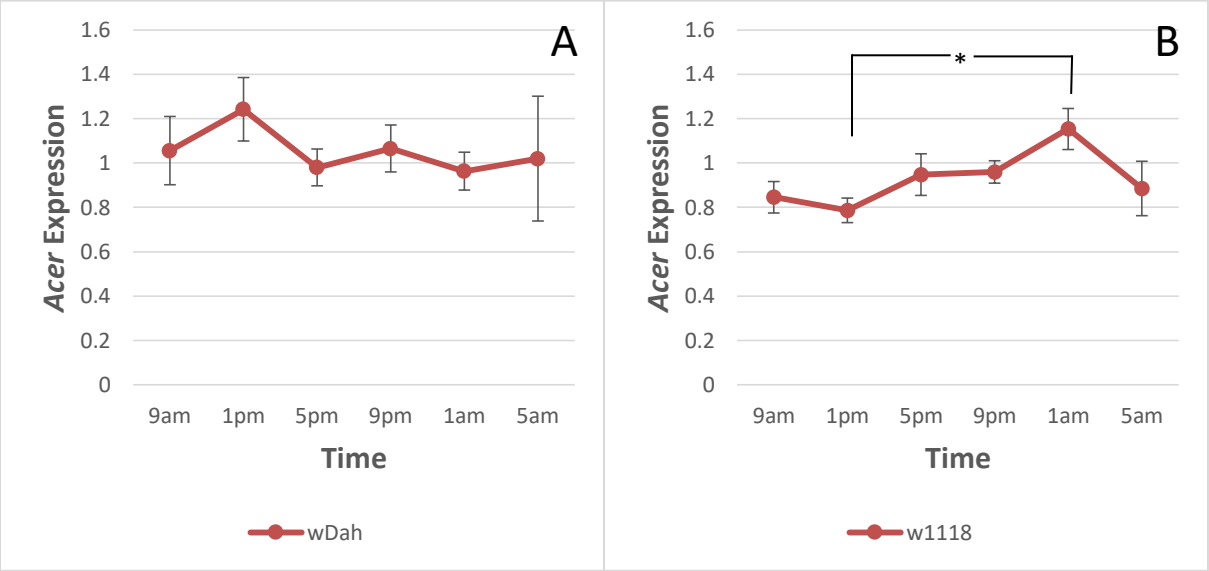

Figure 63: *Acer* expression over 24 hours after 4 days feeding on DR food in w^{Dah} and w^{1118} female flies. Day = 9am – 5pm. Night = 9pm – 5am. (A) *Acer* expression in the w^{Dah} background. N = 4. (B) *Acer* expression in the w^{1118} background. N = 4. Data was checked for normality and was found to be normally distributed. For mean comparison statistics see Table 83. *Indicates significant difference between genotypes (P=<0.05).

The results showed that *Acer* expression fluctuated in the w^{Dah} background over 24 hours but the difference was not significant. In the w^{1118} background over 24 hours *Acer* expression differed significantly between 1pm and 1am with a higher level of expression at 1am (Figure 63B) for female flies on DR food (Table 83).

Comparison of the backgrounds showed that at 1pm the *Acer* expression in w^{1118} females was significantly lower than in w^{Dah} females (p=0.0141) but there was no difference in expression at any other time-point. The mean cumulative expression

across all time-points showed that Acer expression is lower in w^{1118} females compared to w^{Dah} females with the result nearing significance (p=0.067).

Genotype	Time comparison	Comparison of *Acer* expression (p-value)
w^{Dah}	9am – 1pm	0.9542
	9am – 5pm	0.9996
	9am – 9pm	1
	9am – 1am	0.9988
	9am – 5am	1
	1pm – 5pm	0.811
	1pm – 9pm	0.9644
	1pm – 1am	0.7653
	1pm – 5am	0.8989
	5pm – 9pm	0.9993
	5pm – 1am	1
	5pm – 5am	1
	9pm – 1am	0.9988
	9pm – 5am	1
	1am – 5am	0.9999
w^{1118}	9am – 1pm	0.9974
	9am – 5pm	0.9592
	9am – 9pm	0.9329
	9am – 1am	0.105
	9am – 5am	0.9998
	1pm – 5pm	0.7374
	1pm – 9pm	0.6737
	1pm – 1am	0.0338*
	1pm – 5am	0.9656
	5pm – 9pm	1
	5pm – 1am	0.4843
	5pm – 5am	0.9964
	9pm – 1am	0.5499
	9pm – 5am	0.9911
	1am – 5am	0.2021

Table 83: Tukey-Kramer HSD comparisons of means comparing *Acer* expression in the fly in w^{Dah} and w^{1118} females after four days feeding on DR food over a 24hr period. *Indicates significant difference (P=<0.05).

The analysis for this experiment is on-going including the analysis of *Acer* expression after four days feeding on FF food for females and analysis of *Acer*

expression after four days feeding on DR and FF foods for males comparing both backgrounds.

7.3: Discussion

In this study it has been previously shown that *Acer* is involved in the response of sleep, glycogen storage, lifespan and stresses to dietary change. The *dilp* expression results show that *Acer* may be involved in the regulation of *dilp* transcription in response to diet.

The nutrient responsive *dilps* are *dilp5* in the head of both male and females and *dilps 4, 5* and *6* in female bodies. In the body the *dilps* appeared to respond to high food with a decrease in transcript levels but *dilp7* clearly increased transcription with increasing nutrients. In male bodies, the *dilps* showed no response to dietary changes although *dilp7* did show a decreasing trend with increasing food. This follows the general lack of response to dietary changes seen in males throughout this study with the exception of glycogen storage.

In the adult head, as has been found previously (Broughton, et al., 2010) *dilp5* is the only *dilp* that shows a response to dietary changes, as diet increases transcription levels increase in both male and female flies. Interestingly, although the result is not significant $Acer^{\Delta}$ females showed a trend towards reduced transcript levels compared to controls whereas $Acer^{\Delta}$ males show a trend towards increased transcript levels for *dilp5*. This suggests that the response of *dilp5* to changing differs between the sexes and *Acer* potentially modulates the response of *dilp5* in the fly head to changing diet.

The expression of *dilp6* in the female body clearly showed a reduction in transcript levels in controls between Starvation and FF food in agreement with other studies showing a heightened expression of *dilp6* to starvation conditions (Slaidina, et al., 2009). $Acer^{\Delta}$ females showed a reduction in *dilp6* expression on both Starvation and FF food compared to controls. In males bodies there was no significant difference in *dilp6* expression between the foods for controls, however a decreasing trend of *dilp6* expression was observed with increasing food. $Acer^{\Delta}$ males showed a significant reduction in *dilp6* signalling on FF food. *Acer*, like *dilp6*, is expressed within the fly fat body and this data suggests that *Acer* potentially modulates the expression of *dilp6* in the fly fat body to diet.

The expression of *dilp7* differs between the female and male bodies. Female expression of *dilp7* increases with increasing food for controls but $Acer^{\Delta}$ females showed significantly reduced expression on DR and FF foods. This suggests that *Acer*

modulates the response of *dilp7* to changing diet. Yang, et al. (2008) have previously shown that *dilp7* is required in the process of female site analysis for egg-laying and that elevated levels of *dilp7* led to increased fecundity. The increase in *dilp7* expression with increasing food levels supports this theory as females lay more eggs when feeding on high yeast food (Skorupa, et al., 2008). Interestingly, $Acer^{\Delta}$ females in the w^{Dah} background showed a reduced fecundity phenotype on both DR and FF foods (Chapter 5) and *dilp7* expression is also reduced on these foods. These data suggest that *Acer* modulates the expression of *dilp7* to changing diet in female flies. In male control flies *dilp7* showed a decreasing trend of expression with increasing diet. $Acer^{\Delta}$ males showed significantly decreased expression of *dilp7* on FF food, suggesting that *Acer* is involved in the response of *dilp7* to high food in male flies. *Acer* has been found within the male testes, therefore it is possible that *Acer* may be involved in courtship behaviour or sperm generation as well as the modulation of *dilp7*.

The number of replicates for this experiment was only three and a higher N will be required to confirm these results. This experiment was conducted in the w^{Dah} background only and as we have seen different phenotypes between the backgrounds for controls and $Acer^{\Delta}$ flies it is important to analyse *dilp* expression in the w^{1118} background. As we have also found different phenotypes on Low food for $Acer^{\Delta}$ flies it is important to test the *dilp* transcription levels on this food in the future.

The immunohistochemistry of the fly brains showed that in w^{1118} control males and females DILP5 protein expression in the mNSCs increased with increasing diet with the increase between Low and FF foods nearing significance. $Acer^{\Delta}$ males and females showed a significantly higher level of DILP5 protein on Low food than controls with the DILP5 protein levels returning to controls levels for DR and FF foods. These results suggest that *Acer* modulates the response of DILP5 protein in the mNSCs to Low food. Interestingly, although the QPCR results showed a lower level of *dilp5* transcription in $Acer^{\Delta}$ female heads for DR and FF food the protein levels showed no difference to controls. DILP5 protein levels were found to be high in the mNSCs of the fly brain on Low food, therefore it is important to test *dilp5* transcription levels on Low food to ascertain whether the higher levels of protein are due to higher transcript levels in $Acer^{\Delta}$ males and females. The immunohistochemistry was only performed in the w^{1118} background and as we have seen differences in the response to the loss of *Acer* expression between the backgrounds, it is important to repeat this experiment in males and females in the w^{Dah} background. For clarity with the transcription data, results on

Starvation food would also be interesting to analyse in both backgrounds for both sexes.

The expression of *Acer* in w^{Dah} and w^{1118} males and females was investigated and it was found that in the w^{Dah} background *Acer* is expression does not change significantly over 24 hours but in the w^{1118} background *Acer* expression is significantly different between 1pm and 1am with higher levels of expression at 1am. This difference in expression may explain the different phenotypes seen of $Acer^{\Delta}$ males and females in the different backgrounds. The analysis for this experiment is on-going and includes the investigation of *Acer* expression after four days on FF food in females and on both foods in both backgrounds for males. Due to $Acer^{\Delta}$ flies' lack of response to diet it will interesting to see if *Acer* expression responds to dietary changes in either of the backgrounds and whether there are differences in expression between male and female flies.

Chapter 8 – ANCE a homologue of human ACE

8.1: Introduction

ANCE (Angiotensin converting enzyme) like ACER, is a *Drosophila* homologue of human ACE. *Ance* shares similar properties with *Acer* including expression within the *Drosophila* heart and both homologues are thought to function as peptidyl-dipeptidases with likely roles in peptide metabolism (Siviter, et al., 2002). Unlike ACER, ANCE is able to cleave angiotensin-I converting it into angiotensin-II and is able to hydrolyse bradykinin which are both important substrates of ACE (Bingham, et al., 2006). However, flies do not have an angiotensin system therefore the *Ance* substrate is unknown in *Drosophila*. *Ance* is also expressed within the *Drosophila* midgut and male reproductive tissues as well as the amnioserosa during embryogenesis (Houard, et al., 1998).

The $Ance^{\Delta}$ is currently unpublished but $Ance^{\Delta}$ flies developed and reproduced normally in this experiment suggesting that *Ance* is not vital to development or reproduction.

In this study, $Acer^{\Delta}$ males and females were found to be involved in the response of lifespan to diet and $Acer^{\Delta}$ females were less fecund than controls in the w^{Dah} background (Chapter 5). In this chapter the effect of the lack of *Ance* on lifespan and fecundity in response to diet was analysed.

8.1.1: Aims

Ance was tested by partial deletion of the *Ance* gene resulting in the lack of *Ance* expression. Lifespan and fecundity were monitored in response to DR to establish any potential link between *Ance* and the nutrient response.

8.1.2: Research Design

The longevity of $Ance^{\Delta}$ males and females in the w^{Dah} background was measured initially on standard food (50g/l of sugar and 100g/l of yeast) before being measured on DR (50g/l of sugar and 50g/l of yeast) and FF (50g/l of sugar and 200g/l

of yeast) food with fecundity recorded for female flies. A heterozygous *Ance*$^{\Delta}$ (*Ance*$^{\Delta}$/+) was added for the DR experiment for males and females.

For the measurement of longevity, 3 day old flies (N=100) were transferred to either DR or FF food for the duration of their life and their lifespan recorded.

For the measurement of fecundity the number of eggs laid by female flies was recorded weekly until the amount of eggs laid was reduced to a minimal level on both DR and FF food.

8.2 Results

8.2.1: Backcrossing and Validation of the *Ance* deletion

Ance is a homolog of *Acer* in *Drosophila melanogaster* and it was backcrossed into the w^{Dah} genetic background in the same way as the *Acer* deletion (see Chapter 2.2). Figure 64 shows the *Ance* deletion in the w^{Dah} background including the *Ance* deletion homozygote, the heterozygote and the w^{Dah} control with *Ance* present.

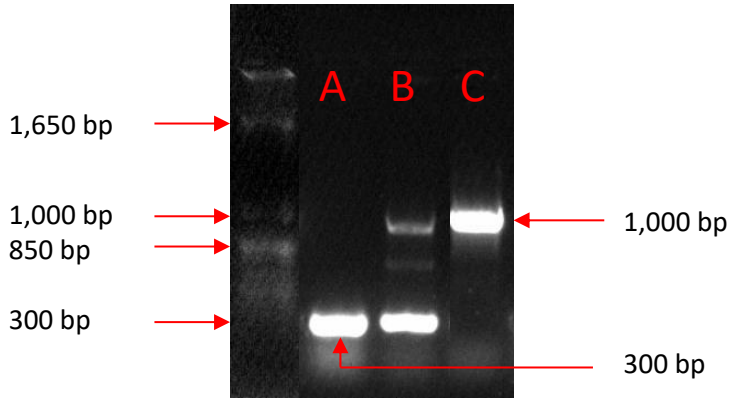

Figure 64: PCR analysis of the *Ance* deletion. (A) The *Ance* deletion homozygote with a strong band at 300 bp. (B) The *Ance* deletion heterozygote with a strong band at 300 bp and a weaker band at 1,000 bp. (C) The w^{Dah} control background with the *Ance* gene present with a strong band at 1,000 bp. (bp = base pairs).

The *Ance* deletion is about 700 base pairs and the *Ance* homozygous deletion can be clearly identified from the heterozygous state and the control (Figure 64). The shortest band clearly identifies the *Ance* deletion homozygote and the longest band identifies the control with *Ance* gene present. The *Ance* deletion heterozygote can be identified with a strong band at the same length as the homozygote deletion and a much weaker band at the same length as the controls. This allowed for identification of the *Ance* deletion homozygote, the heterozygote state and the control with the presence of the *Ance* gene. Like the *Acer* deletion, the *Ance* deletion is a null mutation and these flies are referred to as $Ance^{\Delta}$ throughout the study.

8.2.2: *Ance* is not involved in the longevity response to DR.

Ance is a different *Drosophila* homolog of human ACE and is related to *Acer*. Here we tested ***Ance's*** nutrient response to lifespan as well as fecundity in the w^{Dah} background.

The results showed that $Ance^{\Delta}$ females (Figure 65 and Tables 84 and 85) responded normally to Dietary Restriction (DR) and dietary changes compared to controls. The heterozygous $Ance^{\Delta}$ females also responded normally to DR and were significantly long-lived compared to $Ance^{\Delta}$ females on DR food with extension nearing significance on FF food. Compared to w^{Dah} controls $Ance^{\Delta}$ heterozygote females were not significantly long-lived but the statistics were approaching significance (Table 85). This data suggests that the heterozygous deletion may be enough to recover the lifespan phenotype to the same level as controls but requires further analysis.

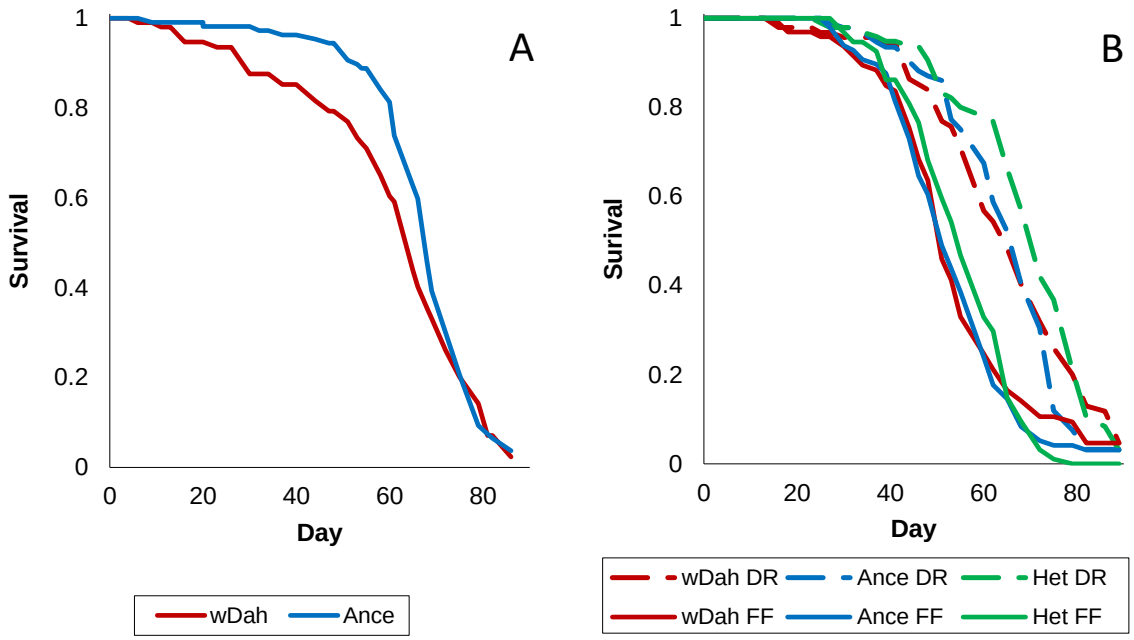

Figure 65: Female Lifespan in the w^{Dah} background for control and $Ance^{\Delta}$ females. (A) Lifespan on standard food. (B) Lifespan on DR and FF food with a heterozygous $Ance^{\Delta}$ added ($Ance^{\Delta}/+$). (A) Median Lifespan: w^{Dah} = 65 days, N = 109; $Ance^{\Delta}$ = 68 days, N = 106. (B) Median Lifespan: w^{Dah}: DR = 65 days, N = 96; FF = 51 days, N = 96. $Ance^{\Delta}$: DR = 68 days, N = 97; FF = 51 days, N = 97. $Ance^{\Delta}/+$: DR = 72 days, N = 97; FF = 55 days, N = 101. Survival analysis was performed in JMP using the log-rank test (Tables 84 and 85).

Genotype	Log-rank comparing DR & FF
w^{Dah}	<.0001*
$w^{Dah};Ance^{\Delta}$	<.0001*
$w^{Dah};Ance^{\Delta}/+$	<.0001*

Table 84: Female comparison of survival curves by genotype for response to DR using the log rank test in JMP for each food comparing controls, *Ance$^{\Delta}$* and *Ance$^{\Delta}$/+* females. *Indicates significant difference (P=<0.05).

Genetic Comparison	Experiment	Food	Log-rank (p-value)
w^{Dah} *to* $Ance^{\Delta}$	A	SF	0.5291
	B	DR	0.2514
	B	FF	0.7555
w^{Dah} *to* $Ance^{\Delta}/+$	B	DR	0.1202
	B	FF	0.1961
$Ance^{\Delta}$ *to* $Ance^{\Delta}/+$	B	DR	0.0003*
	B	FF	0.05

Table 85: Female comparison of survival curves by genotype for standard, DR and FF diets using the log rank test in JMP for each food comparing controls, *Ance$^{\Delta}$* and *Ance$^{\Delta}$/+* females. *Indicates significant difference (P=<0.05).

Ance is unlikely to be involved in the nutrient response to lifespan as no difference in lifespan was observed between controls and $Ance^{\Delta}$ females on either food. However, these experiments were only conducted once on each food and therefore repetition is required to confirm this phenotype.

Fecundity was tested alongside lifespan for the DR and FF foods in the second experiment (Figure 66 and Tables 86 and 87).

The results showed that all genotypes decreased fecundity in an age-related manner on both DR and FF foods (p=<.0001) and that all genotypes laid fewer eggs on DR food than FF food (p=<.0001).

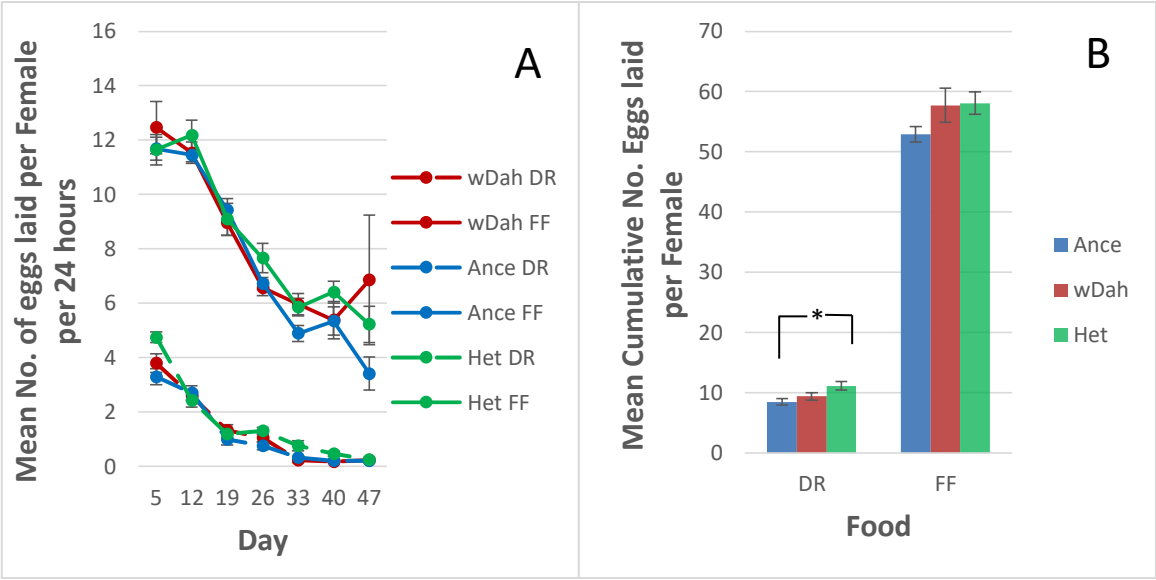

Figure 66: Fecundity for female controls, *Ance*$^{\Delta}$ homozygotes and *Ance*$^{\Delta}$ heterozygotes on DR and FF diets in the *w*^{Dah} background. (A) Mean no. of eggs laid per female per 24 hours: *w*^{Dah}: DR N = 10; FF N = 10. *Ance*$^{\Delta}$: DR N = 10, FF N = 10. *Ance*$^{\Delta}$*/+*: DR N= 10; FF N = 10. (B) Mean cumulative no. of eggs laid per female: *w*^{Dah}: DR N = 10; FF N = 10. *Ance*$^{\Delta}$: DR N = 10, FF N = 10. *Ance*$^{\Delta}$*/+*: DR N= 10; FF N = 10. Data was checked for normality and was found to be normally distributed. For mean comparison and variance statistics see Tables 86 and 87. * Indicates significant differences between genotypes (P=<0.05).

		Day p-value						
Genetic Comparison	Food	5	12	19	26	33	40	47
w^{Dah} *to Ance*$^{\Delta}$	DR	0.4311	0.9313	0.3691	0.3168	0.811	0.9652	0.9965
	FF	0.6986	0.9878	0.776	0.9506	0.0758	0.9987	0.2427
w^{Dah} *to Ance*$^{\Delta}$*/+*	DR	0.0615	0.8491	0.8242	0.4176	0.0087*	0.0125*	0.9678
	FF	0.6737	0.501	0.9792	0.109	0.97	0.3927	0.719
Ance$^{\Delta}$ *to Ance*$^{\Delta}$*/+*	DR	0.0032*	0.6411	0.7196	0.0268*	0.0373*	0.0228*	0.9441
	FF	0.9991	0.4158	0.8783	0.1902	0.1205	0.3675	0.6627

Table 86: Tukey-Kramer HSD comparisons of means of eggs laid per female per 24 hours with age in the *w*^{Dah} background for controls, *Ance*$^{\Delta}$ and *Ance*$^{\Delta}$*/+* females. *Indicates significant difference (P=<0.05).

For egg-laying on both DR and FF food *Ance*$^{\Delta}$ females responded normally to dietary changes compared to controls, however flies heterozygous for the *Ance*$^{\Delta}$ laid significantly more eggs on DR food than *Ance*$^{\Delta}$ females but laid similar amounts on

FF food. Similarly to lifespan, the heterozygotes were shown to be more fecund than *Ance*$^{\Delta}$ females, however no difference in fecundity was seen between the double *Ance* mutant and controls, suggesting that *Ance* is unlikely to be involved in reproduction. Repetition is required for a more conclusive outcome.

Genetic Comparison	Food	Mean cumulative no. of eggs laid per Female (p-value)
w^{Dah} *to* *Ance*$^{\Delta}$	DR	0.5715
	FF	0.232
w^{Dah} *to* *Ance*$^{\Delta}$*/+*	DR	0.1424
	FF	0.9915
Ance$^{\Delta}$ *to* *Ance*$^{\Delta}$*/+*	DR	0.0162*
	FF	0.189

Table 87: ANOVA/Pooled t-test measure of variance of mean cumulative eggs laid per female in the w^{Dah} background for controls, *Ance*$^{\Delta}$ and *Ance*$^{\Delta}$*/+* females. *Indicates significant difference (P=<0.05).

The results showed that *Ance*$^{\Delta}$ males (Figure 67 and Tables 88 and 89) responded to DR normally with an extended lifespan and were significantly short-lived compared to controls on the DR and FF foods.

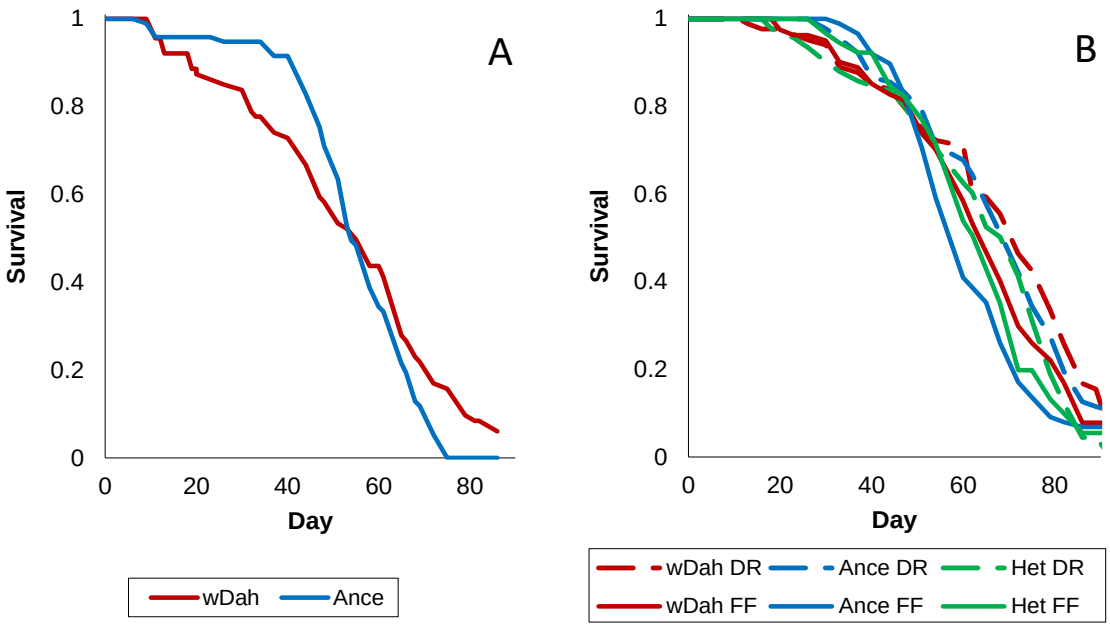

Figure 67: Male Lifespan in the w^{Dah} background for controls and *Ance*$^{\Delta}$ males. (A) Lifespan on standard food. (B) Lifespan on DR and FF food with a heterozygous *Ance*$^{\Delta}$ added (*Ance*$^{\Delta}$*/+*). (A) Median Lifespan: w^{Dah} = 54 days, N = 107; *Ance*$^{\Delta}$ = 54 days, N = 110. (B) Median Lifespan: w^{Dah}: DR =

72 days, N = 98; FF = 62 days, N = 94. *Ance*$^{\Delta}$: DR = 68 days, N = 91; FF = 60 days, N = 94. *Ance*$^{\Delta}$*/+*: DR = 72 days, N = 101; FF = 62 days, N = 95. Survival analysis was performed in JMP using the log-rank test (Tables 88 and 89).

However, the flies with the heterozygous deletion responded normally when compared with controls. This suggests, like females, that the recovery of one *Ance* allele may be enough to recover the control phenotype on FF food. No significant difference was seen on the standard food between *Ance*$^{\Delta}$ males and controls but *Ance*$^{\Delta}$ males did appear to have a shorter maximum lifespan. These are the results of one lifespan on each food and therefore repetition is required to confirm the outcome of these experiments.

Genotype	Log-rank comparing DR & FF	Wilcoxon comparing DR & FF
w^{Dah}	0.0025*	0.0367*
w^{Dah}*;Ance*$^{\Delta}$	0.0001*	0.0038*
w^{Dah}*;Ance*$^{\Delta}$*/ +*	0.0056*	0.00517

Table 88: Male comparison of survival curves by genotype for response to DR using the log rank test in JMP for each food comparing controls, *Ance*$^{\Delta}$ and *Ance*$^{\Delta}$*/+ males*. *Indicates significant difference (P=<0.05).

Genetic Comparison	Food	Log-rank Comparison
w^{Dah} *to Ance*$^{\Delta}$	SF	0.3095
	DR	0.0272*
	FF	0.0144*
w^{Dah} *to Ance*$^{\Delta}$*/+*	DR	0.1185
	FF	0.419
Ance$^{\Delta}$ *to Ance*$^{\Delta}$*/+*	DR	0.4755
	FF	0.0529

Table 89: Male comparison of survival curves by genotype for standard, DR and FF diets using the log rank test for each food comparing controls, *Ance*$^{\Delta}$ and *Ance*$^{\Delta}$*/+* males. *Indicates significant difference (P=<0.05).

Ance does not appear to be involved in the dietary response of male flies to longevity with *Ance*$^{\Delta}$ males responding normally to dietary changes compared to controls.

8.3: Discussion

Ance, like *Acer*, is a homologue of human *Ace*. In this study, *Acer* has been found to demonstrate a role modulating the response of various phenotypes to changing nutrition, therefore *Ance* was tested for a potential role in the modulation of longevity and fecundity to changing diet. $Ance^{\Delta}$ males and females responded to DR normally with a lifespan extension on DR food, as did flies with the heterozygous deletion. There was no difference in lifespan or fecundity between controls and $Ance^{\Delta}$ flies, suggesting that *Ance*, unlike *Acer*, is not likely to be involved in the dietary response. The lifespan and fecundity experiments require repeating to confirm this phenotype.

These results showed that *Ance* is unlikely to be involved in the response of lifespan to nutrition. $Acer^{\Delta}$ flies have already shown a sleep fragmentation at night (Carhan, et al., 2010) and are resistant to starvation in the w^{Dah} background (Chapter 6) independent of diet, therefore testing $Ance^{\Delta}$ flies' response to sleep and stress would be interesting. A double $Acer^{\Delta}$ and $Ance^{\Delta}$ mutant would highlight any links between the genes and the knockdown of *Ance* specifically in the male reproductive tissues may highlight any role for *Ance* in reproduction.

Chapter 9: Discussion

The data presented in this study showed a defective nutrient response of *Acer*$^{\Delta}$ flies for the nutrient responsive phenotypes of sleep, nutrient storage, lifespan and fecundity which strongly suggests that *Acer* normally plays a role in metabolic and physiological responses to dietary changes.

Acer's response to nutrition was also affected by genetic background as different phenotypes were seen for *Acer*$^{\Delta}$ males and females between the w^{Dah} and w^{1118} backgrounds. The w^{Dah} background is an equatorial population and the w^{1118} background is a temperate population (Ziehm, et al., 2013) and differences in the response to the loss of *Acer* within these backgrounds was seen in the phenotypes that responded to nutrition (Figure 68).

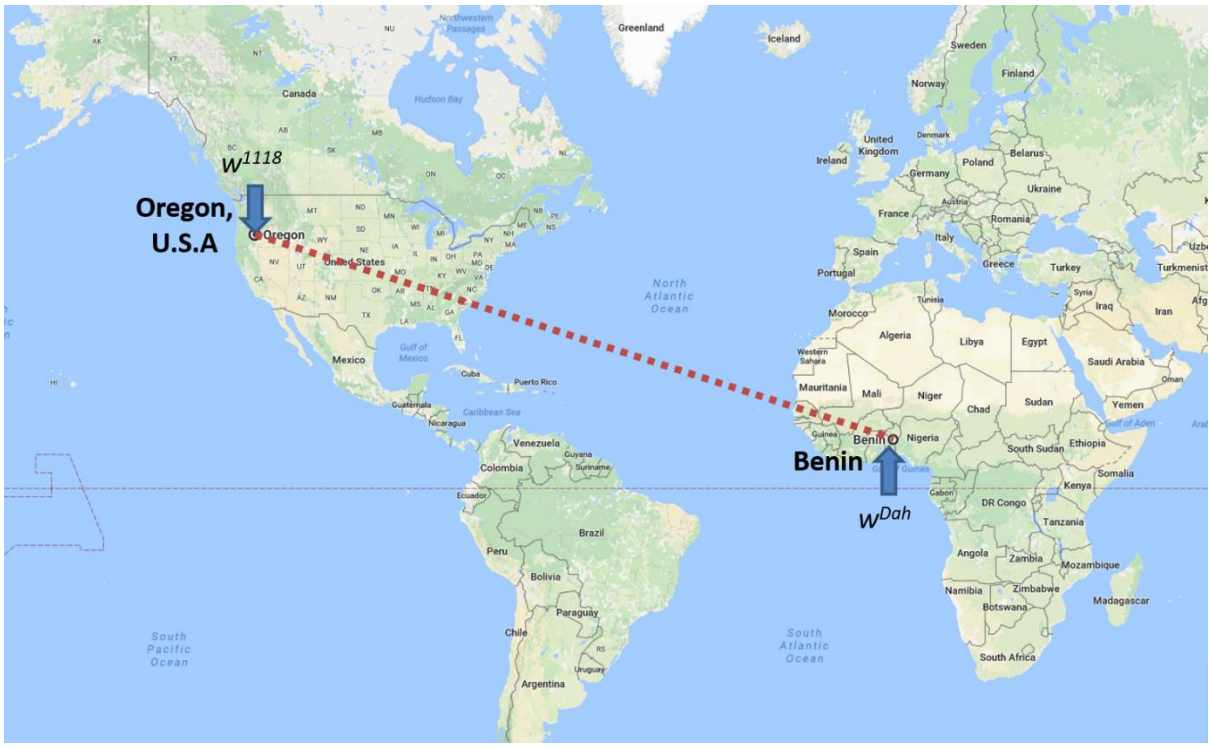

Figure 68: Location Map of w^{1118} and w^{Dah} origins. The w^{1118} strain (temperate) was developed from the Oregon R. strain sourced from the West Coast of the United States of America. The w^{Dah} strain (equatorial) was developed from the Dahomey strain sourced from Benin in the West of Africa (Ziehm, et al., 2013). Map was sourced from Google Maps.

In this investigation it was found that *Acer* expression in the w^{Dah} background does not change significantly over 24 hours but in the w^{1118} background *Acer*

expression was significantly higher at 1am than at 1pm in female flies, suggesting that the control of *Acer's* expression varies with genetic background (Chapter 7). This correlates with a previous study that found variation in *Acer* expression within different genetic and environmental conditions (Svetec, et al., 2015). The analysis of this experiment is incomplete and the results shown are for females in both backgrounds on DR food only. The analysis of *Acer* expression for females on FF food and for male flies on both DR and FF foods in both backgrounds is on-going. In this study *Acer* has been found to modulate the response to starvation and low food conditions, therefore future experiments may test the expression of *Acer* on Starvation and Low food as well as DR and FF food.

Equatorial populations are generally not subjected to as many changes in climate as temperate populations meaning that temperate populations are often more adaptable to changing climate (Kobey & Montooth, 2013). This may be a reason for *Acer's* difference in expression between the two backgrounds and warrants further investigation using different genetic backgrounds with a range of temperate and equatorial populations analysed. Furthermore, due to temperature differences between the two original locations of the genetic backgrounds, it would be interesting to test *Acer's* expression at different temperatures, such as 28ºC and 18ºC, in both backgrounds to establish whether temperature has an effect on the expression of *Acer*. Also it would interesting to analyses $Acer^{\Delta}$ flies to see if they show any difference in lifespan compared to controls at these different temperatures indicating whether *Acer* is needed to adapt to temperature.

Sleep in flies is similar to sleep in humans as flies sleep more at night and are more active during the day as they have similar circadian rhythms to humans (Shaw, et al., 2000). $Acer^{\Delta}$ flies in both backgrounds and for both sexes showed normal circadian rhythms when compared to controls. This is in agreement with Carhan et al., (2010) which also saw no difference in the circadian pattern. In this study some sleep phenotypes responded to changing diet in control flies and results differed between the backgrounds and the sexes (Chapter 3 and Figure 69).

As expected, when activity levels were higher sleep levels were lower and vice-versa as the two phenotypes mirror-image each other. All flies slept more in the dark than the light regardless of food concentration, genotype, sex and background. Figures 69A and 69B show that for total activity and total sleep in female flies that

there is a difference between the genetic backgrounds in the response of sleep and activity to changing diet.

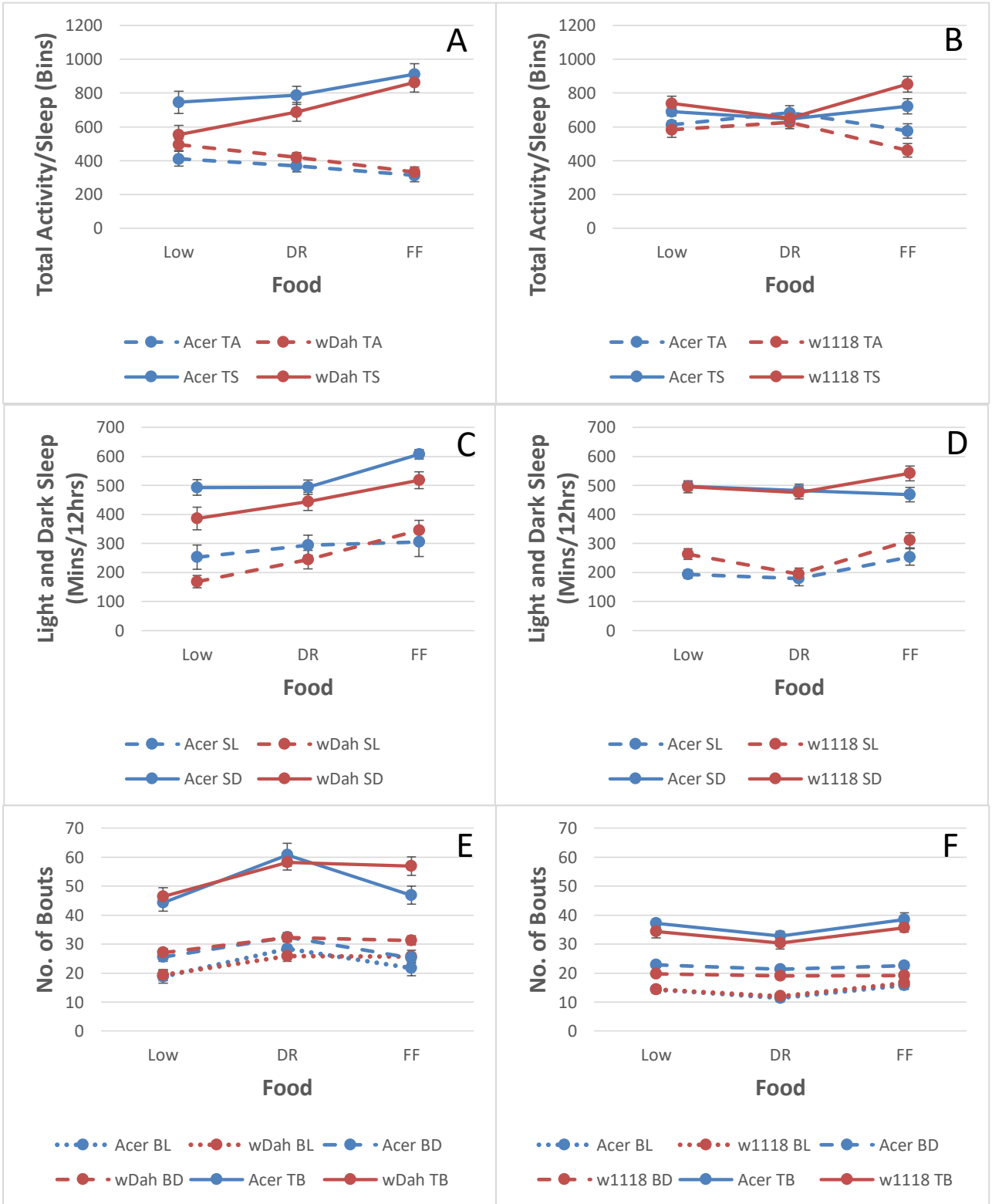

Figure 69: Summary of Female Sleep Data. I Bin = I minute. (A and B) Total Activity (TA) and Total Sleep (TS) for w^{Dah} and w^{Dah};$Acer^{\Delta}$ (A) and w^{1118} and w^{1118};$Acer^{\Delta}$ (B). (C and D) Sleep in the Light (SL)

and Dark (SD) for w^{Dah} and $w^{Dah};Acer^{\Delta}$ (C) and w^{1118} and $w^{1118};Acer^{\Delta}$ (D). (E and F) Total Sleep Bouts (TB) and Sleep Bouts in the Light (BL) and the Dark (BD) for w^{Dah} and $w^{Dah};Acer^{\Delta}$ (E) and w^{1118} and $w^{1118};Acer^{\Delta}$ (F).

In the w^{Dah} background (Figure 69A, C and E), sleep and activity were closest on Low food with total sleep levels increasing and activity levels decreasing as food levels increased. Both dark and light sleep responded to increasing diet with an increase in sleep and bouts of sleep increased between Low and DR food before levelling off at the same level as DR food for FF food. In the w^{1118} background (Figures 69B, D and F), total activity and total sleep were at similar levels on DR food with peak activity and the lowest level of sleep intersecting on DR food. Like w^{Dah} controls the highest level of sleep and lowest level of activity were seen on FF food. Light and dark sleep in the w^{1118} background differed with sleep in the dark showing no difference between Low and DR food but an increase in sleep was seen on FF food. Light sleep showed a decrease in sleep between Low and DR food before sleep rose again on FF food. Sleep bout numbers in total and in the light and dark showed no change with food increase.

$Acer^{\Delta}$ females did not show the same sleep response to dietary changes when compared to controls in both backgrounds. In the w^{Dah} background, $Acer^{\Delta}$ females showed no significant change in total activity or total sleep levels with changing diet. However, when broken down into light and dark sleep $Acer^{\Delta}$ females did significantly increase sleep levels with increasing food in the dark but showed no difference between the foods in the light. However, $Acer^{\Delta}$ females slept significantly longer in the dark on Low food and FF food than w^{Dah} controls. In the w^{1118} background $Acer^{\Delta}$ females did not show any response of sleep in the dark with changing food but slept significantly less on FF food and in the light $Acer^{\Delta}$ females slept significantly less on Low food than controls. The 24 hour analysis suggested that the main differences between controls in both backgrounds and $Acer^{\Delta}$ females occurred in the transition hours between light and dark. This suggests that *Acer* modulates the response of sleep to changing diet and potentially the effect of diet on the transition between light and dark sleep and activity in female flies.

Male controls in both backgrounds did not respond to dietary changes for sleep and activity (Figure 70). In the w^{Dah} background $Acer^{\Delta}$ males also showed no sleep or activity response to nutrition but in the w^{1118} background $Acer^{\Delta}$ males responded to diet with an increase in activity and a decrease in sleep with increasing food. This

resulted in significantly increased activity and decreased sleep on FF food for *Acer*$^{\Delta}$ males in the *w*^{1118} background.

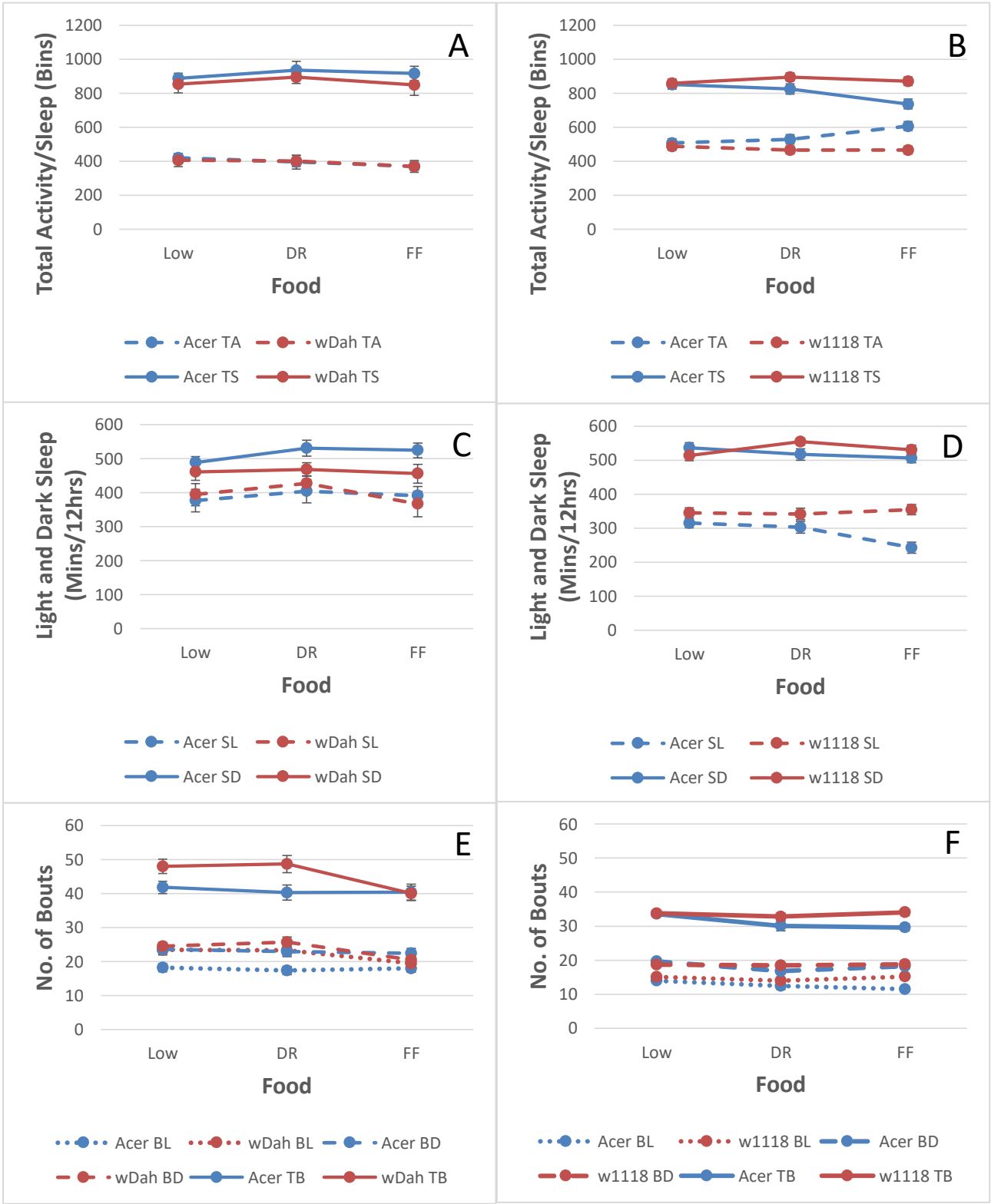

Figure 70: Summary of Male Sleep Data. I Bin = I minute. (A and B) Total Activity (TA) and Total Sleep (TS) for *w*^{Dah} and *w*^{Dah}*;Acer*$^{\Delta}$ (A) and *w*^{1118} and *w*^{1118}*;Acer*$^{\Delta}$ (B). (C and D) Sleep in the Light (SL) and

Dark (SD) for w^{Dah} and $w^{Dah};Acer^{\Delta}$ (C) and w^{1118} and $w^{1118};Acer^{\Delta}$ (D). (E and F) Total Sleep Bouts (TB) and Sleep Bouts in the Light (BL) and the Dark (BD) for w^{Dah} and $w^{Dah};Acer^{\Delta}$ (E) and w^{1118} and $w^{1118};Acer^{\Delta}$ (F).

Total sleep in the light and the dark showed no significant difference in male controls in either background. In the w^{Dah} background $Acer^{\Delta}$ males showed no response of sleep in the dark or light to diet. However, $Acer^{\Delta}$ males slept significantly more in the dark on DR food and showed increased sleep on FF food that was nearing significance. No difference was seen between the genotypes in the light. In the w^{1118} background $Acer^{\Delta}$ males showed no response of sleep in the dark to changing diet and no difference to controls. However in the light, sleep significantly decreased for $Acer^{\Delta}$ males on FF food compared to Low and DR food, resulting in $Acer^{\Delta}$ males sleeping less on FF food than controls. The 24 hour results confirmed the lack of overall response to diet in male controls. However, the differences seen at separate time points between controls and $Acer^{\Delta}$ males, like females, occurred at the transition time points between light and dark. This suggests a potential role for *Acer* in modulating the dietary response of sleep and activity in the transition between light and dark.

Total bouts of sleep as well as bouts of sleep in the dark and light did not respond to dietary changes for male controls in the w^{1118} background. However, control males in the w^{Dah} background showed a decrease in bouts on FF food in total and in the dark. $Acer^{\Delta}$ males in the w^{Dah} background did not respond to changing diet and a decrease in bouts on Low and DR food in total bouts and bouts in the light was seen compared to controls. In the w^{1118} background, $Acer^{\Delta}$ males showed a significant reduction in total sleep bouts and bouts in the light between Low and FF food. This resulted in significantly reduced total bouts and bouts in the light on FF food for $Acer^{\Delta}$ males. This suggests that *Acer* may modulate the dietary response of sleep bouts in the light in male flies but that the direction is dependent genetic background.

The differences in sleep and activity between the genetic backgrounds is most likely due to the strains' origins (Figure 68). The 12 hour light/dark cycle is much closer to the light/dark pattern found near the equator and is therefore more representative of the conditions for the w^{Dah} background than the w^{1118} background. The light cycle for the w^{1118} background would change more with the seasons and would therefore need to be more adaptable. Although the 24 hour *Acer* expression analysis is

incomplete in this study, it did propose that *Acer* expression remained constant in the w^{Dah} background but cycled in the w^{1118} background with peak expression at 1am and lowest expression at 1pm. This is interesting as in most light/dark patterns 1am would almost always be dark and 1pm would almost always be light. The expression analysis has so far only been conducted in females and on DR food. Further analysis for males on DR food as well as males and females on FF food may help in the understanding of whether *Acer* itself is affected by dietary change and whether expression levels of *Acer* may indicate the differences seen in the sleep phenotype.

Male and female flies showed differences in their response of sleep and activity to changing diet. Females were more reactive to dietary change while males showed very little response to the change in their diet. The most likely reason for this is that female flies lay eggs which is an energy consuming process. Female flies are known to lay more eggs on high yeast food than on lower yeast food (Skorupa, et al., 2008) and that has proved the same in this study (Chapter 5). For female flies searching for food for the energy required for egg laying is important and therefore activity is likely to increase when food is scare and reduced to conserve energy when food is plentiful. Males do not possess this behaviour and are therefore more likely to keep energy and sleep levels constant. If *Acer* modulates dietary responses then more prominent differences are likely to be seen in female flies rather than male flies due to females heightened response to dietary changes.

The sleep results in this study suggest that *Acer* modulates the response of sleep to changing diet, however the effects of the loss of *Acer* showed that *Acer*'s role is background and sex dependent. The sleep experiment was conducted once in each background, therefore repetition is needed to confirm the results. Carhan, et al. (2010) also found that flies fed the ACE inhibitor Fosinopril, which also inhibits ACER, showed disturbed sleep similar to $Acer^{\Delta}$ mutants. Repeating the Fosinopril experiment, as well as testing other ACE inhibitors such as Enalapril, on the different foods would establish whether the phenotypes seen in this study were due to the loss of *Acer* expression. Thimgan, et al. (2015) found that *Acer* expression was up-regulated when flies were sleep deprived, therefore analysing *Acer* expression in controls that are sleep deprived and comparing their sleep deprivation behaviour to $Acer^{\Delta}$ flies may help to understand *Acer's* role in sleep and whether *Acer* is only involved in the response to nutrition or has other roles in the sleep phenotype. Masek, et al. (2014) has previously found that starvation resistant fly strains slept for longer than strains that were not starvation

resistant. *Acer*$^{\Delta}$ females showed increased sleep on Low food but analysis of sleep behaviour under starvation conditions was not measured in this study. *Acer* is up-regulated under starvation conditions (Thimgan, et al., 2015) and *Acer*$^{\Delta}$ mutants are starvation resistant in the w^{Dah} background and starvation sensitive in the w^{1118} background (Chapter 6), therefore analysis of the sleep behaviour of *Acer*$^{\Delta}$ flies under starvation conditions would establish a role, if any, for *Acer* in the response to starvation. This study was conducted at young ages and as *Acer* appears to be involved in sleep regulation it would interesting to test whether this is just an initial response or whether it continues throughout life at all ages. Sleep in flies has been found to increase and fragment with age with a decrease in sleep at night contrasted by increased sleep during the day (Bushey, et al., 2010; Metaxakis, et al., 2014). These ageing phenotypes are similar to those seen in humans making *Drosophila* a good model to test sleep (Cirelli, 2009). Reduced levels of IIS have been found to decrease day time sleep and ameliorate the age-related fragmentation in *Drosophila* (Metaxakis, et al., 2014). *Acer*$^{\Delta}$ females showed increased in total sleep on Low food and have also shown altered day time sleep compared to controls. The response of sleep to nutrition appears to differ between the genetic backgrounds and the sexes and with different patterns of sleep in the light and the dark. Further investigation into sleep is needed to establish *Acer's* role in this phenotype.

The reduced response to diet in sleep for *Acer*$^{\Delta}$ flies followed the same trend for glycogen storage where *Acer*$^{\Delta}$ males and females showed altered glycogen storage levels (Chapter 4).

In control females (Figure 71) and males (Figure 72) glycogen storage levels increased with increasing food levels. A drop in glycogen storage was seen on FF food for female flies with the reduction on FF food most likely to be caused egg laying. Egg laying is a high energy process, which increases on the higher yeast foods (Burger, et al., 2007) (Chapter 5), therefore fewer eggs are remaining inside the ovaries resulting in a reduction in glycogen storage on high yeast food that is not observed in male flies. The confirm this the assay could be repeated using flies that have had their ovaries removed or in female flies that do not have ovaries to establish whether the decrease in glycogen levels on FF food for control flies was truly due to egg-laying.

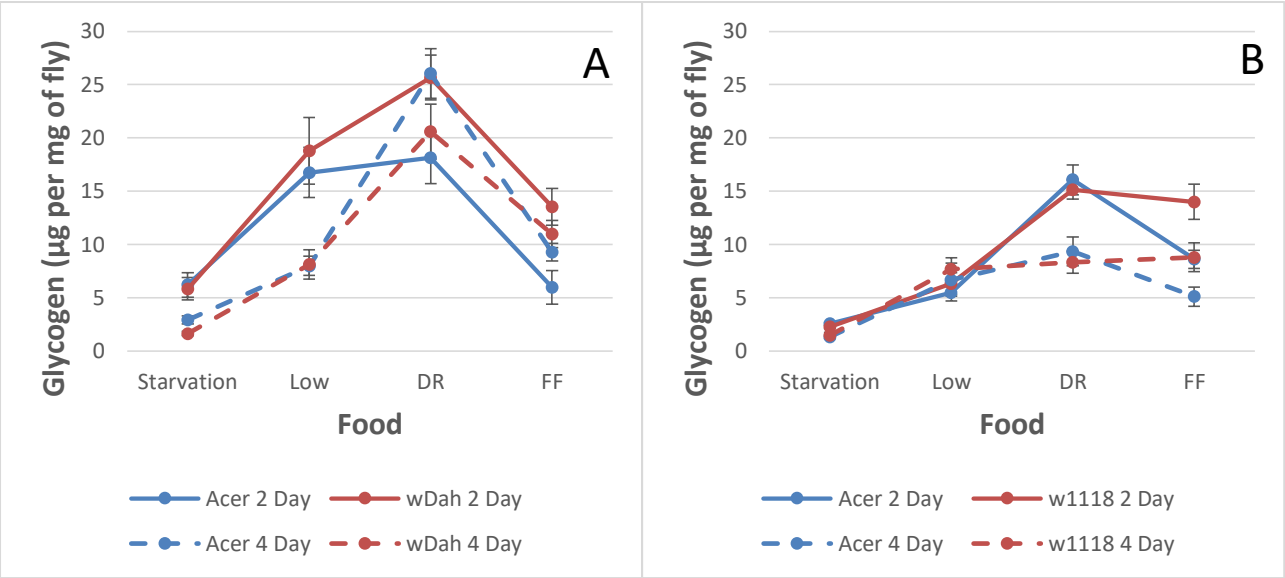

Figure 71: Summary of Female Glycogen levels on Starvation, Low, DR and FF foods after feeding for 2 Days (2 Day) and 4 Days (4 Day). A) w^{Dah} and w^{Dah};$Acer^{\Delta}$ B) w^{1118} and w^{1118};$Acer^{\Delta}$.

In the w^{Dah} background $Acer^{\Delta}$ males and females stored less glycogen than controls on DR and FF foods after two days feeding on the different diets but the glycogen levels returned to normal after four days feeding on these foods due to a reduction in control stores and an increase in $Acer^{\Delta}$ stores. $Acer^{\Delta}$ females in this background also showed higher storage levels of glycogen in response to starvation after four days with only water and no food. This data suggests that *Acer* modulates the glycogen response to dietary changes especially at higher food levels (DR and FF). In the w^{1118} background control females did not show the same reduction of glycogen stores on FF food as w^{Dah} controls. Control w^{1118} females are not as fecund as w^{Dah} females (Chapter 5) which agrees with the previous thought that the reduction in glycogen levels on FF food was due to higher energy use for egg-laying in the w^{Dah} background. $Acer^{\Delta}$ females in the w^{1118} background showed a reduced level of glycogen storage after two and four days feeding. This data suggests that the effect of the loss of *Acer* on glycogen storage differs between the two backgrounds in female flies. $Acer^{\Delta}$ males in the w^{1118} background appeared to show a delayed response of the effect of the loss of *Acer* as glycogen levels were normal after two days feeding. However, these flies still showed a reduction in glycogen storage on high food after four days feeding on the different diets.

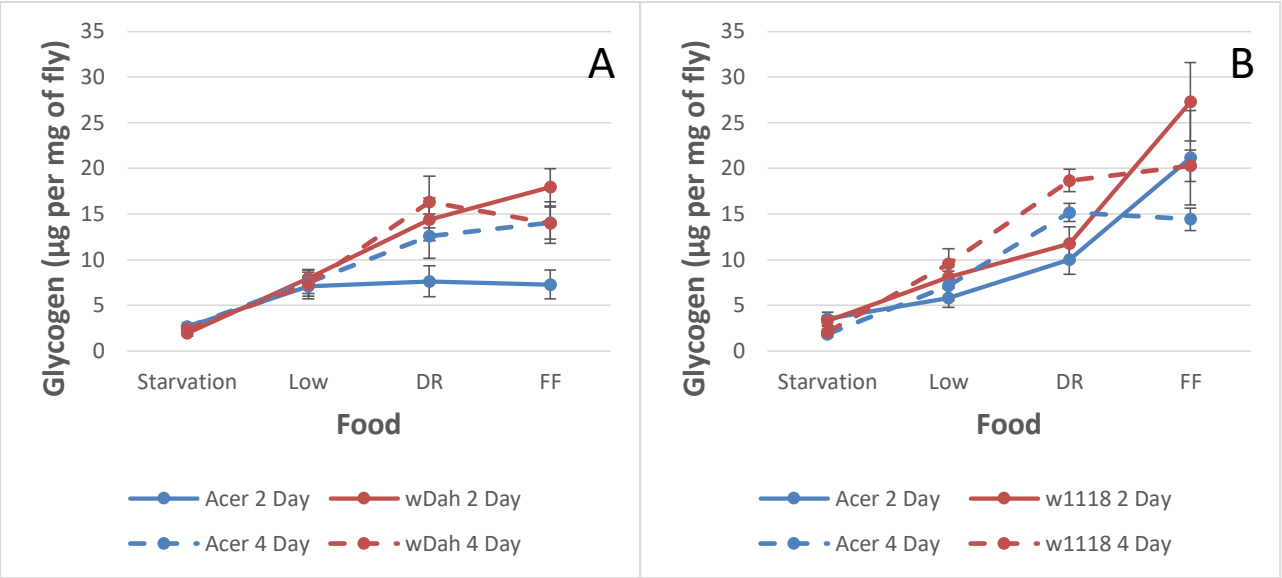

Figure 72: Summary of Male Glycogen levels on Starvation, Low, DR and FF foods after feeding for 2 Days (2 Day) and 4 Days (4 Day). A) w^{Dah} and w^{Dah};$Acer^{\Delta}$ B) w^{1118} and w^{1118};$Acer^{\Delta}$.

Glycogen is a form of carbohydrate storage therefore levels in the fly would be expected to increase as dietary sugar levels rose. Higher levels of carbohydrate within the food flies consume has been found to increase the intake of food (Skorupa, et al., 2008), so any rise in the levels of glycogen could be due to increased food intake. However, the data in this study suggests that yeast levels are more important for glycogen storage as there was still a rise in stored glycogen when the sugar content remained the same for control flies at 50g/l while yeast levels increased from 50g/L (DR) to 200g/l (FF).

The altered storage of glycogen on the higher yeast foods suggests that *Acer*$^{\Delta}$ flies may be unable to store as much glycogen as controls or that *Acer* flies use more of their stored glycogen than controls, which may be the result of higher activity. However, it is unlikely that the lower stores are due to higher activity as male and female flies in the w^{Dah} background showed no difference in activity and sleep on high food. Although *Acer*$^{\Delta}$ males in the w^{1118} background did show increased activity levels on high food females did not suggesting that the cause of lower glycogen stores is not activity related. The difference seen in glycogen storage suggests a link with the IIS pathway as insulin-like ligands are involved in converting sugar into glycogen storage (Broughton & Partridge, 2009). Xu et al (2008) found that flies which lack a clock gene to regulate gene expression within the fat body are more likely to store lower levels of glycogen and this is a similar phenotype shown by *Acer*$^{\Delta}$ mutants. Xu et al (2008) also

found that flies without a clock gene were more susceptible to starvation and consumed more food, however the results in this study found that *Acer*$^{\Delta}$ males and females fed normally in the both backgrounds, suggesting that the decrease in glycogen was not due to decreased feeding. As the results showed differing levels of glycogen after two days feeding it is important in the future to assess *Acer*$^{\Delta}$ male and female feeding on different foods after two days feeding to assess whether feeding patterns are altered between two and four days feeding on the different diets. It is possible that the reduction in glycogen storage over two days is due to initial reduced feeding for *Acer*$^{\Delta}$ mutants, however this is unlikely as lipid levels are unchanged between *Acer*$^{\Delta}$ mutants and controls.

Acer has been selected for by evolution and therefore must be needed for flies to continue its expression, otherwise *Acer* would have been naturally deselected over time. The evidence in this study suggests that *Acer* may be required for flies to maximise glycogen storage when diet is plentiful.

Lipid metabolism was not affected by changing diet and *Acer* appears to play no role in lipid metabolism except, like glycogen storage, *Acer*$^{\Delta}$ females in the *w*^{Dah} background appeared to utilise lower amounts of stored lipid in response to starvation compared to controls.

Higher lipid and glycogen storage levels are thought to protect against starvation (Ballard, et al., 2008; Xu, et al., 2008) and interestingly *Acer*$^{\Delta}$ females in the *w*^{Dah} background showed increased lipid and glycogen stores on Starvation food and were found to be starvation resistant compared to controls (Chapter 6). *Acer*$^{\Delta}$ females in the *w*^{1118} background were found to be slightly sensitive to starvation but did not show increased levels of lipid and glycogen storage under starvation conditions. These data suggest that *Acer*$^{\Delta}$ females are resistant to starvation due to higher lipid and glycogen levels. However, *Acer*$^{\Delta}$ males are also resistant to starvation but did not show increased levels of lipid and glycogen stores on Starvation food after four days feeding. This data suggests that *Acer* modulates the response to starvation resistance independent of lipid and glycogen stores or the effect of the loss of *Acer* on starvation resistance is different in male and female flies. A likely reason for this difference between the sexes in the *w*^{Dah} background is the high egg laying phenotype of control females. In this study, *Acer*$^{\Delta}$ females laid fewer eggs than control females (Chapter 5), and although egg-laying was not monitored on Starvation food due to very low numbers, it is possible that the increase in glycogen and lipid stores observed on

Starvation food after four days feeding for *Acer*$^{\Delta}$ females could be fecundity related. This would explain why *Acer*$^{\Delta}$ males do not show the same increased glycogen and lipid stores and why no difference between the genotypes was observed in the *w*^{1118} background as *w*^{1118} females lay much fewer eggs.

In this study, nutrient storage was tested by analysing lipid and glycogen storage levels after two and four days feeding on different diets. Repetition is required to confirm these phenotypes after two and four days feeding in both backgrounds. Flies also store carbohydrate as trehalose which is stored in the fly fat body and the haemolymph (Becker, et al., 1996). Due to the reduced glycogen phenotype it is important to analyse trehalose levels to determine whether *Acer* is involved in all carbohydrate storage or if it is specific to glycogen storage. *Acer's* presence in the fat body indicates that trehalose may be potentially linked with *Acer* (Carhan, et al., 2010). ACE inhibitors that also inhibit ACER, such as Fosinopril, have already been shown to cause similar effects to the loss of *Acer* in the sleep phenotype (Carhan, et al., 2010). The reduction in glycogen storage could be tested by feeding flies ACE inhibitors such as Fosinopril and Enalapril to see if a reduced glycogen response is observed with the inhibition of ACER, resulting in a similar phenotype to *Acer*$^{\Delta}$ mutants. Different dosages of the inhibitors may also indicate the threshold at which glycogen storage is affected by the inhibition of ACER. As *Acer* is expressed within the fly fat body and glycogen is also stored in the fat body, a knockdown of *Acer* expression within the fat body only may indicate whether the loss of *Acer* expression in the fat body is responsible for the reduced glycogen phenotype that was observed in this study.

The wet weight of *Acer*$^{\Delta}$ females responded normally after four days feeding on the different diets in both backgrounds. However after two days feeding *Acer*$^{\Delta}$ females were heavier than *w*^{Dah} controls on Starvation and Low food and lighter on the DR and FF foods in the *w*^{1118} background. The decrease in weight on the FF food does correlate with the reduced glycogen levels observed in the *w*^{1118} background but does not correlate with the normal glycogen levels on the DR food. The increased weight in the *w*^{Dah} background does not correlate with the finding for lipid and glycogen storage. These data suggest that the differences in weight for *Acer*$^{\Delta}$ females is not due to glycogen and lipid stores. Protein, another form of energy storage in the fat body, storage was not analysed in this study. Protein levels are important in the mediation of DR (Bass, et al., 2007) and protein storage is independent of carbohydrate storage

(Skorupa, et al., 2008), therefore it is interesting that differences were seen between *Acer*$^{\Delta}$ flies and controls when dietary yeast concentrations were changed (DR and FF foods). Therefore analysis of protein levels would establish whether *Acer*$^{\Delta}$ flies store and utilise protein normally and any difference may lead to a link to the TOR signalling pathway which is activated by amino acids.

Protein is also an important factor in fecundity as female flies lay a higher number of eggs on yeast rich medium (Burger, et al., 2007) but this phenotype appears to be traded-off with DR as fecundity levels are not optimal under DR conditions (Fontana, et al., 2010). *Acer*$^{\Delta}$ females in both backgrounds responded to DR normally by laying fewer eggs on the DR food compared to the FF food (Burger, et al., 2007). In the w^{Dah} background *Acer*$^{\Delta}$ females laid fewer eggs than controls on both DR and FF food (Chapter 5), suggesting that *Acer* is involved in the fecundity response independent of diet. In the w^{1118} background there was no difference in fecundity between *Acer*$^{\Delta}$ females and controls on either food. Interestingly, w^{Dah} control females laid significantly more eggs than w^{1118} females across both foods indicating that fecundity is dependent on genetic background and that the loss of *Acer* was potentially magnified in the more fecund background.

Reduced fecundity in *Acer*$^{\Delta}$ females in the w^{Dah} background led to the analysis of fecundity by reciprocal mating to establish whether the reduction in egg-laying was the result of the lack of *Acer* in male or female flies. A trend towards reduced fecundity for *Acer*$^{\Delta}$ females was seen but *Acer*$^{\Delta}$ males appeared to have no role in the reduced fecundity phenotype as reciprocally mated control female fecundity was not affected by mating with *Acer*$^{\Delta}$ males. This experiment was conducted on standard food and as the reduction in egg-laying was most prominent on FF food it would wise to conduct a repeat reciprocal mating experiment on both DR and FF foods.

The *Acer*$^{\Delta}$ mutant response to longevity with dietary changes was mixed (Chapter 5) but *Acer*$^{\Delta}$ mutants responded to DR with a lifespan extension, therefore *Acer* is not required for lifespan extension to DR. In the w^{Dah} background, *Acer*$^{\Delta}$ females were short-lived on FF food compared to controls but in the w^{1118} background the result was mixed with a lifespan extension on FF food seen as well as no difference in lifespan on the same food. In both backgrounds *Acer*$^{\Delta}$ females showed no difference in lifespan on DR food compared to controls. The results suggest that *Acer* may be involved in the response of lifespan to high food and that the direction is background dependent. Repetition is needed to clarify the effect of the loss of *Acer* on lifespan.

The lifespan results for *Acer*$^{\Delta}$ males was variable between the experiments and the backgrounds. In both backgrounds *Acer*$^{\Delta}$ males were long-lived on DR and FF foods compared to controls, suggesting a possible role for *Acer* in the DR response and the response to high food. However in both backgrounds a second experiment found no significant difference between *Acer*$^{\Delta}$ males and controls on both foods. The lifespan response of *Acer*$^{\Delta}$ males requires repetition to firmly establish if *Acer* modulates the response of lifespan to diet in male flies.

The response lifespan to the loss of *Acer* to Low food did not differ between the sexes but did differ between the backgrounds. In the *w*^{1118} background *Acer*$^{\Delta}$ males and females responded normally but in the *w*^{Dah} background *Acer*$^{\Delta}$ males and females were short-lived on Low food. These data suggest that *Acer* modulates the lifespan response to low food in the *w*^{Dah} background but not the *w*^{1118} background.

This investigation has found that *Acer* is not involved in regulating the cold stress response and that the cold response to nutrition is variable. The equatorial *w*^{Dah} population was sensitive to cold compared to the temperate *w*^{1118} population.

Heat stress was measured at 37ºC and 39ºC and control flies in both backgrounds were significantly shorter-lived at the higher temperature. Control females in the *w*^{Dah} background were the only flies to respond to changing diet with an increase in survival time on FF food compared to DR food at 39ºC, suggesting that high food was protective against high levels of heat stress. *Acer*$^{\Delta}$ females did not respond to diet and were significantly short-lived on FF food compared to controls. In the *w*^{1118} background control females showed no response of heat stress resistance to changing diet at either temperature, however *Acer*$^{\Delta}$ females were long-lived on DR food compared to controls. Male controls in both backgrounds did not respond to dietary change at either temperature, but *Acer*$^{\Delta}$ males were short-lived at 39ºC. These data suggest that Acer modulates the response of high heat stress to high food in the *w*^{Dah} background but had little effect in the *w*^{1118} background.

As previously mentioned, *Acer*$^{\Delta}$ males and females were resistant to starvation in the *w*^{Dah} background but were sensitive to starvation in the *w*^{1118} background, suggesting that *Acer* modulates the response of lifespan to starvation but the direction is background dependent.

Oxidative stress was measured using hydrogen peroxide under DR and FF conditions and the results differed with genetic background (Chapter 6). Initially *Acer*$^{\Delta}$ flies were found to be resistant to oxidative stress in the *w*^{Dah} background for male and

female flies. This was confirmed under DR conditions which were shown to be protective in the response to oxidative stress for controls but *Acer*$^{\Delta}$ mutants showed no response to diet in the response to oxidative stress with median lifespan almost identical for the two foods. In the *w*^{1118} background DR did not affect oxidative stress resistance for controls and *Acer*$^{\Delta}$ mutants responded normally. These data suggest that *Acer's* role in oxidative stress is dependent on genetic background.

Due to *Acer's* role in the response of sleep and glycogen storage to nutrition a potential link to the nutrient sensing IIS pathway was investigated. The data on *Acer* suggests a link to the IIS pathway based on similar phenotypes including resistance to starvation and oxidative stress in the *w*^{Dah} background. However, the data presented here suggests a more complex situation as unlike mNSC-ablated flies *Acer*$^{\Delta}$ mutants are not long-lived on low food or high food and are not cold stress (Broughton, et al., 2005).

Dilps (*Drosophila* insulin-like peptides) are part of the IIS pathway in flies and there are eight different *dilps* that are expressed throughout the fly body, including the fat body and the head of the fly. ACER is known to be expressed within the fat body of flies (Carhan, et al., 2010) and specifically *dilp6* is also expressed in the fat body (Slaidina, et al., 2009). DILPs 2, 3 and 5 are expressed within the median neurosecretory cells (mNSCs) of the brain and ablation of these cells results in reduced levels of insulin-like signalling (Broughton, et al., 2005). Overexpression of *dilp6* in the fat body increases lifespan and the transcription factor dFOXO has been found to regulate the expression of *dilp6* within this tissue (Bai, et al., 2012; Slaidina, et al., 2009). Both ACER and *dilp6* are also expressed within the fly head as well as the fat body suggesting a possible link between ACER and *dilp6* expression.

The results testing *dilp* transcription in the fly head and body found, as previously discovered (Broughton, et al., 2010), that *dilp5* responded to diet in the fly head. Although the N of this experiment was small (N=3) a trend towards reduced levels of transcription for *dilp5* in *Acer*$^{\Delta}$ females was seen but repetition is required to confirm this lower level of expression. In *Acer*$^{\Delta}$ males the trend was towards increased levels of *dilp5* suggesting that *Acer* may have a different role in response to *dilp5* between the sexes. In the fly bodies, *dilp6* and *dilp7* responded significantly to diet in control females with a decrease in transcription with increasing food for *dilp6* and a significant increase in *dilp7* with increasing food. Control males showed a decreasing trend of both *dilp6* and *dilp7* with increasing food. *Acer*$^{\Delta}$ females showed a reduction

in transcription of *dilp6* on Starvation and FF food and on FF food only for *Acer*$^{\Delta}$ males. This data is in agreement with previous findings for *dilp6* (Slaidina, et al., 2009). *Dilp7* in control females showed a significant increase in transcription with increasing food levels but *Acer*$^{\Delta}$ females showed a significant lack of response to diet on both the DR and FF foods (Figure 73).

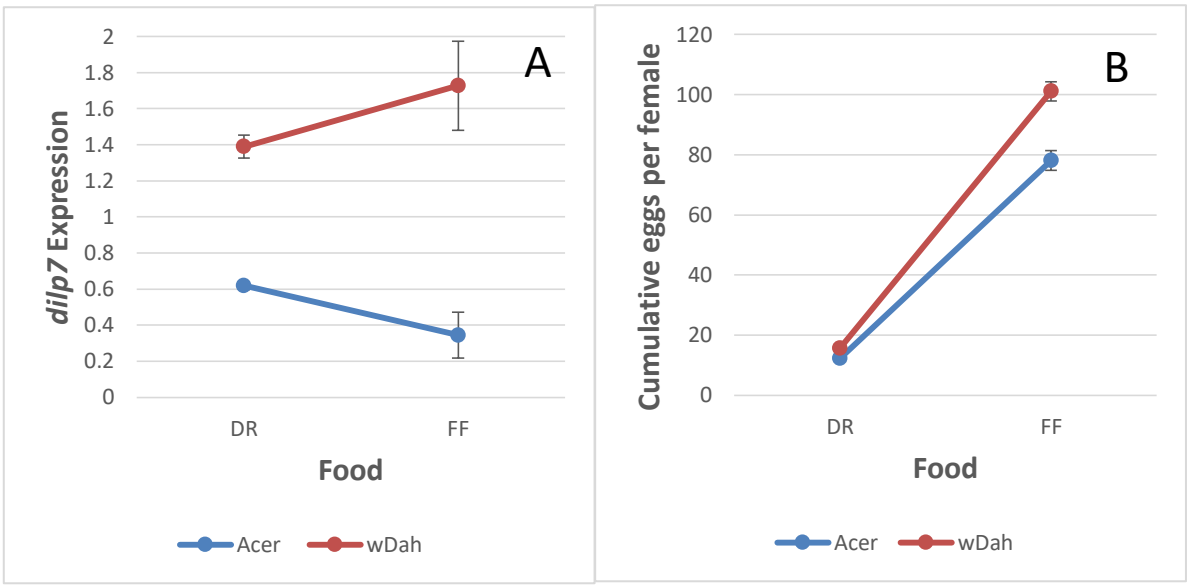

Figure 73: Comparison of *dilp7* expression and fecundity for *Acer*$^{\Delta}$ females and controls in the *w*^{Dah} background on DR and FF food. A) *dilp7* expression on DR and FF food for *w*^{Dah} controls and *w*^{Dah}*;Acer*$^{\Delta}$ females. B) Cumulative egg laying per fly on DR and FF food for *w*^{Dah} controls and *w*^{Dah}*;Acer*$^{\Delta}$ females.

Interestingly in males, *dilp7* transcription levels decreased with increasing food for controls but *Acer*$^{\Delta}$ males showed a lack of response to diet with decreased transcription levels on FF food. The lack of response of *dilp7* in *Acer*$^{\Delta}$ females is interesting as *dilp7* has been connected with female site analysis for egg-laying (Yang, et al., 2008). The results showed an increase in *dilp7* transcription with increasing yeast and female flies are known to increase egg-laying on higher yeast foods (Bass, et al., 2007). *Acer*$^{\Delta}$ females laid fewer eggs on DR and FF foods than controls (Chapter 5 and Figure 73) and showed a decreased level of *dilp7* transcription on DR and FF foods (Chapter 7). Together these data suggest that *Acer* may modulate the expression of *dilp7* causing reduced fecundity in the absence of *Acer*. With DILP7 potentially affecting egg laying in female flies it is possible that DILP7 may be involved in reproduction in male flies. Courtship behaviour could possibly be controlled by

DILP7 levels which are then modulated by ACER. In this study courtship behaviour was not analysed therefore analysis of courtship behaviour of controls and *Acer*$^{\Delta}$ males may indicate whether ACER is involved in courtship and potentially DILP7 as well.

ACER and DILP6 are both expressed within the fly fat body, therefore analysis of *dilp6* expression when *Acer* is knocked-down specifically in the fly fat body may reveal if *Acer* modulates the response of *dilp6* in this tissue. Immunohistochemistry of *dilp6* in the fly fat body and *dilp7* in the abdominal neuromeres in *Acer*$^{\Delta}$ mutants may establish whether *Acer* modulates the expression of these *dilps* in these tissues.

The lack of response of *dilp* transcription in *Acer*$^{\Delta}$ flies to diet in the nutrient responsive *dilps* suggests a role for *Acer* in modulating the response of nutrient responsive *dilps* to changing diet. However, the Ns for the expression experiments were low and were only conducted in the w^{Dah} background and therefore needs to be repeated to confirm our results and also conducted in the w^{1118} background as we have previously seen differences between the genetic backgrounds.

Immunohistochemistry of DILP5 in the mNSCs of the fly brain showed that DILP5 protein levels, like transcription levels (Chapter 7), increase with increasing food in control flies in the w^{1118} background. DILP5 protein expression was found to be normal on the DR and FF foods in *Acer*$^{\Delta}$ males and females but DILP5 levels were significantly higher on the Low food compared to controls. *Dilp* transcription was not analysed on Low food in this study, therefore with the significantly higher level of DILP5 protein in *Acer*$^{\Delta}$ mutants transcription levels of *dilp5* needs to be analysed on Low food. If transcription levels of *dilp5* were higher than controls on Low food this would agree with the immunohistochemistry data, however if transcription levels were no different to controls this would suggest that *Acer* modulates the release of DILP5 to low food in the mNSC's. This link may be more complex than just DILP5 expression as mNSC-ablated flies are long-lived on low food (Broughton, et al., 2005) whereas *Acer*$^{\Delta}$ males and females are short-lived on low food.

Wolbachia has been found to increase levels of insulin-like signalling (Ikeya, et al., 2009) and with *Acer's* potential involvement of the modulation of *dilps* it would interesting to analyse *Acer's* expression in the presence of Wolbachia and without Wolbachia to see if *Acer* expression is also increased.

ACER and ANCE are *Drosophila* homologs of human ACE which work as peptidyl-dipeptidases (Siviter, et al., 2002). ACE is involved in the human Renin-

Angiotensin System (RAS) which regulates blood pressure in a feedback loop mechanism (Guang, et al., 2012). ACE expression has also been found in human adipose tissue but little is known about ACE's role in this tissue (Jonsson, et al., 1994). This is particularly interesting as ACER is expressed within the fly fat body which acts similarly to human liver and adipose cells (Carhan, et al., 2010). *Drosophila* do not have an angiotensin system and therefore the substrates for ACER and ANCE cannot be angiotensin -1 and bradykinin which are the substrates of ACE in humans (Siviter, et al., 2002). Siviter et al., (2002) investigated peptides than may be suitable substrates for ACER and ANCE. It was found that ACER and ANCE were likely to have multiple substrates with ACER's substrate specificity thought to be more relaxed than ANCE's. Leucokinin-I (LK) was found to be the best substrate for ACER but compared to ANCE's best substrate (Lom TK-1) hydrolysis was 10-fold lower. LK was also cleaved by ANCE, but with a lower affinity. The ability to cleave the same peptides could indicate that ACER and ANCE work in conjunction with each other in *Drosophila*. LK is involved in the regulation of the sleep pattern and is downstream of the circadian clock proteins (Cavey, et al., 2016). $Acer^{\Delta}$ flies have shown differing sleep compared to controls but this has differed with diet and genetic background. In this study *Acer* expression was investigated but the experiment remains unfinished. The results suggested that on DR food females in the w^{Dah} background expressed *Acer* linearly throughout a 24 hour period, whereas in the w^{1118} background *Acer* expression showed more of a cyclic pattern with peak expression at 1am. Analysis of LK levels in ACER and ANCE mutants may indicate whether LK expression is influenced by ACER and ANCE and whether this could be the cause of the differences seen between $Acer^{\Delta}$ flies and controls in the sleep phenotype.

Further analysis is required on the *Ance* gene which does not appear to be involved in the response of lifespan and fecundity to diet (Chapter 8), although the lifespan and fecundity experiments require repetition. Double homozygous deletion *Acer/Ance* mutants may indicate if the expression and formations of *Ance* and *Acer* are connected if these flies are viable. It is possible that one gene compensates for the loss of the other and that the loss both is lethal when the loss of only one is not. This could also be achieved by investigations into *Ance* expression in controls and in $Acer^{\Delta}$ flies to see if there is compensatory increase in *Ance* expression in the absence of *Acer*. $Acer^{\Delta}$ mutants showed altered sleep and glycogen storage therefore

analysing *Ance*$^{\Delta}$ mutant sleep and glycogen storage would establish whether *Ance*, like *Acer*, is involved in regulating sleep and glycogen.

For neurosecretion to occur receptor proteins are essential links in the pathway and Methuselah (MTH) is a G-coupled protein receptor which is potentially a peptide receptor that could bind a product of *Acer* cleavage (Gimenez, et al., 2013). Reduced signalling of *mth* throughout the fly body and specifically within the brain of the fly has been shown to increase lifespan in *mth* nulls, possibly by reducing IIS overall. However, the lifespan increase may be due to FOXO (transcription factor) activation in insulin producing cells (IPCs). It is also possible that *mth* has links to the JNK pathway which regulates oxidative stress and can also activate FOXO, which in turn can reduce the level of IIS signalling (Gimenez, et al., 2013).

Acer$^{\Delta}$ mutants are resistant to oxidative stress in the *w*^{Dah} background (Chapter 6), therefore *Acer* may be linked to the JNK pathway. Analysis of *mth* expression in response to different diets in *Acer*$^{\Delta}$ and *Ance*$^{\Delta}$ mutants may reveal a link to *mth* signalling as would the levels of *Acer* and *Ance* expression in *mth* nulls. With *mth* being linked to the IIS and JNK pathways it would interesting to examine the levels of *dilp* expression in *mth* nulls which would indicate whether *mth* is involved in the IIS pathway. In this study *Acer*$^{\Delta}$ flies appeared to show reduced levels of *dilps* overall and reduced *dilp5* in the head and *dilps* 6 and 7 in the body on DR and FF food. If similar phenotypes were shown in *mth* nulls this may further indicate a link with *Acer*. Fecundity of the *mth* nulls could be measured to see if the loss of *mth* reduces fecundity like the loss of *Acer* and therefore indicate a possible link between the two genes in the regulation of egg laying. Sleep has proved to be complex and affected by sex, diet and genetic background. Altered sleep in *mth* nulls may also establish a link to *Acer* and whether the *mth* receptor itself is involved in regulating sleep.

It is possible that ACER may have multiple substrates which would explain the variety of different phenotypes seen in this study (Table 90 and Figure 74). These substrates may also lead to ACER being involved in multiple signalling pathways making ACER complex to investigate. *Acer* expression may be higher in female flies which may be why the loss of *Acer* appears to show greater affects in female flies than males. *Acer*$^{\Delta}$ flies also show a reduced response to changing diet compared to controls, usually to low or high diet. Further investigation into *Acer* expression may reveal whether ACER itself is responsive to dietary change as well as potentially linking it to ANCE.

	Genotype				
Phenotype	w^{Dah}		w^{1118}		Diet
	Female	Male	Female	Male	
Sleep					
Total Activity	4	1	3	3	4
Total Sleep	4	1	3	3	4
Sleep in the Dark	3	0	3	0	2
Sleep in the Light	2	0	3	3	4
Total Bouts of Sleep	2	3	1	2	2
Bouts of Sleep in the Dark	4	1	4	1	3
Bouts of Sleep in the Light	2	4	2	4	2
Median Bout length in the Dark	4	1	4	0	1
Median Bout Length in the Light	3	1	4	0	2
Glycogen	5	5	5	5	5
Lipid	0	0	0	0	0
Wet Weight	3	3	0	0	2
Feeding	0	0	0	0	0
Lifespan	3	3	1	2	3
Fecundity	5	-	1	-	5
Cold Stress	1	1	1	1	1
Heat Stress	2	2	1	0	0
Oxidative Stress	4	4	2	2	4
Starvation	5	5	5	4	-
Dilp Expression					
dilp2 (Head)	0	0	-	-	0
dilp3 (Head)	0	0	-	-	0
dilp5 (Head)	3	3	-	-	3
dilp6 (Head)	1	0	-	-	1
dilp4 (Body)	0	0	-	-	0
dilp5 (Body)	2	2	-	-	2
dilp6 (Body)	4	3	-	-	4
dilp7 (Body)	5	3	-		5
DILP5 Immunohistochemistry	-	-	4	4	4

Table 90: Summary of ACER analysis in the phenotypes tested in this study and the likely involvement of ACER in those phenotypes to diet. Scale: 0 = ACER is not involved in modulating this phenotype. 5 = ACER is highly involved in modulating this phenotype.

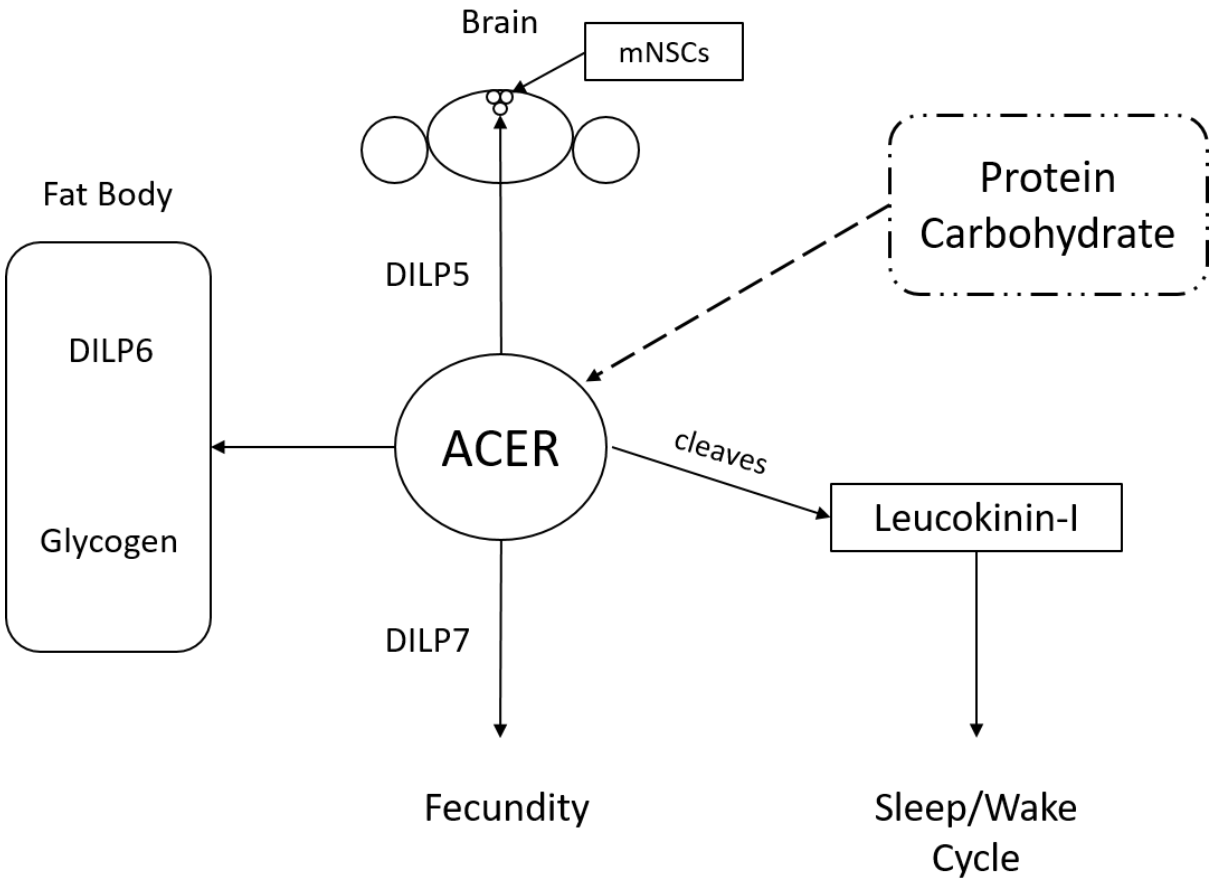

Figure 74: Summary of ACER'S potential role in *Drosophila melanogaster*. In this study, the loss of ACER has often been found in response to diet and the main components of the diet in flies are protein (yeast) and carbohydrate (sugar) (Chapter 2.9 Table 3). Therefore it is likely that ACER responds to diet in some way, either to carbohydrate, protein or both. Due to ACER's ability to cleave Leucokinin (Siviter, et al., 2002), which is involved in the sleep wake process, it is possible that this is the reason for the differences in the sleep phenotypes observed in Chapter 3. Fecundity was reduced in *Acer*$^{\Delta}$ females (Chapter 5) and recently DILP7 was found to be involved in site analysis for egg-laying (Yang, et al., 2008). The reduction in fecundity coincided with a reduction in *dilp7* expression in the body on both DR and FF food (Chapter 7), suggesting that ACER affects *dilp7* transcription and that this potentially was the cause of reduced fecundity in *Acer*$^{\Delta}$ females. ACER's presence in the fat body is interesting as this is where nutrients are stored in flies. Glycogen and lipid were tested in this study and glycogen was found to be reduced in *Acer*$^{\Delta}$ flies on DR and FF food. DILP6 is also present in the fly fat body and transcription levels of *dilp6* in the body were found to be reduced on FF food in *Acer*$^{\Delta}$ flies (Chapter 7). This suggests that ACER in the fat body has more than one function. Reduced *dilp5* transcription in the head of the fly continues to link ACER to the IIS pathway (Chapter 7.2.1), along with DILP6 and DILP7 mentioned above. Although *dilp5* transcript

levels were reduced in the fly head of *Acer*$^{\Delta}$ females, DILP5 protein levels in the median neurosecretory cells (mNSCs) was much higher on Low food than controls (Chapter 7.2.2). This suggests that ACER may modulate the release of DILP5 protein in the mNSCs in response to low food levels. ACER appears to have a very complex role within the fly which may be the result of multiple substrates and mechanisms.

In this study, behaviour as a measure of healthspan, such as walking and negative geotaxis, was not monitored in *Acer*$^{\Delta}$ flies which may be affected by the loss of *Acer*. *Acer*$^{\Delta}$ females in the w^{Dah} background slept more on Low food and were short-lived, therefore their health may have been affected by the loss of *Acer*. In the w^{1118} background, on FF food *Acer*$^{\Delta}$ flies were more active and slept less than controls which is in contrast to the w^{Dah} background. Activity and walking behaviour is likely to differ between backgrounds and sexes, therefore a more in depth analysis of the activity and sleep behaviour of the genetic backgrounds may lead to an indication about the role of *Acer* and why that role differs between the backgrounds and sexes.

Behavioural analysis such as walking, negative geotaxis (innate reaction of flies to move upwards in the response to gravity) and smell aversion may help indicate whether *Acer*$^{\Delta}$ mutants are not as heathy as their control counterparts. Most of the experiments in this study were conducted at an early stage of life therefore it is not known whether the lack of response to nutrition for *Acer*$^{\Delta}$ flies continues across the lifespan of the fly or if it is an initial response in early life which is later compensated for. Ageing is more likely to increase the differences seen between *Acer*$^{\Delta}$ flies and controls especially in terms of behaviour that senesces over time, therefore repeating the phenotypes in which the loss of *Acer* has affected the normal response to nutrition, such as sleep and glycogen storage, need to be repeated throughout the lifetime of the fly. This will assess whether the loss of *Acer* impacts the entire life of the fly or that the loss is compensated for in some way by another gene if phenotypes return to the level of the controls.

To determine whether *Acer* is a novel gene for ageing, experiments must be conducted over the lifespan of the fly and with measures of healthy ageing, such as walking and negative geotaxis. Many of the experiments conducted in this study require repetition to confirm *Acer's* role in the response to changing nutrition. Genetic background and sex appears to be important in determining *Acer's* role within the fly and the response to nutrition, therefore results from the on-going experiment on *Acer*

expression levels in the w^{Dah} and w^{1118} backgrounds may shed light upon the differences observed between the two backgrounds and whether *Acer* itself responds to changing diet.

9.1: Conclusions

The defective nutrient response of $Acer^{\Delta}$ mutants for sleep and glycogen storage suggests that *Acer* may normally play a primary role in metabolic and physiological responses to dietary changes. Lipid storage concentration was unaffected by the lack of *Acer*, therefore *Acer* is does not modulate the storage of lipid. Like reduced insulin signalling mNSC-ablated flies, $Acer^{\Delta}$ mutants' showed resistance to starvation and oxidative stress in the w^{Dah} background. This suggests a link to the nutrient-sensing IIS pathway. Furthermore, the reduction in *dilp5* transcript levels in the head and reduced *dilp6* and *dilp7* transcript levels in the body indicate that a link to IIS is likely. Fecundity was reduced in $Acer^{\Delta}$ females which may be due to lack of *Acer* expression or a consequence of the lower levels of *dilp7* expression, which is related to egg-laying, in $Acer^{\Delta}$ females.

Differences observed between the genetic backgrounds and sexes also suggests that *Acer's* role is complex and in some phenotypes its role is background and sex dependent which may be due to expression levels. The likelihood of multiple substrates also makes its role difficult to determine and further investigation in many areas is required.

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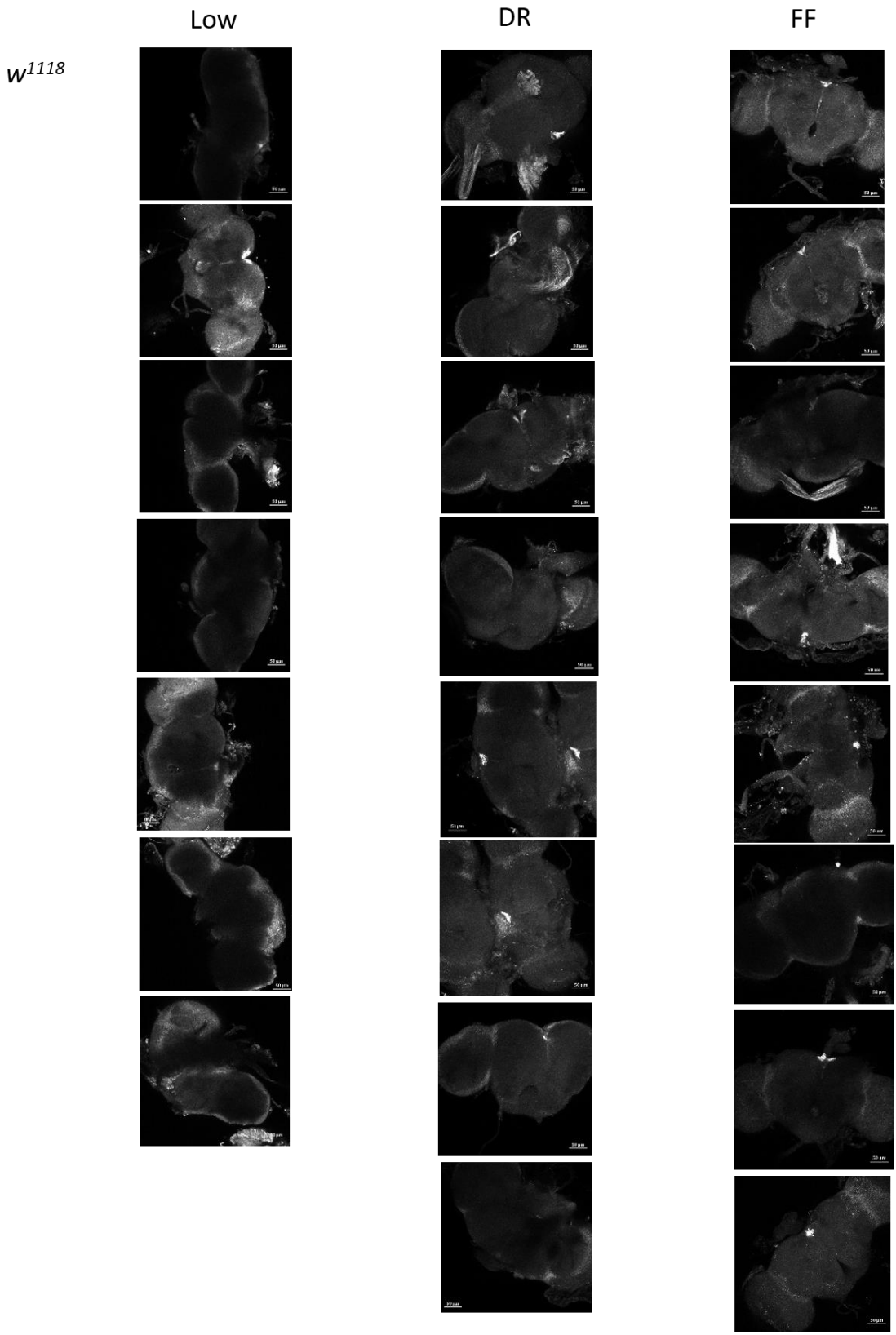
Appendix 1: Immunohistochemistry of fly brains

Females



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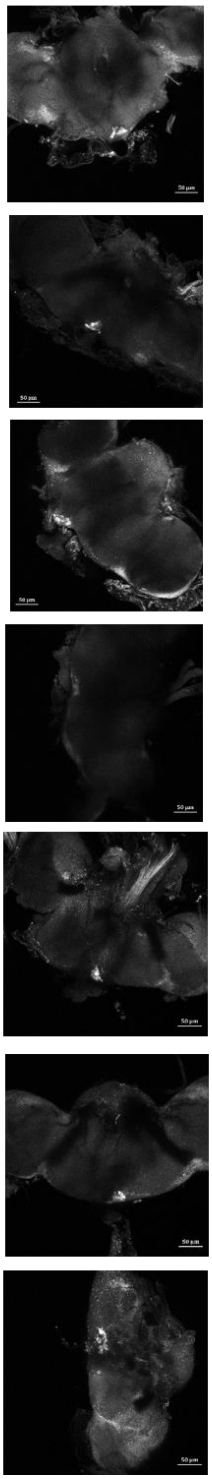
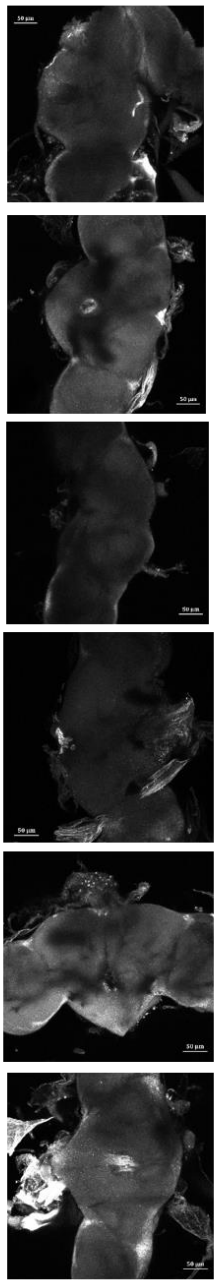
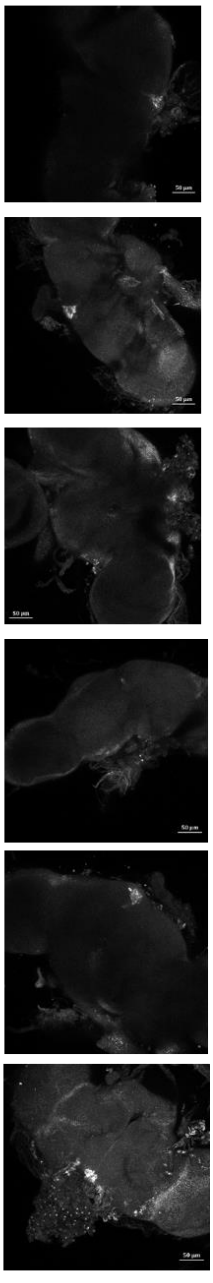
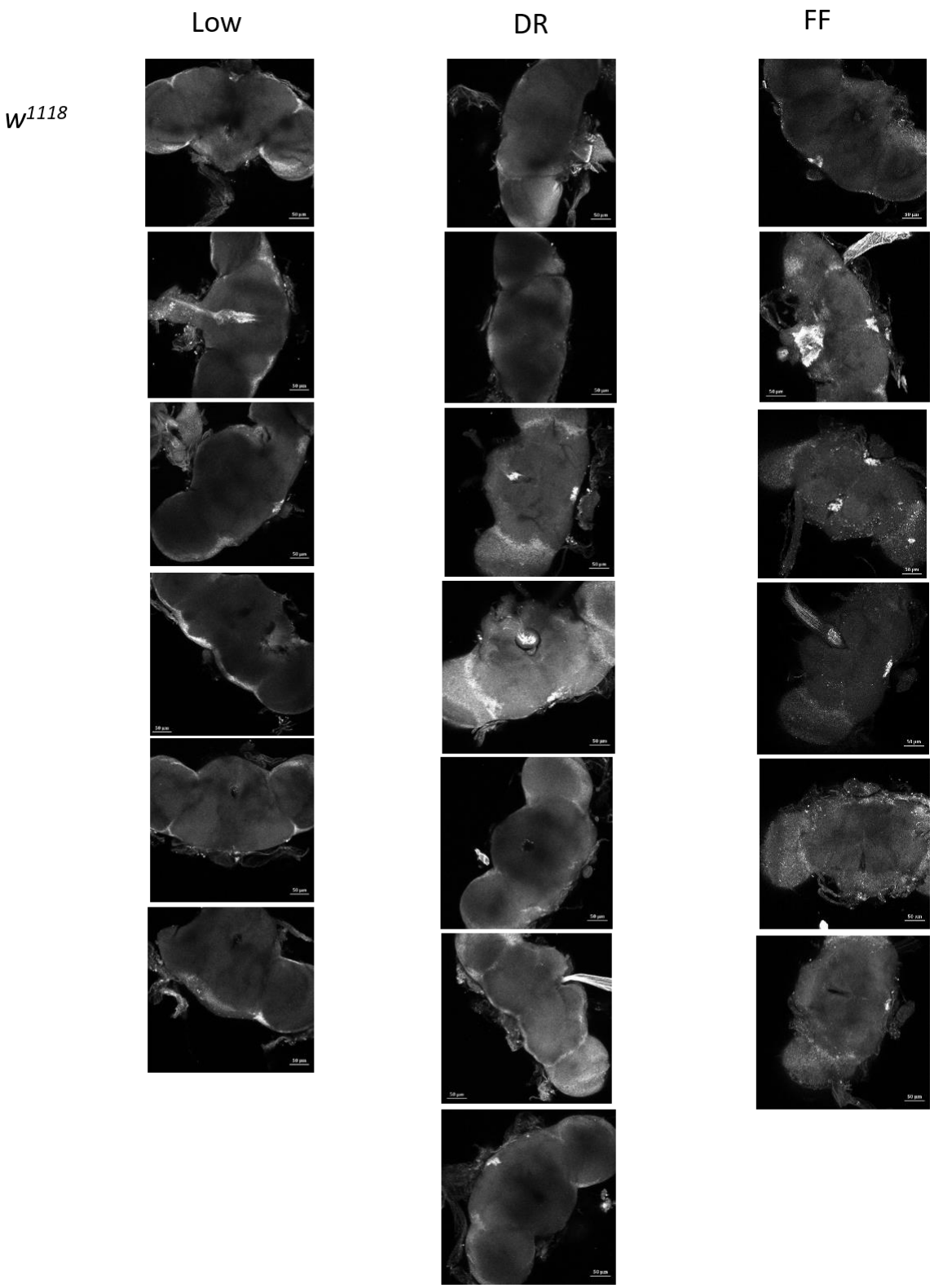
Acer$^{\Delta}$			

Males



	Low	DR	FF
Acer$^{\Delta}$