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Loss of Angiotensin-converting enzyme-related (ACER) peptidase disrupts behavioural and metabolic responses to diet in *Drosophila melanogaster*

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Summary statement: Little is known about how organisms perceive and respond to changes in nutrient availability, and this paper provides evidence for a novel role of *Drosophila* Acer in behavioural and metabolic responses to diet.

Abstract

Drosophila Acer (*Angiotensin-converting enzyme-related*) encodes a member of the angiotensin-converting enzyme (ACE) family of metallopeptidases that in mammals play roles in the endocrine regulation of blood homeostasis. ACE is also expressed in adipose tissue where it is thought to play a role in metabolic regulation. *Drosophila* Acer is expressed in the adult fat body of the head and abdomen and is secreted into the haemolymph. *Acer* null mutants have previously been found to have reduced night time sleep and greater sleep fragmentation. Acer may thus be part of a signalling system linking metabolism with sleep. To further understand the role of Acer in response to diet, we measured sleep and other nutrient-responsive phenotypes in *Acer* null flies under different dietary conditions. We show that loss of Acer disrupts the normal response of sleep to changes in nutrition. Other nutrient sensitive phenotypes, including survival and glycogen storage, were also altered in the *Acer* mutant but lipid storage was not. Although the physiological substrate of the Acer peptidase has not been identified, an alteration of the normal nutrient dependent control of *Drosophila* insulin-like peptide 5 protein in the *Acer* mutant suggests insulin/IGF-like signalling as a candidate pathway modulated by Acer in the nutrient-dependent control of sleep, survival and metabolism.

Introduction

Mammalian angiotensin I-converting enzyme (ACE) is a dipeptidyl carboxypeptidase, which plays a key role in the renin-angiotensin system (RAS) by converting angiotensin I into the vasoconstrictor angiotensin II. ACE therefore plays a key role in the regulation of blood homeostasis (Corvol and Soubrier, 2004) and ACE inhibitors are widely prescribed to treat hypertension and other cardiovascular diseases (Hoogwerf, 2010, Slagman et al., 2010). ACE, and other components of the RAS, have also been shown to be present in adipose tissue where a role in body fat deposition, glucose clearance and energy expenditure has been suggested (de Kloet et al., 2009, de Kloet et al., 2010, Segura and Ruilope, 2007, Jayasooriya et al., 2008, Santos et al., 2008, Weisinger et al., 2009). ACE knockout mice have reduced body mass, despite normal food intake, and increased lipid metabolism compared to control littermates (Jayasooriya et al., 2008). Studies with transgenic mice have shown that increasing the ACE gene dosage decreases fat deposition on a high fat diet (Heimann et al., 2005) and that under fasting conditions these mice have reduced adiposity compared to animals with a single ACE gene per haploid genome (Fonseca-Alaniz et al., 2017). The apparently contradictory results from knockout and over-expression studies may be explained in part by the diversity of roles of the RAS, the ability of ACE to both activate and inactivate regulatory peptides, and secondary effects caused by loss of renal function in the knockout model (Fonseca-Alaniz et al., 2017).

The first invertebrate ACE was identified in 1994 in the house fly, *Musca domestica* (Lamango and Isaac, 1994). Shortly thereafter, genes encoding homologues of mammalian ACE were identified in *Drosophila melanogaster* (Cornell et al., 1995, Tatei et al., 1995, Taylor et al., 1996) and other insects (Burnham et al., 2005, Yan et al., 2017). Genome sequencing has since revealed ACE-like genes throughout the animal kingdom and in a small number of bacterial species (Riviere et al., 2007) but, so far, none have been identified in protists, fungi or plants. Where biochemical studies have been undertaken the enzymes encoded by these genes have been found to have similar catalytic activity to mammalian ACE (Houard et al., 1998, Riviere et al., 2007). Humans and other vertebrates have a single ACE gene which codes for a somatic form of the enzyme with two catalytic domains and a testicular form with a single domain. ACE2 is a related enzyme but with catalytically distinct carboxypeptidase activity (Donoghue et al., 2000, Tipnis et al., 2000). In contrast, invertebrates usually have multiple ACE-like genes which can be grouped into distinct families according to sequence similarity (Isaac et al., 2007). Gene duplication in some cases has also led to tandem clusters of ACE-like genes; for example *Anopheles gambiae* has a cluster of six ACE-like genes on chromosome 3 (Burnham et al., 2005). *Drosophila melanogaster* has six ACE-like genes, two of which (*Ance* and *Acer*) encode catalytically active enzymes (Coates et al., 2000). The

proteins encoded by the other four genes (*Ance-2, Ance-3, Ance-4* and *Ance-5*) lack essential active site amino acids and are predicted to be catalytically inactive (Coates et al., 2000). Despite the conservation of the ACE family across the animal kingdom, little is known about the function of ACE-like proteins in invertebrates and no *in vivo* substrates have been identified, although there are a number of candidates from *in vitro* studies (Siviter et al., 2002).

In situ hybridisation and immunolocalisation studies have shown that *Drosophila Ance* is expressed in adult male reproductive tissues (Hurst et al., 2003, Rylett et al., 2007) but no fat body expression has been reported. *Acer*, on the other hand, is expressed strongly in adipose tissue (fat body) of the adult head and abdomen, and is secreted into the haemolymph (Carhan et al., 2011). *Acer* null mutants have been found to have reduced night time sleep and greater sleep fragmentation (Carhan et al., 2011), but little is known about the mechanism by which Acer modulates sleep. Sleep is a process that is conserved across the animal kingdom and studies on fruit flies, rodents and humans have demonstrated the importance of sleep to maintain a healthy existence (Cirelli and Tononi, 2008, Killgore, 2010). Sleep in *Drosophila* is thought to be regulated by at least some of the biochemical pathways known to affect sleep in mammals (for review see (Bushey and Cirelli, 2011)) and the fly has thus become a principal model organism to investigate the mechanisms of sleep. Numerous systems including diet (Broughton et al., 2010, Catterson et al., 2010b, Yamazaki et al., 2012, Linford et al., 2012) and the nutrient sensing insulin/IGF-like signalling (IIS) pathway (Metaxakis et al., 2014, Cong et al., 2015) modulate sleep in flies. Similarly to humans, sleep patterns in flies are sexually dimorphic and sex-specific sleep is controlled by the central nervous system and by the fat body (Khericha et al., 2016). The fat body also plays an important role in nutrient sensing and communication with insulin-like peptide (ILP) neurosecretory cells (Rajan and Perrimon, 2012, Delanoue et al., 2016). The fat body of the fly is an important, multifunctional organ involved in metabolism and hormone secretion with functional similarities to vertebrate liver and adipose tissue (Liu et al, 2009). Co-expression of *Acer* with *dilp 6*, *Clk* and *Cyc* in this organ suggests a potential role for Acer in the regulation of sleep by nutrition and/or IIS (Xu et al., 2008, Santos et al., 2009). It is possible that Acer functions in the fat body or haemolymph to process fat body-derived signalling peptides.

To investigate further Acer's role in sleep and other nutrient-sensitive responses we measured phenotypes including sleep, longevity and nutrient storage in response to changes in diet in *Acer* null flies. Dietary restriction (DR) is an evolutionarily conserved intervention that extends lifespan in many organisms from yeast to mammals (Jiang et al., 2000, Lin et al., 2000, Houthoofd et al., 2003, Klass, 1977, Lakowski and Hekimi, 1998, Kaeberlein et al., 2006, Magwere et al., 2004, Mair et al., 2003, Masoro, 2005). In *Drosophila*, DR is achieved by dilution of yeast in the food medium

such that lifespan peaks at an intermediate concentration of yeast (DR diet) and decreases at a high yeast concentration (Fully Fed (FF) diet) (Bass et al., 2007). At lower food levels, lifespan decreases because of starvation. The response of *Acer* null flies to lifespan extending Dietary Restriction and to mild and complete starvation diets was determined.

We show that loss of Acer disrupts the normal response of sleep, survival and glycogen storage to changes in nutrition. Although the substrate of the Acer peptidase has not been identified, an alteration of the normal nutrient dependent control of *Drosophila* insulin-like peptide 5 protein in the insulin-like peptide-producing cells (IPCs) in the brain of the *Acer* mutant suggests insulin/IGF-like signalling as a candidate pathway modulated by Acer in the nutrient-dependent control of sleep, survival and metabolism. We suggest that ACE may play an evolutionarily conserved role in adipose tissue as a mediator of nutrient signalling pathways.

Materials and Methods

Fly Stocks and Maintenance

The $Acer^{\Delta 168}$ deletion (Carhan et al., 2011) was backcrossed 6 times into the $white^{Dahomey}$ outbred backgrounds, as previously described (Broughton et al., 2005). Stocks were maintained and experiments conducted at 25˚C on a 12h:12h light:dark cycle at constant humidity. Flies for all analyses were raised on standard sugar/yeast medium (Bass et al., 2007) before transfer to the appropriate diet, as described later. Flies for all experiments were reared at standard larval density on standard sugar/yeast food, as previously described (Broughton et al., 2005). Eclosing adults were collected over a 12 hour period and mated for 48 hours before sorting into single sexes.

Dietary manipulations

The dietary manipulations were: Starvation (0% sugar and yeast), Low (1% sugar and yeast), Dietary Restriction (DR = 5% sugar and yeast) and Fully Fed (FF= 5% sugar and 20% yeast). Standard food for maintenance of stocks and rearing experimental flies contained 5% sugar and 10% yeast. Recipes for all diets are shown in Table 1. Dietary restriction was achieved using an optimised regime with sugar at a constant 5% as described in (Bass et al., 2007). Mild starvation was achieved using 1% sugar and yeast (Low diet) (Broughton et al., 2010). Complete starvation (0) was achieved using 1.5% agar medium.

Acer expression analysis

Following backcrossing to the w^{Dah} genetic background, the presence of the $Acer^{\Delta 168}$ deletion was confirmed by PCR and Western blot. Single fly genomic PCR was performed using screening primers, as described in (Carhan et al., 2011). The primer sequences were: Forward TGTCCGGAATGCGGGTGTTCC and Reverse: TCGATCATGGCCTGGCGATTC. Protein was extracted from 5 bodies per sample and Western blots performed using the protocol described in (Broughton et al., 2008) using 10% SDS gels and an anti-Acer antibody (Carhan et al, 2011)_at 1/2000 dilution.

Lifespan

Lifespan analyses were carried out as described in (Clancy et al., 2001) and (Mair et al., 2003). Lifespan was measured in once-mated female and male flies kept at 10 ⁄ vial on the indicated food medium and transferred to new food three times a week. Deaths were scored 5–6 times in every 7 days.

Sleep

Flies were generated as for lifespan experiments on standard food and entrained at 25°C in a 12:12h light/dark cycle. Flies were transferred to Low, DR or FF diets at 5 days old in groups of 10 flies/vial, and maintained under the same conditions for 2 days. At 7 days old individual flies (n=12-18) were placed in Trikinetics *Drosophila* Activity Monitors in tubes (5mm x 65 mm made of polycarbonate plastic) containing the appropriate food medium and activity was monitored in 1 minute bins for 3 days at 25°C in a 12h light/dark cycle. Sleep was defined as 5 minutes of inactivity, as described in (Shaw et al., 2000), and activity and sleep parameters calculated using BeFLY! Analysis Tools v7.23 (Ed Green) in Excel. Analyses were performed using data collected from days 2-3.

RNA Extraction and cDNA synthesis

RNA was extracted from 20 heads of 10 day old flies following 48-h feeding on the indicated diet, with three to six independent head RNA extractions performed per genotype per diet. RNA was extracted using Tri Reagent (Sigma), in 1.7mm Zirconium Bead Ribolyser tubes (OPS DIAGNOSTICS), according to the manufacturer's instructions. mRNA in total RNA was reverse transcribed using oligo(dT) primers and Superscript III First-Strand Synthesis System (Invitrogen).

QPCR

dilp5 transcript levels were measured by QPCR using SYBR Green (Sigma), and *dilp5* expression was normalised to 3 reference genes: actin5C (Broughton et al. 2005), tubulin and Rpl32 (Ponton et al., 2011). QPCR reactions were performed in 20 µl total volumes with 2 µ of cDNA, 100 nM of each primer and SYBR Green master mix (Sigma) in 96-well optical plates (Bio-Rad). The cycling conditions were: incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 30s, 58°C for 30s and 72°C C for 30s. QPCR relative expression values were determined by the $2^{-\Delta\Delta CT}$ method, following confirmation of PCR efficiency.

Immunohistochemistry

Immunohistochemical analysis of DILP5 protein in whole mount brains of 10-day-old females following 48-h feeding on the indicated food was performed as described in (Lee et al., 2000). Anti-DILP5 primary antibody (Broughton et al., 2010) was used at a dilution of 1:50 followed by a Fluorophor 488 labelled anti-rat secondary antibody (Molecular Probes) at 1:500 dilution. Confocal imaging of Fluorophor 488 fluorescence was carried out on an LSM 880 confocal microscope using the same settings for each sample. Confocal image stacks were converted to projections and relative quantifications of DILP5 levels in the Insulin-producing cells (IPCs) were performed in Image J (NIH, Bethesda, MD, USA) by measuring integrated density over a defined area encompassing the IPC cluster in each brain image examined.

Lipid and Glycogen analysis

The glycogen and lipid content of individual, adult female flies were measured in 10 day old flies as described in (Broughton et al., 2005). Data are expressed relative to the fresh body weight of each fly.

Feeding Assay

Direct quantification of food consumption (brilliant blue dye quantification), was carried out as described in (Wong et al., 2009).

Statistics

All statistical analyses were performed using JMP (version 8) software (SAS Institute Inc., Cary, NC, USA), $p<0.05$. Lifespan data were subjected to survival analysis (Log Rank tests). Other data (glycogen, lipid, QPCR, sleep, feeding and DILP levels) were tested for normality using the Shapiro-

Wilk W test on studentised residuals (Sokal & Rohlf, 1998) and found to be normally distributed. Analyses of variance (ANOVA) were performed to test for significant effects (diet and genotype), $P<0.05$. Planned comparisons of means were made using Tukey–Kramer HSD.

Results

The *Acer* deletion Δ168 (Carhan et al., 2011) was backcrossed six times into the w^{Dah} genetic background, and PCR and Western blot analyses confirmed the presence of the deletion in the w^{Dah};Acer$^{\Delta}$ homozygote following backcrossing (Fig. S 1 and Fig. S2). We then investigated how loss of Acer affects sleep and other phenotypes that are responsive to changes in diet.

Loss of Acer disrupts the normal dietary modulation of sleep.

Activity and sleep were measured in 7 day old w^{Dah};Acer$^{\Delta}$ and w^{Dah} male and female flies following transfer to Low, DR and FF diets 2 days prior to testing.

As expected diet had a significant effect on total activity and total sleep over a 24 hour period in control w^{Dah} female flies (Fig. 1, Table S1), which displayed significantly lower activity (Fig. 1A) and longer sleep duration (Fig. 1B, Table S1) on the FF diet compared to the Low diet. This response of total sleep duration to diet was due to a similar effect of diet on day and night sleep duration – control w^{Dah} females slept longer during both the day and night periods on the FF diet compared to the Low diet (Fig. 1E,F, Table S1). The longer sleep duration of w^{Dah} control females on FF food compared to Low food was predominantly due to a significantly longer mean sleep bout length on FF compared to Low and DR diets (Fig. 1D, Table S1). The number of sleep bouts of controls responded less strongly to diet, with a significant difference between Low and DR diets (Fig. 1C, Table S1). w^{Dah};Acer$^{\Delta}$ females, however, did not show the normal response of total activity, total sleep and day time sleep to diet (Fig. 1A, B and F, Table S1). w^{Dah};Acer$^{\Delta}$ females were less active and slept more than w^{Dah} controls on the Low diet, but behaved similarly to control w^{Dah} females on DR and FF diets. Night sleep duration did respond significantly to diet in w^{Dah};Acer$^{\Delta}$ females with significantly longer sleep duration on the FF diet compared to the DR and Low diets (Fig. 1E and Table S1). However, although night sleep duration in w^{Dah};Acer$^{\Delta}$ females showed a normal change in response to diet, w^{Dah};Acer$^{\Delta}$ females slept significantly longer than w^{Dah} controls on the Low and FF diets (Fig. 1F). Thus, underlying the observed lack of a dietary response of total sleep duration over a 24 hour period in w^{Dah};Acer$^{\Delta}$ females was a differential effect of loss of Acer on day and night time

sleep duration. Sleep bout parameters in w^{Dah};Acer$^{\Delta}$ females showed an altered response to diet compared to w^{Dah} controls (Fig. 1C-F, Table S1). Number of sleep bouts per day was significantly altered by diet in w^{Dah};Acer$^{\Delta}$ females between Low and DR diets and between DR and FF diets (Table S1), but w^{Dah};Acer$^{\Delta}$ females did not show the same response as w^{Dah} controls, performing fewer bouts of sleep than w^{Dah} controls on the FF diet (Figure 1C). The mean sleep bout duration of w^{Dah};Acer$^{\Delta}$ females responded to diet (Figure 1D, Table S1), but again displayed a different response than w^{Dah} controls with w^{Dah};Acer$^{\Delta}$ females displaying longer sleep bouts than controls on Low and FF diets. These data indicate that: (1) w^{Dah};Acer$^{\Delta}$ females sleep longer than w^{Dah} controls on Low food due to an increase in sleep bout duration; and (2) the apparently normal total 24 hour sleep behaviour of w^{Dah};Acer$^{\Delta}$ females on the FF diet (Figure 1B) was the result of w^{Dah};Acer$^{\Delta}$ females performing fewer, but longer bouts of sleep than w^{Dah} controls on this diet (Figure 1C and D).

The effect of diet and Acer deletion on sleep and activity in male flies was similarly analysed. In contrast to w^{Dah} control females, sleep and activity parameters in w^{Dah} control males were less responsive to diet (Fig. 2). Only the number of sleep bouts per day responded significantly to diet, with w^{Dah} males displaying fewer sleep bouts on FF food compared to Low and DR foods (Fig. 2C, Table S2). All sleep and activity parameters, including number of sleep bouts per day, did not respond significantly to diet in w^{Dah};Acer$^{\Delta}$ males (Fig. 2A-F). In addition, compared to w^{Dah} control males, w^{Dah};Acer$^{\Delta}$ males performed significantly fewer and longer total sleep bouts per day and slept longer during the night.

Together, these data indicate that Acer is involved in the normal dietary modulation of sleep and activity in male and female *Drosophila*.

Loss of Acer alters the dietary modulation of lifespan.

The disruption of the diet responsiveness of sleep in w^{Dah};Acer$^{\Delta}$ flies led us to consider the effect of loss of Acer on other diet responsive phenotypes, and we next measured the effect of the *Acer* deletion on the dietary modulation of lifespan.

The survival of w^{Dah};Acer$^{\Delta}$ female flies compared to controls on starvation, Low, DR and FF diets in 2 independent experiments is shown in Figure 3. Dietary restriction (DR) is known to extend lifespan compared to full feeding (FF diet), and the w^{Dah} control females showed the expected response of lifespan to these diets. That is, DR significantly extended the lifespan of w^{Dah} females compared to the FF diet (Fig. 3 A and B), and w^{Dah} females were short lived on starvation and Low diets (Fig. 3 C and D). w^{Dah};Acer$^{\Delta}$ flies responded normally to Dietary Restriction, with extended

lifespan on DR compared to FF (Fig. 3 A and B), but they showed an altered response to the Low diet compared to controls. w^{Dah};Acer$^{\Delta}$ females were short-lived compared to w^{Dah} control females on the Low diet in both replicate experiments (Fig. 3 C and D).

The survival of w^{Dah};Acer$^{\Delta}$ male flies compared to controls on starvation, low, DR and FF diets in 2 independent experiments is shown in Figure 4A-D. w^{Dah} control males showed the expected response of lifespan to these diets, as w^{Dah} control males responded to DR with a small but significant lifespan extension, and were short lived on low and starvation diets (Fig. 4A-D). w^{Dah};Acer$^{\Delta}$ males responded normally to DR with extended lifespan on DR compared to FF diets (Fig. 4A-B). In contrast to the effect of loss of *Acer* in females, w^{Dah};Acer$^{\Delta}$ males were longer lived than controls on the DR diet (Fig. 4A-B). Similarly to females, w^{Dah};Acer$^{\Delta}$ males were shorter lived than controls on the Low diet (Fig. 4C-D).

These data show that Acer is not required for extension of lifespan due to dietary restriction (DR) in both males and females, but Acer does modulate the effect of DR in males. The data further indicate that Acer influences the response of lifespan to nutrient stress (Low diet) in both sexes.

Loss of Acer alters the dietary modulation of glycogen storage.

Stored levels of lipid and glycogen in *Drosophila* are known to respond to dietary intake of sugar and yeast (Skorupa et al., 2008). Levels of these stored energy sources, as well as fly weight, were measured in w^{Dah};Acer$^{\Delta}$ and w^{Dah} control flies following transfer to starvation, Low, DR and FF diets 2 days prior to testing.

Glycogen levels in w^{Dah} control females and males displayed a significant response to diet after 2 days of feeding (Fig. 5 A and B). In control females, glycogen levels increased with increasing dietary intake of sugar and yeast between starvation and DR diets but then showed a decrease on the FF diet (Fig. 5 A). In w^{Dah} control males, glycogen levels increased with increasing dietary intake between starvation and FF diets (Fig. 5 B). w^{Dah};Acer$^{\Delta}$ males and females had normal levels of glycogen on the starvation diet compared to controls but displayed significantly lower levels of glycogen than controls on DR and FF foods (Fig. 5 A and B). The response of w^{Dah};Acer$^{\Delta}$ flies to increasing dietary intake was therefore weakened compared to controls, indicating that Acer is involved in the normal dietary modulation of stored glycogen.

The effect of diet on lipid levels in w^{Dah} control males and females is shown in Figure 5C and D. In contrast to the effect on glycogen storage, loss of Acer in w^{Dah};Acer$^{\Delta}$ flies had no effect on the normal nutrient responsive control of lipid levels (Fig. 5 C and D). The weights of w^{Dah};Acer$^{\Delta}$ males

and females compared to w^{Dah} controls showed a normal increase from starvation to FF diets (Fig. 5E and F), but female w^{Dah};Acer$^{\Delta}$ flies were significantly heavier than controls after 2 days of starvation (Fig. 5E).

Loss of Acer alters the dietary modulation of *Drosophila* insulin-like peptide 5 (Dilp5).

The data presented indicate that Acer plays a role in specific nutrient responsive phenotypes, but the mechanism of its action is unknown. Given that Acer is expressed in the fat body (Carhan et al., 2011) and is involved in the dietary modulation of sleep, lifespan and glycogen storage, we speculated that Acer may modulate *Drosophila* insulin-like peptides (Dilps) in response to nutrition. Transcription of the 8 *dilps* in adult *Drosophila* respond in different ways to varying the protein:carbohydrate ratio in the diet (Post and Tatar, 2016). A previous study analysed the response of *dilps* 2, 3 and 5 produced in the IPCs (insulin-producing cells) of the fly brain to varying diet (Broughton et al., 2010) using a similar dietary regime to that used here. In control female adult flies in the w^{Dah} genetic background, it was found that *dilp* 5 (both transcript and protein) was modulated by diet (Broughton et al., 2010). Therefore, we measured the effect of diet on *dilp5* transcript and protein levels in w^{Dah};*Acer*$^{\Delta}$ flies to determine if this nutrient responsive phenotype is modulated by Acer.

Similarly to the previous study (Broughton et al., 2010), *dilp5* transcript levels in heads of w^{Dah} control flies were low under starvation and Low diet conditions and increased significantly on the FF diet (Fig. 6A). In w^{Dah};*Acer*$^{\Delta}$ flies, levels of *dilp5* transcript responded to diet in a similar way – levels were low under starvation and low diet conditions and showed a significant increase on the FF diet (Fig. 6A). Thus, Acer is not required for the dietary control of *dilp5* transcription.

However, Dilp5 protein levels in the brain IPCs of w^{Dah};*Acer*$^{\Delta}$ flies did not respond to diet in the same way as controls. Dilp5 protein levels in w^{Dah} control IPCs were low under Low food conditions and increased significantly under DR and FF feeding (Fig. 6B and C, Fig. S2). Under starvation conditions, w^{Dah} control flies showed the expected high levels of Dilp5 protein in the IPCs, despite low transcription of the gene. However, Dilp5 protein levels in w^{Dah};*Acer*$^{\Delta}$ IPCs did not show a significant dietary response across Starvation, Low, DR and FF diets (Fig. 6B and C, Fig. S3). Interestingly, under starvation conditions, w^{Dah};*Acer*$^{\Delta}$ flies had significantly lower levels of Dilp5 in the IPCs compared to controls (Fig. 6B and C). These data show that Acer is not required for the normal response of *dilp5* transcription to diet but is involved in the normal dietary control of Dilp5 protein levels in adult IPCs.

Loss of Acer disrupts the dietary response of feeding behaviour in females but not males.

Feeding behaviour in *Drosophila* is influenced by nutritional needs as well as food palatability and quality (Dus et al., 2011, Broughton et al., 2010). Feeding was therefore measured in 7 day old w^{Dah};$Acer^{\Delta}$ and w^{Dah} males and females on low, DR and FF diets. As expected, the feeding of w^{Dah} control females and males responded to diet, with control flies eating more of the lower quality Low diet than the higher quality FF diet (Fig. 7A and B). w^{Dah};$Acer^{\Delta}$ males ate a similar amount of each diet as controls and showed a normal response to diet (Fig. 7 B). These data show that Acer in males is not required for the normal response of feeding to diet, and indicate that the defective dietary responses of other phenotypes in w^{Dah};$Acer^{\Delta}$ males were not due to differences in the quantity of each food consumed. The feeding of female w^{Dah};$Acer^{\Delta}$ flies, however, did not respond to diet in the same way as controls (Fig. 7A). w^{Dah};$Acer^{\Delta}$ females ate a similar quantity of Low food as controls but they did not decrease their feeding on the DR and FF diets.

Discussion

Previous research has shown that *Drosophila* Acer is involved in the control of night time sleep (Carhan et al., 2011). The data presented here support this role in sleep regulation and further reveal that Acer is in fact involved in the normal response of both day and night time sleep to changes in nutrition. Other nutrient-responsive phenotypes are altered in the Acer mutant indicating that Acer is part of a wider nutrient-responsive mechanism that may involve regulation of the *Drosophila* insulin-like peptide (Dilp)-producing neurosecretory cells (IPCs) in the fly brain. Little is known about how organisms perceive and respond to changes in nutrient availability, and the data presented here provide evidence of a novel role for Acer in behavioural and metabolic responses to diet.

Carhan et al (2011) measured sleep and activity in flies on a 5% sucrose diet and found that Acer null mutants had reduced night time sleep and increased sleep fragmentation compared to controls. The effect of loss of Acer in the present study was to generally increase sleep duration and decrease fragmentation. However, the diets used here containing varying concentrations of both sugar and yeast (1% to 20%) are not comparable to that used in (Carhan et al., 2011) which contained only sugar. Yeast in the diet is a source of protein, levels of which greatly influence lifespan and sleep (Linford et al., 2012, Broughton et al., 2010). Given the role of Acer identified here

in responding to dietary levels of sugar and yeast, the apparently contrasting effect of the Acer mutation on sleep between the two studies is likely due to the different diets used. It is also possible that the different genetic backgrounds of the flies in the two studies contributed to the differences in sleep patterns.

Diet has been shown to play an important role in the modulation of sleep in *Drosophila* (Catterson et al., 2010a, Takahama et al., 2012, Linford et al., 2012). Interestingly, the IPCs, which are known to promote sleep as part of a neural circuit regulating sleep and arousal (Yurgel et al., 2015), have been suggested to be involved in mediating the response of night time sleep to low food intake (Broughton et al., 2010). IPC-ablated flies on Low food are more active and display less night-time sleep than control flies (Broughton et al., 2010). The role of Acer identified here in the dietary regulation of Dilp5 protein levels in the IPCs identifies Acer as a novel regulator of these cells and raises the possibility that Acer modulates sleep in response to diet via this regulation of the IPCs. These data further raise the question of why Dilp5 protein levels in Acer IPCs did not respond normally to diet. The low levels of *dilp5* transcript but high levels of Dilp5 protein in the IPCs under starvation conditions may indicate that Acer flies are defective in releasing Dilp5 protein. However, further research is needed to determine if the IPCs in Acer flies have defects in the dietary modulation of *dilp5* translation and/or storage.

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. Acer female flies showed an increase in night sleep duration on Low and FF diets and w^{Dah};Acer$^{\Delta}$ males showed an increase in night sleep duration on DR and FF diets. Although not consistent across all diets, the increased night time sleep of Acer mutants may have influenced their survival under nutrient stress. However, w^{Dah};Acer$^{\Delta}$ males and females were shorter lived than controls on the mild starvation (Low) diet (1% sugar and yeast) and survived similarly to controls under complete starvation indicating that in Acer null flies sleep quality does not correlate with nutrient stress resistance.

Further research is needed to understand Acer's mechanism of action but we can speculate that Acer's influence on Dilp5 in the IPCs may also be involved in other diet-responsive phenotypes including DR lifespan extension. Dietary Restriction without malnutrition is an evolutionarily conserved intervention that extends lifespan and modulates ageing in model organisms (for review see (Fontana et al.). The dietary regime used in this study (DR vs FF) is a well-established means of achieving DR lifespan extension in *Drosophila* (Wong et al., 2009, Grandison et al., 2009, Broughton et al., 2010). The mechanisms by which DR extends lifespan and improves health in model organisms are not fully understood but a number of neuronal, systemic, cell autonomous and tissue specific

mechanisms are thought to be involved (Fontana et al.). In particular, the modulation of nutrient-sensing signalling pathways such as the Insulin/IGF-like (IIS) and TOR network are closely linked to the lifespan effects of DR in flies (Piper and Partridge, 2007). Interestingly, the IPCs in the brain are required to mediate the response of lifespan to full feeding (FF diet) (Broughton et al., 2010). Although both w^{Dah};Acer$^{\Delta}$ males and females showed a normal lifespan extension on the DR diet compared to the FF diet, male w^{Dah};Acer$^{\Delta}$ flies were consistently longer lived than controls on the DR diet. Thus, although Acer is not required for lifespan extension by DR it does have a novel, sex-dependent effect on the dietary modulation of ageing that we speculate may be mediated via regulation of the IPCs. The IPCs exist in the *pars intercerebralis* (PI) region of the fly brain, an area which is known to control sexually dimorphic locomotory behaviour (Belgacem and Martin, 2006, Gatti et al., 2000).

Although Acer plays a role in only a subset of diet responsive phenotypes, it was possible that altered feeding behaviour could have been involved in the altered dietary responses of w^{Dah};Acer$^{\Delta}$ flies. The feeding of w^{Dah};Acer$^{\Delta}$ males, however, showed a normal response to diet, indicating that the altered responses of sleep, glycogen storage and survival in males were not due to differences in the quantity of each diet consumed. In females, although the response of feeding to diet in w^{Dah};Acer$^{\Delta}$ flies was different to controls, the lack of response to diet did not correlate with the defects in other diet responsive phenotypes. For example, despite eating more than controls on DR and FF diets, w^{Dah};Acer$^{\Delta}$ females stored less glycogen than controls and showed no difference in weight, lipid and Dilp5 levels on these diets. In addition, w^{Dah};Acer$^{\Delta}$ females ate the same quantity of Low food as controls, but showed higher levels of Dilp5 in IPCs on this diet. Thus, it is unlikely that the altered dietary responses of w^{Dah};Acer$^{\Delta}$ females were due to their altered feeding. Of particular interest in this respect is the effect of the Acer deletion on glycogen storage. w^{Dah};Acer$^{\Delta}$ flies stored less glycogen than controls on DR and FF foods, but stored normal levels on Low and starvation diets. Glycogen levels in the fat body, along with hemolymph levels of trehalose and glucose, have been shown to decrease after prolonged starvation, which in turn triggers an internal taste-independent metabolic sensing pathway controlling food preferences (Dus et al., 2011). The fat body (where Acer is expressed) and the IPCs have been suggested to be involved in this regulation of feeding behaviour (Erion and Sehgal, 2013, Xu et al., 2008). It is possible that the lower levels of glycogen in w^{Dah};Acer$^{\Delta}$ females compared to controls on DR and FF diets were sufficient to trigger a metabolic response to increase feeding, in turn suggesting that the altered feeding in w^{Dah};Acer$^{\Delta}$ females is an indirect effect due to their lowered glycogen storage.

The demonstrated substrate specificity of the dipeptidyl peptidase activity of the purified enzyme (Houard et al., 1998, Siviter et al., 2002) suggests that Acer performs its role in modulating diet-sensitive responses by cleaving at the carboxy terminus of a small peptide substrate, leading to either activation or inactivation of the peptide by altering its affinity for its receptor. Acer is made by the fat body and secreted into the haemolymph so it could potentially act within the secretory pathway to process a peptide made by the fat body itself but it could also act on peptides secreted by the fat body or other tissues into the haemolymph. Our results suggest that a substrate, or product, of Acer modulates Dilp5 production and/or secretion from the IPCs in response to diet. Of the known fat body signals that are known to act on the IPCs, the *Unpaired2*, *Stunted* and *Eiger* gene products are too large to be likely Acer substrates. CCHamide2 (GCQAYGHVCYGGHamide) is unlikely to be cleaved by Acer owing to the blocked C-terminus and the cyclic nature resulting from the disulphide bond between the cysteines. Another fat body signal that controls Dilp release from the IPCs in response to diet is Dilp6. Starvation leads to increased Dilp6 release from the fat body which signals to the IPCs in the brain, repressing dilp2 and dilp5 (Slaidina et al., 2009). Dilp6, however, is also unlikely to be a direct target of Acer owing to its size and disulphide bonds. Although none of the identified fat body signals is likely to be an Acer substrate, Acer may play an upstream regulatory role in the release of one or more of these signals, or another unidentified signal, from the fat body or another tissue. Another possibility is that Acer itself acts as a secreted signal from the fat body. Interestingly, the 'ACE' protein of the nematode *C. elegans* lacks enzyme activity but still plays a vital role in development (Brooks et al., 2003). The low activity of *Drosophila* Acer against most peptides tested *in vitro* (Siviter et al., 2002) may indicate that its enzyme activity is irrelevant to its function in modulating diet responses.

The presence of ACE or ACE-like enzymes in adipose tissue of both vertebrates and insects and the requirement of these enzymes for appropriate responses to changes in diet, suggests a conserved, and possibly ancient, function for ACE. If this were the case, ACE-like enzymes should be present in the adipose tissue of other invertebrates. Our analysis of sequenced insect genomes reveals that Acer homologues are only present in the Brachyceran suborder of the Diptera (data not shown); however insects have multiple ACE-like enzymes and it is possible that Acer's role is fulfilled by another enzyme in insects outside the Brachycera. The lepidopterans, *Bombyx mori* and *Spodoptera littoralis* have both been shown to possess an ACE that is expressed in fat body (Yan et al., 2017, Lemeire et al., 2008). The evolution of Acer in the Brachycerans may be a consequence of mutation and loss of function of an ACE previously fulfilling the diet-response role.

Summary

Drosophila Acer modulates a subset of behavioural and metabolic responses to diet. Although the mechanism of its action is currently unknown, its role in the normal dietary control of Dilp5 protein suggests that Acer may be involved in modulating some nutrient responsive phenotypes via regulation of the *Drosophila* insulin-producing neurosecretory cells.

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Author Contributions

Conceived and designed the experiments: SJB and AS. Performed the experiments: SJB, ZG, MDH, ND, JC, HA. Analyzed the data: SJB, ZG, MDH. Wrote the paper: SJB and AS.

Competing Interests

No competing interests declared.

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Figures

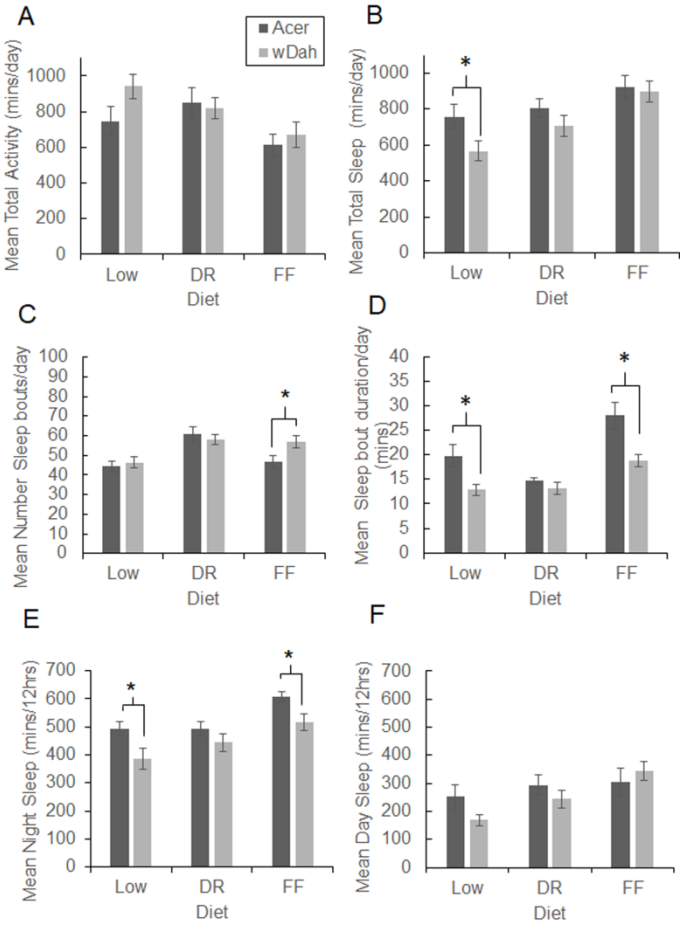

Figure 1. Activity and sleep behaviour of w^{Dah};Acer$^{\Delta}$ females compared to w^{Dah} controls under Low, DR and FF diets. (A) Total 24hr activity duration (mins/day). **(B)** Total 24hr sleep duration (mins/day). **(C)** Mean number of sleep bouts per day. **(D)** Mean sleep bout duration per day (mins/bout). **(E)** Night sleep duration (mins/12hrs). **(F)** Day sleep duration (mins/12hrs). N=18 for w^{Dah} on each food and w^{Dah};Acer$^{\Delta}$ on DR diet. N=15 for w^{Dah};Acer$^{\Delta}$ on FF diet and N=12 for w^{Dah};Acer$^{\Delta}$ on Low diet. Data are presented as means ±SEM. Data were analysed by two-way ANOVA (genotype and diet effects) and planned comparisons of means performed using Tukey HSD. ANOVA found that diet was a significant effect ($p<0.05$) for all activity and sleep parameters, and planned comparisons of means for the effect of diet on each genotype were performed using Tukey HSD (p values are given in Table S1). ANOVA found that genotype was a significant effect ($p<0.05$) for total and night sleep duration, and mean sleep bout length. * indicates significant differences ($p<0.05$) for planned comparisons of means of these parameters between w^{Dah};Acer$^{\Delta}$ and w^{Dah} control genotypes on each diet.

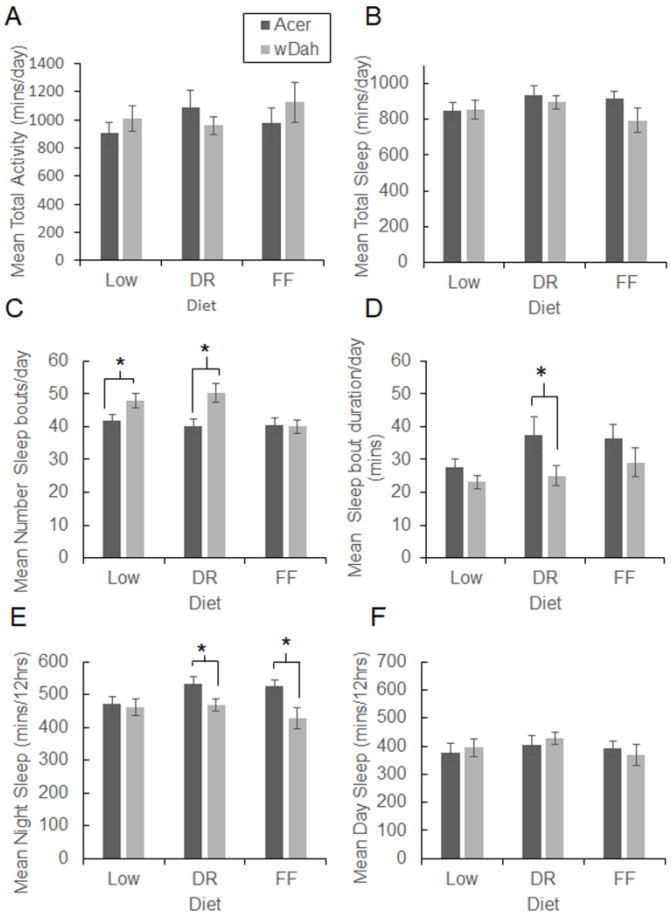

Figure 2. Activity and sleep behaviour of w^{Dah};Acer$^{\Delta}$ males compared to w^{Dah} controls under Low, DR and FF diets. (A) Total 24hr activity duration (mins/day). **(B)** Total 24hr sleep duration (mins/day). **(C)** Mean number of sleep bouts per day. **(D)** Mean sleep bout duration per day (mins/bout). **(E)** Night sleep duration (mins/12hrs). **(F)** Day sleep duration (mins/12hrs). N=18 for w^{Dah} on each diet and w^{Dah};Acer$^{\Delta}$ on DR and FF diet. N=14 for w^{Dah};Acer$^{\Delta}$ on Low diet. Data are presented as means ±SEM. Data were analysed by ANOVA (genotype and diet effects) and planned comparisons of means performed using Tukey HSD. ANOVA found that diet was a significant effect ($p<0.05$) for mean number of sleep bouts per day, and planned comparisons of means for the effect of diet on each genotype were performed using Tukey HSD (p values are given in Table S2). ANOVA found that genotype was a significant effect ($p<0.05$) for number of sleep bouts, mean sleep bout length, and night sleep duration. * indicates significant differences ($p<0.05$) for planned comparisons of means of these parameters between w^{Dah};Acer$^{\Delta}$ and w^{Dah} control genotypes on each diet.

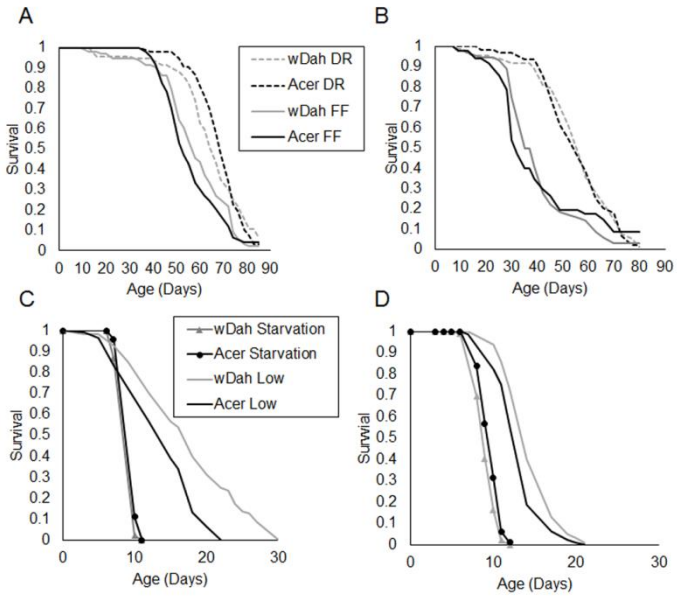

Figure 3. Survival of female w^{Dah};Acer$^{\Delta}$ compared to w^{Dah} controls under starvation, Low, DR and FF diets in 2 replicate experiments. (A-B) Survival of once mated females under DR and FF diets. **(A) Replicate experiment 1.** Median lifespans and sample sizes were: w^{Dah};Acer$^{\Delta}$ DR = 69 days, N=97; w^{Dah};Acer$^{\Delta}$ FF = 53 days, N=96; w^{Dah} DR = 64 days, N=94; and w^{Dah} FF = 58 days, N=98. Both w^{Dah};Acer$^{\Delta}$ and w^{Dah} females showed an increased survival from FF to DR diet (Log rank test, P<0.05). w^{Dah};Acer$^{\Delta}$ was shorter lived than w^{Dah} on the FF diet (Log rank test, P=0.0139). **(B) Replicate experiment 2.** Median lifespans and sample sizes were: w^{Dah};Acer$^{\Delta}$ DR = 54 days, N=93; w^{Dah};Acer$^{\Delta}$ FF = 31 days, N=98; w^{Dah} DR = 54 days, N=95; and w^{Dah} FF = 38 days, N=97. Both w^{Dah};Acer$^{\Delta}$ and w^{Dah} females showed an increased survival from FF to DR diet (Log rank test, P<0.05). **(C-D)** Survival of once mated females under starvation and Low diets. **(C) Replicate experiment 1.** Median lifespans and sample sizes were: w^{Dah};Acer$^{\Delta}$ Starvation = 8.5 days, N=100; w^{Dah};Acer$^{\Delta}$ Low = 13.5 days, N=124; w^{Dah} Starvation = 8.5 days, N=100; and w^{Dah} Low = 17 days, N=123. w^{Dah};Acer$^{\Delta}$ was shorter lived than w^{Dah} on the Low diet (Log rank test, P<0.0001). **(D) Replicate experiment 2.** Median lifespans and sample sizes were: w^{Dah};Acer$^{\Delta}$ starvation = 9.5 days, N=100; w^{Dah};Acer$^{\Delta}$ Low = 13 days, N=101; w^{Dah} Starvation = 8.5 days, N=100; and w^{Dah} Low = 13 days, N=99. w^{Dah};Acer$^{\Delta}$ was longer lived than w^{Dah} on the starvation diet (Log rank test, P=0.0025). w^{Dah};Acer$^{\Delta}$ was shorter lived than w^{Dah} on the Low diet (Log rank test, P=0.0081).

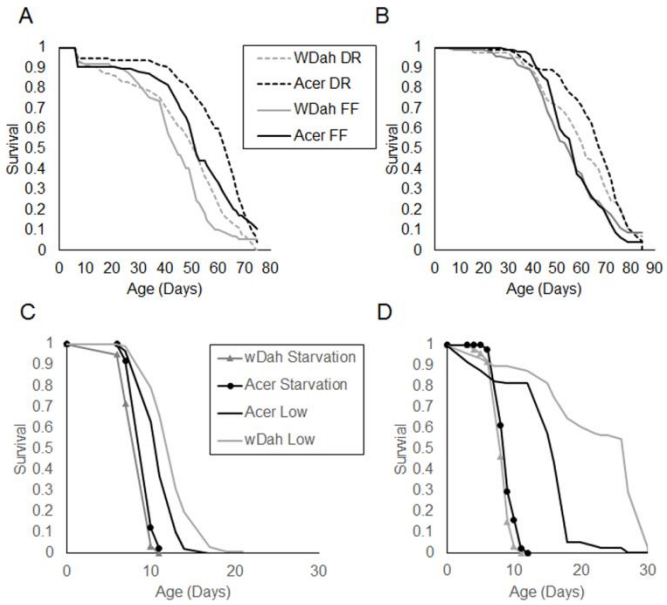

Figure 4. Survival of male w^{Dah};Acer$^{\Delta}$ compared to w^{Dah} controls under starvation, Low, DR and FF diets in 2 replicate experiments. (A-B) Survival of males under DR and FF diets. **(A) Replicate experiment 1.** Median lifespans and sample sizes were: w^{Dah};Acer$^{\Delta}$ DR = 64 days, N=100; w^{Dah};Acer$^{\Delta}$ FF = 50.5 days, N=92; w^{Dah} DR = 50.5 days, N=104; and w^{Dah} FF = 43 days, N=98. Both w^{Dah};Acer$^{\Delta}$ and w^{Dah} males showed an increased survival from FF to DR diet (Log rank test, P<0.05). w^{Dah};Acer$^{\Delta}$ was longer lived than w^{Dah} on the DR diet (Log rank test, P=0.0002) and the FF diet (Log rank test, P=0.003). **(B) Replicate experiment 2.** Median lifespans and sample sizes were: w^{Dah};Acer$^{\Delta}$ DR = 68 days, N=100; w^{Dah};Acer$^{\Delta}$ FF = 56.5 days, N=97; w^{Dah} DR = 61 days, N=97; and w^{Dah} FF = 54 days, N=94. Both w^{Dah};Acer$^{\Delta}$ and w^{Dah} males showed an increased survival from FF to DR diet (Log rank test, P<0.05). **(C-D)** Survival of males under Starvation and Low diets. **(C) Replicate experiment 1.** Median lifespans and sample sizes were: w^{Dah};Acer$^{\Delta}$ Starvation = 8.5 days, N=100; w^{Dah};Acer$^{\Delta}$ Low = 10.5 days, N=101; w^{Dah} Starvation = 8.5 days, N=98; and w^{Dah} Low = 12 days, N=99. Both w^{Dah};Acer$^{\Delta}$ and w^{Dah} males showed an increased survival from DR to FF diet (Log rank test, P<0.05). w^{Dah};Acer$^{\Delta}$ was longer lived than w^{Dah} on the starvation diet (Log rank test, P<0.001). w^{Dah};Acer$^{\Delta}$ was shorter lived than w^{Dah} on the Low diet (Log rank test, P<0.001). **(D) Replicate experiment 2.** Median lifespans and sample sizes were: w^{Dah};Acer$^{\Delta}$ Starvation = 8.5 days, N=100; w^{Dah};Acer$^{\Delta}$ Low = 15.5 days, N=121; w^{Dah} Starvation = 7 days, N=100; and w^{Dah} Low = 26.5 days, N=120. w^{Dah};Acer$^{\Delta}$ was longer lived than w^{Dah} on the starvation diet (Log rank test, P<0.001). w^{Dah};Acer$^{\Delta}$ was shorter lived than w^{Dah} on the Low diet (Log rank test, P<0.001).

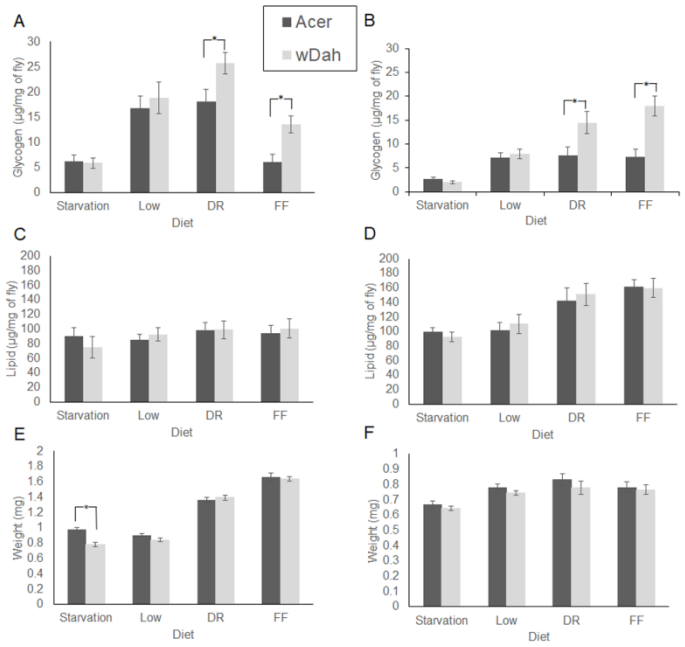

Figure 5. Glycogen and lipid levels and weights of 10 day old w^{Dah};Acer$^{\Delta}$ flies compared to w^{Dah} controls after 2 days feeding on Starvation, Low, DR and FF diets. (**A**) Mean glycogen levels of w^{Dah};Acer$^{\Delta}$ and w^{Dah} females. (**B**) Mean glycogen levels of w^{Dah};Acer$^{\Delta}$ and w^{Dah} males. (**C**) Mean lipid levels of w^{Dah};Acer$^{\Delta}$ and w^{Dah} females. (**D**) Mean lipid levels of w^{Dah};Acer$^{\Delta}$ and w^{Dah} males. (**E**) Mean weight of w^{Dah};Acer$^{\Delta}$ and w^{Dah} females. (**F**) Mean weight of w^{Dah};Acer$^{\Delta}$ and w^{Dah} males. Data are shown as means of 10 flies (N = 10) ± SEM. Data were analysed by ANOVA and, for glycogen storage, both genotype and diet were significant effects ($p<0.05$). Planned comparisons of means were performed using Tukey HSD. *indicates significant difference between indicated genotypes, $P<0.05$, for each genotype.

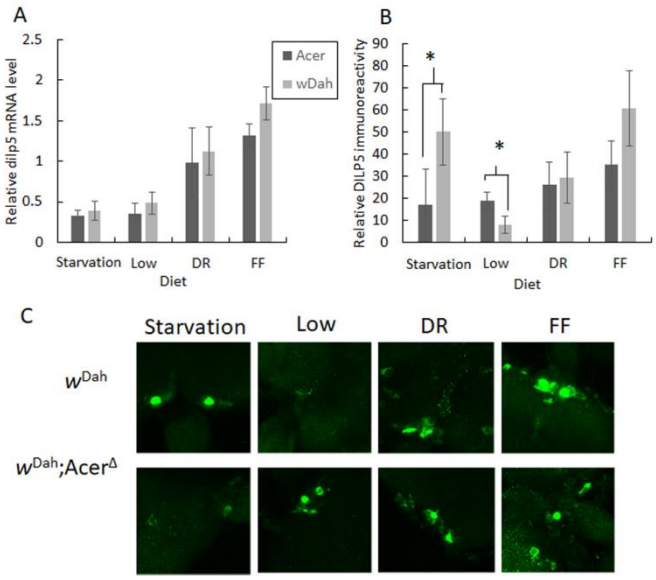

Figure 6. Dilp5 transcript and protein levels of w^{Dah};Acer$^{\Delta}$ flies compared to w^{Dah} controls after 2 days feeding on Starvation, Low, DR and FF diets. (**A**) Relative mRNA abundance of *dilp5* from adult heads of 10 day old w^{Dah};Acer$^{\Delta}$ and w^{Dah} females following 48 h treatment with Starvation (0), Low, DR and FF diets was measured by quantitative RT-PCR and normalised to the abundance of actin5C, tubulin and Rpl32. Data are shown as means of 3-6 independent experiments, ± SEM. Sample sizes were: Starvation N= 5, Low N=3, DR N=6, FF N=6. Data were analysed by two-way ANOVA (diet and genotype) and only diet was found to be a significant effect ($p<0.0001$), with no effect of genotype ($p=0.27$). Planned comparisons of means by diet were performed for each genotype using Tukey HSD ($p<0.05$) and in both w^{Dah};Acer$^{\Delta}$ and w^{Dah} the abundance of dilp5 on FF diet was significantly greater than on Low and Starvation diets. (**B**) Immunohistochemical analysis of DILP5 protein in w^{Dah};Acer$^{\Delta}$ and w^{Dah} 10 day old female brains following 48 h treatment with Starvation (0), Low, DR and FF diets. Sample sizes for w^{Dah} were: Starvation N= 8, Low N=13, DR N=11, FF N=8. Sample sizes for w^{Dah};Acer$^{\Delta}$ were: Starvation N= 6, Low N=9, DR N=9, FF N=11. Quantification of Dilp5 levels were performed using Image J on the confocal microscope images shown in Supplementary Figures S2 and S3. Data are shown as mean relative fluorescence ± SEM. Data were analysed by ANOVA and diet was found to be a significant effect, with a significant interaction with genotype ($p<0.05$). Planned comparisons of means by genotype on each diet were performed by Tukey HSD and *indicates significant difference between genotypes, $P < 0.05$. (**C**) Representative images of anti-DILP5 staining. w^{Dah} control (See Supplementary Fig. S2 for images of all w^{Dah} brains examined). w^{Dah};Acer$^{\Delta}$ (See Supplementary Fig. S3 for images of all w^{Dah};Acer$^{\Delta}$ brains examined).

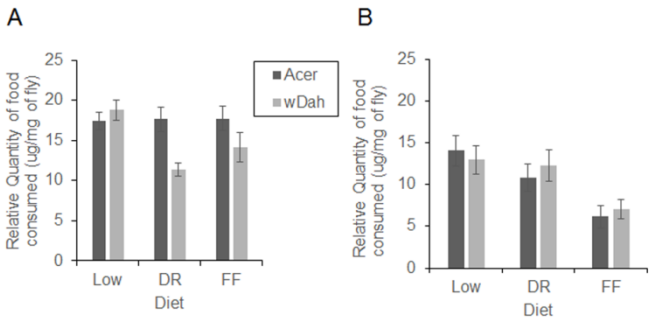

Figure 7. The effect of diet on feeding in male and female w^{Dah};Acer$^{\Delta}$ flies. Relative quantity of food consumed by (**A**) Female and (**B**) Male flies of the indicated genotype after 30 mins feeding on Low, DR and FF diets containing blue dye. Direct quantification of Brilliant Blue dye consumed was carried out by colour spectrophotometry. The data are presented as mean µg of food consumed per mg of fly, ± SEM, N = 33-35 (vials of five flies) per genotype, per food. ANOVAs were performed and food, genotype and sex were significant effects. Diet had a significant effect on the quantity of food consumed by control w^{Dah} males and females and by w^{Dah};Acer$^{\Delta}$ males ($p<0.05$). There was no significant effect of diet on the quantity of food consumed by w^{Dah};Acer$^{\Delta}$ female flies ($p=0.99$).

Tables

Table 1: Dietary manipulations.

	Standard	Starvation	Low	Dietary Restriction (DR)	Fully Fed (FF)
Agar (g/L)	**15**	**15**	**15**	**15**	**15**
Sugar (g/L)	50	0	10	50	50
Yeast (g/L)	100	0	10	50	200

Supplementary Figure 1

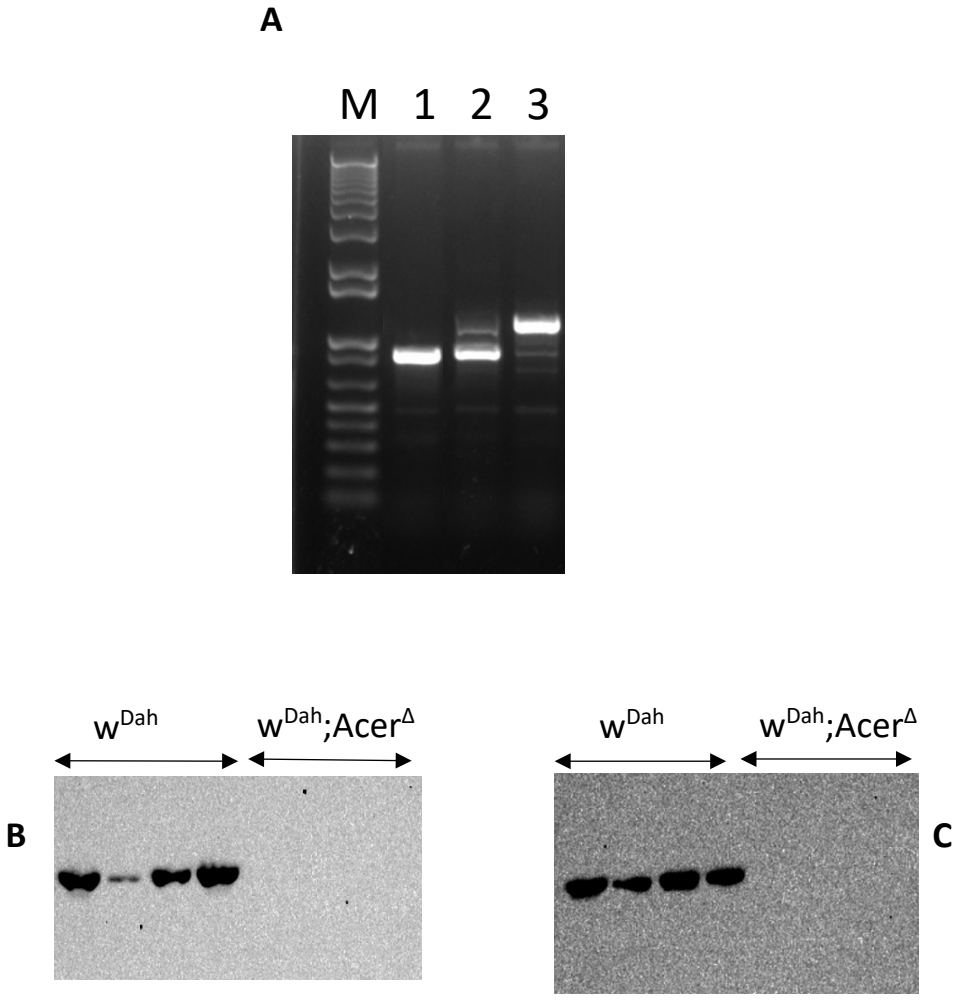

Figure S1: (A) PCR analysis of the *Acer* deletion in the w^{Dah} background. Lane M: marker. Lane 1: The $Acer^{\Delta 168}$ deletion homozygote with a strong band at 850 bp. Lane 2: The $Acer^{\Delta 168}$ deletion heterozygote with a strong band at 850 bp and a weaker band at 1,150 bp. Lane 3: The w^{Dah} control background with a strong band at 1,150 bp. (bp = base pairs). **(B-C)** Western blot analysis of the Acer $^{\Delta 168}$ deletion in the w^{Dah} background showing absence of Acer protein in w^{Dah};$Acer^{\Delta}$ males and females. Four independent protein extractions per genotype and sex were performed using 5 flies per sample. **(B)** Females. **(C)** Males.

Supplementary Figure 2

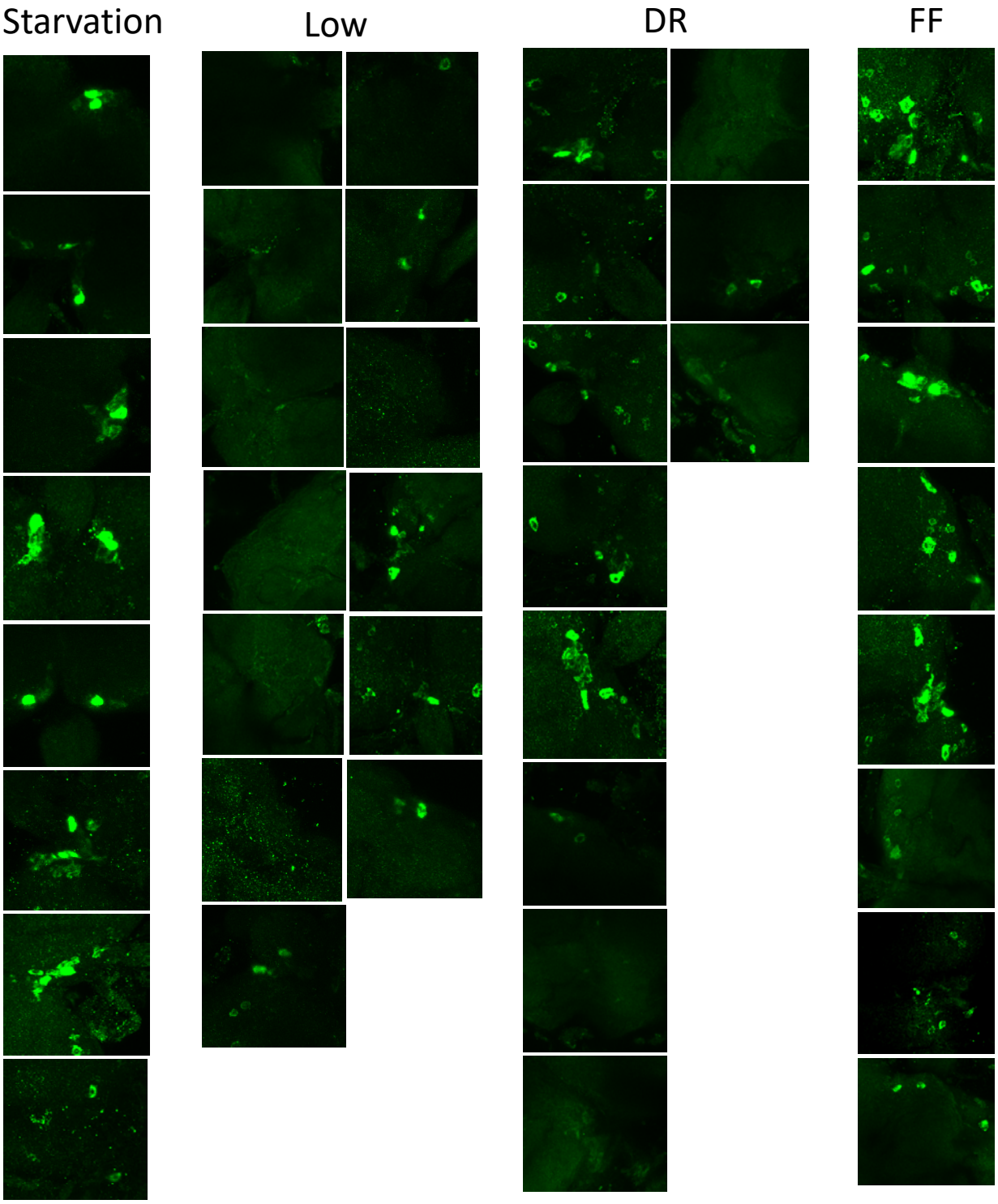

Figure S2. Immunohistochemical analysis of DILP5 protein in w^{Dah} 10 day old female brains following 48 h treatment with Starvation, Low, DR and FF diets.

Supplementary Figure 3

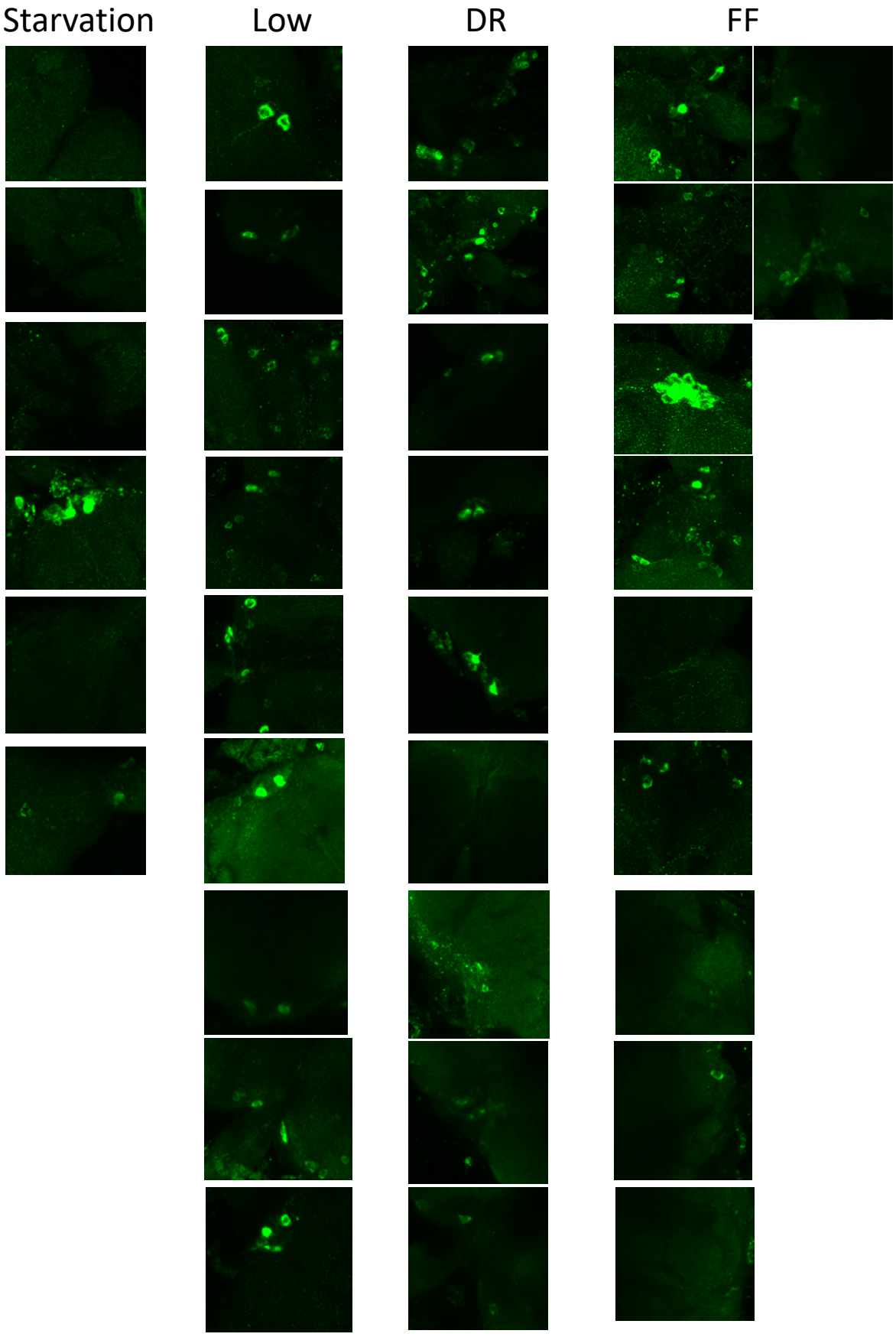

Figure S3. Immunohistochemical analysis of DILP5 protein in w^{Dah};Acer$^{\Delta}$ 10 day old female brains following 48 h treatment with Starvation, Low, DR and FF diets.

Table S1: Statistical analysis of female sleep data presented in Figure 1. P values of planned comparisons of means for the effect of diet on sleep parameters in w^{Dah};Acer$^{\Delta}$ and w^{Dah} females, performed using Tukey HSD. Numbers in bold indicate significant differences ($p<0.05$).

Genotype	Food comparison	Activity/day	Total Sleep/day	Day-time sleep	Night-time sleep	Number of bouts	Bout duration
wDah	Low-DR	0.3932	0.2001	0.1636	0.4309	**0.0102**	0.9681
	Low-FF	**0.0152**	**0.0004**	**0.0003**	**0.0212**	0.2113	**0.0019**
	DR-FF	0.2641	0.054	**0.0497**	0.2823	0.3849	**0.0039**
w^{Dah};Acer$^{\Delta}$	Low-DR	0.9714	0.6333	0.7747	0.9997	**0.0053**	0.0529
	Low-FF	0.1709	0.0799	0.6831	**0.0054**	0.8687	**0.014**
	DR-FF	0.0702	0.3109	0.9793	**0.0026**	**0.0016**	**<0.0001**

Table S2: Statistical analysis of male sleep data presented in Figure 2. P values of planned comparisons of means for the effect of diet on sleep parameters (Number of bouts) in w^{Dah};Acer$^{\Delta}$ and w^{Dah} males, performed using Tukey HSD. Numbers in bold indicate significant differences ($p<0.05$).

Genotype	Food comparison	Number of bouts
wDah	Low-DR	**0.025**
	Low-FF	**0.0392**
	DR-FF	0.9738
w^{Dah};Acer$^{\Delta}$	Low-DR	0.8802
	Low-FF	0.8953
	DR-FF	0.9991