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RESEARCH ARTICLE

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

Zoe Glover*, Matthew D. Hodges*, Nikolett Dravecz, Jack Cameron, Helen Askwith, Alan Shirras and Susan J. Broughton‡

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.

KEY WORDS: Insulin/IGF-like signalling, Neurosecretory cells, Nutrition, Sleep

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 (Bernstein et al., 2005) 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, 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 with 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 with 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 (Rivière 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; Rivière 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 melanogaster 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*, in contrast, 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

Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster LA1 4YQ, UK.
*These authors contributed equally to this work

‡Author for correspondence (s.j.broughton@lancaster.ac.uk)

Z.G., 0000-0001-9313-9674; M.D.H., 0000-0002-6815-751X; A.S., 0000-0002-1377-5134; S.J.B., 0000-0001-8168-5387

Received 11 October 2018; Accepted 29 March 2019

Journal of Experimental Biology (2019) 222, jeb194332. doi:10.1242/jeb.194332

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. Similar to those in 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 *dilp6*, *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. We determined the response of *Acer* null flies to lifespan-extending DR and to mild and complete starvation diets.

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 background, as previously described (Broughton et al., 2005) to create the *Acer* null mutants. Stocks were maintained and experiments conducted at 25°C on a 12 h:12 h 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 below. 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 h period and mated for 48 h before sorting into single sexes.

Dietary manipulations

The dietary manipulations were: starvation (0% sugar and yeast), low (1% sugar and yeast), DR (5% sugar and yeast) and 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. DR 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 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 TCGATC-ATGGCCTGGCGATTC. Protein was extracted from five bodies per sample and western blots were 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 per vial on the indicated food medium and transferred to new food 3 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 on a 12 h:12 h light:dark cycle. Flies were transferred to low, DR or FF diets at 5 days old in groups of 10 flies per 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 (5 mm×65 mm made of polycarbonate plastic) containing the appropriate food medium and activity was monitored in 1 min bins for 3 days at 25°C on a 12 h:12 h light:dark cycle. Sleep was defined as 5 min of inactivity, as described in Shaw et al. (2000), and activity and sleep parameters were 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 1.7 mm Zirconium Bead Ribolyser tubes (OPS Diagnostics, Lebanon, NJ, USA), 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).

Table 1. Diet composition

	Standard	Starvation	Low	DR	FF
Agar (g l^{-1})	15	15	15	15	15
Sugar (g l^{-1})	50	0	10	50	50
Yeast (g l^{-1})	100	0	10	50	200

DR, dietary restriction; FF, fully fed.

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qPCR

Drosophila insulin-like peptide 5 (*dilp5*) transcript levels were measured by qPCR using SYBR Green (Sigma), and *dilp5* expression was normalised to that of three reference genes: *actin5C* (Broughton et al., 2005), *tubulin* and *Rpl32* (Ponton et al., 2011). qPCR reactions were performed in 20 µl total volume with 2 µl of cDNA, 100 nmol l^{-1} 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 30 s, 58°C for 30 s and 72°C for 30 s. 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 quantification of DILP5 levels in the IPCs was performed in ImageJ (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 mass 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), and significance was assumed at $P<0.05$. 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 and Rohlf, 1998) and found to be normally distributed. Analyses of variance (ANOVA) were performed to test for significant effects (diet and genotype), and significance was assumed at $P<0.05$. 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 (*Acer* null mutant) following backcrossing (Fig. S1). 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 *Acer* null mutant and w^{Dah} control 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 h period in control 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 with 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 females slept longer during both the day and night periods on the FF diet compared with the low diet (Fig. 1E,F; Table S1). The longer sleep duration of control females on the FF diet compared with the low diet was predominantly due to a significantly longer mean sleep bout duration on the FF compared with the 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). *Acer* null mutant females, however, did not show the normal response of total activity, total sleep and daytime sleep to diet (Fig. 1A,B,F; Table S1). *Acer* null mutant females were less active and slept more than controls on the low diet, but behaved similarly to control females on DR and FF diets. Night sleep duration did respond significantly to diet in *Acer* null mutant females, with significantly longer sleep duration on the FF diet versus the DR and low diets (Fig. 1E; Table S1). However, although night sleep duration in *Acer* null mutant females showed a normal change in response to diet, these females slept significantly longer than controls on the low and FF diets (Fig. 1E). Thus, underlying the observed lack of a dietary response of total sleep duration over a 24 h period in *Acer* null mutant females was a differential effect of loss of Acer on day and night-time sleep duration. Sleep bout parameters in *Acer* null mutant females showed an altered response to diet compared to controls (Fig. 1C–F; Table S1). The number of sleep bouts per day was significantly altered by diet in *Acer* null mutant females between low and DR diets and between DR and FF diets (Table S1), but these females did not show the same response as controls, performing fewer bouts of sleep than controls on the FF diet (Fig. 1C). The mean sleep bout duration of *Acer* null mutant females responded to diet (Fig. 1D; Table S1), but again displayed a different response from controls, with *Acer* null mutant females displaying longer sleep bouts than controls on low and FF diets. These data indicate that: (1) *Acer* null mutant females sleep longer than controls on low food because of an increase in sleep bout duration; and (2) the apparently normal total 24 h sleep behaviour of *Acer* null mutant females on the FF diet (Fig. 1B) was the result of these females performing fewer but longer bouts of sleep than controls on this diet (Fig. 1C,D).

The effect of diet and ACER deletion on sleep and activity in male flies was similarly analysed. In contrast to control females, sleep and activity parameters in control males were less responsive to diet (Fig. 2). Only the number of sleep bouts per day responded significantly to diet, with control males displaying fewer sleep bouts on the FF diet versus the low and DR diet (Fig. 2C; Table S2). All sleep and activity parameters, including the number of sleep bouts per day, did not respond significantly to diet in *Acer* null mutant males (Fig. 2A–F). In addition, compared with control males, *Acer* null mutant 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 *Acer* null mutant flies led us to consider the effect of loss of ACER on other diet-responsive phenotypes, and we next measured the effect of *Acer* deletion on the dietary modulation of lifespan.

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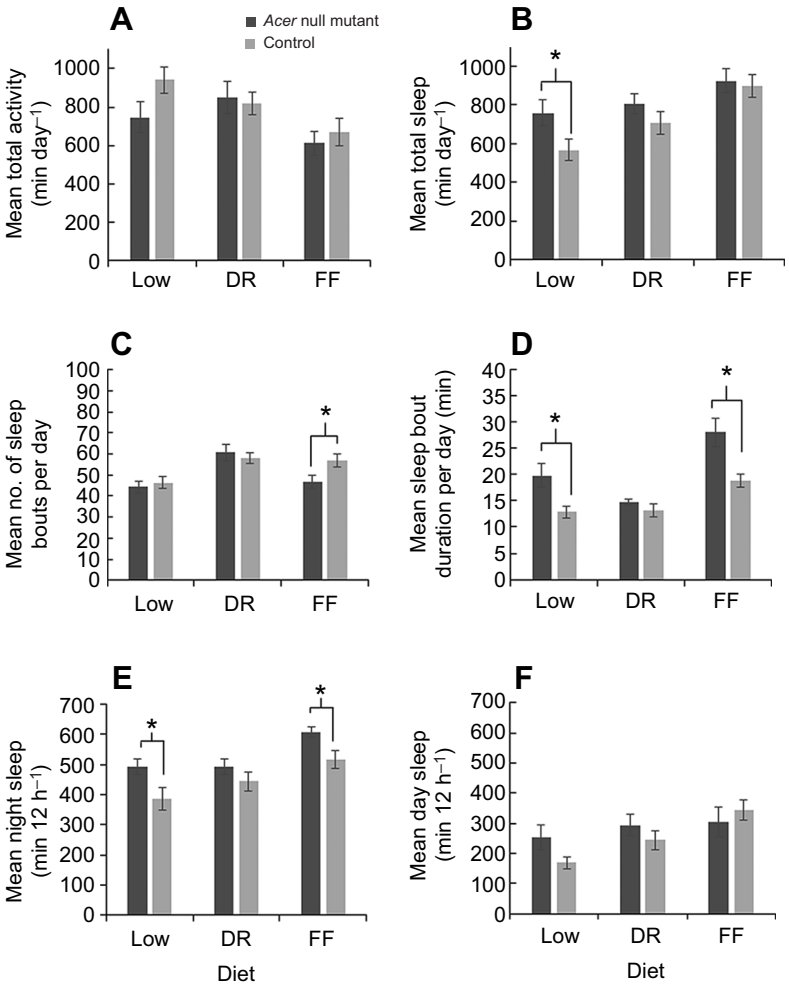

Fig. 1. Activity and sleep behaviour of *Acer* null mutant (w^{Dah}; $Acer^{\Delta}$) females compared with controls (w^{Dah}) under low, dietary restriction (DR) and fully fed (FF) diets. (A) Total 24 h activity duration. (B) Total 24 h sleep duration. (C) Mean number of sleep bouts per day. (D) Mean sleep bout duration per day. (E) Night sleep duration. (F) Day sleep duration. *N*=18 for controls on each food and *Acer* null mutants on DR diet; *N*=15 for *Acer* null mutants on FF diet; and *N*=12 for *Acer* null mutants on low diet. Data are presented as means±s.e.m. Data were analysed by two-way ANOVA (genotype and diet effects) and planned comparisons of means were 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 had a significant effect ($P<0.05$) for total and night sleep duration, and mean sleep bout length. *Significant differences ($P<0.05$) for planned comparisons of means of these parameters between the *Acer* null mutant (w^{Dah};$Acer^{\Delta}$) and control (w^{Dah}) genotypes on each diet.

The survival of *Acer* null mutant female flies compared with controls on starvation, low, DR and FF diets in two independent experiments is shown in Fig. 3. DR is known to extend lifespan compared with FF, and the control females showed the expected response of lifespan to these diets; that is, DR significantly extended the lifespan of control females compared with the FF diet (Fig. 3A,B), and control females were short lived on starvation and low diets (Fig. 3C,D). *Acer* null mutant flies responded normally to DR, with an extended lifespan on the DR compared with the FF diet (Fig. 3A,B), but they showed an altered response to the low diet compared with controls. *Acer* null mutant females were short lived compared with control females on the low diet in both replicate experiments (Fig. 3C,D).

The survival of *Acer* null mutant male flies compared with controls on starvation, low, DR and FF diets in two independent experiments is shown in Fig. 4. Control males showed the expected response of lifespan to these diets, as control males responded to DR with a small but significant lifespan extension, and were short lived on low and starvation diets (Fig. 4). *Acer* null mutant males responded normally to DR with an extended lifespan on the DR compared with the FF diet (Fig. 4A,B). In contrast to the effect of loss of *Acer* in females, *Acer* null mutant males were longer lived than controls on the DR diet (Fig. 4A,B). Similar to females, *Acer* null mutant 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 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 mass, were measured in *Acer* null mutant and control flies following transfer to starvation, low, DR and FF diets 2 days prior to testing.

Glycogen levels in control females and males displayed a significant response to diet after 2 days of feeding (Fig. 5A,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. 5A). In control males, glycogen levels increased with increasing dietary intake between starvation and FF diets (Fig. 5B). *Acer* null mutant males and females had normal levels of glycogen on the starvation diet compared with controls but displayed significantly lower levels of glycogen than controls on the DR and FF diet (Fig. 5A,B). The response of *Acer* null mutant flies to increasing dietary intake was therefore weakened compared with that of controls, indicating that ACER is involved in the normal dietary modulation of stored glycogen.

The effect of diet on lipid levels in control males and females is shown in Fig. 5C,D. In contrast to the effect on glycogen storage, loss of ACER in *Acer* null mutant flies had no effect on the normal

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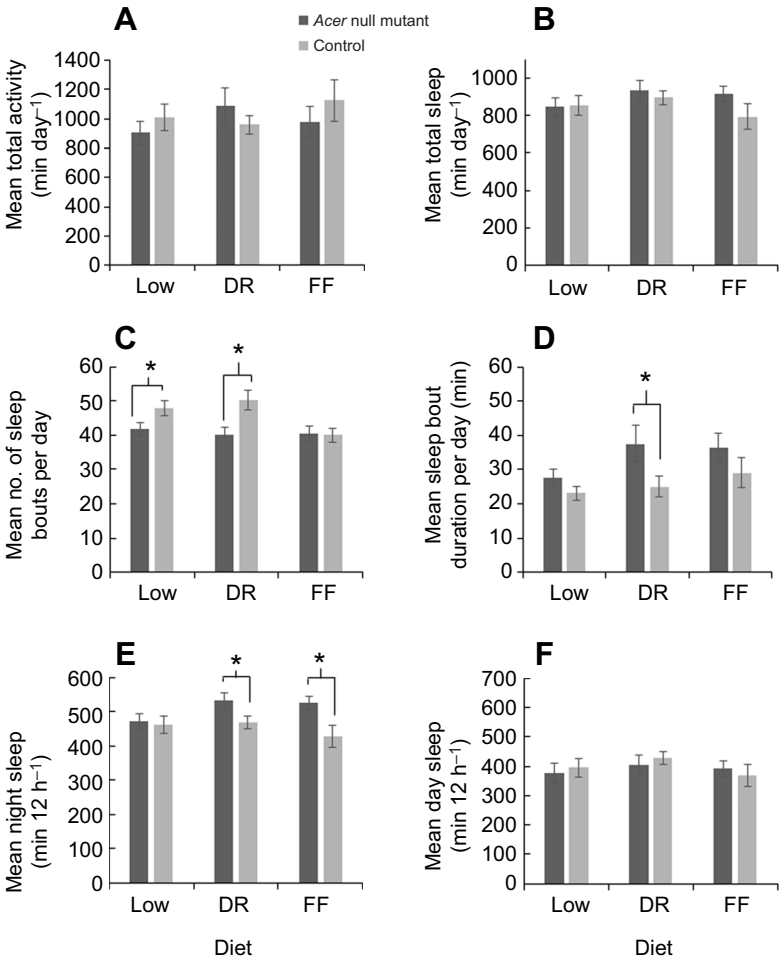

Fig. 2. Activity and sleep behaviour of *Acer* null mutant males compared with controls under low, DR and FF diets. (A) Total 24 h activity duration. (B) Total 24 h sleep duration. (C) Mean number of sleep bouts per day. (D) Mean sleep bout duration per day. (E) Night sleep duration. (F) Day sleep duration. N=18 for controls on each diet and *Acer* null mutants on DR and FF diet; and N=14 for *Acer* null mutants on low diet. Data are presented as means±s.e.m. Data were analysed by ANOVA (genotype and diet effects) and planned comparisons of means were 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 duration and night sleep duration. *Significant difference (P<0.05) for planned comparisons of means of these parameters between the *Acer* null mutant (w^{Dah}; $Acer^{\Delta}$) and control (w^{Dah}) genotypes on each diet.

nutrient-responsive control of lipid levels (Fig. 5C,D). The mass of *Acer* null mutant males and females compared with that of controls showed a normal increase from starvation to FF diet (Fig. 5E,F), but female *Acer* null mutant flies were significantly heavier than controls after 2 days of starvation (Fig. 5E).

Loss of ACER alters the dietary modulation of 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 eight *dilps* in adult *Drosophila* responds in different ways to varying the protein:carbohydrate ratio in the diet (Post and Tatar, 2016). A previous study analysed the response of *dilp2*, *3* and *5* produced in the IPCs 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 DILP5 (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 *Acer* null mutant flies to determine whether this nutrient-responsive phenotype is modulated by ACER.

Similarly to the previous study (Broughton et al., 2010), *dilp5* transcript levels in heads of control flies were low under starvation and low diet conditions and increased significantly on the FF diet (Fig. 6A). In *Acer* null mutant 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 *Acer* null mutant flies did not respond to diet in the same way as in controls. DILP5 protein levels in control IPCs were low under low diet conditions and increased significantly under the DR and FF diet (Fig. 6B,C; Fig. S2). Under starvation conditions, control flies showed the expected high levels of DILP5 protein in the IPCs, despite low transcription of the gene. However, DILP5 protein levels in *Acer* null mutant IPCs did not show a significant dietary response across starvation, low, DR and FF diets (Fig. 6B,C; Fig. S3). Interestingly, under starvation conditions, *Acer* null mutant flies had significantly lower levels of DILP5 in the IPCs compared with that in controls (Fig. 6B,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 *Acer* null mutant and control males and females on low, DR and FF diets. As expected, the feeding of control females and males responded to diet, with flies eating more of the low diet than the higher quality FF diet (Fig. 7). *Acer* null mutant males ate a similar amount of each

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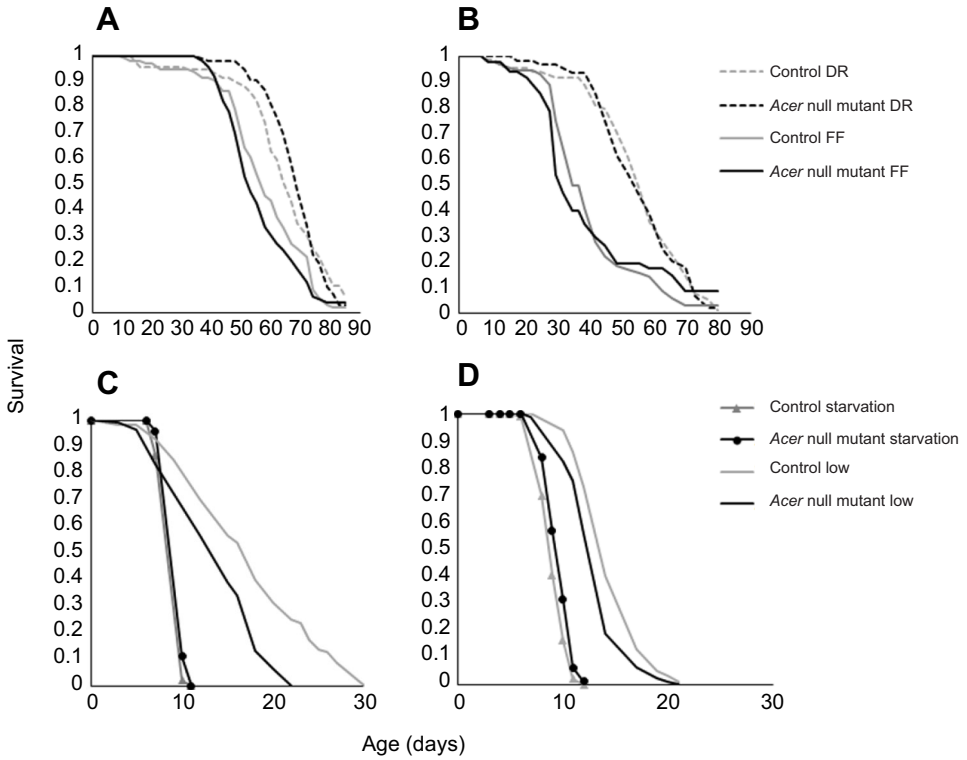

Fig. 3. Survival of female *Acer* null mutants versus controls under starvation, low, DR and FF diets in two replicate experiments. (A,B) Survival of once-mated females under DR and FF diets. (A) Replicate experiment 1. Median lifespans and sample sizes were: *Acer* null mutants, DR 69 days, *N*=97; FF 53 days, *N*=96; and controls, DR 64 days, *N*=94; FF 58 days, *N*=98. Both *Acer* null mutant and control females showed an increased survival from FF to DR diet (log rank test, $P<0.05$). *Acer* null mutants were shorter lived than controls on the FF diet (log rank test, $P=0.0139$). (B) Replicate experiment 2. Median lifespans and sample sizes were: *Acer* null mutants, DR 54 days, *N*=93; FF 31 days, *N*=98; and controls, DR 54 days, *N*=95; and FF 38 days, *N*=97. Both *Acer* null mutant and control 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: *Acer* null mutants, starvation 8.5 days, *N*=100; low 13.5 days, *N*=124; and controls, starvation 8.5 days, *N*=100; low 17 days, *N*=123. *Acer* null mutants were shorter lived than controls on the low diet (log rank test, $P<0.0001$). (D) Replicate experiment 2. Median lifespans and sample sizes were: *Acer* null mutants, starvation 9.5 days, *N*=100; low 13 days, *N*=101; and controls, starvation 8.5 days, *N*=100; low 13 days, *N*=99. *Acer* null mutants were longer lived than controls on the starvation diet (log rank test, $P=0.0025$). *Acer* null mutants were shorter lived than controls on the low diet (log rank test, $P=0.0081$).

diet to controls and showed a normal response to diet (Fig. 7B). 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 *Acer* null mutant males were not due to differences in the quantity of each diet consumed. The feeding of female *Acer* null mutant flies, however, did not respond to diet in the same way as controls (Fig. 7A). *Acer* null mutant females ate a similar quantity of the low diet to controls but they did not decrease their intake 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 daytime 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 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 with 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 probably 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 diet 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.

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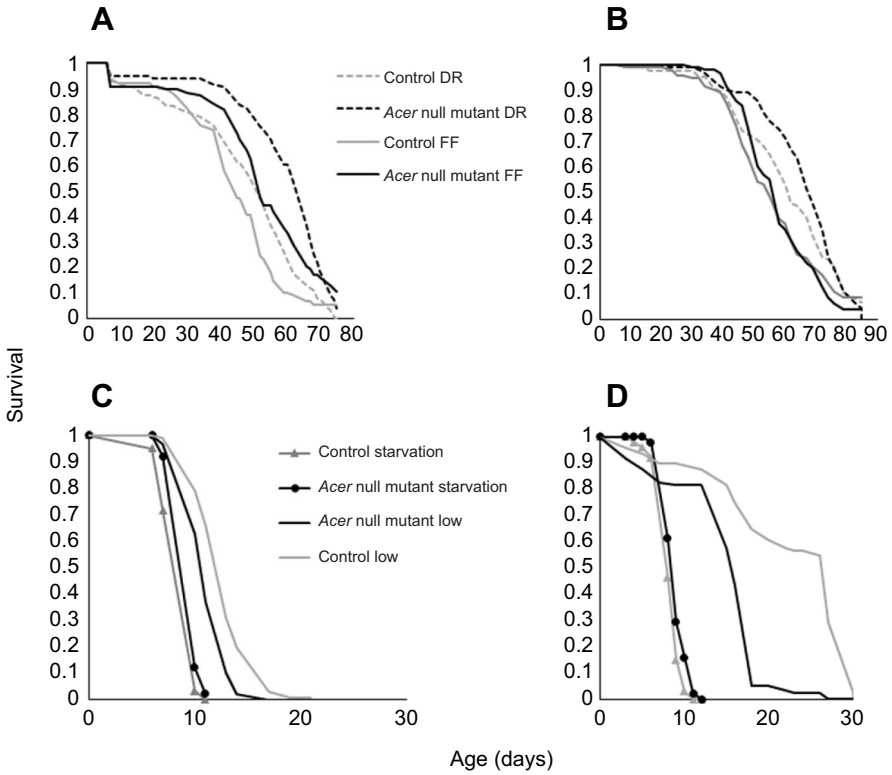

Fig. 4. Survival of male *Acer* null mutants versus controls under starvation, low, DR and FF diets in two replicate experiments. (A,B) Survival of males under DR and FF diets. (A) Replicate experiment 1. Median lifespans and sample sizes were: *Acer* null mutants, DR 64 days, *N*=100; FF 50.5 days, *N*=92; and controls, DR 50.5 days, *N*=104; FF 43 days, *N*=98. Both *Acer* null mutant and control males showed an increased survival from FF to DR diet (log rank test, $P<0.05$). *Acer* null mutants were longer lived than controls 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: *Acer* null mutants, DR 68 days, *N*=100; FF 56.5 days, *N*=97; and controls, DR 61 days, *N*=97; FF 54 days, *N*=94. Both *Acer* null mutant and control 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: *Acer* null mutants, starvation 8.5 days, *N*=100; low 10.5 days, *N*=101; and controls, starvation 8.5 days, *N*=98; low 12 days, *N*=99. Both *Acer* null mutant and control males showed an increased survival from DR to FF diet (log rank test, $P<0.05$). *Acer* null mutants were longer lived than controls on the starvation diet (log rank test, $P<0.001$). *Acer* null mutants were shorter lived than controls on the low diet (log rank test, $P<0.001$). (D) Replicate experiment 2. Median lifespans and sample sizes were: *Acer* null mutants, starvation 8.5 days, *N*=100; low 15.5 days, *N*=121; and controls, starvation 7 days, *N*=100; low 26.5 days, *N*=120. *Acer* null mutants were longer lived than controls on the starvation diet (log rank test, $P<0.001$). *Acer* null mutants were shorter lived than controls on the low diet (log rank test, $P<0.001$).

These data further raise the question of why DILP5 protein levels in *Acer* null mutant 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* null flies are defective in releasing DILP5 protein. However, further research is needed to determine whether the IPCs in *Acer* null 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* null female flies showed an increase in night sleep duration on low and FF diets and *Acer* null mutant 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, *Acer* null mutant 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 the mechanism of action of ACER 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. DR without malnutrition is an evolutionarily conserved intervention that extends lifespan and modulates ageing in model organisms (for review, see Fontana et al., 2010). The dietary regime used in this study (DR versus 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., 2010). In particular, the modulation of nutrient-sensing signalling pathways such as the 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 *Acer* null mutant males and females showed a normal lifespan extension on the DR diet compared with the FF diet, male *Acer* null mutant 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 region of the fly brain, an area which is known to control sexually dimorphic locomotory behaviour (Belgacem and Martin, 2006; Gatti et al., 2000).

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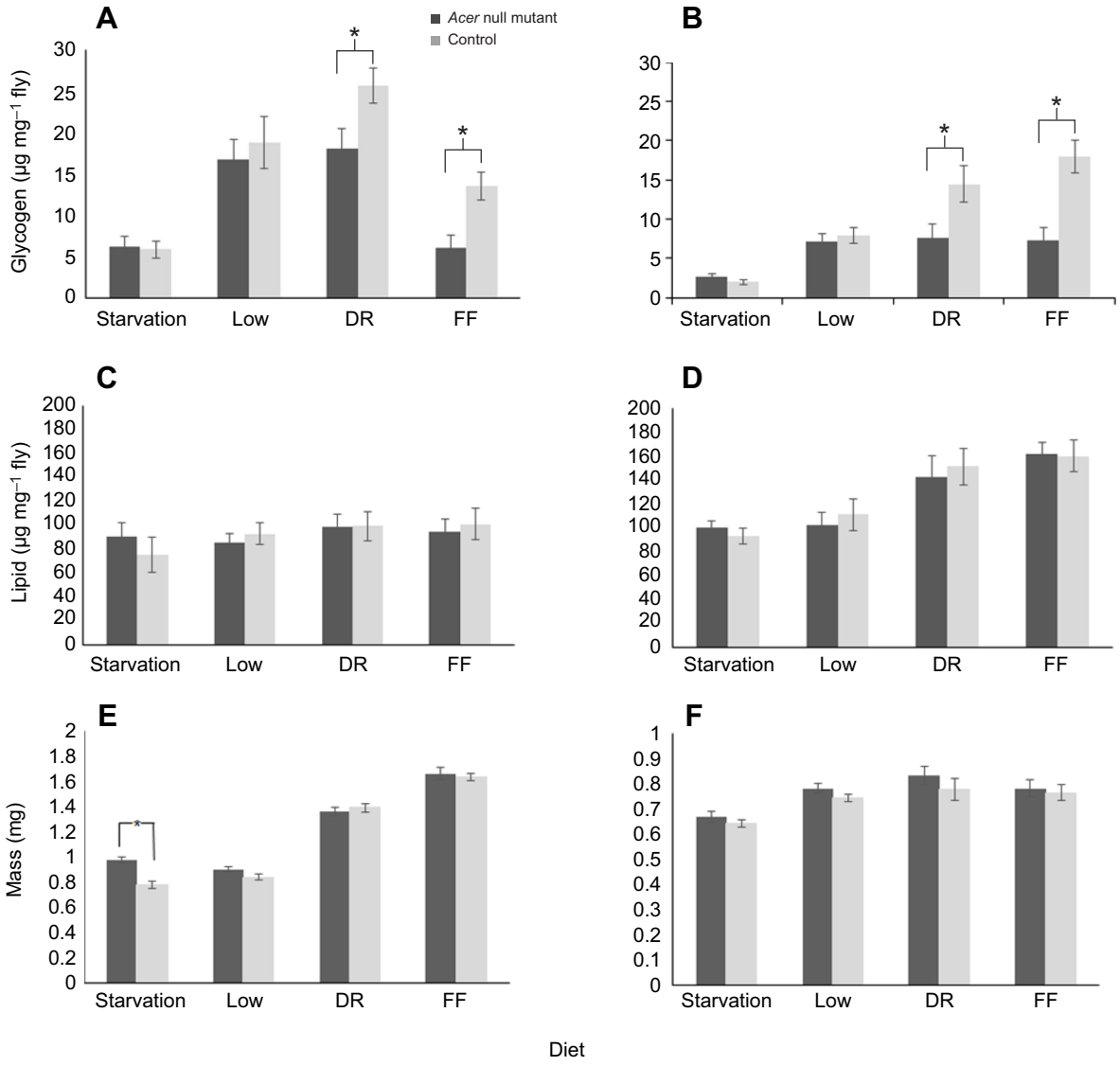

Fig. 5. Glycogen and lipid levels and mass of 10 day old *Acer* null mutant flies versus controls after 2 days on the starvation, low, DR and FF diets. (A,B) Mean glycogen levels of *Acer* null mutant and control females (A) and males (B). (C,D) Mean lipid levels of *Acer* null mutant and control females (C) and males (D). (E,F) Mean mass of *Acer* null mutant and control females (E) and males (F). Data are shown as means±s.e.m. *N*=10 flies. 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. *Significant difference between indicated genotypes ($P<0.05$).

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 *Acer* null mutant flies. The feeding of *Acer* null mutant 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 *Acer* null mutant flies was different from that of 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, *Acer* null mutant females stored less glycogen than controls and showed no difference in mass, lipid and DILP5 levels on these diets. In addition, *Acer* null mutant 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 response of *Acer* null mutant females was due to their altered feeding. Of particular interest in this respect is the effect of the ACER deletion on glycogen storage. *Acer* null mutant 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 haemolymph 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 *Acer* null mutant females compared with controls on DR and FF diets were sufficient to trigger a metabolic response to increase feeding, in turn suggesting that the altered feeding in *Acer* null mutant females is an indirect effect of their lowered glycogen storage.

The demonstrated substrate specificity of the dipeptidyl peptidase activity of the purified enzyme (Houard et al., 1998; Siviter et al.,

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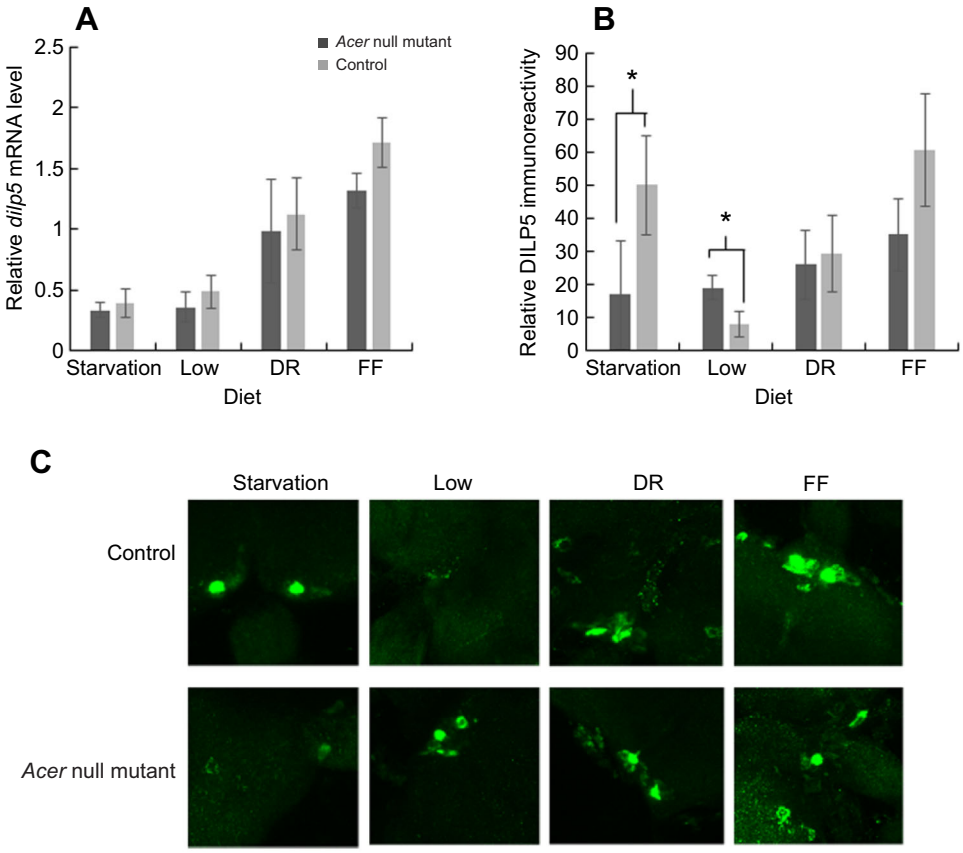

Fig. 6. DILP5 transcript and protein levels of *Acer* null mutant flies versus controls after 2 days on the starvation, low, DR and FF diets. (A) Relative mRNA abundance of *dilp5* from adult heads of 10 day old *Acer* null mutant and control females following 48 h treatment with complete starvation, low, DR and FF diets, as measured by quantitative RT-PCR and normalised to the mRNA abundance of *actin5C*, *tubulin* and *Rpl32*. Data are shown as means±s.e.m. of three to six independent experiments. 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 *Acer* null mutants and controls the abundance of *dilp5* on FF diet was significantly greater than that on the low and starvation diets. (B) Immunohistochemical analysis of DILP5 protein in *Acer* null mutant and control 10 day old female brains following 48 h treatment with complete starvation, low, DR and FF diets. Sample sizes were: controls, starvation N=8, low N=13, DR N=11, FF N=8; *Acer* null mutants, starvation N=6, low N=9, DR N=9, FF N=11. Quantification of DILP5 levels was performed using ImageJ on the confocal microscope images shown in Figs S2 and S3. Data are shown as mean±s.e.m. relative fluorescence. 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. *Significant difference between genotypes, P<0.05. (C) Representative images of anti-DILP5 staining for controls (see Fig. S2 for images of all brains examined) and *Acer* null mutants (see Fig. S3 for images of all brains examined).

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 fat body signals

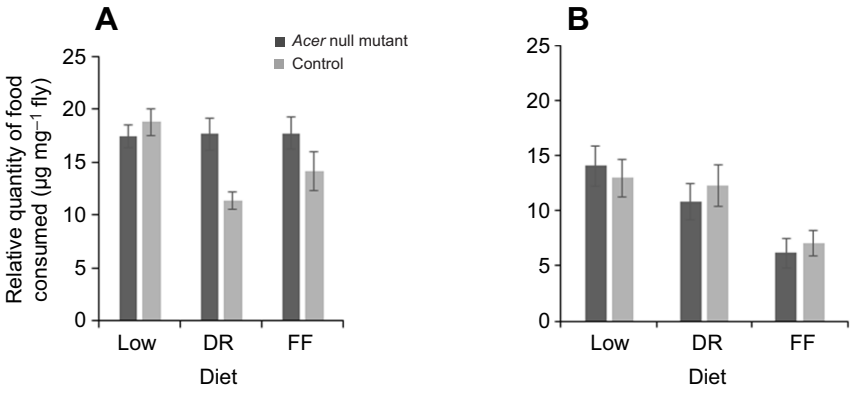

Fig. 7. Effect of diet on feeding in male and female *Acer* null mutant flies. Relative quantity of food consumed by (A) female and (B) male flies of the indicated genotype after 30 min feeding on low, DR and FF diets containing Brilliant Blue dye. Direct quantification of dye consumed was carried out by colour spectrophotometry. The data presented are means±s.e.m., N=33–35 (vials of five flies) per genotype, per diet. ANOVA were performed and food, genotype and sex were significant effects. Diet had a significant effect on the quantity of food consumed by control males and females and by *Acer* null mutant males (P<0.05). There was no significant effect of diet on the quantity of food consumed by *Acer* null mutant female flies (P=0.99).

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 its 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 *Caenorhabditis 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 Brachycera 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.

Conclusions

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.

Acknowledgements

We thank Elwyn Isaac for a critical reading of the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.S., S.J.B.; Methodology: A.S., S.J.B.; Validation: S.J.B.; Formal analysis: Z.G., M.D.H., S.J.B.; Investigation: Z.G., M.D.H., N.D., J.C., H.A., S.J.B.; Resources: S.J.B.; Data curation: S.J.B.; Writing - original draft: A.S., S.J.B.; Writing - review & editing: A.S., S.J.B.; Supervision: S.J.B.; Project administration: S.J.B.; Funding acquisition: S.J.B.

Funding

This work was supported by Lancaster University (PhD Studentship to Z.G.) and The Royal Society (RG110541 to S.J.B).

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.194332.supplemental

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Supplementary Figure 1

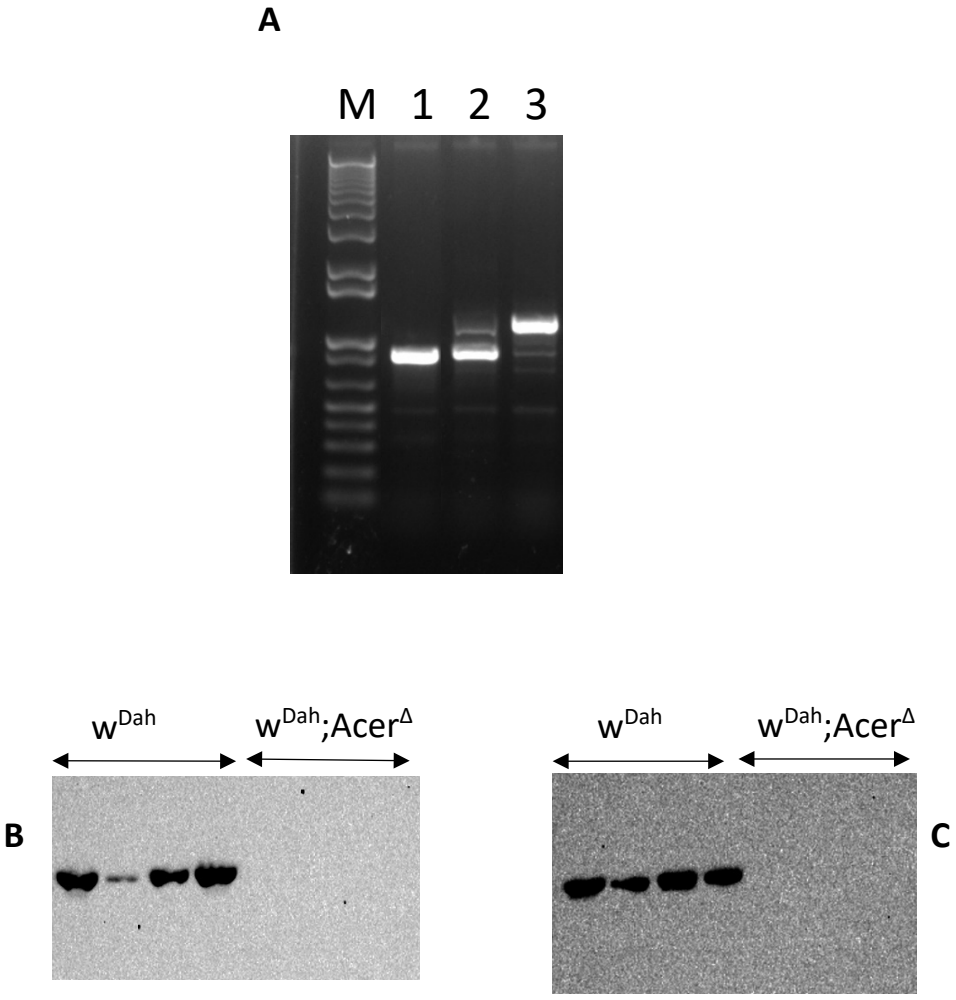

Figure S1: (A) PCR analysis of the *Acer* deletion in the w^{Dah} background. Lane M: marker. Lane 1: The $Acer^{\Delta 168}$ deletion homozygote with a strong band at 850 bp. Lane 2: The $Acer^{\Delta 168}$ deletion heterozygote with a strong band at 850 bp and a weaker band at 1,150 bp. Lane 3: The 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

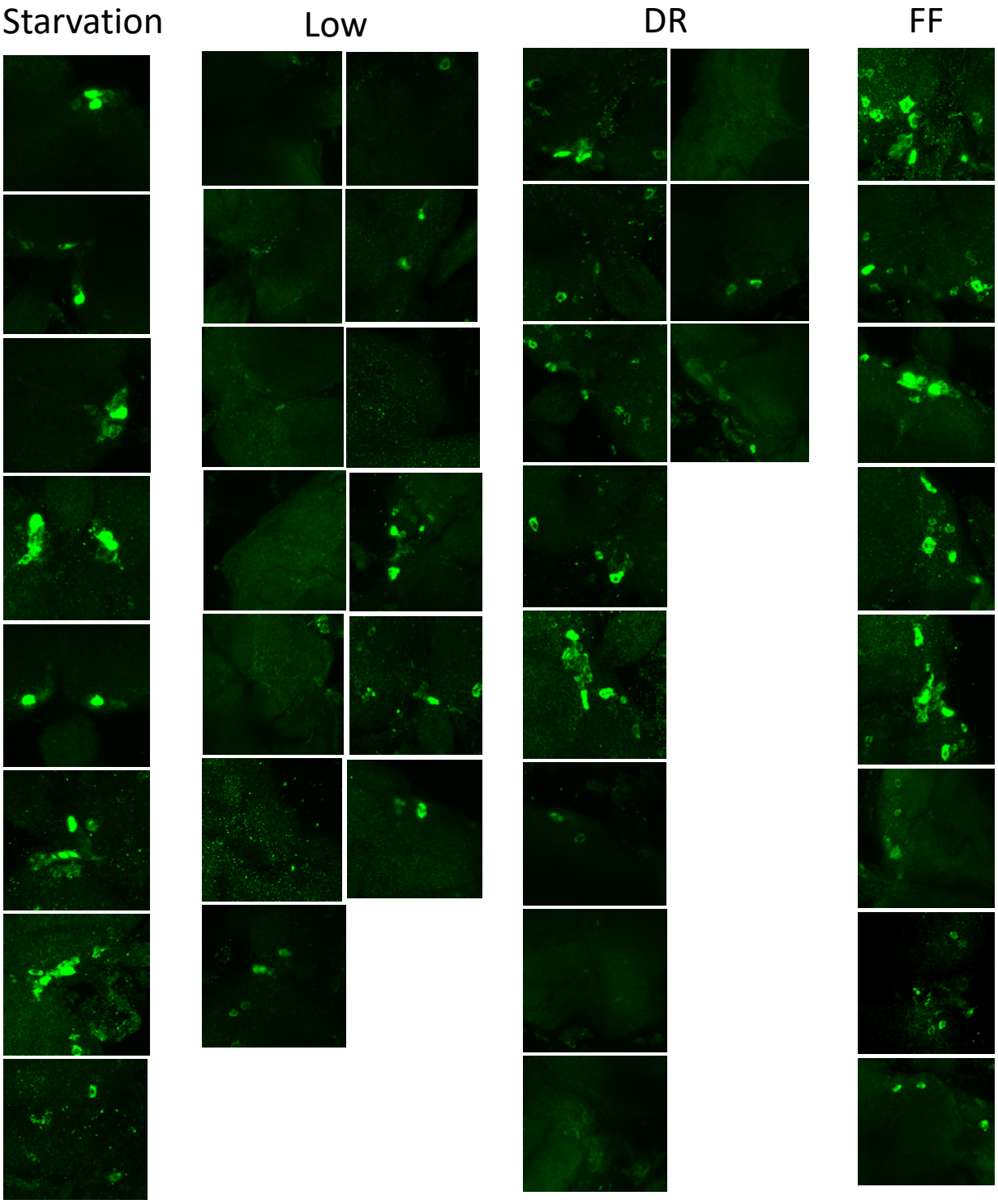

Figure S2. Immunohistochemical analysis of DILP5 protein in w^{Dah} 10 day old female brains following 48 h treatment with Starvation, Low, DR and FF diets.

Supplementary Figure 3

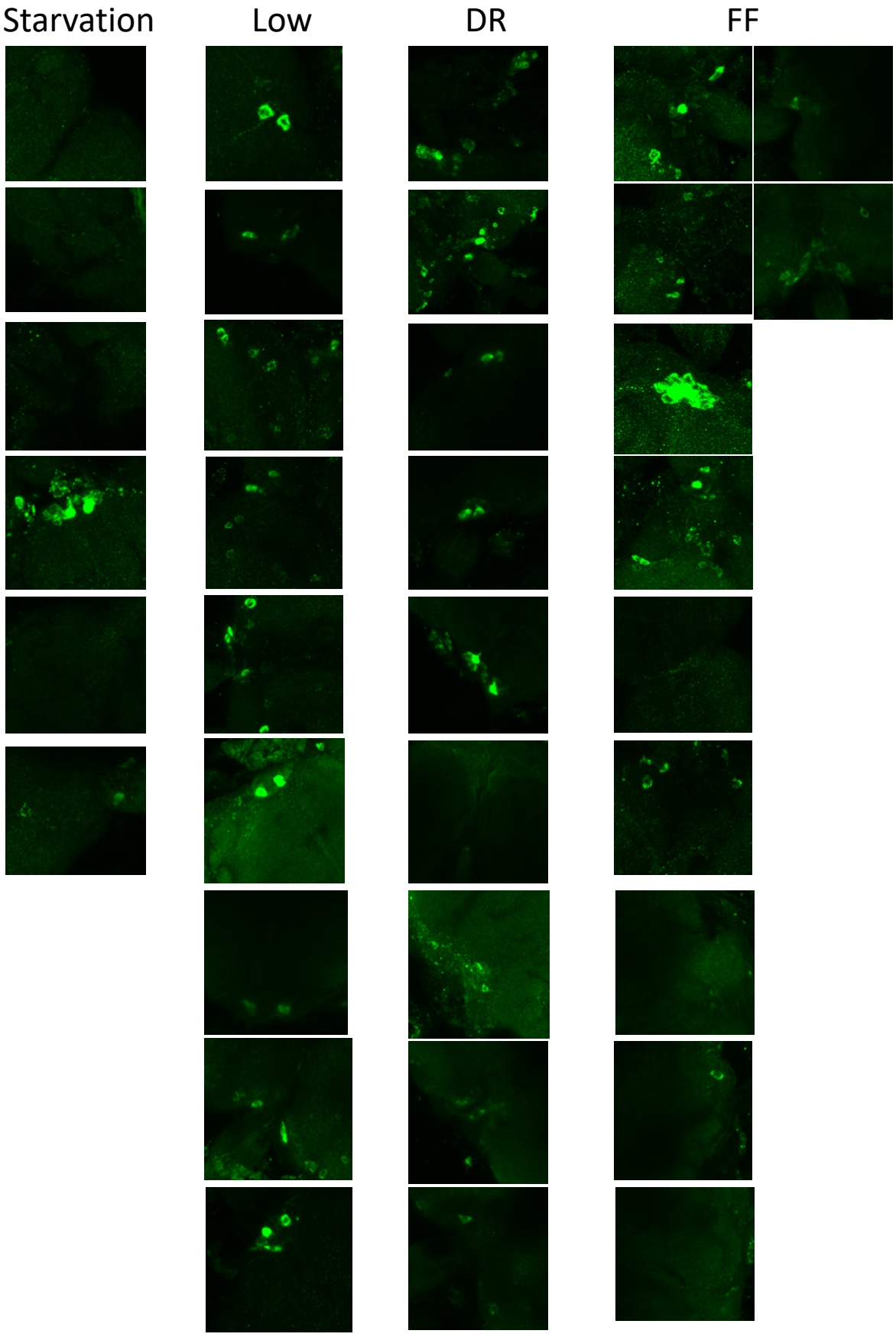

Figure S3. Immunohistochemical analysis of DILP5 protein in w^{Dah};Acer$^{\Delta}$ 10 day old female brains following 48 h treatment with Starvation, Low, DR and FF diets.

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