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Natural variation in plasticity of glucose homeostasis and food intake

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SUMMARY

Balancing the acquisition, allocation and storage of energy during periods of food deprivation is critical for survival. We show that natural variation in the *foraging* (*for*) gene, which encodes a cGMP-dependent protein kinase (PKG) in the fruit fly *Drosophila melanogaster*, affects behavioral and physiological responses to short-term food deprivation. Rover and sitter, natural allelic variants of *for*, differ in their stored carbohydrate reserves as well as their response to short-term deprivation. Fewer carbohydrates are stored in the fat body of rovers compared with sitters, and more labeled glucose is allocated to lipid stores compared with carbohydrate stores during a short feeding bout. Short-term food deprivation decreases hemolymph glucose levels in rovers but not in sitters. After food deprivation, rovers increase their food intake more slowly than sitters, and rover hemolymph levels take longer to respond to re-feeding. Finally, rovers have lower adipokinetic hormone (akh) mRNA levels than sitters. Our data suggest that *for* mediates larval responses to short-term food deprivation by altering food intake and blood glucose levels.

Key words: adipokinetic hormone, behavior genetics, cGMP-dependent protein kinase, food intake, foraging, glucose homeostasis.

INTRODUCTION

The ability to balance the acquisition and storage of energy is essential for survival in nature because the abundance and quality of food can change drastically over time and this can result in periods of food deprivation. When food is limited, the homeostatic control system regulates energy balance and storage (Stubbs and Tolkamp, 2006). Despite tight regulation of energy homeostasis, there are individual differences in energy balance (Speakman, 2004). This is due, in large part, to interactions between the nutritive environment and the genes that contribute to variation in energy balance (Speakman, 2004). Energy acquisition, use and storage all contribute to energy balance.

The *foraging* (*for*) gene in *Drosophila melanogaster* (L.) provides a rare example of a naturally occurring genetic polymorphism that contributes to individual differences in energy acquisition (Kaun et al., 2007). *for* encodes a *D. melanogaster* cGMP-dependent protein kinase (PKG); two naturally occurring alleles of *for* are rover (for^R) and sitter (for^s) (de Belle et al., 1989). In *Drosophila*, the adult heads and larval nervous systems of rovers have higher PKG activity and *for*-transcript levels than those of sitters (Osborne et al., 1997). *for* affects larval food acquisition in an environmentally dependent fashion (Kaun et al., 2007). When food is plentiful, rover larvae have lower food intake and a higher proportion of glucose absorbed than sitters. When food is scarce, rover and sitter larvae increase food intake to a common maximal level with rovers retaining their increased glucose absorption. These phenotypes affect larval survival and development in nutritionally depleted environments (Kaun et al., 2007).

Interestingly, PKG has also been associated with human disorders in energy balance, including obesity and diabetes. High levels of *cGK1*, the mammalian ortholog of *for*, have been associated with obesity in mammals (Engeli et al., 2004; Su et al., 2003) whereas low levels have been associated with high glucose concentrations and diabetes (Wang et al., 2002; Zanetti et al., 2005).

During short periods of food deprivation, organisms use their stored carbohydrate reserves to supply energy for survival. In insects, the mobilization of these stored carbohydrates is mediated by peptide hormones called adipokinetic hormones (AKHs) (Gade and Auerswald, 2003). In *Drosophila*, one of these peptides is called AKH or dAKH. It is encoded by the *akh* gene and it mobilizes glucose after starvation (Kim and Rulifson, 2004; Lee and Park, 2004). Targeted cell ablation of *akh*-producing cells decreases hemolymph sugar levels whereas over-expression of *akh* increases them (Isabel et al., 2005; Kim and Rulifson, 2004; Lee and Park, 2004). A decrease in *akh* expression is also associated with a lack of starvation-induced hyperactivity and resistance to starvation-induced death (Isabel et al., 2005; Lee and Park, 2004). Thus, *akh* appears to mediate hemolymph sugar levels and starvation-induced changes in behavior.

In the present study, we explore how natural variation in *for* affects glucose homeostasis by investigating the physiological and behavioral responses to short-term food deprivation. Firstly, we investigate whether *for* affects the absorption and storage of carbohydrates. Secondly, we examine whether *for* affects glucose expenditure following short periods of food deprivation. Finally, we investigate whether different levels of *akh* mRNA are found in the rover and sitter variants.

MATERIALS AND METHODS

Strains

The wild-type rover and sitter strains used are isogenic for chromosomes *2* and *3* and homozygous for the for^R or for^s alleles, respectively (deBelle and Sokolowski, 1987). To ensure that any differences found between the two natural variants was specific to *for*, the same measurements were made using for^{s2}, a sitter mutant generated on a rover genetic background (deBelle et al., 1989; Pereira and Sokolowski, 1993). Flies were maintained in 170 ml plastic culture bottles with 40 ml of standard culture medium at 25±1°C and a 12L:12D photocycle. Standard culture medium

contained: 50 g Baker's yeast; 100 g sucrose; 16 g agar; 0.1 g KPO_4; 8 g $KNaC_4H_4O_6 \cdot 4H_2O$; 0.5 g NaCl; 0.5 g $MgCl_2$; and 0.5 g $Fe_2(SO_4)_3$ per liter of tap water. Larvae were reared from egg-hatch to mid-third-instar (96±2 h post-hatch) in 25°C at densities of 100 larvae per 35 ml of medium in 100mm×15mm Petri dishes.

Glucose absorption and allocation

The glucose absorption protocol was modified from Riha and Luckinbill (Riha and Luckinbill, 1996) and is described in Kaun et al. (Kaun et al., 2007). Glucose allocation experiments were modified from Bligh and Dyer (Bligh and Dyer, 1959) and Westphal and Jann (Westphal and Jann, 1965) as described below. Groups of 30 larvae were homogenized and lipids, carbohydrates and proteins were extracted in layers from the same homogenate. Sample sizes were 30 larvae per vial with six vials per strain. Larvae were removed from –80°C and homogenized in 200 µl of 2:1 chloroform:methanol in a 1.5 ml tube using a hand-held motorized mortar with plastic pestle. An additional 800 µl of 2:1 chloroform:methanol was added, samples were then vortexed and centrifuged at 10,000 ***g*** for 5 min at 4°C. The chloroform phase, composed of soluble material containing the lipid fraction, was removed and transferred to scintillation vials. 500 µl of phosphate-buffered saline solution (PBS: 2.5 mmol l^{-1} NaH_2PO_4; 8.5 mmol l^{-1} Na_2HPO_4; and 175 mmol l^{-1} NaCl; pH 7.4) was added to the remaining precipitate and methanol phase. Samples were vortexed, 500 µl of phenol was added and samples were heated at 65°C in a water bath for 1 h with occasional vortexing. Samples were cooled on ice for 1 h and then centrifuged at 3000 ***g*** for 30 min at 4°C for biphasic separation. The lower phenol layer, composed of soluble material containing the protein fraction, was removed and transferred to scintillation vials. The upper layer, containing the aqueous carbohydrate fraction, was transferred to separate scintillation vials. The remaining interphase was also transferred to scintillation vials. We then added 10 ml scintillation fluid to each scintillation vial, and samples were vortexed for 30 s, shaken for two hours then left at room temperature for 24 h. The amount of ^{14}C in each vial was calculated using counts observed over 60 s per sample in a scintillation counter (Wallac 1409 Liquid Scintillation Counter, Perkin Elmer Life Sciences, Woodbridge, ON, Canada).

Prior to calculating the specific activity (fmoles) of intake and absorption of [^{14}C]-6-glucose per larva, a conversion factor taking into account the specific activity of the radiolabeled substance was calculated. Preferential storage of glucose consumed in the form of one macronutrient over the other, was calculated as percentage of ^{14}C absorbed per strain compared with the total ^{14}C absorption per strain, where total ^{14}C was defined as the sum of ^{14}C in lipids, carbohydrates, proteins and interphase.

Protein assays

Protein levels were determined using the bicinchoninic acid (BCA) method [modified from Marron et al. (Marron et al., 2003)]. Individual larvae were homogenized in 300 µl double-distilled water (ddH_2O) and centrifuged at 10,000 ***g*** for 2 min. After centrifugation, 10 µl of the supernatant was removed from each sample and placed in a SPECTRAplate^{TM} Quartz UV Transparent 96-well Microplate (Molecular Devices, Sunnyvale, CA, USA) with 100 µl of Sigma BCA Protein Assay Reagent (Sigma Chemical Co., St Louis, MO, USA). Samples were incubated overnight at room temperature and protein concentrations were determined by comparing the absorbance at 562 nm with standard curves. Standard curves were constructed using bovine serum albumin (BSA; Sigma Chemical Co.) with concentrations ranging from 0.20 mg ml^{-1} to 0.45 mg ml^{-1}.

Lipid assays

Lipid levels were determined by hydrolyzing triglycerides and then measuring the resulting glycerol levels [modified from Lee and Park (Lee and Park, 2004)]. Individual larvae were homogenized on ice in 150 µl 0.1% Tween-20 in PBS in a 1.5 ml tube using a hand-held motorized mortar with plastic pestle. Tween-20 is a non-ionic surfactant that is used to disperse and emulsify, and acts to disperse lipids into globules, which are suspended in the water solution. Samples were heated at 70°C for 5 min to inactivate endogenous enzymes, then vortexed briefly. 30 µl of the homogenate was removed and incubated on a rocking platform at 37°C overnight in a 1.5 ml tube with 30 µl of 2 mg ml^{-1} *Candida rugosa* lipase (Benjamin and Pandey, 1998) (Sigma Chemical Co.) suspended in 1×PBS. The lipase acts to hydrolyze triglycerides resulting in the production of free fatty acids and glycerol. Samples were then vortexed briefly and centrifuged at 13,000 ***g*** for 10 min. 10 µl of the supernatant was removed and incubated with 100 µl of Sigma Free Glycerol Reagent (Sigma Chemical Co.) at 37°C for 2 h in a SPECTRAplate^{TM} Quartz UV Transparent 96-well Microplate (Molecular Devices). Glycerol concentrations were determined by comparing absorbance at 540 nm with standard curves. Standard curves were constructed using Sigma glycerol standards (Sigma Chemical Co.) with concentrations ranging from 0.20 mg ml^{-1} to 0.45 mg ml^{-1}. Ten larvae per food deprivation condition per strain were assayed.

Carbohydrate assays

Whole larval assays

Whole animal carbohydrate assays were as in Marron et al. (Marron et al., 2003). Individual larvae were homogenized in 300 µl ddH_2O in a 1.5 ml tube using a hand-held motorized mortar with plastic pestle. 10 µl of *Rhizopus* mold amyloglucosidase (8 mg ml^1 suspended in ddH_2O; Sigma Chemical Co.) was added to 10 µl of homogenate in a SPECTRAplate^{TM} Quartz UV Transparent 96-well Microplate (Molecular Devices). *Rhizopus* amyloglucosidase catalyzes the conversion of glycogen and trehalose into glucose (Parrou and Francois, 1997). Samples were left overnight at room temperature. 100 µl of Sigma Glucose Assay Reagent (Sigma Chemical Co.) was added and samples were left for 1 h. Glucose was assayed using the hexokinase and G6PDH reactions and measuring the increase in absorbance at 340 nm due to $NADP^+$ reduction to NADH. Glucose concentrations were determined by comparing absorbance by NADH at 340 nm with standard curves. Standard curves were constructed using Sigma glucose standards (Sigma Chemical Co.) with concentrations ranging from 0.05 mg ml^{-1} to 0.30 mg ml^{-1}. Ten larvae per food deprivation condition per strain were assayed.

Larval hemolymph carbohydrate assays

Hemolymph carbohydrate assays were performed as above with the following changes [modified from Kim and Rulifson, and Lee and Park (Kim and Rulifson, 2004; Lee and Park, 2004)]. Ten groups of five mid-third-instar larvae were washed with distilled water (dH_2O) and blot dried. Hemolymph was extracted by tearing the cuticle and allowing the hemolymph to bleed out onto a glass slide. 2 µl of hemolymph was rapidly withdrawn and mixed with 38 µl 1×PBS. The sample was vortexed and centrifuged for 10 min to precipitate blood cells and tissue debris. 10 µl of supernatant from each sample plated with 10 µl of 8.0 mg ml^{-1} *Rhizopus* amyloglucosidase (Sigma Chemical Co.) was left overnight at room temperature. 100 µl of Sigma Glucose Assay Reagent (Sigma Chemical Co.) was added and left for 1 h, after which, glucose concentrations were determined as above.

Larval fat body carbohydrate assays

Fat body carbohydrate assays were performed as for the whole larval carbohydrate assays with the following modifications. Fat bodies were dissected from individual animals in 1×PBS and transferred to 1.5 ml vials with 50 µl 0.1% Tween-20, which were kept on ice. Samples were homogenized using a hand-held motorized mortar with plastic pestle. 20 µl of 8.0 mg ml^{-1} *Rhizopus* amyloglucosidase was added, and samples were vortexed briefly. Samples were left overnight in 37°C, vortexed again, then centrifuged for 5 min at 13,000 ***g*** to precipitate tissue debris. 10 µl of supernatant was plated with 90 µl of Sigma Glucose Assay Reagent (Sigma Chemical Co.) and left for 1 h. Glucose concentrations were determined as above using standard curves with concentrations ranging from 0.01 mg ml^{-1} to 0.25 mg ml^{-1}. Glucose concentrations were standardized by protein concentration from the same samples, determined using the BCA method described above. Eight larvae per food deprivation condition per strain were assayed.

Acute food deprivation and re-feeding

Larvae were removed from food plates, washed in dH_2O, and placed in groups of 30–40 in 45 mm×10 mm Petri plates with three 20 mm×3 mm plugs of 1.4% agar on the bottom. Lids were held firmly on top by a 0.2 kg weight. Larvae were left for 1, 2 or 3 h on agar before testing for nutrient storage or food intake. For re-feeding assays, larvae were removed from agar plates to 45 mm×10 mm Petri plates with three 20 mm×3 mm plugs of standard laboratory fly culture medium for 30 min, 1 or 2 h.

Food intake

Food intake protocols were performed as in Kaun et al. (Kaun et al., 2007). Briefly, larvae were removed from food plates, washed in dH_2O, and groups of ten were placed into circular wells (86 mm in diameter and 0.5 mm deep) previously filled with yeast paste (2:1 water:yeast) mixed with 0.08% Brilliant Blue R dye (Sigma Chemical Co.). The wells were then covered with 9 cm Petri plate lids. Larvae remained on this dyed yeast paste for varying amounts of time depending on the experiment. They were then boiled for 10 s, aligned on a microscope slide, placed under a dissecting microscope (Zeiss, Toronto, ON, Canada) and imaged using Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada). Food intake was measured as the number of pixels (square pixels were used for quantification) in the image colored by the dye relative to the total number of pixels in the whole larval body taken as a percentage. Image J software was used (ImageJ v. 1.28j, 2002 and ImageJ v. 1.32j, 2004; http://rsweb.nih.gov.ij) for the digital quantification. Thirty larvae per food deprivation condition per strain were assayed.

Quantitative real-time PCR (qRT-PCR)

RNA extraction

RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Larvae were collected and frozen in liquid nitrogen in groups of ten, then stored at –80°C until RNA extraction was performed. Ten larvae per strain per condition were homogenized on ice in 500 µl TRIzol Reagent in a 1.5 ml tube using a hand-held motorized mortar with RNAse-free plastic pestle. They were left for 5 min at room temperature. 100 µl of chloroform was then added and samples were shaken vigorously for 15 s and then left for 3 min at room temperature. Samples were centrifuged at 12,000 ***g*** for 15 min at 4°C. The supernatant was then extracted and placed in a 1.5 ml RNase-free microtube. 250 µl of isopropanol was added, samples were shaken gently, and left at room temperature for 10 min. Samples were then centrifuged at 12,000 ***g*** for 10 min at 4°C and the supernatant was disposed of. 500 µl of 75% ethanol was added, samples were vortexed briefly, then centrifuged at 7500 ***g*** for 5 min at 4°C. Supernatant was disposed of and samples were left for 15 min in the fumehood to dry. 50 µl of RNase-free water was added and samples were heated to 55°C for 10 min. Concentration of RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Reverse transcript synthesis

Transcripts were synthesized using SuperScript II Reverse Transcriptase (Invitrogen). 2 µg of RNA was added to 10 µl of RNase-free water, samples were then treated with DNase by adding 0.5 µl of DNase (200 U µl^{-1}, Invitrogen) and 1 µl of 10X DNase Buffer (Invitrogen) and left at room temperature for 15 min. 1 µl of 25 mmol l^{-1} EDTA was added and samples were incubated at 75°C for 5 min to inactivate the enzyme. Samples were centrifuged briefly (13,000 ***g***), then 0.4 µl random heximers (Qiagen, Mississauga, ON, Canada) and 1 µl of Biolase dNTP (Bioline, Randolph, MA, USA) were added. Samples were incubated at 65°C for 5 min, quick chilled on ice and centrifuged briefly (13,000 ***g***). 2 µl of 0.1 mol DTT (Invitrogen) and 4 µl of 5×Superscript II Buffer (Invitrogen) was mixed in with gentle pipetting. Samples were left at room temperature for 2 min after which 1 µl of Superscript II Reverse Transcriptase (200 U µl^{-1}, Invitrogen) was gently pipetted into each sample. Samples were left at room temperature for 10 min and then incubated at 42°C for 50 min. The reaction was stopped by incubating samples at 70°C for 15 min.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed with the SYBR green method (Eurogentec, San Diego, CA, USA) using the ABI 7700 Sequence Detection (Applied Biosystems, Foster City, CA, USA). cDNA was diluted 1:10 and plated as 5 µl samples with 2.5 µl each of 3 µm forward (5′-GGCAACTGCAAGACCTCCAA-3′) and backward (5′-TGTGCCTGAGATTGCACGAA-3′) *akh* primers, 2.5 µl ddH_2O and 12.5 µl SYBR-green Master Mix (Eurogentec). A standard curve using mixed cDNA of the samples was composed using dilutions 1:2, 1:5, 1:10, 1:20, 1:50 and 1:100. Samples were standardized against *Rp49* using 50 nmol l^{-1} final concentration for forward (5′-ATCGGTTACGGATCGAACAA-3′) and backward (5′-GACAATCTCCTTGCGCTTCT-3′) primers. Three wells per sample were plated on a 96 well-plate (Applied Biosystems) and a mean of these wells was taken as a sample size of one. Relative expression was calculated using Relative Expression Software Tool and Multiple Condition Solver REST-MCS (v. 2, http://bioinformatics.gene-quantification.info). Sample size was six independent groups of larvae per strain.

Statistical analysis

JMP/IN 5.1 was used for all statistical analyses (SAS Institute, Cary, NC, USA). Means ± s.e.m. are presented on all graphs. One-way and two-way analyses of variance (ANOVA) were performed when sample sizes were larger than six per condition per strain and when tests for unequal variances showed non-significance. Equality of variance was analysed using two tests, i.e. Levene's test and Bartlett's test. Pairwise parametric comparisons were performed with Student Neuman–Keuls (SNK) *post hoc* tests. Non-parametric Kruskal–Wallis tests were followed with non-parametric Wilcoxon two-group tests. $P<0.05$ was considered significant. All experiments were replicated at least once.

RESULTS

Rovers absorb more total [^{14}C]-6-glucose compared with sitters and the sitter mutants; this was true for each macronutrient (protein, $F_{2,15}$=15.09, P=0.0003; lipid, $F_{2,15}$=196.79, P<0.0001; carbohydrate, $F_{2,15}$=18.18, P<0.0001) (Fig. 1A). Compared with sitters, rover larvae store a larger proportion of ingested ^{14}C in total lipid stores and a smaller proportion in total carbohydrate stores. There were no significant differences in the proportion of ingested ^{14}C in total protein stores (lipids, Kruskal–Wallis $\chi^2_{2,18}$=9.93, P=0.007, and Wilcoxon for^R *vs* for^s P=0.03, for^R *vs* for^{s2} P=0.004, for^s *vs* for^{s2} P=0.3; carbohydrates, $\chi^2_{2,18}$= 12.54, P=0.002, for^R *vs* for^s P=0.004, for^R *vs* for^{s2} P=0.004, for^s *vs* for^{s2} P=0.1; proteins, $\chi^2_{2,18}$=0.25, P=0.9).

Well-fed, mid-third-instar rover (for^R) and sitter (for^s and for^{s2}) larvae differ in their total body carbohydrates but not in their lipid or protein stores (Fig. 1B) (carbohydrate, $F_{2,57}$=18.11, P=0.0001; SNK, for^R *vs* for^s P=0.02, for^R *vs* for^{s2} P=0.0003, for^s *vs* for^{s2} P=0.1; lipid, $F_{2,57}$=0.27, P=0.8; protein, $F_{2,57}$=0.33, P=0.7). Rover larvae have significantly lower carbohydrate levels in fat body than sitters (Fig. 1C) ($F_{2,21}$=4.34, P=0.03; SNK, for^R *vs* for^s P=0.03, for^R *vs* for^{s2} P=0.01, for^s *vs* for^{s2} P=0.8). No differences in carbohydrate levels are found in larval hemolymph of the variants (Fig. 1D) ($F_{2,57}$=0.80, P=0.5).

Acute food deprivation does not affect total protein levels (Fig. 2A) (strain, $F_{2,104}$=0.92, P=0.40; food deprivation, $F_{3,104}$=0.48, P=0.70; strain by food deprivation interaction, $F_{6,104}$=1.16, P=0.33) or total carbohydrate levels (Fig. 2C) (strain, $F_{2,105}$=11.51, P<0.0001; food deprivation, $F_{3,105}$=0.86, P=0.47; strain by food deprivation, $F_{6,105}$=1.16, P=0.33) in rovers or sitters. Lipid levels are decreased in rovers but not in sitters after three hours of food deprivation (Fig. 2B) ($F_{2,26}$=4.12, P=0.03, for^R *vs* for^s P=0.03, for^R *vs* for^{s2} P=0.01, for^s *vs* for^{s2} P=0.6).

Acute food deprivation decreases hemolymph carbohydrate levels in rover but not in sitter larvae (Fig. 2D) (strain, $F_{2,107}$=0.70, P=0.50; food deprivation, $F_{3,107}$=25.78, P<0.0001; strain by food deprivation, $F_{6,107}$=9.67, P<0.0001). By contrast, food deprivation does not affect carbohydrate levels in the fat body of either variant (Fig. 2E) (strain, $F_{2,84}$=5.84, P=0.004; food deprivation, $F_{3,84}$=0.09, P=0.97; strain by food deprivation, $F_{6,84}$=0.20, P=0.97).

After one hour of food deprivation, sitter larvae increase their food intake more than rover (Fig. 3A) (strain, $F_{2,348}$=5.74, P=0.004; food deprivation, $F_{3,348}$=57.36, P<0.0001; strain by food deprivation, $F_{6,348}$=5.14, P<0.0001). There are no differences in food intake between the strains after two or three hours of food deprivation (Fig. 3A) (2 h, $F_{2,87}$=0.44, P=0.65; 3 h, $F_{2,87}$=0.60, P=0.55).

We examined recovery from acute food deprivation by re-feeding larvae following a period of acute food deprivation. Upon re-feeding, sitter food intake returns to initial well-fed levels sooner than in rover (Fig. 3B) (strain, $F_{2,339}$=4.01, P=0.02; time spent re-feeding, $F_{3,339}$=22.23, P<0.0001; strain by time spent re-feeding, $F_{6,339}$=2.57, P=0.02). For example, after three hours of food deprivation and 30 min of re-feeding, food intake levels of sitters return to initial well-fed levels (for^s, $F_{1,58}$=2.91, P=0.09; for^{s2}, $F_{1,56}$=2.86, P=0.10); however, after two hours of re-feeding, rover food intake levels are still higher than rover well-fed food intake levels ($F_{1,58}$=14.89, P<0.0003). Similar recovery profiles are found after one and two hours of food deprivation (data not shown). Hemolymph carbohydrate levels in rover larvae return to well-fed levels more slowly than in sitters (Fig. 3C) (strain, $F_{2,80}$=11.90, P<0.0001; time spent re-feeding, $F_{2,80}$=7.39, P=0.001; strain by time spent re-feeding, $F_{4,80}$=2.89, P=0.03).

Relative abundance of *akh* RNA using qRT-PCR shows that *akh* RNA is more abundant in well-fed sitters compared with rovers

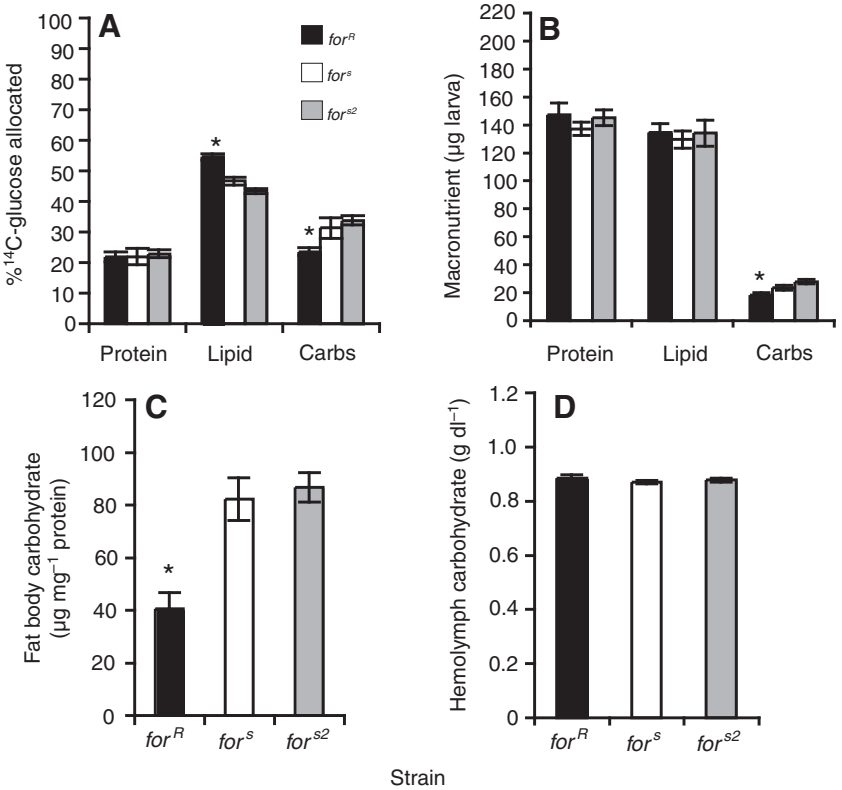

Fig. 1 Natural variation in nutrient storage due to *for*. (A) Rover (for^R) larvae store a larger proportion of ingested ^{14}C in their lipid stores whereas sitter larvae (for^s and for^{s2}) store a larger proportion in their carbohydrate stores. (B) Well-fed mid-third-instar for^R, for^s and for^{s2} larvae differ in total body carbohydrate but not lipid or protein stores. (C) Well-fed for^R larvae have significantly lower carbohydrate levels in fat body but not in hemolymph (D) compared with for^s and for^{s2} larvae. Error bars indicate means ± s.e.m.

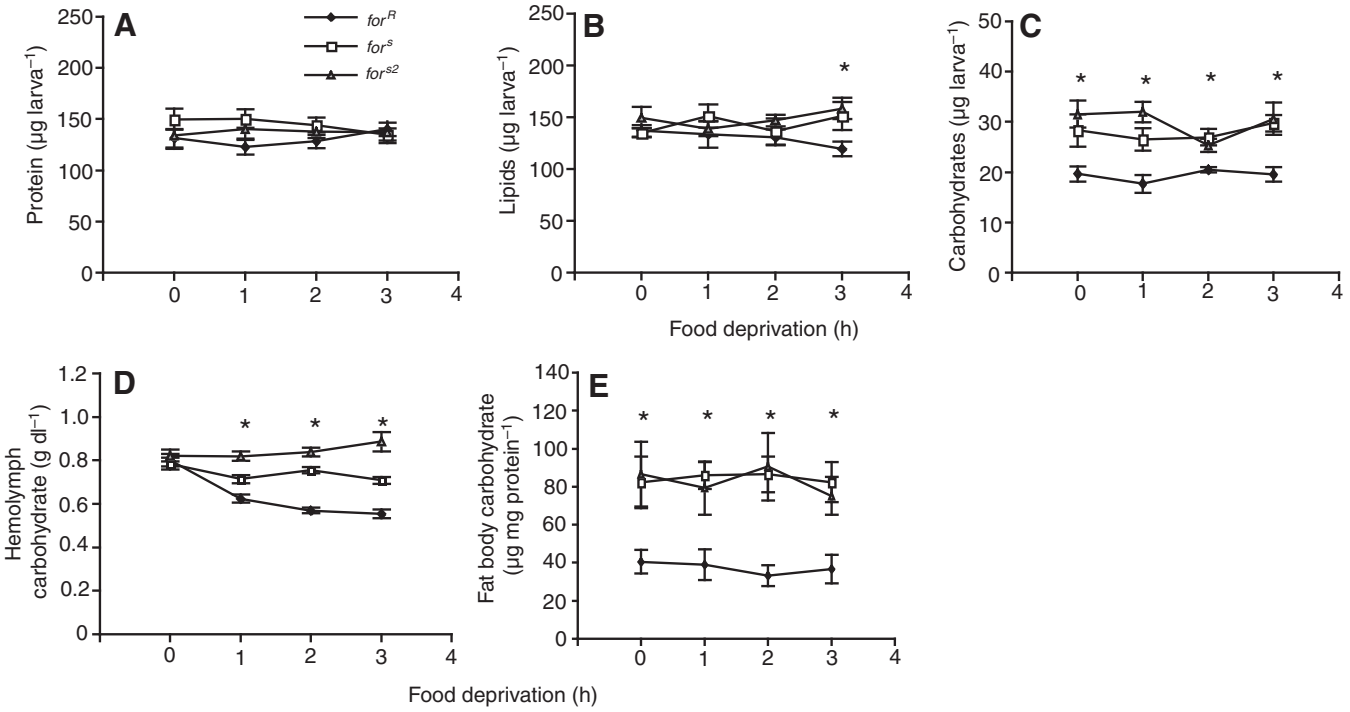

Fig. 2 Natural variation in nutrient store expenditure following acute food deprivation. (A) Acute food deprivation does not affect total protein levels in rovers (*for*^{R}) or sitters (*for*^{s} and *for*^{s2}). (B) Three hours of acute food deprivation significantly decreases total body lipid levels in rover but not in sitter larvae. (C) Acute food deprivation does not affect whole body carbohydrate levels despite rover larvae showing significantly lower total body carbohydrate levels compared with sitter larvae. (D) Acute food deprivation decreases hemolymph carbohydrate levels in *for*^{R} larvae but not *for*^{s} or *for*^{s2} larvae. (E) Acute food deprivation does not affect fat body carbohydrate levels; *for*^{R} larvae have significantly less carbohydrate in their fat bodies than *for*^{s} or *for*^{s2} larvae. Error bars indicate means ± s.e.m.

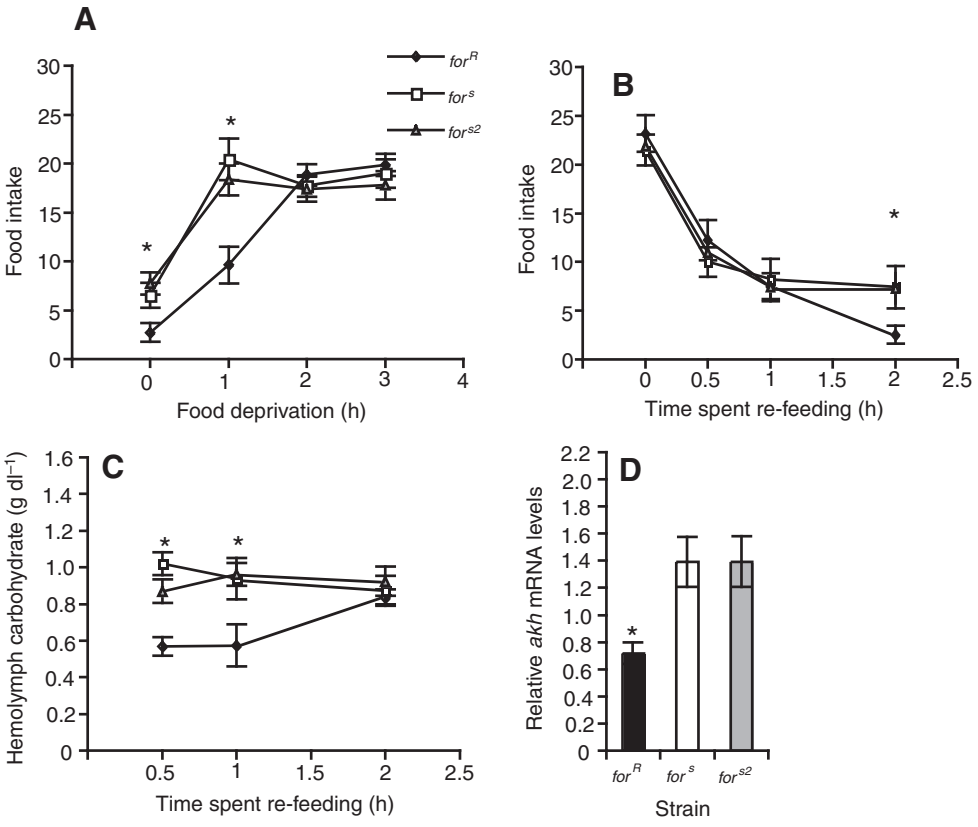

Fig. 3 *for* affects recovery from acute food deprivation. (A) After one hour of food deprivation, sitter (*for*^{s} and *for*^{s2}) larvae increase their food intake to a greater extent than rover (*for*^{R}) larvae. After two and three hours of food deprivation, there were no significant differences in food intake between rovers and sitters. (B) Re-feeding experiments show that after three hours of acute food deprivation, followed by 0, 30 min, 1 h or 2 h of re-feeding, food intake levels in *for*^{R} larvae return to their initial well-fed food intake levels more slowly than in *for*^{s} and *for*^{s2} larvae. (C) Hemolymph carbohydrate levels in *for*^{R} larvae returned to well-fed levels after 2 h of re-feeding, whereas hemolymph carbohydrate levels in *for*^{s} and *for*^{s2} were not changed by acute food deprivation (see Fig. 2D) or re-feeding. (D) Well-fed *for*^{R} larvae have significantly lower *akh* mRNA levels than *for*^{s} and *for*^{s2}. Relative *akh* mRNA levels were measured using quantitative real-time PCR and standardized against *for*^{R} larvae. Error bars indicate means ± s.e.m.

(Fig. 3D) ($F_{2,17}$=6.17, P=0.01; SNK, for^R vs for^s P=0.007, for^R *vs* for^{s2} P=0.007, for^s *vs* for^{s2} P=1.0).

DISCUSSION

The ability to balance the acquisition, storage and use of energy is essential for all organisms. We previously showed that well-fed rovers ingest less food but absorb more glucose than well-fed sitters (Kaun et al., 2007). In the present study, we show that *for* affects the allocation and storage of food in well-fed animals and hemolymph carbohydrate reserves in food-deprived animals. Together, these data suggests that *for* plays an integral role in energy homeostasis in *Drosophila.*

Our measures of energy allocation and storage in fed animals suggest that rovers and sitters exhibit differences in their metabolic strategies. Rovers tend towards fat metabolism and sitters towards carbohydrate metabolism. Sitters allocated more of their ingested ^{14}C-glucose to carbohydrate reserves and had higher total fat body carbohydrate stores. We did not find higher total lipid stores in rovers than in sitters. Further studies of lipid storage and metabolism in the larval fat body are needed to understand *for*'s affect on lipid levels. These differences in nutrient metabolism may be linked to the foraging behavior of rover and sitter larvae. Rovers move more on and between food substrates compared with sitters and, thus, may need stored energy resources that can sustain longer bouts of locomotion. In other organisms, such as locusts, sustained flight results in mainly lipid metabolism (Candy et al., 1997). There is some evidence suggesting a role for PKG in lipid metabolism (Lafontan et al., 2005; Langdin, 2006). Atrial natriuetic peptides (ANP) increase intracellular cGMP, which, in turn, activates PKG leading to lipase phosphorylation and lipolysis (Lafontan et al., 2005).

Homeostatic regulation of blood sugar levels is a fundamental physiological process in both invertebrates and vertebrates. Disruptions in glucose homeostasis are associated with health problems such as diabetes (Rosen and Speigelman, 2006). In humans, diabetes-related disorders are associated with low PKG levels. Our results with *D. melanogaster* suggest a conserved role for PKG in glucose homeostasis.

Our results also hint at possible mechanisms through which PKG may mediate glucose homeostasis. In insects such as locusts, AKHs mobilize carbohydrates from the fat bodies *via* binding to a G_q-dependent receptor thus stimulating a phospholipase C (PLC). The resulting inositol triphosphate (IP_3) releases Ca^{2+} from internal stores (Gade and Auerswald, 2003). This cascade activates glycogen phosphorylase leading to release of stored carbohydrates into the hemolymph.

How might PKG affect *akh*? It could act directly or indirectly on *akh* by changing its transcription (Pilz and Broderick, 2005). Alternatively, PKG could indirectly affect *akh* by acting on molecules that disrupt glucose homeostasis, such as insulin or neuropeptide F (*npf*). In *Drosophila*, the insulin signaling pathway is integral for proper storage of carbohydrates and lipids, especially during growth and development (Mirth and Riddiford, 2007). *npf* signaling has been linked to insulin signaling and starvation-induced differences in behavior (Wu et al., 2005a; Wu et al., 2005b). PKG may be linked to *npf*-dependent food acquisition (Kaun, 2007). Thus, disruption of insulin signaling could potentially disrupt glucose homeostasis and indirectly affect *akh*.

PKG is also known to directly phosphorylate PLC-β, leading to inhibition of PLC-β3 activity (Xia et al., 2001). Inhibition of PLC could decrease glycogen phosphorylase activity, potentially slowing the release of stored carbohydrates into the hemolymph. This is consistent with rovers showing decreased hemolymph sugar levels after food deprivation compared with sitters. It would be interesting to determine if PKG and AKH proteins interact and co-localize in *Drosophila* tissue.

The difference in both foraging behavior and response to food deprivation between rover and sitter *Drosophila* bears an intriguing resemblance to the polyphenism seen in *Locusta migratoria migratoriodes* gregarious and solitary locusts, which also differ in adipokinetic strategies (Pener et al., 1997). Parallels can be drawn between rovers and gregarious locusts, and sitters and solitary locusts. Both rovers and gregarious locusts move greater distances to forage. Gregarious locusts and potentially rovers preferentially metabolize lipids over carbohydrates (Pener et al., 1997). Solitary locusts have higher resting hemolymph sugar levels similar to sitters after a short period of food deprivation (Pener et al., 1997). Solitary locusts also have higher AKH levels similar to the higher *akh* mRNA levels found in sitters (Pener et al., 1997). Whether *for* plays a role in foraging behavior or adipokinetic balance in solitary and gregarious locusts remains to be determined.

for has been implicated in food-related behaviors in a variety of organisms, including *Caenorhabditis elegans* (Fujuwara et al., 2002; You et al., 2008), honey bees (Ben-Shahar et al., 2002), ants (Ingram et al., 2005) and *Drosophila* (Osborne et al., 1997). Whether and how PKG plays a role in energy balance in these organisms remains to be investigated.

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