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Natural variation in food acquisition mediated *via* a *Drosophila* cGMP-dependent protein kinase

Karla R. Kaun[1], Craig A. L. Riedl[1], Munmun Chakaborty-Chatterjee[1], Amsale T. Belay[1], Scott J. Douglas[1], Allen G. Gibbs[2] and Marla B. Sokolowski[1,3]

[1]*Department of Biology, University of Toronto, 3359 Mississauga Road, Mississauga, Ontario, L5L 1C6, Canada and* [2]*School of Life Sciences, University of Nevada, 4505 Maryland Parkway, Las Vegas, NV 89154-4004, USA*

*Author for correspondence (e-mail: marla.sokolowski@utoronto.ca)

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Summary

In natural environments where food abundance and quality can change drastically over time, animals must continuously alter their food acquisition strategies. Although genetic variation contributes to this plasticity, the specific genes involved and their interactions with the environment are poorly understood. Here we report that natural variation in the *Drosophila* gene, *foraging* (*for*), which encodes a cGMP-dependent protein kinase (PKG), affects larval food acquisition in an environmentally dependent fashion. When food is plentiful, the wild-type rover (for^R) allele confers lower food intake and higher glucose absorption than both the wild-type sitter (for^s) allele and the mutant for^{s2} allele. When food is scarce, for^R, for^s and for^{s2} larvae increase food intake to a common maximal level, but for^R larvae retain their increased absorption efficiency. Changes in *for* expression can induce corrective behavioral modifications in response to food deprivation. When reared in environments with low food levels, for^R larvae have higher survivorship and faster development than for^s and for^{s2} larvae. Together, these results show that natural variation in *for* has far reaching implications affecting a suite of phenotypes involved in the regulation of food acquisition.

Key words: *Drosophila*, feeding, foraging, cGMP-dependent, protein kinase.

Introduction

Animals must feed to survive, but foraging often involves a delicate balance of benefits and risks. The benefits of nutrient acquisition are weighted against elevated exposure to threats from predators, parasites, exhaustion or toxins, for example. Many risk-mitigation adaptations, which help to increase the foraging benefit-to-risk ratio, have evolved among animals. These include behavioral strategies, such as foraging at times when predators are less active or abundant, or when food is less well defended. Other adaptations include altered metabolic or physiological traits. Some animals, for example, may gorge themselves to rapidly increase fat supplies when food is plentiful and then deplete these reserves in times of famine. Alternatively, other animals, such as reptiles, adjust their metabolism or gut absorption levels to maintain body mass in response to food scarcity (Stubbs and Tolkamp, 2006; Wang et al., 2006).

Within a species, individuals may exhibit polymorphic and/or plastic responses to fluctuating food availability. Naturally varying genes involved in plastic responses to changes in food availability are difficult to identify, often due to the subtlety of their influence. Nevertheless, within-species genetic polymorphisms affecting foraging behaviors have been identified and characterized in the fruit fly, *Drosophila melanogaster* (Osborne et al., 1997), and the nematode, *Caenorhabditis elegans* (de Bono and Bargmann, 1998). Here we establish a novel role for the *D. melanogaster* gene, *foraging* (*for*), which influences foraging locomotory behaviors, in the mediation of food acquisition strategies and plastic responses to food availability.

The fly gene, *for*, encodes a cGMP-dependent protein kinase (PKG), and natural allelic variation in *for* affects rover- or sitter-type foraging locomotion (de Belle et al., 1989). Larvae with the wild-type rover (for^R) allele travel further when feeding and move more between food patches than those with only sitter (for^s) alleles (Osborne et al., 1997; de Belle et al., 1989; Sokolowski et al., 1983; Sokolowski, 1980). Well-fed rovers have higher PKG enzyme activities compared to sitters (Osborne et al., 1997; de Belle et al., 1989), and transgenic expression of *for* in sitters is sufficient to induce rover-like elevations of PKG activity and foraging locomotion (Osborne et al., 1997).

We speculated that the increased foraging locomotion of rovers may be energetically costly (Berrigan and Lighton, 1993), and thus, we hypothesized that the rover variants would require more energy and thereby differ in food acquisition compared to the sitter variants. Food acquisition was assessed by measuring rates of both food intake and nutrient absorption. Intriguingly, rover allelic variants have lower food intake than sitters despite having similar sizes and metabolic and

developmental rates. This apparent paradox is clarified by our further observation that rovers may counterbalance their reduced intake with increased rates of nutrient absorption.

Despite different foraging styles, rovers and sitters occur at stable frequencies in natural populations (70% rovers: 30% sitters) (Sokolowski et al., 1997). However, selection experiments have demonstrated a selective advantage of the rover allele (for^R) compared to the sitter allele (for^s) when rover and sitter larvae are reared together for many generations in crowded conditions (Sokolowski et al., 1997). We postulated that this rover advantage may stem in part from an increased ability to tolerate or adjust to the more depleted medium found in crowded culture conditions. Thus, we hypothesized (1) that rovers will have enhanced survival compared to sitters in nutrient-restricted conditions, and (2) that this advantage may arise from differences in nutrient uptake. Indeed, our results indicate that, when reared in environments with relatively low nutrient levels, rover larvae have higher survivorship and faster development than sitter larvae. Moreover, changes in *for* expression can induce corrective behavioral modifications in response to food. That is, when food is scarce both rover and sitter larvae express similarly reduced PKG activity and food intake is elevated to a common maximal level. Rover larvae, however, retain their relatively elevated nutrient absorption efficiency, perhaps contributing to their survival advantage under such conditions.

The mechanisms by which changes in gene function and expression are naturally selected to confer adaptive behavioral responses to varying environments is a central question in behavioral genetics. This work uses the *D. melanogaster* rover/sitter polymorphism to investigate this question by addressing the role of *for* in mediating plasticity in food acquisition strategies.

Materials and methods

Strains

The rover and sitter strains used in these experiments are homozygous for the for^R or for^s alleles on chromosome-2 and share co-isogenic third chromosomes from the for^R strain (de Belle and Sokolowski, 1987). The X-chromosomes shared by these strains were mostly from the for^R background. for^{s2} is a sitter mutant generated on the rover genetic background and does not differ from for^R aside from the induced mutation within the *for* gene (Osborne et al., 1997; de Belle et al., 1989). In order to increase the expression of *for*, a DNA fragment encoding the complete *for*T2 amino acid sequence (Kalderon and Rubin, 1989) was subcloned into the transformation vector, pUAST, and transformed into w^1 embryos using standard techniques (Spradling and Rubin, 1982). The sitter second chromosome (containing the *for* locus) was substituted into the transgenic strain, creating $w^1;for^s;UASforT2$. These were then crossed to $w^1;for^s;hsGAL4$, resulting in an increase in expression of *forT2* in an otherwise for^s genetic background. We relied on leaky expression of the *hs* promoter (e.g. Bainton et al., 2005); experiments were done at 23°C and no heat shock was applied.

Flies (*Drosophila melanogaster*) were maintained in 170 ml plastic culture bottles with 40 ml of standard culture medium at 25±1°C and a 12 h:12 h L:D photocycle. Standard culture medium contained 50 g Baker's yeast, 100 g sucrose, 16 g agar, 0.1 g KPO_4, 8 g sodium potassium tartrate, 0.5 g NaCl, 0.5 g $MgCl_2$ and 0.5 g $Fe_2(SO_4)_3$ per litre of tapwater. Larvae were reared from egg-hatch to mid-third instar (96±2 h post-hatch) at densities of 100 larvae per 35 ml of medium in 100 mm×15 mm Petri dishes.

Food intake

Method 1: image analysis

Whole body measurement. Larvae were removed from food plates, washed in distilled water and groups of 10 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, Mississauga, ON, Canada). 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 1.28, 2002 and ImageJ V 1.32j, 2004) for the digital quantification. Thirty larvae/strain/condition combinations were assayed. Initially food intake was measured after feeding larvae for 10, 15 and 20 min. Further experiments were done using the 15 min feeding period, which was representative of strain differences. All larvae were staged to mid-third instar prior to testing food intake (Demerec, 1994). When larvae were reared in high-quality food conditions this was 96±2 h post-hatch. When larvae were reared in 25% food this was 144–168 h post-hatch, and when larvae were reared in 15% food this was 168–192 h post-hatch, depending on humidity in the rearing incubator.

Fructose–agarose and glucose–agarose intake. Fructose–agarose and glucose–agarose food intake experiments were performed as above with the exception of food substrate. For fructose–agarose food intake experiments, 100 mm×15 mm Petri plates containing 1% agarose, 2 mol l^{-1} fructose and 0.5% Carmine dye (Sigma) that were prepared 4 h prior to the test. For glucose–agarose food intake experiments, 45 mm×10 mm plates were made 24 h prior to testing with 2.3% agarose, 10% glucose and 0.5% Carmine dye. Small cuts were made in glucose–agarose plates to aid in feeding on a solid substrate. Larvae were left on sugar–agarose food substrate for 15 min before food intake was measured.

Gut measurement. Larvae were reared as above and fed dyed yeast paste for 15 min. Guts were dissected in phosphate-buffered saline (PBS) and gently elongated on a Sylgard™ base for imaging. Gut food content was quantified by calculating the amount of dye-colored pixels as a percentage of the total number of pixels in the gut. Twenty larval guts/strain/condition combinations were measured.

Method 2: spectrophotometric analysis

Methods for spectrophotometric analysis of food intake were modified from Edgecomb et al. (Edgecomb et al., 1994). Briefly, groups of 50 larvae were reared, as above, and fed yeast

paste with 0.16% Erioglaucine dye (aka FD&C Blue No. 1, Sigma) for 15 min. After feeding, each group of larvae was washed 3× in distilled water, placed in 1.5 ml tubes and immediately frozen in liquid nitrogen. Larvae were then homogenized in 250 μl distilled water, centrifuged at 13 ***g*** for 10 min, and 225 μl of supernatant was transferred to a new 1.5 ml tube containing 50 μl 100% ethanol. Tubes were vortexed for 30 s and re-centrifuged for 10 min. 250 μl of supernatant was placed in a new tube, which was centrifuged at 13 ***g*** for 5 min. Then, 200 μl supernatant was placed in a 96-well crystal plate and the OD^{633} read (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA). Nine groups of 50 larvae per strain were analyzed.

Mouthhook contractions

Mouthhook contractions were measured for 15 min per larva on a glucose–agarose substrate described above. Contractions were measured when larvae were scraping the glucose–agarose substrate in and/or along a crack in the substrate. Individual larvae were immediately boiled following mouthook measurements and food intake was measured.

Body size and mass measurements

Length and width measurements were obtained from the digital images using ImageJ software. To establish dry mass, groups of ten larvae were placed in 1.5 ml tubes and dried for 48 h in a desiccator, after which masses were determined for 30 groups. All larvae were staged to mid-third instar prior to measurement.

Food quality manipulation

Larvae were reared in food qualities defined as 100%, 75%, 50%, 25% or 15% until mid-third instar (Demerec, 1994) for food intake tests and size measurement. Food quality was manipulated by decreasing the proportion of both yeast and sucrose in the standard culture medium (see recipe above) while keeping all other constituents constant.

Survivorship and developmental time

Twenty newly hatched larvae were placed in a 9.5 mm×25 mm diameter glass vial with 10 ml of food medium (100% 75%, 50%, 25% or 15% quality). Animals were grown at 25°C on a 12 h:12 h L:D cycle with lights on at 08:00 h. Eclosed adult flies in each vial were counted and removed once every 24 h for 26 days.

Gut contractions

Third-instar larvae were removed from food plates, washed gently and stuck to a clear microscope slide using double-sided stick tape, ventral side facing up. The number of gut contractions in the anterior midgut, acidic region, and posterior midgut were measured for 2 min. This number was divided by two to yield the number of gut contractions per minute.

The anterior midgut was defined as the region of the gut immediately following the proventriculus. The acidic midgut was defined as the narrow region of midgut following the wide foregut region and is characterized by a low pH marked by a color change from blue to yellow after ingestion of Bromophenol Blue. The posterior midgut was defined as the muscular area of the midgut immediately following the acidic portion and preceding the hindgut.

Excretion rate

Third-instar larvae were removed from food plates, washed gently, then placed on a 100 mm×15 mm Petri dish with 5 ml yeast paste with 0.08% Fast Green FCF dye for 30 min. Larvae that had food in their gut were selected. Larvae were then placed on an agarose substrate for 3 h. The number of excretion spots were counted every 15 min and then added for a total number after 3 h. Total concentration of excretion was measured by soaking feces–agarose in 1 ml distilled water for 12 h then measuring concentration of dye at 625 nm in a spectrophotometer.

PKG assays

PKG enzyme assays were performed on 96 h post-hatch whole larval homogenates. Ten whole larvae were homogenized in 25 mmol l^{-1} Tris (pH 7.4), 1 mmol l^{-1} EDTA, 2 mmol l^{-1} EGTA, 5 mmol l^{-1} β-mercaptoethanol, 0.05% Triton X-100 and protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada) and microcentrifuged for 5 min. The supernatant was removed and total protein levels were quantified. Supernatants containing equal amounts of total protein were analysed for cGMP-dependent protein kinase (PKG) activity. The reaction mixture contained (at final concentration): 40 mmol l^{-1} Tris-HCl (pH 7.4), 20 mmol l^{-1} magnesium acetate, 0.2 mmol l^{-1} [g^{32}P]ATP (500–1000 c.p.m. $pmol^{-1}$) (Amersham Pharmacia Biotech, Baie D'Urfe, QC, Canada), 113 mg ml^{-1} heptapeptide (RKRSRAE), 3 mmol l^{-1} cGMP (Promega, Burlington, ON, Canada) and a highly specific inhibitor of cAMP-dependent protein kinase (5-24, Calbiochem, San Diego, CA, USA). The reaction mixtures were incubated at 30°C for 10 min, followed by termination of the reaction by spotting 70 μl of the reaction mix onto Whatman P-81 filters, which were then soaked with 75 mmol l^{-1} H_3PO_4 for 5 min and washed three times with 75 mmol l^{-1} H_3PO_4 to remove any unreacted [γ^{32}P]ATP. Filters were rinsed with 100% ethanol and air dried before quantification. For quantification of PKG activity, counts were taken in a Wallac 1409 Liquid Scintillation Counter (Perkin Elmer, Woodbridge, ON, Canada) using universal scintillation cocktail (ICN). Specific activity of PKG was expressed as pmol of ^{32}P incorporated into the substrate min^{-1} mg^{-1} protein.

Larval respiration rate

Respiration rates were measured by indirect calorimetry (Gibbs et al., 2003). Groups of ten mid-third-instar larvae were placed in 5 ml glass–aluminum respirometry chambers containing a strip of medium, approximately 1 cm×2 cm× 0.2 cm. Larvae exhibited apparently normal behavior, eating and crawling in and around the food. The chambers were placed in a Sable Systems (Las Vegas, Nevada, USA) TR-2 respirometer, and CO_2-free air was pumped through the chamber at 100 ml min^{-1}. Placement of the larvae in the respirometer was staggered, so that they had been in place approximately 60 min before data were collected. Rates of CO_2 release were measured over a 15 min period with a Li-Cor LI-6262 infrared CO_2 sensor. Data acquisition and analysis were done using Datacan V software (Sable Systems, Las Vegas, NV,

USA). Control readings from chambers containing medium alone indicated no CO_2 release from microbial contaminants.

Glucose and leucine absorption

Larvae were removed from food plates, washed in distilled water and placed in groups of 70 in 9 cm×1 mm circular wells. Wells were filled with dead yeast paste (2:1 water:yeast, autoclaved 20 min) mixed with 0.08% Brilliant Blue R dye (Sigma) and 2 μCi ml^{-1} ^{14}C-6-glucose (specific activity 58 μCi mmol l^{-1}, Amersham Biosciences) or 2 μCi ml^{-1} L-[U-^{14}C]leucine (specific activity 318 μCi mmol l^{-1}, Amersham Biosciences). After 15 min of feeding, larvae were removed and washed gently with a constant stream of 50 ml distilled water. Groups of ten larvae were then placed into 1.5 ml tubes, frozen in liquid nitrogen and stored at –80°C. For absorption experiments [protocol modified from Riha and Luckinbill (Riha and Luckinbill, 1996)] larvae were purged of the radio-labeled yeast paste prior to collection by placing them on unlabeled, undyed, heat-killed yeast paste for 3 h, the time it took for larvae to have no visible dye remaining in their guts (data not shown). We found no differences in the rate of passage of food through the rover or sitter guts (data not shown). Larvae were then carefully removed from the yeast paste, washed, placed in groups of ten into 1.5 ml tubes, and frozen in liquid nitrogen, as above.

Twenty-four hours later, larvae were removed from –80°C, placed in scintillation vials in groups of ten and solubilized at 70°C for 10 min in 200 μl Solvable (PerkinElmer Life Sciences, Woodbridge, ON, Canada) with 100 μl perchloric acid (Sigma). Next, 100 μl H_2O_2 was added and samples were vortexed for 30 s. We then added 10 ml scintillation fluid, and samples were vortexed again for 30 s and shaken for 2 h, then left at room temperature for 24 h. Samples were then vortexed again, left for 2 h at room temperature, and the amount of ^{14}C in each vial calculated using counts observed over 60 s per sample in a scintillation counter (Wallac 1409 Liquid Scintillation Counter). Sample sizes were ten larvae/vial with six vials/strain.

Prior to calculating the specific activity (fmol) of intake and absorption of ^{14}C-6-glucose or L-[U-^{14}C]leucine per larva, a conversion factor taking into account the specific activity of the radio-labeled substance was calculated. First the amount of specific activity (fmol) per 1 μl of radio-labeled substance was calculated by dividing the concentration supplied in the vial (mCi ml^{-1}) by the specific activity of the batch (mCi mmol l^{-1}). Next, the number of counts per minute (c.p.m.) in 1 μl of radioactive substance was divided by the number of fmol per 1 μl of radio-labeled substance to give the specific activity conversion factor. Each experimental c.p.m./larva was then divided by this conversion factor to reveal the specific activity of the sample (fmol).

Western blot

Western blots were carried out as described (Belay et al., 2007) with the following changes. Protein was extracted from fly heads of 3–7 day-old flies reared at 23°C. 20 μg of protein extract was electrophoretically separated on SDS–10% polyacrylamide gels. To control for equal protein loading and transfer, blots were probed with a 1:5000 diluted monoclonal anti-β-actin antibody (Sigma).

Statistical analysis

The *y*-axis in all figures represents mean ± s.e.m. unless

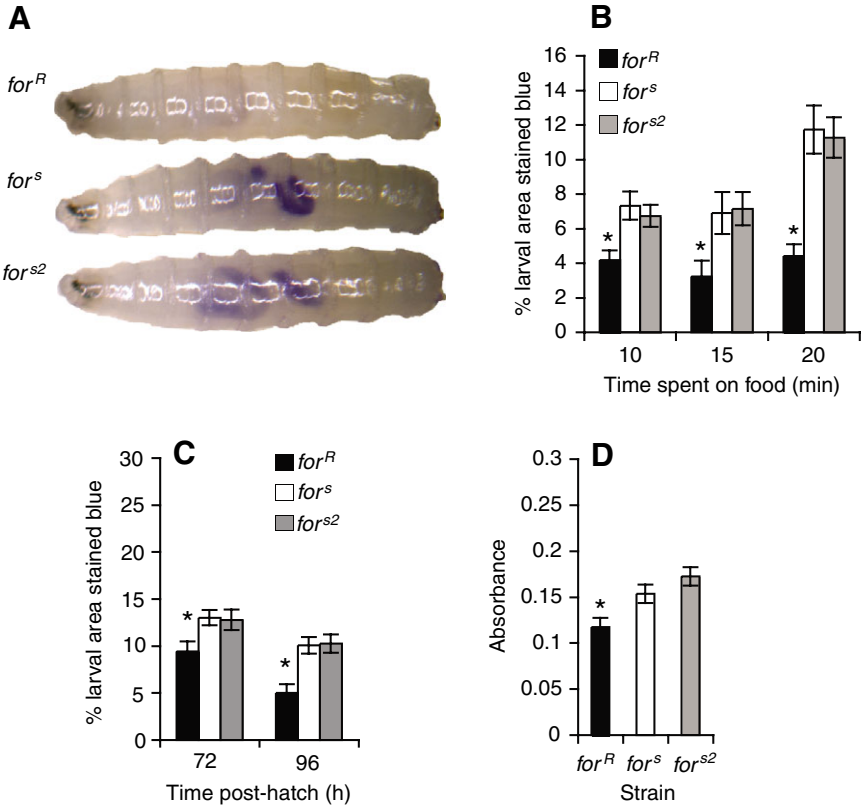

Fig. 1. *for* affects food intake. (A) Mid-third-instar rover (*for*^{R}), sitter (*for*^{s}) and sitter mutant (*for*^{s2}) larvae with ingested dye visible through the ventral cuticle. (B) Well-fed rovers (*for*^{R}) had significantly lower food intake than well-fed sitters after 10 min (ANOVA, $F_{(2,84)}$=12.03, P<0.0001), 15 min ($F_{(2,85)}$=11.46, P<0.0001) and 20 min ($F_{(2,85)}$=7.09, P<0.001) on dyed yeast paste. A two-way ANOVA on strain (*for*^{R}, *for*^{s}, *for*^{s2}) and time (10, 15, 20 min) showed a significant effect of strain ($F_{(2,254)}$=12.12, P<0.001), no effect of time ($F_{(2,254)}$=0.13, P=0.9) and no strain-by-time interaction ($F_{(2,254)}$=0.23, P=0.9). (C) Rovers had significantly less blue area than sitters at early third ($F_{(2,89)}$=4.24, P=0.02) and mid-third-instar ($F_{(2,87)}$=10.52, P<0.0001) stages. (D) Spectrophotometric quantification of homogenates from larvae fed a Erioglaucine (FD and C Blue No. 1) dyed yeast paste for 15 min showed that *for*^{R} larvae ingest significantly less dye than *for*^{s} or *for*^{s2} larvae ($F_{(2,26)}$=7.88, P<0.01).

otherwise stated. SAS or JMP/IN 5.1 was used for all statistical analyses (SAS Institute Inc., Cary, NC, USA). Two-way and one-way analyses of variances (ANOVA) were performed followed by Student–Neuman–Keuls pairwise *post-hoc* comparisons using $P<0.05$ as significant. Non-parametric Kruskal–Wallis tests were performed followed by Wilcoxon two-group comparisons using $P<0.05$ as significant when sample size was smaller than six.

Results and Discussion

for affects food acquisition

We first asked whether variation in *for* elicits differences in food intake and absorption in well-fed rovers and sitters. To determine if the effects we saw were specific to *for*, we also measured food intake and absorption in the sitter mutant (for^{s2}), which was generated on a rover genetic background (Pereira and Sokolowski, 1993).

To measure food intake, well-fed third instar larvae were placed in food, a yeast paste colored with blue dye, and the amount of dye ingested was quantified. The amount of dye in the gut seen through the cuticle of each larva relative to the larval body area was used to estimate food intake (Fig. 1A). We quantified food intake in the natural rover (for^R) and sitter (for^s) allelic variants as well as the sitter mutant (for^{s2}) to establish the connection between *for*-PKG and food intake. Well-fed rovers (for^R) had significantly lower food intake than well-fed sitters after 10, 15 and 20 min on dyed yeast paste (Fig. 1B,C).

These data were confirmed using several assays including spectrophotometric quantification of dye (Fig. 1D), and visual quantification of dye in dissected guts (Fig. 2A). Quantification of the rate at which the gut filled with food calculated between 5 and 30 min of feeding on a yeast paste substrate demonstrated that sitter (for^s and for^{s2}) larvae fill their gut at a higher rate than rover (for^R) larvae (Fig. 2B). After approximately 30 min of feeding, the midguts of all larvae were filled with blue yeast paste, thus limiting further quantification of food intake. for^R larvae also showed decreased food intake compared to for^s and for^{s2} larvae on glucose–agarose and fructose–agarose substrates, suggesting that the decreased food intake of for^R larvae is a general response to all food types (Fig. 2C).

Traditionally, mouthhook movements have been used to quantify food intake in larvae (Sewell et al., 1975; Wu et al., 2005). However, our observations of larval behavior suggested that larvae use their mouthhooks for both food ingestion and locomotion. We found no significant correlation between mouthhook movements and amount of dye ingested when larvae fed on a glucose–agarose substrate (Fig. 2D). In summary, our results clearly show that *for* affects the amount of food consumed in well-fed larvae, where variants of *for* associated with higher PKG activity confer lower food intake than variants of *for* associated with lower PKG activity.

A paradox in energy consumption and energy use

Rovers were originally distinguished from sitters by their increased foraging locomotion (Sokolowski, 1980). We confirmed that after 15 min exposure to food, rovers travel significantly farther on yeast than do sitters ($P<0.0001$)

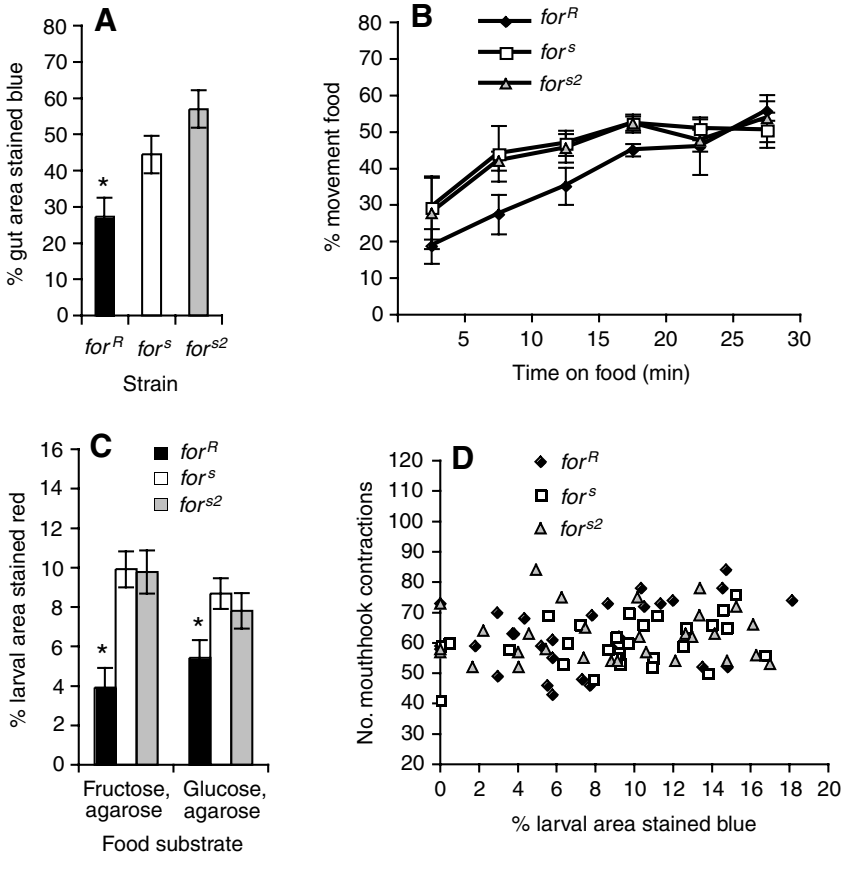

Fig. 2. *for* affects feeding rate and food acquisition. (A) Gut dissections from larvae fed blue yeast paste for 15 min also indicated that for^R larvae ingested significantly less food than for^s or for^{s2} larvae ($F_{(2,26)}=5.32$, $P<0.05$). (B) The rate that the gut filled with food was measured by feeding larvae on a yeast paste with Brilliant Blue dye, extracting the gut, and comparing the length of the gut with food to the length of the entire gut. Gut size did not differ significantly between strains ($F_{(2,67)}=0.86$, $P=0.43$). Sitter (for^s and for^{s2}) larvae filled their gut more quickly than rover (for^R) larvae (10–20 min feeding, two-way-ANOVA $F_{(8,36)}=70.53$ $P=0.0008$; effect of strain, $F_{(2,36)}=5.82$, $P=0.007$; effect of time, $F_{(2,36)}=5.52$, $P=0.008$; one-way ANOVAs: 5 min, $P=0.82$; 10 min, $P=0.10$; 15 min, $P=0.05$; 20 min, $P=0.04$). This difference stabilized after about 25–30 min of feeding when the midguts of all larvae become saturated with the blue yeast paste, resulting in no measurable difference in food intake (one-way ANOVAs: 25 min, $P=0.85$; 30 min, $P=0.68$). (C) Rovers showed significantly less food intake on fructose–agarose ($F_{(2,87)}=12.20$, $P<0.0001$; for^R *vs* for^s, $P<0.0001$; for^R vs for^{s2}, $P<0.0002$; for^s *vs* for^{s2}, $P=0.92$) and glucose–agarose ($F_{(2,87)}=3.98$, $P=0.02$; for^R *vs* for^s, $P=0.0078$; for^R *vs* for^{s2}, $P=0.048$; for^s *vs* for^{s2}, $P=0.47$) when food intake was measured using Carmine dye. (D) *Drosophila* larvae use their mouthhooks for both food ingestion and locomotion. We found no significant correlation between mouthhook movements and amount of dye ingested when larvae fed on a glucose–agarose substrate ($R^2=0.18$, $P=0.11$).

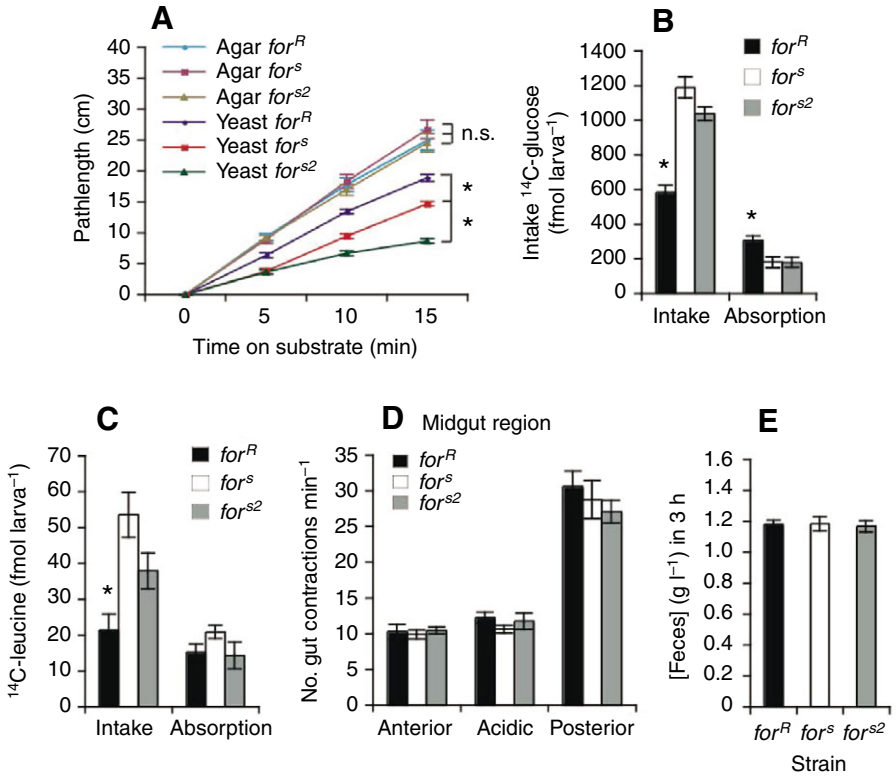

Fig. 3. *for* affects glucose absorption third-instar larvae. (A) No differences in path length between rover (for^R), sitter (for^s) and sitter mutant (for^{s2}) larvae were found during a 15 min test on a non-nutritive substrate (agar; $F_{(2,56)}$=0.34, P=0.71). However, as expected, rovers showed significantly longer foraging trails than sitter and sitter mutants during a 15 min test on a yeast substrate ($F_{(2,53)}$=35.77, P<0.0001). (B) After 15 min of feeding on a yeast–water paste containing ^{14}C-labeled glucose, rover larvae ingested significantly less ^{14}C than sitters ($F_{(2,17)}$=52.66, P<0.001). When subsequently exposed to unlabeled medium for 3 h, rovers retained significantly more ^{14}C label, indicating a higher level of glucose absorption compared to sitters ($F_{(2,17)}$=7.36, P<0.006). (C) After 15 min of feeding on a yeast–water paste containing ^{14}C-labeled L-U-leucine, well-fed rover larvae ingested significantly less ^{14}C than sitters ($F_{(2,15)}$=11.03, P=0.001; for^R *vs* for^s, P=0.0003; for^R *vs* for^{s2}, P=0.03; for^s *vs* for^{s2}, P=0.04). When subsequently exposed to unlabeled medium for 3 h, rovers did not differ significantly from sitters in amount of ^{14}C label ($F_{(2,15)}$=1.92, P=0.18). (D) Rovers (for^R) and sitters (for^s and for^{s2}) do not differ significantly in the number of contractions of the anterior midgut ($F_{(2,27)}$=0.17, P=0.85), acidic region ($F_{(2,27)}$=1.83, P=0.18) or posterior midgut ($F_{(2,27)}$=1.37, P=0.27). (E) No significant differences were found in the amount excreted by for^R, for^s or for^{s2} larvae ($F_{(2,57)}$=0.049, P=0.95). In addition, no significant interaction between the number of fecal spots and strain was found with a logistic regression analysis on excreted food concentration ($F_{(2,5)}$=2.40, P=0.100).

(Fig. 3A). This difference is expressed in the presence of a nutritive (yeast) but not a non-nutritive (agar) substrate (Fig. 3A). Thus, it was unclear how this increased locomotion could be sustained by lower food intake especially since, when food is plentiful, rover and sitter larvae develop at similar rates, reach similar sizes (length, width, mass) and have respiration rates that do not differ (Table 1, Fig. 4A).

Intriguingly, despite rovers showing increased locomotion on food, the similarity in metabolic rate between the natural variants suggests similar energy output. Notably, there was no significant behavioral difference observed in mouthhook extensions (Fig. 2D), which could be used for both locomotion and feeding. This suggests that active feeding in a smaller area by sitter larvae equalizes the total activity and therefore metabolic demand between the two natural variants. Thus for *D. melanogaster* larvae, reaching out and grabbing a mouthful of food seems to require as much energy as moving to a new location.

This was our first indication that even though rovers ingested less food than sitters, they do not suffer a fitness deficit relative to sitters. Thus, we hypothesized that rovers may absorb their food more efficiently than sitters.

for enhances glucose absorption

We hypothesized that rovers may offset their reduced food intake with enhanced glucose absorption. We tested this using ^{14}C-6-glucose-labeled food to compare ingestion and absorption (Riha and Luckinbill, 1996; Carvalho et al., 2005) (see Materials and methods). We measured the total amount of ^{14}C ingested and the total amount of ^{14}C absorbed.

As described above, for^R larvae have lower food intake than sitter larvae, as measured by total ^{14}C uptake levels with ^{14}C-6-

Table 1. *Mid-third instar rover (for^R), sitter (for^s) and sitter mutant (for^{s2}) D. melanogaster larval phenotypes when reared in a high food level (100%) medium*

Phenotype	for^R	for^s	for^{s2}
Body length (mm)	4.26±0.04 (30)	4.23±0.03 (30)	4.24±0.04 (30)
Body width (mm)	0.74±0.04 (30)	0.75±0.05 (30)	0.74±0.09 (30)
Dry mass (mg)	458±18 (30)	430±11 (30)	474±19 (28)
Respiration rate (μl CO_2 h^{-1})	7.1±0.7 (13)	8.4±0.6 (14)	6.9±0.6 (11)

Values are means ± s.e.m. (*N*).

ANOVAs show no significant strain differences in body length ($F_{(2,87)}$=0.62, P=0.5), width ($F_{(2,87)}$=1.15, P=0.3), dry mass ($F_{(2,85)}$=1.94, P=0.2) or respiration rates ($F_{(2,37)}$=1.89, P=0.2).

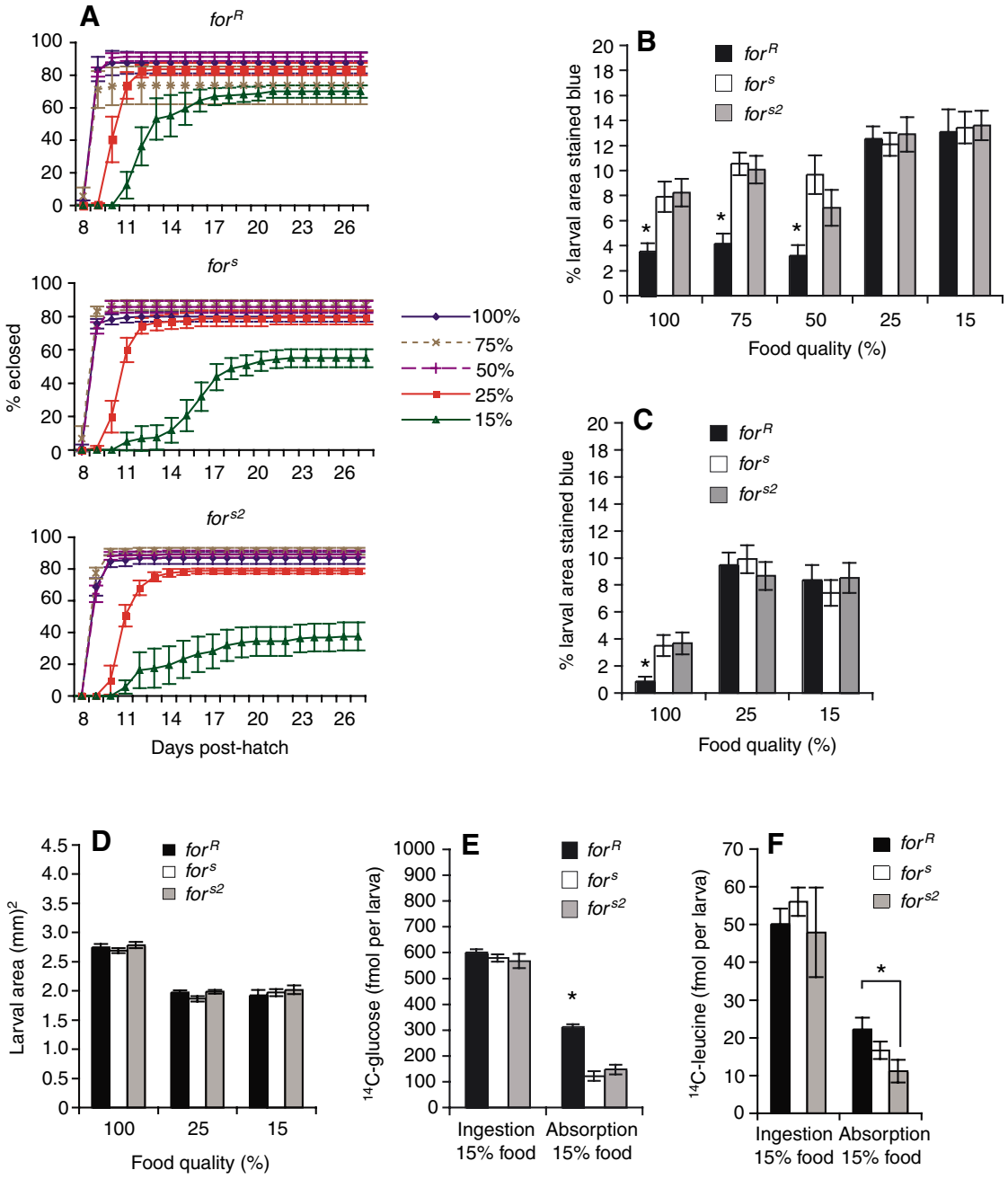

Fig. 4. See next page for legend.

glucose (Fig. 3B). Notably, chasing the radio-labeled medium with unlabeled food resulted in significantly higher ^{14}C counts in *for*^{R} animals relative to *for*^{s} (Fig. 3B). Thus, *for*^{R} larvae absorbed approximately 50% of the ^{14}C-6-glucose ingested whereas *for*^{s} and *for*^{s2} larvae absorbed approximately 15% of the ^{14}C-6-glucose ingested. Rovers retain more of the radioactive label after clearing their guts with unlabeled medium, indicating higher glucose absorption in rovers than in sitter and sitter mutant larvae.

To determine if rovers and sitters differed in amino acid absorption we repeated the above experiment with L-[U-^{14}C]leucine (Fig. 3C). Leucine was chosen because it is one of the most abundant amino acids in yeast, a major food source of *D. melanogaster* larvae (Yamada and Sgarbiera, 2005). No absorption differences were found with L-[U-^{14}C]leucine in well-fed larvae (Fig. 3C). As for glucose, rovers had lower intake of radiolabelled leucine than sitters. This suggested that the *for*-mediated differences in absorption in well-fed larvae may be specific to carbohydrates. It is not known how rovers absorb more glucose in their guts than sitters. Measures of excretion concentration, gross gut morphology and gut contraction rate did not show obvious differences between *for*^{R}, *for*^{s} or *for*^{s2} (Fig. 3D,E). Together, these results provide evidence that the rover/sitter natural polymorphism is a food-related phenomenon involving food intake and absorption.

Fig. 4. Food level affects survivorship to eclosion, development rate and food intake in rovers and sitters. (A) Larvae were reared from egg-hatch on media containing 100%, 75%, 50%, 25% or 15% of the yeast and sucrose content of standard medium (100%; see Materials and methods). Values are means ± s.e.m. Between-strain survivorship analysis revealed that differences were not significant at 100% ($F_{(2,21)}$=0.91, P=0.4), 75% ($F_{(2,20)}$=1.70, P=0.2), 50% ($F_{(2,21)}$=1.17, P=0.3) or 25% ($F_{(2,21)}$=0.73, P=0.5) food levels, but at 15% rovers differed significantly from for^{s} ($F_{(1,14)}$=6.00, P<0.03) and for^{s2} ($F_{(1,14)}$=13.29, P<0.003). Between-strain comparisons of developmental delay revealed that rovers differed from both for^{s} and for^{s2} at 15% ($F_{(2,21)}$=4.33, P<0.03) and rovers differed from for^{s2} at 100% ($F_{(2,21)}$=4.42, P<0.03), 50% ($F_{(2,21)}$=7.06, P<0.005) and 25% ($F_{(2,21)}$=3.80, P<0.04) food levels but not at 75% ($F_{(2,20)}$=2.58, P=0.1). (B) Rovers (for^{R}) ingested less yeast paste after 15 min feeding than sitters (for^{s} and for^{s2}) when raised on high levels of food (100%, 75%, 50%), but not when reared on low levels of food (25%, 15%) [two-way ANOVA for strain ($F_{(2,435)}$=5.13, P<0.006) and strain-by-food level ($F_{(8,435)}$=2.28, P<0.02); one-way ANOVA on 100% ($F_{(2,87)}$=6.86, P<0.002), 75% ($F_{(2,87)}$=14.33, P<0.0001), 50% ($F_{(2,87)}$=6.34, P<0.003), 25% ($F_{(2,87)}$=0.14, P=0.9), 15% ($F_{(2,87)}$=0.11, P=0.9)]. (C) Rovers and sitters differ in food intake measured after 5 min feeding on yeast paste when reared at 100%, food quality but not when food-deprived (reared on 25% and 15% food) [ANOVA, at 100%, $F_{(2,87)}$=5.53, P<0.0006, 25% $F_{(2,87)}$=0.69, P=0.50), 15% $F_{(2,87)}$=0.33, P=0.7]. (D) All larvae reared under low food levels were staged to mid-third instar prior to measuring food intake. Rover and sitter larvae reared in dilute (15% and 25%) food were smaller in size at mid-third instar compared to larvae reared in normal (100%) food. However, no within-strain differences were found at any food level [two-way ANOVA, $F_{(8,260)}$=56.50, P<0.0001; strain, $F_{(2,260)}$=0.19, P=0.82; food quality, $F_{(2,260)}$=73.25, P<0.0001, with significantly smaller larvae at 25% (P<0.0001) and 15% (P<0.0001) compared to 100%; strain×food quality $F_{(2,4)}$=0.23, P=0.92]. (E) At 15% food, rovers, sitters and sitter mutants ingested similar amounts of ^{14}C-labeled media in15 min ($F_{(2,15)}$=0.67, P=0.3), but rovers absorbed more than sitter and sitter mutants ($F_{(2,17)}$=45.51, P<0.0001). for^{R} larvae had a twofold increase in absorption compared to for^{s} and for^{s2} larvae at 15% food levels ($F_{(2,12)}$=41.96, P<0.0001; for^{R} *vs* for^{s}, P<0.0001; for^{R} *vs* for^{s2}, P<0.0001; for^{s} *vs* for^{s2}, P=0.12). (F) After 15 min of feeding on a yeast–water paste containing ^{14}C-labeled L-U-leucine, rover larvae reared on 15% food did not differ significantly from sitters in amount of ^{14}C ingested ($F_{(2,15)}$=0.37, P=0.70). When subsequently exposed to unlabeled medium for 3 h, rovers absorbed significantly more ^{14}C label compared to sitter mutants ($F_{(2,15)}$=4.55, P=0.03; for^{R} *vs* for^{s}, P=0.15; for^{R} *vs* for^{s2}, P=0.009; for^{s} *vs* for^{s2}, P=0.53).

for affects survivorship and development time under low-nutrient conditions

Our results demonstrate that *for* affects the nutrient acquisition strategies of well-fed larvae, where rovers have decreased food intake and higher glucose absorption compared to sitters. We hypothesized that these differences in energy acquisition could affect larval responses to changes in food availability. Because the opportunity for larval food deprivation in nature is high (Atkinson, 1979) we investigated whether larvae exhibited plastic responses to food deprivation and if so whether this plasticity was mediated by *for*.

In order to determine appropriate food deprivation conditions we reared larvae from egg-hatch to pupation on media containing 100%, 75%, 50%, 25% or 15% of the yeast and sucrose content of standard medium (see Materials and methods). We hypothesized that the differences in food acquisition between variants of *for* affect larval development and survivorship. Larval development and survivorship of for^{R}, for^{s} and for^{s2} did not differ at 100%, 75% or 50%, suggesting that these food levels were not limiting. However, all strains were developmentally delayed when reared on 25% and 15%, and survivorship to eclosion also tended to be reduced (Fig. 4A). As a result, we used 25% and 15% as our food deprived conditions. Within-strain comparisons showed that all strains developed more slowly at 25% and 15%, and had significantly lower survival to eclosion at 15%. The magnitude of these changes was greatest in for^{s} and the for^{s2} sitter mutant larvae. Between-strain survivorship analysis revealed that differences were not significant at 100%, 75%, 50% or 25% food levels, but at 15% rovers differed significantly from for^{s} and for^{s2}. Between-strain comparisons of developmental delay revealed that rovers differed from both for^{s} and for^{s2} at 15% and rovers differed from for^{s2} at 100%, 50% and 25% food levels, but not at 75%. These results suggest variation in *for* affects larval survivorship under low food conditions.

for affects plasticity of food intake in food-depleted environments

When larvae are reared to third instar under food deprivation conditions and then placed on a dyed yeast paste, for^{R}, for^{s} and for^{s2} larvae exhibit plasticity in food intake, and this plasticity is mediated by *for* (see below). The food intake of all strains was significantly elevated when larvae were reared to mid-third instar on media containing 25% or 15% food compared to those reared on 100% food (Fig. 4B; two-way ANOVA for food level, $F_{(4,435)}$=18.94). Rovers (for^{R}) ingested less yeast paste than sitters when (for^{s} and for^{s2}) when raised on high levels of food (100%, 75%, 50%), but not when reared on low levels of food (25%, 15%) whether they were feeding for 15 min (Fig. 4B) or 5 min (Fig. 4C). When for^{R}, for^{s} and for^{s2} larvae reared in food-deprived conditions (25% and 15%) reached mid-third instar, their sizes did not significantly differ from each other, despite being developmentally delayed and smaller than larvae reared in abundant food (50%, 75% and 100%) (Fig. 4D); for all food intake measures, larvae were staged to mid-third instar (see Materials and methods).

Glucose absorption differences persisted when larvae were reared under food-deprivation conditions (25% or 15%) demonstrating the persistence of increased glucose absorption by rovers compared to sitters and sitter mutants (Fig. 4E). As expected, in 15% food, for^{R}, for^{s} and for^{s2} larvae ingested similar amounts of ^{14}C-labeled media in 15 min. However, for^{R} still absorbed more than for^{s} and for^{s2} larvae. Since both rovers and sitters ingest food at the same rate when reared under poor food conditions, these results also suggest that rovers are able to absorb more nutrients per unit food ingested, and differences in absorption are not due to the amount of food in the gut.

Intriguingly, the absorption efficiency, defined as the percentage of ^{14}C-absorbed/^{14}C-ingested remained at about 50% for for^{R} larvae and 17% for for^{s} and for^{s2} larvae, regardless of the quality or dilution of food in which the larvae are reared. Although it looks as if the amount of ^{14}C absorbed increased from 100% to 15% food in for^{R} larvae, their total food ingestion also increases, resulting in an unchanged absorption efficiency.

We also found a nearly significant difference in absorption of leucine between rovers and sitters when reared under 15% food (Fig. 4F), suggesting that larvae may alter their leucine absorption in response to food deprivation but further experiments are required to assess this hypothesis. Overall, both rovers and sitters ingest food at the same rate after being reared under food deprivation conditions; however, rovers absorb more glucose than sitters. This may contribute to their enhanced fitness under 25% and 15% food deprivation conditions.

From the data above, we conclude that food ingestion rates of *D. melanogaster* larvae are sensitive to the nutritional state of the individual. Larvae do not eat at maximal rates when well fed but may approach ceiling ingestion rates when placed on yeast paste after having been raised in the 25% and 15% food conditions provided here. Furthermore, we find that rover food intake is not fixed at lower levels than sitters; rather, the differential varies depending on the degree of food deprivation. Since maximal rates of food intake do not appear to differ between the variants, the greater plasticity of rovers can be explained by their lower intake rates under well-fed conditions, which may be balanced by their higher glucose absorption under both well-fed and food-deprived conditions. These data may help explain why long-term selection at high larval density favors rovers (Sokolowski et al., 1997).

Variation in for mediates food acquisition strategies

As the larval cells in which FOR are expressed are not yet known, we chose to manipulate *for* by ubiquitously increasing expression of *for* in sitters. To this end, we employed the transgenic sitter strain carrying a T2 transgene (*UASforT2*), which was expressed ubiquitously in a sitter genetic background using the leaky expression of a heat-shock promoter (*hsGAL4*). Such expression of *forT2* in a sitter genetic background has been previously shown to result in rover-like foraging locomotion (Osborne et al., 1997) and increases the amount of protein produced by *forT2* (Fig. 5A).

When sitters expressing *forT2* were examined, rover-like food intake and glucose absorption patterns were observed (Fig. 5B–D). Consistently, expression of *forT2* in sitters also significantly decreased food intake and increased the glucose absorption rate. Assessment of the survivorship to eclosion of *hs-GAL4*, *UASforT2* larvae over a range of food levels demonstrated that variation in *for* also accounts for differences between rover and sitter survivorship and development rates (Fig. 6A). Expression of *forT2* was sufficient to increase developmental rate and survivorship of food-deprived sitters raised on low nutrient media (25% or 15%). These data provide further proof that variation in *for* levels can account for the distinct rover and sitter food acquisition types.

Regulation of for is responsive to food deprivation

Our goal in the present study was to test the hypothesis that *for* plays a role in food-related phenotypes. Follow-up investigations will address the cellular mechanisms through which PKG acts to mediate this plasticity. To further establish the connection between *for*-PKG and plasticity in food intake, we assayed PKG activity in rovers and sitters reared on 100%, 25% and 15% food levels. When food was not limited (100%), rovers ingest less and have higher PKG activity than sitters or for^{s2} sitter mutants (Fig. 6B). In contrast, when for^{R}, for^{s} and for^{s2} larvae were grown under food deprivation conditions (25% or 15%), food intake levels increased to a similar level. Concomitantly, as food deprivation increased, PKG enzyme activities dropped to comparable levels, reducing rover–sitter differences in food intake (Fig. 6B,C). Rovers (for^{R}) had significantly higher PKG activity than sitters (for^{s} and for^{s2}) when reared on high food levels, but this difference diminished when larvae were food deprived. Thus, food intake and PKG enzyme activity are negatively correlated, suggesting that *for* is sensitive to changes in nutritional state.

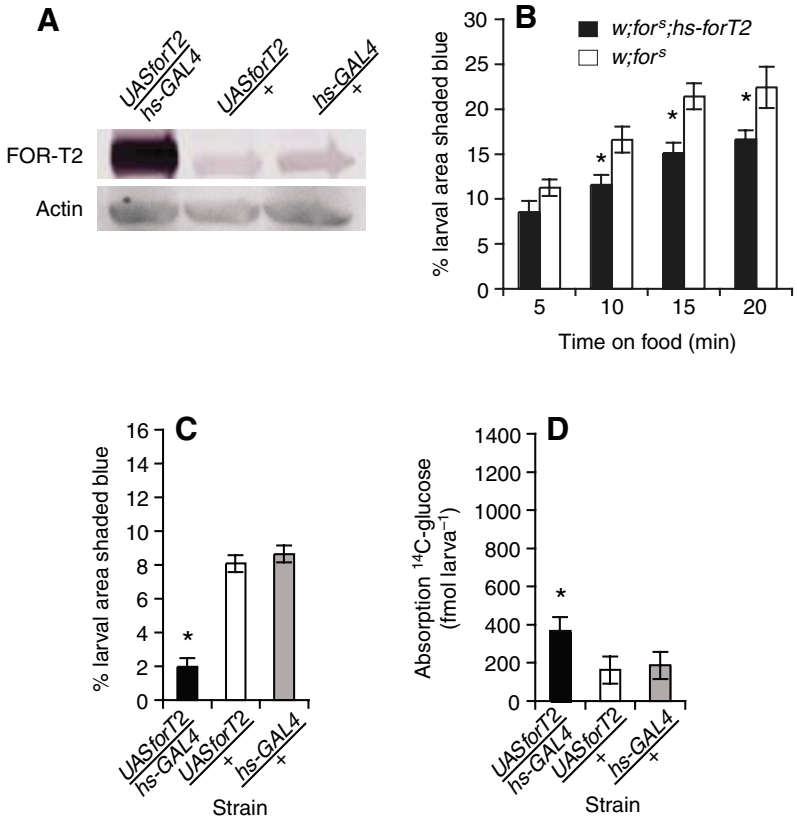

Fig. 5. Variation in *for* mediates nutrient acquisition strategies. (A) for^{s}*;hs-GAL4/UASforT2* flies reared at 23°C show an increase in T2 protein level (lane 1) compared to control flies reared under the same conditions (lanes 2 and 3). The actin bands show that equal amounts of protein were loaded in each lane. (B) Expression of a *forT2* transgene in sitters resulted in a rover-like decrease in food intake (ANOVA: at 5 min, $F_{(1,57)}$=3.03, P=0.09; 10 min, $F_{(1,58)}$=7.60, P=0.008; 15 min, $F_{(1,58)}$=11.92, P=0.001; 20 min, $F_{(1,57)}$=5.46, P=0.02). (C) Ubiquitous expression of *forT2* in sitters using a leaky *hs-GAL4* transgene significantly decreased food intake ($F_{(2,89)}$=19.56, P<0.0001) and (D) increased glucose absorption rate ($F_{(2,17)}$=47.51, P<0.0001).

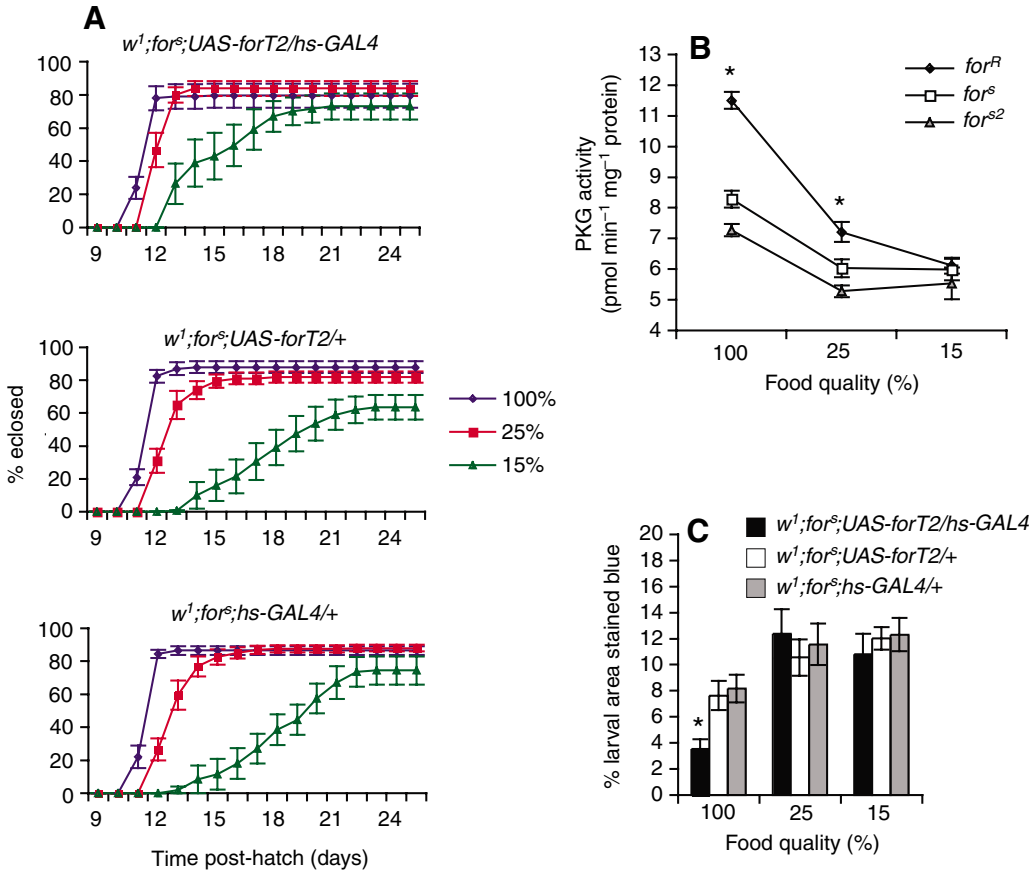

Fig. 6. Variation in *for* affects response to food quality. (A) Expression of *forT2* was sufficient to increase developmental rate and survivorship in food-deprived sitter larvae raised on low nutrient media (25% or 15%) [development time: at 25% ($F_{(2,27)}$=17.78, P<0.0001) and at 100% ($F_{(2,26)}$=0.95, P=0.4); survivorship: at 15% ($F_{(2,29)}$=4.07, P<0.03) and at 100% ($F_{(2,28)}$=0.66, P=0.5)]. (B) PKG enzyme activity is inversely related to food intake. Rovers (for^R) had significantly higher PKG activity than sitters (for^s and for^{s2}) when reared on high food levels, but this difference diminished when larvae were food-deprived [two-way ANOVA: for strain ($F_{(2,63)}$=57.36, P<0.0001), food level ($F_{(2,63)}$=95.26, P<0.0001), strain-by-food level ($F_{(4,63)}$=11.67, P<0.0001); ANOVA: for 100% ($F_{(2,21)}$=87.9, P<0.0001), 25% ($F_{(2,21)}$=14.39, P<0.0001), 15% ($F_{(2,21)}$=0.70, P=0.5) food level]. (C) Sitter larvae expressing a *forT2* transgene expressed a rover-like pattern of food [two-way ANOVA: for strain ($F_{(2,261)}$=3.78, P=0.02), food level ($F_{(2,261)}$=2.85, P=0.06), strain-by-food level ($F_{(4,261)}$=1.60, P=0.2); one-way ANOVA by strain: for 100% ($F_{(2,87)}$=6.87, P<0.002), 25% ($F_{(2,87)}$=0.32, P=0.7), 15% ($F_{(2,87)}$=0.42, P=0.7) food level].

Furthermore, sitter larvae expressing a *forT2* transgene had rover-like reduced food intake when food was plentiful, but not when reared in lower food levels (Fig. 6C). Together these data suggest that the regulation of *for* responds to food deprivation, and that changes in its expression can induce plasticity in food intake.

Interestingly, unlike food intake, the increased rate of glucose absorption in rovers did not vary in a food-dependent manner. Regardless of food level, higher levels of glucose absorption were found in rovers and sitters expressing the *forT2* transgene compared with sitters and the sitter mutant. Elevated glucose absorption in rovers might partially account for their increased survivorship compared to sitters at 25% and 15%, despite similar PKG enzyme activity levels.

Our data do not address whether *for* acts throughout development or in an immediate nature to mediate food acquisition and plasticity in food intake. Thus, several possibilities could explain why glucose absorption does not vary with food level in a similar way as food intake and PKG activity. For example, food deprivation may not downregulate *for* activity in cell types important for glucose absorption, or the effects of PKG on glucose absorption may be fixed early in development. Future studies on specific expression of *for* and targeting manipulation of *for* in the cells in which *for* is expressed may elucidate both the developmental timescale and cellular mechanism through which *for* acts to mediate these responses.

Conclusions

The implications of this study are twofold. Firstly we demonstrate that natural variation in PKG affects food acquisition, and secondly we show *for*'s role in plastic compensatory food intake responses to food deprivation. The rover/sitter system provides an enticing model for further investigations into the effects of 'thrifty' genes that regulate efficiency of nutrient homeostasis (Zimmet and Thomas, 2003; Shmulewitz et al., 2006). Alleles of such genes may promote individual fitness under restrictive food regimes (Neel, 1962) just as *for*-PKG regulates normal individual differences in food intake. The role of *for* in food-specific behaviors is conserved

in several species including honeybees, nematodes and harvester ants (Ben-Shahar et al., 2002; Fujiwara et al., 2002; Ingram et al., 2005). Future studies will determine whether similar gene-by-environment interactions prevail in the *for* orthologs identified in other species.

Intriguingly, a phylogeny constructed from available *for*-PKG protein sequences suggests that despite broad taxonomic breadth (nematodes to humans), there may be a widespread conserved association between PKG and food-related behaviors (Fitzpatrick and Sokolowski, 2004). If the role of *for* in the plasticity of food intake and regulation of food absorption is conserved in other animals including humans, then it may play an important role in regulation of energy homeostasis essential in maintaining a healthy body mass. The human homolog of *for, cGK1,* plays an important role in intestinal muscle function and thus may affect passage of intestinal content and ability to absorb food (Hofmann, 2005). Changes in PKG are also associated with disorders such as obesity and diabetes. For example, high expression of *cGK1* has been associated with obesity, and a reduction of PKG has been associated with diabetes and high glucose concentrations (Wang et al., 2002; Wang et al., 2003; Wang et al., 2004; Engeli et al., 2004; Su et al., 2003; Zanetti et al., 2005; Chang et al., 2004). Although a polymorphism in *cGKI* has not yet been found between obese and healthy mass populations (Zakharkin et al., 2005), the evidence from *D. melanogaster* larvae suggests that *cGKI* may help sustain a healthy mass by maintaining a balance between energy input and output. Future research will help resolve whether a natural polymorphism in *for* also contributes to individual differences in energy balance in humans and across diverse taxa.

Little is known of the genetic or molecular basis for phenotypic plasticity, as genetic variability has intentionally been removed or reduced during the establishment of model organisms used in genetic analyses (DeWitt and Scheiner, 2004). Our findings provide a rare example of a naturally varying gene that affects plasticity providing a unique opportunity to investigate both the genetic and functional aspects of plasticity.

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