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## RESEARCH ARTICLE

# Contribution of larval nutrition to adult reproduction in *Drosophila melanogaster*

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### SUMMARY

**Within the complex life cycle of holometabolous insects, nutritional resources acquired during larval feeding are utilized by the pupa and the adult. The broad features of the transfer of larval resources to the pupae and the allocation of larval resources in the adult have been described by studies measuring and tracking macronutrients at different developmental stages. However, the mechanisms of resource transfer from the larva and the factors regulating the allocation of these resources in the adult between growth, reproduction and somatic maintenance are unknown. *Drosophila melanogaster* presents a tractable system in which to test cellular and tissue mechanisms of resource acquisition and allocation because of the detailed understanding of *D. melanogaster* development and the experimental tools to manipulate its tissues across developmental stages. In previous work, we demonstrated that the fat body of *D. melanogaster* larvae is important for survival of starvation stress in the young adult, and suggested that programmed cell death of the larval fat cells in the adult is important for allocation of resources for female reproduction. Here, we describe the temporal uptake of larval-derived carbon by the ovaries, and demonstrate the importance of larval fat-cell death in the maturation of the ovary and in fecundity. Larvae and adults were fed stable carbon isotopes to follow the acquisition of larval-derived carbon by the adult ovaries. We determined that over half of the nutrients acquired by the ovaries in 2-day-old adult females are dependent upon the death of the fat cells. Furthermore, when programmed cell death is inhibited in the larval fat cells, ovarian development was depressed and fecundity was reduced.**

Key words: resource allocation, energetics, fat body, fecundity, stable isotope.

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### INTRODUCTION

The acquisition and allocation of resources across life stages to support growth, somatic tissue maintenance and reproduction are central to life history evolution. In holometabolous insects, distinct developmental stages are tightly linked to feeding and non-feeding periods, thus the different phases of the life cycle can potentially act upon each other. The larval stage is characterized by feeding, while metamorphosis is a non-feeding stage during which the larva is transformed into the adult, and the adult itself may not feed. Holometabolous insects eclose to adulthood containing energy acquired during larval stages that often represents a significant fraction of the adult energy budget (Zera and Harshman, 2001; Fischer et al., 2004; Boggs and Freeman, 2005; Boggs, 2009). In saturniid moths and the Ephemeroptera, adult metabolism and reproductive effort must be supported entirely by energy acquired as larvae. A less extreme example includes autogenous mosquitoes, in which the first clutch of eggs is laid before the female takes her first blood meal (Telang and Wells, 2004).

The mechanisms by which larval energy reservoirs are tagged and then released for use in later developmental stages, and by which they are allocated for somatic maintenance and reproduction, are not well understood, but programmed cell death (PCD) or autophagy of the larval fat cells appear to be involved. In the silkworm moth *Bombyx mori*, PCD begins in the early pupal stage (Kaneko et al., 2011), when egg maturation occurs. A different process occurs during metamorphosis of the skipper butterfly, *Calpodes ethlius*. Individual fat cells reorganize into nodular clumps surrounding the tracheoles, and then undergo autophagy, with intracellular remodeling taking place. Mitochondria, rough endoplasmic reticulum and other structures are sequestered in organelle-specific autophagic vacuoles and are destroyed by hydrolytic enzymes, but the cells retain their integrity (reviewed by Hoshizaki, 2005).

The fat body has a complex life history. During the last 3 days of larval development, *Drosophila melanogaster* increases 200-fold in mass (Church and Robertson, 1966), with nutrient reserves primarily accumulated in the larval fat body. Before metamorphosis, the larva stops feeding and a 12–24 h ‘wandering’ phase begins, during which the animal searches for a suitable pupariation site (Riddiford, 1993). During metamorphosis, the imaginal cells proliferate to give rise to the adult tissues, while the larval tissues undergo PCD (Lee and Baehrecke, 2001). A striking exception to the loss of larval tissues is the fat body, which undergoes tissue remodeling into individual cells during the pre-pupal stages of metamorphosis (Nelliot et al., 2006). These cells survive metamorphosis (Aguila et al., 2007) and are present in the newly eclosed adult. The larval fat cells presumably support the *D. melanogaster* pupae, which do not feed, and the newly eclosed adult, which remains inactive for approximately 8 h until the wings expand and the cuticle tans (Chiang, 1963; Edgecomb et al., 1994). Thus, larvae must acquire enough nutrients not only to fuel the developmental re-organization of the pupa but also to survive the late larval and early adult periods.

In the adult, the larval fat cells undergo PCD. Within 24 h of eclosion, 85% of the fat cells are absent, and by 3 days the larval fat cells have been replaced by the adult fat cells. Surprisingly, newly eclosed adult females are three times more starvation resistant than mature females (3 or 10 days old). This difference is not age dependent *per se*, because retention of the larval fat cells by inhibition of PCD increases the starvation resistance of older adults (Aguila et al., 2007). Thus, larval fat cells represent an important and accessible energy reservoir in the adult female, yet once these reserves are released (and presumably taken up by adult tissues) they are not available to maintain the adult during starvation.

In this study, we tracked transfer of larval- and adult-derived sugar into the ovaries to directly measure the contribution of these nutrients to the ovaries. Furthermore, we tested whether PCD of the larval fat cells is important for the allocation of larval-derived energy reserves and whether these reserves are important for the maturation of the ovaries. We took advantage of natural differences in stable carbon isotopes of cane and beet sugar to assess the relative contributions of larval- and adult-derived sugar carbon to the developing ovaries. The sugar beet undergoes C3 photosynthesis and therefore contains a lower ratio of $^{13}C$ relative to $^{12}C$ than sugar cane, which utilizes C4 photosynthesis (Farquhar et al., 1989). By measuring carbon isotope ratios in ovaries of flies fed different sugar sources as larvae and adults, we were able to determine the contributions of larval- *versus* adult-derived sugar carbon to the ovaries of adult females over a 10 day period. Over half of the sugar carbon in ovaries of 2-day-old females was derived from larval feeding, but this was replaced rapidly and completely by adult-derived sugar carbon by day 7. When PCD was genetically inhibited in the larval fat cells, ovaries of 2-day-old feeding adults contained approximately half of the normal larval-derived sugar carbon. Furthermore, overall ovarian development was delayed, and fecundity was depressed. Our data demonstrate a novel use of PCD to mobilize larval resources for uptake and maturation of the ovaries.

## MATERIALS AND METHODS

### *Drosophila melanogaster* husbandry and genetic crosses

*Drosophila melanogaster* Meigen 1830 were raised at 25°C on a corn-meal–sugar–yeast medium (42.6 g $l^{-1}$ corn meal, 68.2 g $l^{-1}$ beet or cane sugar, 23.9 g $l^{-1}$ baker's yeast, 7.9 g $l^{-1}$ agar and 4.5 ml $l^{-1}$ propionic acid), supplemented with dry yeast.

Genetically constructed flies were used in assays of energy content, ovary mass, fecundity and stable isotope ratios. Briefly, ectopic expression of the cell death inhibitor gene, *Drosophila inhibitor of apoptosis 1* (*diap1*), in the larval fat body was achieved using the GAL4/UAS system (Brand and Perrimon, 1993). Construction of flies with the genotype *y w; P{Lsp2-GAL4.H}, P{w+mc=UAS-n-syb.eGFP}3* (abbreviated *Lsp2-GAL4*) is described by Aguila et al. (Aguila et al., 2007). A stock carrying the *UAS-diap1* transgene, *P{w[+mC]={UAS-DIAP1.H}1* (abbreviated *UAS-diap*), was obtained from the Bloomington Stock Center (Bloomington, IN, USA) and crossed to *Lsp2-GAL4* to drive ectopic expression of *diap1* specifically in the larval fat cells, thereby inhibiting PCD in these cells (Aguila et al., 2007). Because fat-cell death was inhibited, these experimental flies are abbreviated FCDI. In all experiments, the two parental stocks (*UAS-diap* and *Lsp2-GAL4*), in which normal PCD occurs, were used as controls.

### Triglyceride, carbohydrate and protein assays

Energetic substrates (lipids, carbohydrates and proteins) were assayed in triplicate for individual flies. Adult female flies were homogenized in a solution containing detergent to solubilize lipids (1% NP-40, 0.5% deoxycholic acid, 0.1% Triton-X 100, 100 mmol $l^{-1}$ NaCl, 0.1 mmol $l^{-1}$ $CaCl_2$, 2 mmol $l^{-1}$ $MgCl_2$, pH 7.6). The homogenates were heated for 5 min at 75°C to inactivate lipases. Triacylglyceride levels were measured using a commercial serum triglyceride kit (no. TR0100-1KT; Sigma-Aldrich, St Louis, MO, USA), and protein content was quantified using the bicinchoninic acid method (Smith et al., 1985). Carbohydrates (glycogen and trehalose) were digested with amyloglucosidase and quantified with a blood glucose kit (no. G7521; Pointe Scientific, Canton, MI, USA), as described by Marron et al. (Marron et al., 2003).

### Photography

Ovaries were dissected from female flies in Dulbecco's phosphate buffered saline (DPBS) (52 mmol $l^{-1}$ NaCl, 40 mmol $l^{-1}$ KCl, 10 mmol $l^{-1}$ Hepes, 1.2 mmol $l^{-1}$ $MgSO_4$, 1.2 mmol $l^{-1}$ $MgCl_2$, 2 mmol $l^{-1}$ $Na_2HPO_4$, 0.4 mmol $l^{-1}$ $KH_2PO_4$, 1 mmol $l^{-1}$ $CaCl_2$, 45 mmol $l^{-1}$ sucrose, 5 mmol $l^{-1}$ glucose, pH 7.2). Ovaries were examined by light microscopy, and photographs were taken using a Canon A620 digital camera coupled to a Zeiss Stemi 2000-C microscope and imported using Canon Zoom Browser EX photo software.

### Fecundity assays

To establish when egg laying began and to determine total egg laying capacity, newly eclosed females (0–2 h) were collected and placed in individual wells of a Fly Condo (no. 59-110; Genesee Scientific, San Diego, CA, USA) containing grape agar and supplemented with yeast paste. Two males were included to ensure mating. The number of eggs laid in each 24 h period was recorded for 10 days.

### Stable isotope studies

Flies for stable isotope analysis were reared on either a cane-sugar- or beet-sugar-based diet as larvae. Note that corn meal, baker's yeast and agar were also potential carbon sources for developing larvae and adult flies. Newly eclosed (0–2 h old) adult females were collected and maintained on the same diet or switched to the other sugar-based diet. Thus four different feeding regimes were used in this study (larval diet/adult diet): cane/cane, cane/beet, beet/beet and beet/cane. We performed stable isotope analyses of ovaries from 1- to 7-day-old virgin female adults to prevent transfer of male carbon sources to the ovaries. Ovaries were dissected on a 25×75 mm glass slide in a drop of DPBS. Approximately 0.5 mg of ovaries (six to 36 ovaries per sample) were placed in 5×9 mm pressed-tin capsules (no. 041061; Costech Analytical Technologies, Valencia, CA, USA) and dried at 50°C for 48 h. The dry mass for each sample was then determined using a Cahn C-30 microbalance (Cahn Instruments, Cerritos, CA, USA) with a precision of 1 μg. These data were used to calculate dry mass per ovary. Carbon isotope ratios were determined by the Las Vegas Isotope Science Laboratory (LVIS; University of Nevada, Las Vegas, NV, USA) using a Costech NA 2000 Elemental Analyzer coupled with a Delta V Plus mass spectrometer (Thermo Scientific, Waltham, MA, USA). Isotope ratios ($\delta^{13}C$) are reported in permil (parts per thousand) relative to Peedee Belemnite (Werner and Brand, 2001). Isotope ratios for internal standards and dietary carbon sources are provided in Table 1.

### Contributions of larval and adult feeding to ovarian carbon

For each sample used in stable isotope analyses, total carbon per ovary was calculated as the product of the dry mass per ovary and the fraction of dry mass consisting of carbon, as indicated by mass spectrometry (mean=50.43% across all samples). To estimate the relative contribution of cane- and beet-sugar diets to ovarian carbon,

Table 1. Stable carbon isotope ratios of internal standards and media components

| Sample | $\delta^{13}C$ (‰) |
|---|---|
| Standards | |
| Acetate | –29.7±0.06 |
| Cabbage | –24.8±0.06 |
| Corn | –11.8±0.06 |
| Media components | |
| Cane sugar | –14.1±0.44 |
| Beet sugar | –24.7±0.27 |
| Yeast | –13.6±0.31 |
| Cornmeal | –13.9±0.23 |
| Agar | –20.3±0.12 |

Data are means ± s.d.

we calculated the fraction ($F$) of carbon from each source as the weighted average of stable isotope ratios for ovaries from females fed a consistent sugar source as larvae and adults:

$$F_{cane} = (\delta^{13}C_{sample} - \delta^{13}C_{beet:beet}) / (\delta^{13}C_{cane:cane} - \delta^{13}C_{beet:beet}), \quad (1)$$

$$F_{beet} = (\delta^{13}C_{sample} - \delta^{13}C_{cane:cane}) / (\delta^{13}C_{beet:beet} - \delta^{13}C_{cane:cane}), \quad (2)$$

where $\delta^{13}C_{cane:cane}$ (−12.4‰) and $\delta^{13}C_{beet:beet}$ (−20.3‰) are mean stable carbon isotope ratios for ovaries from all females fed cane sugar or beet sugar, respectively, throughout their lifespan, and $\delta^{13}C_{sample}$ equals the carbon isotope ratio for a given sample.

Absolute contributions of cane- and beet-derived carbon were calculated as the product of carbon per ovary (see above) and the fraction of cane- and beet-derived carbon, respectively, from Eqns 1 and 2.

### Statistics and data analysis

Data were analyzed using Statistica 7.0 (SAS Institute, Cary, NC, USA). In most cases, the cutoff for accepting statistical significance was set at $P<0.05$. In the case of the stable isotope experiments, we performed a total of 12 ANOVAs. We therefore used a Bonferroni correction to minimize the possibility of Type I error. Means ± s.e.m. are reported unless otherwise indicated.

## RESULTS

### Disruption of PCD of the fat cells does not affect energy storage

Tissue-specific expression of the PCD inhibitor gene, *Drosophila inhibitor of apoptosis*, in the larval fat cells results in increased starvation resistance in adults (Aguila et al., 2007). It is possible that increased starvation resistance is not due to the retention of the larval fat cells, but rather to increased energy storage in the fat body or other tissues. To test this possibility, we measured the triacylglyceride, glycogen and protein content of 1- to 10-day-old adults in which fat-cell death was inhibited (FCDI) and in parental controls (*Lsp2-GAL4* and *UAS-diap*) (Fig. 1). At eclosion, the initial macronutrient content was unaffected by genotype (i.e. no difference between FCDI and parental controls); thus inhibition of larval fat-cell death did not alter the energy stores brought forward into the adults. Triacylglyceride levels were unaffected by genotype and age ($P>0.5$ for both factors; Fig. 1). Protein levels were also unaffected by genotype or age, although a statistically significant genotype–age interaction effect was noted ($F_{8,101}=2.58$, $P<0.02$). Furthermore, glycogen content increased in all genotypes as flies matured, and then declined (genotype–age interaction, $F_{4,101}=3.96$, $P<0.005$). To summarize, the inhibition of larval fat-cell death did not affect the initial energy stores in the adult, and the overall pattern of macronutrient content during the first 10 days of adult life was similar in FCDI flies and parental controls.

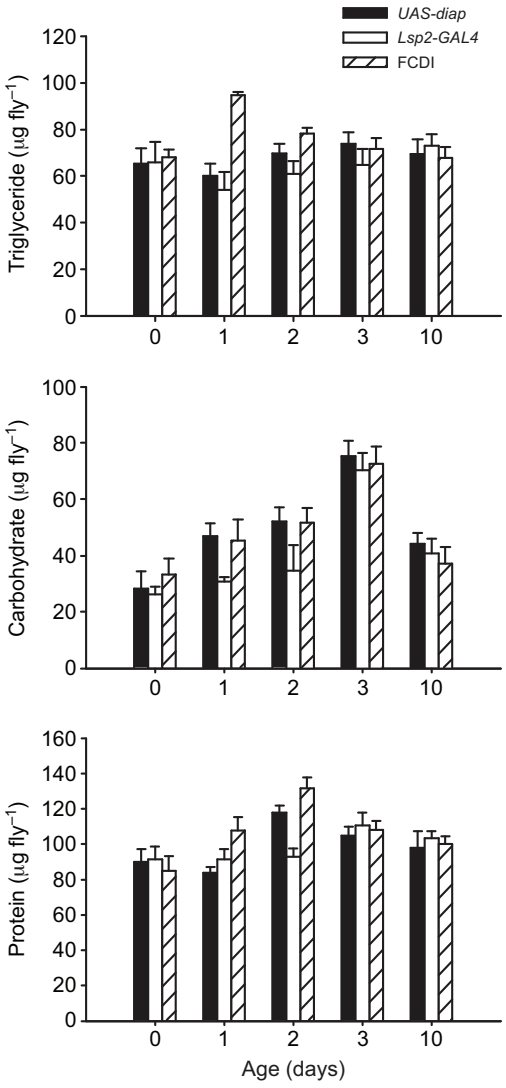


Fig. 1. Effects of inhibiting larval fat-cell death on energy stores in adult female *Drosophila melanogaster*. *Lsp2-GAL4* and *UAS-diap* are female parental controls, and the FCDI flies are F1 females from the *Lsp2-GAL4* and *UAS-diap* cross, in which larval fat-cell death was inhibited. Data are means ± s.d.; $N$=24 for 0-, 2- and 3-day-old adult females; $N$=20 for 10-day-old adult females. Zero-day-old adult females were collected within 30 min of eclosion.

### Ovary mass and egg number

Inhibition of PCD of the larval fat cells did not affect the macronutrient content of the adults (Fig. 1), yet it has been shown to increase starvation resistance (Aguila et al., 2007). The increase is likely due to changes in the allocation pattern of the larval resources within the adult. Qualitative differences in ovary size between FCDI and parental control flies were readily apparent (Fig. 2). Ovarian dry mass in control flies tripled from day 1 to day 2, and then declined and stabilized by day 4 (Fig. 3). In contrast, ovaries from experimental (FCDI) females attained only half the dry mass of ovaries from the parental lines on day 2, but eventually achieved the same dry mass as ovaries from parental controls after

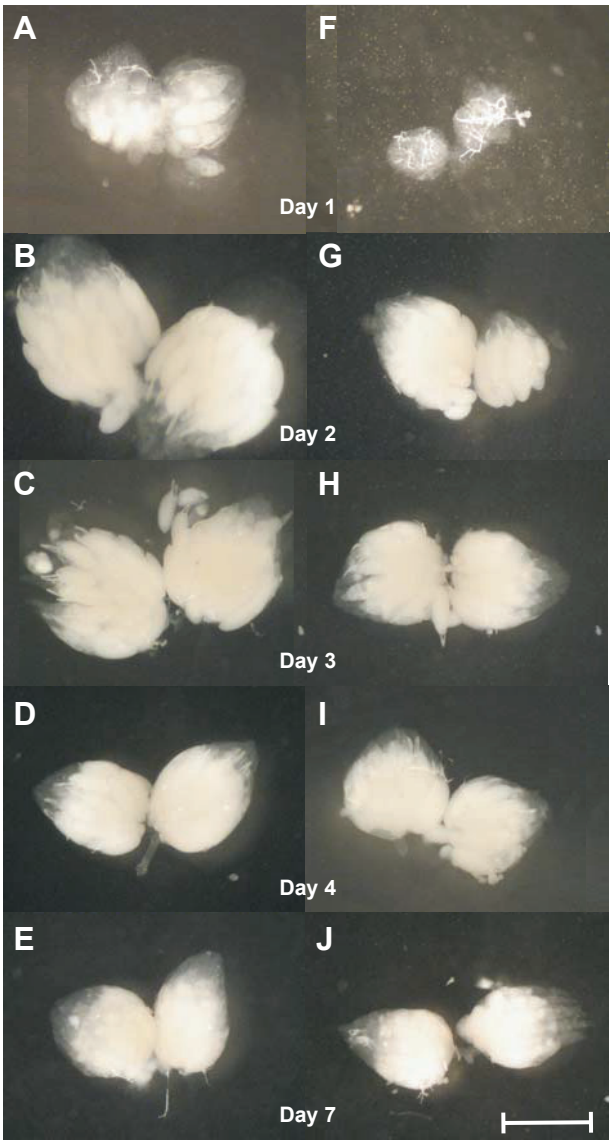


Fig. 2. Effects of age on ovary appearance in adult female *Drosophila melanogaster*. Ovaries from FCDI females (A–E) appeared smaller than ovaries from the *Lsp2-GAL4* control females (F–J) during the first 1–2 days. Scale bar, 10 μm.

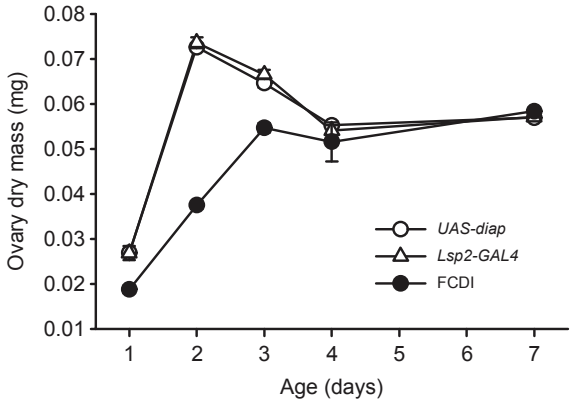


Fig. 3. Effects of age on ovary dry mass in *Drosophila melanogaster*. Data are means ± s.e.m.; *N*=3. Six to 36 ovaries were combined for each measurement, depending on the mass of the individual ovaries for each genotype.

day 4 (Fig. 3). These data suggest that PCD of the larval fat cells contributes to the rapid maturation of the ovaries.

To test whether the apparent delay in ovarian maturation affected fecundity, we determined the number of eggs laid by mated FCDI and parental control females. Egg laying commenced by day 2 post-eclosion for all females (Fig. 4A), but overall fecundity of the FCDI females was lower. FCDI females produced ~200 eggs within the first 10 days of adult life, compared with ~300 eggs for the parental control females ($F_{2,27}$=158, $P<10^{-6}$; Fig. 4A). The daily rate of egg laying was reduced in young FCDI females but approached the control rate during the latter half of the egg laying period (days 6–10; Fig. 4B). Thus, PCD is associated with both rapid maturation of the ovaries and efficient egg production.

### Larval nutrient uptake by the ovaries

Our data are consistent with a model in which PCD serves as a cellular mechanism to allow the rapid release of nutrients from the larval fat cells for uptake by the ovaries. To test this idea, we first measured the uptake of larval-derived carbon by the ovaries, using stable carbon isotopes, and then tested whether uptake was dependent upon PCD. Larvae were raised on a cane- or beet-sugar based diet, and newly eclosed females were either maintained on the larval diet (control treatment) or switched to a diet containing the other sugar (experimental treatment). Because these sugars contain different $^{13}C$:$^{12}C$ ratios, we were able to estimate the relative contributions of ovarian carbon assimilated from larval and adult sugar sources.

One day after eclosion, ovaries from flies maintained on the cane/cane diet had a carbon isotope ratio of $\delta^{13}C$=–12.2±0.2‰ (Fig. 5), similar to the carbon signature of the cane sugar used in our experiments (−11.2±0.3‰). Ovaries from flies reared on the beet/beet diet had a $\delta^{13}C$ value of −20.4±0.5‰ (Fig. 5), similar to that of the beet sugar used to prepare media (−23.6±0.5‰). The differences between the $\delta^{13}C$ values of the pure sugars and the ovaries were primarily caused by the presence of additional carbon sources in the diet, especially corn meal and yeast (Table 1).

As expected, stable isotope ratios in the ovaries remained consistent over 7 days when flies were reared on the same larval and adult diets. In contrast, carbon isotope $\delta^{13}C$ ratios of the ovaries changed when adults were switched to a different adult sugar source (Fig. 5). An initial ANOVA, using genotype, age, and larval and adult diets as factors, revealed highly significant differences for all main effects and most interactions ($P<10^{-6}$). To simplify analysis, at each age we performed an ANOVA for flies fed the same adult diet. We also compared 1-day-old females fed different larval diets but the same adult diet. Because of the large number of ANOVAs performed, we used a Bonferroni correction, with $P<0.004$ required to reject the null hypothesis. This analysis allowed us to address our main hypothesis, that PCD is a primary driver of larval nutrient uptake by the adult ovaries.

### Larval cane to adult beet diet

Ovaries from 1-day-old females from parental control lines reared on a larval cane-sugar diet and switched to an adult beet-sugar diet (cane/beet) initially had a cane-sugar-like carbon isotope ratio of $\delta^{13}C$=–12.9±0.3‰ (Fig. 5A). As expected, ovaries from cane/beet adults gradually acquired a carbon isotope $\delta^{13}C$ ratio similar to that of beet sugar. In contrast, when programmed fat-cell death was blocked, the ovaries more quickly acquired an adult diet signature,

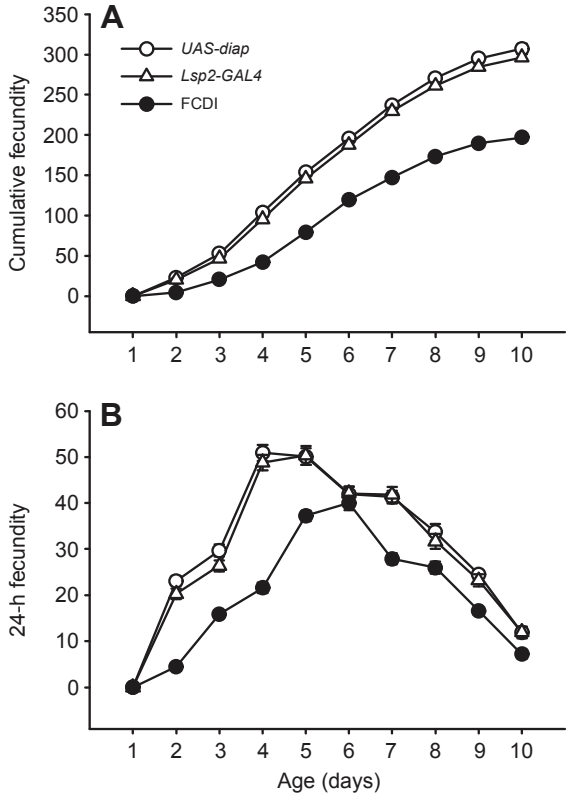


Fig. 4. Effects of inhibition of larval fat-cell death on (A) total and (B) daily fecundity in *Drosophila melanogaster*. The daily number of eggs produced from mated FCDI and control females was determined for 10 days. Data are means ± s.e.m.; *N*=9–10 adult females.

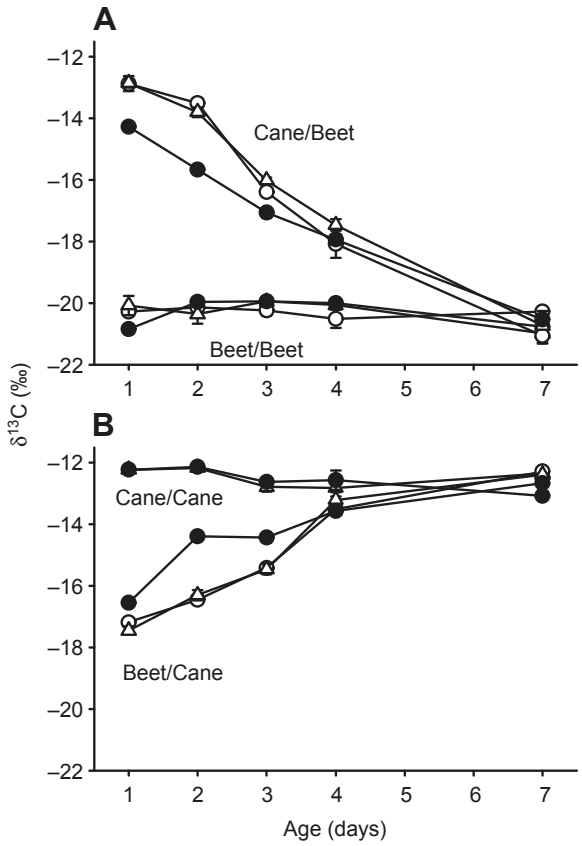


Fig. 5. Stable isotope analysis of dissected ovaries from aged female *Drosophila melanogaster*. Flies were reared on one of four diet regiments (larval diet/adult diet): cane/cane, cane/beet, beet/beet and beet/cane. (A) Larvae reared on cane- (upper lines) or beet-sugar medium (lower lines), and then reared on beet-sugar medium as adults. (B) Larvae reared on beet- (lower lines) or cane-sugar (upper lines) medium, and then reared on cane-sugar medium as adults. Points represent mean ± s.e.m. $\delta^{13}C$ values of dissected ovaries; *N*=3. Open circles, ovaries from *UAS-diap* controls; open triangles, ovaries from *Lsp2-GAL4* controls; filled circles, ovaries from F1 females in which larval fat-cell death was inhibited (FCDI).

as reflected by a more beet-like signature at day 1 ($\delta^{13}C$=—14.3±0.2‰; Fig. 5A). An ANOVA for all 1-day-old flies reared on a larval cane-sugar diet revealed significant effects of genotype ($F_{2,12}$=15.49, $P$<0.0005), adult diet ($F_{1,12}$=87.27, $P<10^{-6}$) and their interaction ($F_{2,12}$=13.7, $P$<0.001) in establishing the carbon isotope $\delta^{13}C$ ratio of the ovaries. Tukey's *post hoc* tests indicated that these differences were driven by the FCDI flies that had been switched to a beet diet ($P$<0.0005 for all pairwise comparisons between this and other experimental treatments on day 1; no other comparisons had $P$-values less than 0.035). Thus, ovaries from 1-day-old FCDI females on a cane/beet diet contained a more adult-like carbon isotope signature, indicative of a significant contribution from adult feeding, relative to control flies.

The acquisition of an adult carbon profile as flies aged was examined by separate ANOVAs on each day. All flies fed beet sugar as adults were included, with genotype and larval diet treated as fixed effects. On day 1, genotype (parental controls and FCDI) and larval diet (beet compared to cane) significantly affected $\delta^{13}C$ (Fig. 5A). Ovaries from the cane/beet parental controls had $\delta^{13}C$ values closer to that of cane sugar than the ovaries from the FCDI flies, which had a more beet-sugar-like isotope signature (Tukey's *post hoc* tests, $P$<0.001). Similar results were obtained on day 2 ($P$<0.001 for main effects of genotype and larval diet), and a significant genotype by larval diet interaction was detected. The same general pattern held on day 3, except that the effect of genotype was marginally significant ($F_{2,12}$=8.8, $P$<0.0045), and FCDI flies differed significantly only from the *Lsp2-GAL4* controls. By day 4, larval diet was the only factor affecting $\delta^{13}C$, and on day 7, $\delta^{13}C$ values were statistically indistinguishable among experimental groups. In summary, ovaries from FCDI flies reared on a cane/beet diet acquired an adult carbon isotope signature more rapidly than the parental controls.

### Larval beet to adult cane diet

In the reciprocal diet experiment (cane sugar as adults), similar results were obtained (Fig. 5B). For 1-day-old flies reared as larvae on beet-sugar medium, adult diet and genotype–diet interactions had significant effects on $\delta^{13}C$ of the ovaries ($P$<0.004 for both factors). Ovaries from the FCDI flies tended to have higher $\delta^{13}C$ values (i.e. more cane-like) than parental controls after exposure to cane-sugar medium for 1 day, although these differences were not statistically significant after Bonferroni correction ($P$>0.02; Fig. 5B). On days 2 and 3, genotype, larval diet and their interaction each significantly affected ovarian carbon isotope signature, with FCDI flies on the beet/cane diet having a more adult-derived carbon signature (less negative $\delta^{13}C$ values) than parental controls. Genotypic differences were gone by day 4, although larval diet still had a significant effect ($P$<0.00002). On day 7, larval diet no longer affected $\delta^{13}C$ ($P$>0.07).

The results of these experiments can be summarized as follows. Carbon isotope signatures of ovaries from 1-day-old flies closely

resembled those of their larval diet, and then changed to match the signature of the adult diet as the flies aged. When PCD was inhibited in the larval fat cells, the ovarian isotope signature of 1-day-old females was more similar to the adult diet than the signature of ovaries from the parental controls. These differences in isotope signatures remained for nearly 3 days. In ovaries from 4-day-old flies, $\delta^{13}C$ did not differ between the parental controls and FCDI flies, although ovaries still retained a significant contribution from larval sugar intake. By day 7, carbon isotope signatures indicated no significant contribution of larval sugar carbon in the ovaries.

### Larval- and adult-derived carbon

Our results demonstrate that carbon acquired during larval feeding is transferred to the ovaries, and identifies PCD of the larval fat cells as an important determinant in the effective uptake of larval nutrients into the ovaries. However, because the isotopic analysis provides only a ratio of $^{13}C$ to $^{12}C$, it is not possible to distinguish between a decrease in the uptake of larval-derived nutrients and a compensatory increase in the uptake of adult-derived nutrients. To address this problem, we calculated the absolute amount of carbon derived from the larval diet based on the $\delta^{13}C$ value. We used the mean $\delta^{13}C$ values for all flies (1–7 days) reared on either beet/beet or cane/cane diets to represent 100% beet diet contribution and 100% cane diet contribution, respectively (see Materials and methods). The actual $\delta^{13}C$ values for flies on beet/cane and cane/beet represent a weighted average of contributions of carbon from larval and adult feeding (Min et al., 2006), allowing us to estimate the relative contributions of larval and adult carbon derived from sugar. From our measurements of ovary dry mass (Fig. 2) and carbon fraction (determined as part of the stable isotope analyses), we then calculated the mass of carbon derived from larval and adult diets (see Materials and methods). It is important to note that sugar is not the only carbon source in the diet, and differences in assimilation of yeast and cornmeal protein could affect these calculations.

Fig. 6 shows the calculated amounts of ovarian carbon from sugar derived from larval and adult feeding. ANOVAs for beet/corn and corn/beet flies revealed significant effects of genotype, age and their interaction on the amount of ovarian carbon derived from larval feeding ($P<0.0001$ for all comparisons), with ovaries from FDCI females having less larval carbon than parental controls. These differences were greatest in 2-day-old females, which is consistent with large differences in ovarian dry mass at this age (Fig. 2). In contrast, the quantity of sugar carbon derived from adult feeding did not differ among genotypes (Tukey's *post hoc* test, $P>0.45$ for all pairwise comparisons), suggesting that FCDI females did not engage in compensatory feeding to offset the reduced availability of nutrients from the larval fat cells. Approximately 50% of ovarian sugar carbon in 2-day-old parental control females was obtained from larval feeding in flies fed the beet/cane diet, compared with 25% in FCDI females. In 2-day-old cane/beet flies, we estimated that larval carbon accounted for 84 and 58% of ovarian carbon in control and FCDI females, respectively.

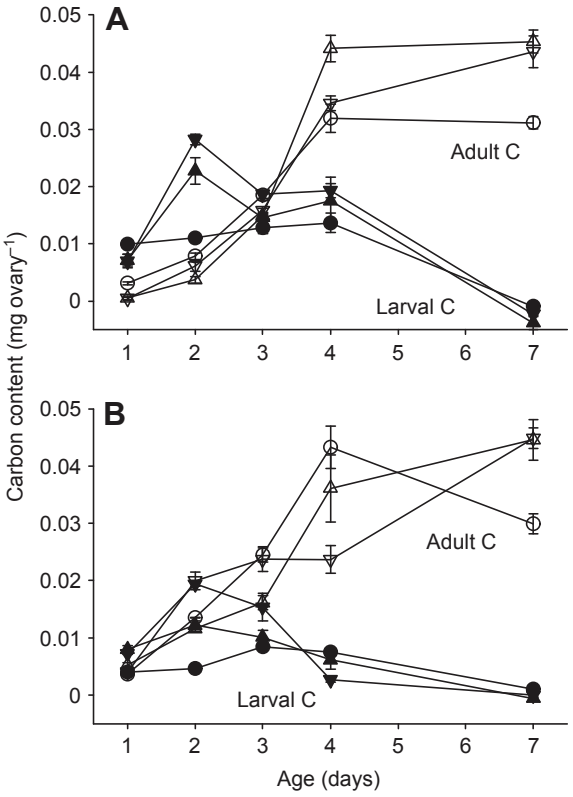


Fig. 6. Carbon content of dissected ovaries from aged female *Drosophila melanogaster*. The carbon content of dissected ovaries was calculated from flies reared on beet/cane or cane/beet diet and based on ovary dry masses, carbon contents and carbon isotope ratios. (A) The amount of carbon in the ovary derived from larval-derived nutrients and from adult nutrients from females reared on a beet/cane diet. (B) The amount of carbon in the ovary derived from larval-derived nutrients and from adult nutrients from females reared on a cane/beet diet. Triangles, ovaries from parental control lines; circles, ovaries from F1 females where larval fat-cell death was inhibited (FCDI). Filled symbols, carbon derived from larval feeding; open symbols, adult-derived carbon.

## DISCUSSION

Energy reserves acquired during the larval stages of holometabolous insects are stored in the larval fat body to fuel the later re-architecture of the animal to the adult form during metamorphosis. Those larval nutrients that are not consumed during metamorphosis are allocated to somatic maintenance and reproduction in the adult (Fischer et al., 2004; Boggs and Freeman, 2005; Min et al., 2006; Boggs, 2009). Previously we reported that the larval fat cells are carried forward into the adult *D. melanogaster*, thus leading to the conclusion that the larval fat cells are the physical transporters of larval nutrients into the adult. Although *D. melanogaster* feed as adults, larval nutrients are assumed to be important for the support of the adult during the first 8 h post-eclosion before feeding commences (Chiang, 1963). The larval fat cells also serve as 'meals-ready-to-eat' in the face of starvation (Aguila et al., 2007), where newly eclosed females that still retain their larval fat cells are over twice as starvation resistant as older adults in which the larval fat cells have undergone PCD.

Rapid maturation of the ovaries corresponds to the window during which larval fat cells normally undergo PCD (Nelliot et al., 2006; Aguila et al., 2007), suggesting a direct connection between these processes in which PCD allows for the rapid release of larval fat cell nutrients for uptake by the ovaries. To test this idea, we disrupted normal larval fat-cell death in the adult and determined the effects on ovarian maturation and fecundity by examining dissected ovaries and determining the number of eggs produced over a 10 day period. Concomitant with this idea is the assumption that larval nutrients represent a major energy source for the maturation of the ovaries in the adult. Thus to determine the larval contribution, we used stable isotopes to establish the ratio of larval- and adult-derived nutrients into the ovaries of females when the larval fat cells underwent normal PCD or when it was inhibited.

### Contribution of larval- and adult-derived nutrients to the ovaries

The stable isotope studies revealed that the $\delta^{13}C$ values of ovaries from 1-day-old female flies resembled those of their larval sugar source (Fig. 5). This is not surprising, because ovarian development begins during metamorphosis (Bodenstein, 1950). In addition, newly eclosed adults do not feed for ~8 h after eclosion (Chiang, 1963), so gonadal growth and development occurring during very early adulthood must rely on larval nutrition. Most of the sugar carbon contained in the ovaries of young flies is derived from larval feeding, but this pattern reverses when PCD is inhibited in the larval fat body. When beet/cane and cane/beet treatments are combined, two-thirds of sugar carbon in ovaries from 2-day-old control flies was derived from the larval diet, compared with 42% in FCDI flies (Fig. 6). These values are only approximations of the relative contributions of larval and adult nutrition, as yeast and corn meal provide significant nutrition during all life stages, and their relative contributions to ovarian carbon may change as flies age. It is clear, however, that in the absence of PCD larval nutrient stores are not readily taken up by the ovaries.

It is possible that FCDI females perform compensatory feeding as adults to offset the lack of availability of energy from the larval fat cells. Ovaries in these flies were smaller than those of parental controls (Fig. 1), suggesting that compensatory feeding could not completely make up for reduced larval energy. More direct evidence against compensatory feeding is provided by the finding that ovaries of 2-day-old females of all genotypes contained similar amounts of adult-derived sugar carbon (Fig. 6B). Thus, we found no indication that FCDI females compensated by eating more or directing more adult energy intake to the ovaries.

It is important to note that nutrients can be mobilized from the larval fat cells, even when PCD is inhibited. Evidence for this is provided by the greater starvation resistance of FCDI females relative to controls (Aguila et al., 2007). It is possible that normal larval fat body energy storage is disrupted in FCDI flies, as expression of the driver we used, *Lsp2-GAL4*, begins early in the third larval instar and continues into adulthood, but we found no significant differences in whole-animal energy content between FCDI flies and parental controls (Fig. 1). Thus, it is unlikely that a reduced capacity of the larval fat body to store nutrients during the larval stages caused decreased fecundity and ovarian development in FCDI flies. We propose that mechanisms for macronutrient mobilization are active within the fat cells but may not be sufficiently rapid to support early ovarian development.

### Larval contribution to adult fecundity

Our results demonstrate that PCD of the larval fat cells is an important mechanism for the reallocation of larval nutrients to the ovaries. Normally, wild-type females contain fully developed eggs ready to be laid approximately 2 days after eclosion, at which time their larval fat cells are absent. In our experiments, egg laying commenced by day 2 post-eclosion for all flies, indicating that some ovarian development occurred, but total fecundity over 10 days decreased by a third in experimental *versus* control flies (Fig. 4A). Even in FCDI flies, fat-cell loss occurs at a slow rate, and by 96 h larval fat cells are nearly gone (Aguila et al., 2007). By this time, larval energy stores have been released, and inhibition of fat-cell death should no longer affect ovarian function. Consistent with this idea, FCDI flies laid numbers of eggs close to parental controls by day 6 post-eclosion, but never quite attained the same daily fecundity (Fig. 4B). Our results support the idea that a critical period exists in early adulthood of female flies when the mobilization of nutrient stores from the larval fat body to the ovaries is necessary for robust gonadal development. If this window of opportunity is disrupted, then overall gonadal development is compromised.

Previous work has shown that early egg production is largely supported by larval nutrition, with half of the carbon content of the eggs derived from dietary sugar (Min et al., 2006; O'Brien et al., 2008). Within 7 days, however, larval-derived sugar carbon provided no detectable contribution to ovarian carbon. When larval fat-cell death was blocked, ovaries acquired an isotope signature similar to that of the adult sugar source even more quickly, because larval-derived nutrients were less available. Min et al. (Min et al., 2006) proposed the existence of a high-turnover 'fast' pool of carbon associated with reproductive effort, and a 'slow' pool of somatic carbon. Our results are consistent with this idea. In addition, we can identify the larval fat body as the primary carbon source for the 'fast' pool during the first few days of adult life. Thus, PCD represents a novel mechanism for the efficient redistribution of larval-derived energy stores to the ovaries.

The timing of PCD of larval fat cells differs among holometabolous insects. In *B. mori*, it begins in the early pupal stage (Kaneko et al., 2011), when egg maturation occurs. Fat-cell autophagy in the adult has also been linked to oogenic cycles in mosquitoes (Bryant and Raikhel, 2011). Although the timing and extent of PCD differ among these species, PCD or autophagy may be generally characteristic of rapid oogenesis in insects. It should be noted, however, that PCD of fat cells begins during the wandering period of the final larval instar in *Manduca sexta* (Müller et al., 2004) and has not been linked to oogenesis. The timing of PCD in *M. sexta* could suggest that energy must be mobilized to support metamorphosis, but this process requires relatively little energy (Merkey et al., 2011).

A remaining conundrum concerns the transport of macronutrients from the fat cells to the ovaries upon fat-cell death. It is not known whether unique mechanisms exist that modify and package macronutrients that are released by cell death for delivery and uptake to the ovaries, or whether circulating lipophorins are sufficient for transport. The utilization of nutrients once they enter the gonads is also unclear. We note that total energetic content of *D. melanogaster* does not change when fat body stores are released and taken up by the ovaries (Fig. 5), yet starvation resistance declines (Aguila et al., 2007). It appears that lipid and carbohydrates are not easily mobilized once incorporated into the ovaries. This is unexpected, as *D. melanogaster* females can reabsorb eggs during nutritional stress (Wilson, 1985).

### Conclusions

Environmental stress, such as starvation, affects tradeoffs between reproduction and survival, but the physiological and molecular mechanisms underlying environmental mediation of these tradeoffs are largely unknown. In an earlier study, we showed that inhibiting normal PCD of the larval fat cells increased starvation resistance in adult *D. melanogaster* (Aguila et al., 2007). In this study, we found that inhibition of PCD reduced the amount of sugar carbon, derived from larval feeding, that entered the ovaries during the first few days of adulthood. Ovarian mass and early fecundity were significantly reduced. Thus, PCD is required for allocation of larval energy stores to the gonads. We propose that when normal fat-cell death is blocked, energy is not rapidly released and is available to other tissues during starvation. In contrast, energy that has entered the gonads following fat-cell death is not readily available to other tissues during starvation.

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