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The role of 20-hydroxyecdysone signaling in *Drosophila* pupal metabolism

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ABSTRACT

In holometabolous insects, the steroid hormone 20-hydroxyecdysone (20E), in coordination with juvenile hormone, regulates the major developmental events that promote larval development and the transition from the larval to the pupal stage. Intimately entwined with the hormonal control of development is the control of larval growth and the acquisition of energy stores necessary for the development of the non-feeding pupa and immature adult. Studies of the coordination of insect development and growth have suggested that the larval fat body plays a central role in monitoring animal size and nutritional status by integrating 20E signaling with the insulin signaling pathway. Previous studies have shown that tissue-specific loss of 20E signaling in the fat body causes pupal lethality (Cherbas et al., 2003). Because the fat body is the major organ responsible for nutrient homeostasis, we hypothesized that the observed pupal mortality is due to a metabolic defect. In this paper we show that disruption of 20E signaling in the fat body does not disrupt nutrient storage, animal size at pupariation, or nutrient utilization. We conclude that 20E signaling in the fat body is not necessary for normal pupal metabolism.

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1. Introduction

A fundamental trade-off exists between increased size and fecundity versus delayed reproduction. In many animals, including *Drosophila*, larger females produce more offspring (Stearns, 1992), but the cost of increased size is often a longer time to reproduction. This trade-off is especially highlighted in insects, where growth does not occur during the adult stage, thus the size of the adult reflects the mass of the larva at the end of larval development (Nijhout, 2003). The length of the larval period is a critical factor in determining adult size because, to a first approximation, the length of the larval period determines the final mass of the larva, which is a determinant in establishing adult size (Davidowitz et al., 2003; Tu and Tatar, 2003; Gefen et al., 2006).

Two hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH), have central roles in controlling the length of the larval stages. In the presence of JH, 20E will initiate a larval molt (Riddiford and Truman, 1993). Completion of the last larval instar is associated with a minor rise in the 20E titer which induces wandering, whereupon the larva stops feeding and searches for a place to pupariate. In *D. melanogaster*, approximately 12–24 h after wandering begins, the JH titer has dropped and a major peak in the 20E titer triggers pupariation (Riddiford and Truman, 1993). The animal ceases wandering and forms a puparium, thus marking the end of larval development and the beginning of metamorphosis. The developmental decision to cease feeding and the commitment to metamorphosis are critical events because they determine the size of the adult and the amount of stored nutrients carried over to the adult from the larval stage (Gefen et al., 2006; Mirth and Riddiford, 2007).

Larval commitment to metamorphosis can only occur after the animal has achieved its critical weight. Critical weight is the larval weight at which a series of physiological events are initiated to trigger pupariation (Davidowitz et al., 2003; Nijhout, 2003; Mirth and Riddiford, 2007). These events include the cessation of JH secretion and the induction of prothoracicotropic hormone (PTTH) secretion. This in turn leads to the secretion of α-ecdysone from the prothoracic gland (PG) (Warren et al., 2002; Gilbert et al., 2002; Gilbert, 2004). α-Ecdysone is converted to 20-hydroxyecdysone (20E, the active form of the hormone) in the peripheral tissues (Petryk et al., 2003; Gilbert, 2004). A rise in the 20E titer triggers the animal to stop feeding, and a second increase in 20E induces puparium formation (Thummel, 1995). In *D. melanogaster* measuring changes in hormone level is difficult, thus most studies of "critical weight" in *D. melanogaster* measure the "minimum weight for viability" (Davidowitz et al., 2003), the smallest mass at which larvae can initiate and successfully complete metamorphosis.

The precise mechanism that allows for cross-talk between tissues to sense the nutritional status of the animal, establish critical weight, and mediate changes in hormonal status has not been determined. However, determinants of critical weight in *Drosophila* are likely to involve the insulin signaling pathway (Brogiolo et al., 2001; Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Several reports

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suggest that cross-talk between the 20E and the insulin signaling pathway coordinate developmental timing with growth. Specifically, it has been proposed that insulin signaling in response to changes in nutritional status can induce secretion of α-ecdysone from the PG thereby modulating larval growth (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Thus, coordination of the insulin and 20E signaling pathways could play a role in determining body size.

Because the fat body is the primary nutrient storage tissue (Beenakkers, 1969; Telfer and Kunkel, 1991; Gronke et al., 2005; Hoshizaki, 2005; Gutierrez et al., 2007) and the central tissue involved in monitoring nutritional status (Colombani et al., 2003; Geminard et al., 2009), it is an ideal site for the integration of 20E and insulin signaling in the control of animal growth. Indeed, 20E is known to induce autophagy (a metabolic process which promotes amino acid mobilization) in the fat body (Rusten et al., 2004), and TOR signaling in the fat body can remotely control insulin release from the insulin producing cells of the brain (Geminard et al., 2009). Of particular significance is a study by Colombani et al. (2005) which demonstrates that disruption of 20E signaling in the fat body results in larger animals at pupariation. Another report, however, by Cherbas et al. (2003) shows that the disruption of 20E signaling in the fat body results in pupal lethality but describes no effect on size. The precise mechanism utilized by the fat body to control animal size has yet to be determined, but it likely involves the coordination of 20E and insulin signaling in nutritional sensing and regulation of fat body metabolism.

We have tested several hypotheses in an effort to determine whether 20E signaling in the fat body might affect pupal survival through a metabolic mechanism. We disrupted 20E signaling in the larval fat body and measured changes in animal size, accumulated energy stores, and the expenditure of energy stores during pupal development. We found that 20E signaling in the fat body is not required for larval nutrient accumulation or pupal expenditure of energy stores but is necessary for completion of pupal development. Moreover, we determined that disruption of 20E signaling in the fat body alone is not sufficient to induce whole-animal size defects.

2. Materials and methods

2.1. Fly stocks

We used the *UAS/Gal4* system (Brand and Perrimon, 1993) to drive ectopic expression of *EcR-F645A*, a dominant-negative form of the ecdysone receptor. Fly strains *Larval serum protein 2-Gal4* (*Lsp2-Gal4*) and *UAS-EcR-F645A* (herein referred to as *UAS-EcR-DN*) were provided by L. Cherbas. The *UAS-gapGFP* stock was acquired from the Bloomington Stock Center. The *pumpless-Gal4* (*ppl-Gal4*) and *cg-Gal4* stocks were provided by M. Pankratz and C. Dearolf, respectively. A stock carrying both the *cg-Gal4* driver and the *UAS-gapGFP* responder was generated and maintained over a second chromosome balancer marked by *CyO* (*cg-Gal4*, *UAS-gapGFP/CyO*; abbreviated in the text as *cg-Gal4/CyO*). This stock was then crossed to the *UAS-EcR-DN* homozygous responder generating two genetically distinct sets of offspring: *UAS-EcR-DN/cg-Gal4*, *UAS-gapGFP* (abbreviated in the text as *UAS-EcR-DN/cg-Gal4*) and *UAS-EcR-DN/CyO*. We were able to distinguish between these two siblings by the expression of green fluorescent protein (GFP). Offspring of the genotype *UAS-EcR-DN/cg-Gal4*, *UAS-gapGFP* expressed GFP while the *UAS-EcR-DN/CyO* offspring, which do not have the *cg-Gal4*, *UAS-gapGFP* transgenes, did not. Three types of control animals were used in this study: the two parental lines, *cg-Gal4*, *UAS-gapGFP/CyO* (abbreviated in the text as *cg-Gal4/CyO*) and homozygous *UAS-EcR-DN*, as well as the *CyO* siblings from the cross, i.e., *UAS-EcR-DN/CyO*. These three controls were compared to the experimental animals (*UAS-EcR-DN/cg-Gal4*). A summary of *Gal4* fly stocks can be found in Table 1. A list of *UAS* responder stocks used here and by others (Cherbas et al., 2003; Colombani et al., 2005) can be found in Table 2, along with a list of phenotypes that result when these responders are crossed to the three *Gal4* stocks (*Lsp2-Gal4*, *cg-Gal4*, and *ppl-Gal4*).

2.2. Microscopy

Wandering third-instar larvae expressing *UAS-GFP* under control of the *pumpless-Gal4* (*ppl-Gal4*) driver were dissected in 1X DPBS (52 mM NaCl; 40 mM KCl; 10 mM Hepes; 1.2 mM $MgSO_4$; 1.2 mM $MgCl_2$; 2 mM Na_2HPO_4; 0.4 mM KH_2PO_4; 1 mM $CaCl_2$; 45 mM sucrose; 5 mM glucose, pH 7.2). Fluorescence imaging was carried out in the School of Life Sciences Imaging Center using a Zeiss Axioplan 2 microscope with Zeiss Axiovision software. The images were compiled in Corel Draw®.

Animals expressing *EcR-F645A* (*UAS-EcR-DN/cg-Gal4*) and control animals (*cg-Gal4/CyO*) were collected at puparium formation as white prepupae. Animals were placed on wet filter paper in a Petri dish and incubated at 25 °C for 90 h. Aged pupae were examined by light microscopy on a Zeiss Stemi 2000-C microscope. A Canon A620 digital camera and Canon Zoom Browser EX software were used to procure the images. The images were compiled in Corel Draw®.

2.3. Dry weights

Ten to twelve animals of each genotype were collected as white prepupae and dried over night at 60 °C. Dried animals were weighed individually on a Cahn C-30 microbalance.

2.4. Protein, glycogen and triglyceride content

Animals were collected at puparium formation as white prepupae and either frozen immediately or placed on wet filter paper in a Petri dish and allowed to develop at 25 °C for 90 h after puparium formation (APF). Two animals for each stage and genotype were homogenized in a total of 60 μL of lysis buffer (1% NP-40, 0.5% deoxycholic acid, 0.1% Triton-X 100, 100 mM NaCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.6). Hydrolases were heat killed by incubating at 70 °C for 5 min followed by centrifugation at 12,000 g for 2 min. Supernatants were diluted as necessary in lysis buffer.

Triacylglyceride levels were measured using a serum triglyceride kit (Sigma Chemical Co., St. Louis, MO, USA). Protein levels were

Table 1
Gal4 driver fly stocks.

Gal4 line	Spatial expression pattern	Temporal expression pattern	References
Lsp2-Gal4	Fat body-specific	Expression starts at third instar continuing throughout development	Cherbas et al., 2003
cg-Gal4	Fat body- and hemocyte-specific	Expression starts at first instar continuing throughout development	Asha et al., 2003 Evans et al., 2009
ppl-Gal4	Fat body, midgut, proventriculus, and salivary gland	Expression begins at third instar. Fat body expression ends 0–2 h after puparium formation (N.B., unpublished results). Expression in salivary gland, midgut and proventriculus continues throughout development until the tissue is histolyzed (N.B., unpublished results)	Fig. 1

Table 2
UAS responder fly stocks.

UAS line	Abbreviation	Construct information	Result with *Lsp2-Gal4*	Result with *cg-Gal4*	Result with *ppl-Gal4*
UAS-EcR-F645A	*UAS-EcR-DN*	Dominant-negative from of the EcR heterodimer partner of the 20E receptor. Generated by point mutation in the ligand binding domain. Expression of this construct results in competition with the wild-type 20E receptor leading to a severe block in 20E mediated transcription (Cherbas et al., 2003)	Pupal lethality (Cherbas et al., 2003). No observed defects in pupariation (N.B., unpublished results).	Pupal lethality (Fig. 2). No observed defects in pupariation (Fig. 3). No defects in energy storage or utilization (Figs. 4–6).	Third-instar larval lethality.
UAS-EcRi^{104}	*UAS-EcRi*	A dsRNA composed of a 663 base pair fragment which is common to all EcR isoforms. Expression of this construct invokes the RNA interference machinery and silences the EcR heterodimer partner leading to a block in 20E-mediated transcription (Colombani et al., 2005).	Pupal lethality; no observed defects in pupariation (N.B., unpublished results).	Pupal lethality; no observed defects in pupariation (N.B., unpublished results).	Larger size at pupariation (Colombari et al., 2005). Pupal lethality (N.B., unpublished results).

quantified using the bicinchoninic acid (BCA) method (Smith et al., 1985), with bovine serum albumin used as a standard. Glycogen was digested with *Rhizopus* amyloglucosidase (Sigma) and glucose levels were quantified using a blood glucose kit (Pointe Scientific, Canton, MI, USA).

2.5. Metabolic rates

Animals were collected at puparium formation as white prepupae and placed on wet filter paper in a Petri dish and allowed to develop at 25 °C. Pupal metabolic rates were measured using flow-through respirometry (Gibbs and Matzkin, 2001; Gibbs et al., 2003). Groups of 5 pupae from each stage/genotype were transferred to a 1 ml glass-aluminum respirometry chamber. Dry CO_2-free air was pumped through the chamber at 50 mL/min to a Li-Cor LI-6262 infrared CO_2 sensor (Li-Cor Biosciences, Lincoln, NE, USA). Metabolic rates were calculated from the release of CO_2 into the air stream. Data acquisition and analysis were performed using Datacan V software (Sable Systems, Las Vegas, NV, USA).

3. Results

3.1. Disruption of 20E signaling in the fat body results in pupal lethality

Disruption of 20E signaling in the fat body was achieved using the *Gal4*/*UAS* system (Brand and Perrimon, 1993) to express *EcR-F645A*, a dominant-negative allele of *EcR* (Cherbas et al., 2003). *EcR* is a subunit of the active, heterodimeric 20E receptor (Koelle et al., 1991). We utilized three *Gal4* drivers, *Lsp2-Gal4*, *ppl-Gal4* and *cg-Gal4* (Table 1) to drive expression of *UAS EcR-F645A* (herein referred to as *UAS-EcR-DN*). *cg-Gal4* is expressed in the fat body and hemocytes beginning in the first larval instar and persists through pupal development (Asha et al., 2003; Evans et al., 2009). The *Lsp2-Gal4* transgene is fat body-specific, and expression is detected early in the third-larval instar and continues throughout pupal development (Cherbas et al., 2003; Nelliot et al., 2006). The *ppl-Gal4* transgene is described as a fat body-specific driver in the larva (Buch et al., 2008) that recapitulates the expression of the endogenous gene which is normally restricted to the fat body (Zinke et al., 1999). We found that expression of the *ppl-Gal4* driver was not exclusive to the larval fat body (Fig. 1) and that expression of *ppl-Gal4* in the fat body did not persist into the pupal stage (N.B., unpublished results).

Disruption of 20E signaling in the fat body and hemocytes throughout larval development via expression of *UAS-EcR-DN* using *cg-Gal4* or *Lsp2-Gal4* resulted in late pupal lethality (Fig. 2, Table 2). These results are in agreement with those of Cherbas et al. (2003), in which disruption of 20E signaling specifically in the fat body starting at the third-larval instar (by expression of *UAS-EcR-DN*; *Lsp2-Gal4*) also caused pupal lethality. Expression of *EcR-DN* directed by *ppl-Gal4* also caused lethality, but this occurred much earlier, during the larval stages (Table 2). Because *ppl-Gal4* expression is not restricted to the larval fat body (Fig. 1), the larval lethality phenotype cannot be attributed to loss of 20E signaling in the fat body alone. It is likely that blocking 20E signaling in tissues other than the fat body (see Fig. 1 for examples) is the cause of the larval lethality observed in *ppl-Gal4*/*UAS-EcR-DN* animals.

From these data we concluded that 20E signaling in the fat body is essential for pupal development but is not required for larval development. Based on these results, we set out to determine how 20E signaling in the fat body affects pupal development.

3.2. Blocking 20E signaling in the fat body does not affect animal size

During the pupal stages vast structural changes occur. To ensure nutrients are acquired and utilized appropriately, metabolism should be tightly regulated during the larval and pupal stages. Because the larval fat body is the central site for energy storage, this tissue is also likely to play a role in coordinating nutrient acquisition and

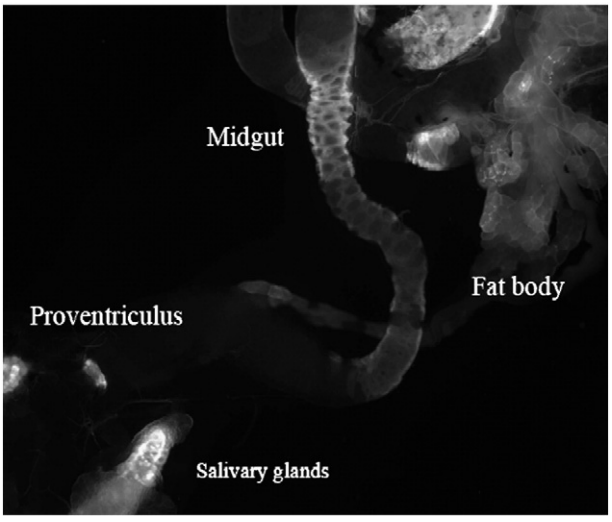

Fig. 1. *ppl-Gal4* expression is not fat body-specific. Third-instar larva expressing *ppl-Gal4*/*UAS-GFP* was dissected and photographed using fluorescent microscopy. *ppl-Gal4* directed expression of GFP in many tissues, including the proventriculus, the salivary gland, the midgut, and the fat body.

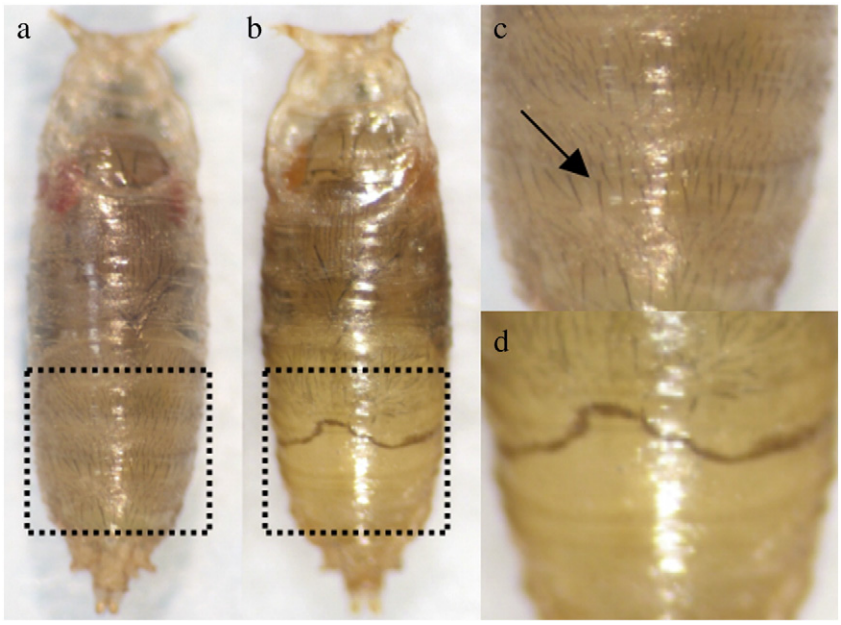

Fig. 2. 20E signaling is required in the fat body for pupal survival. (a,c) Control (*cg-Gal4/CyO*) pupa at 90 h APF with abdominal bristles highlighted by black outline (inset magnified in c, arrow indicating an abdominal bristle). (b,d) Experimental animal (*UAS-EcR-DN/cg-Gal4*) imaged at 90 h APF. Abdominal bristles did not develop and the pupa died before eclosion. Abdominal area lacking bristles is highlighted by black outline (inset magnified in d).

utilization. Thus, in searching for a fat body-specific process that might be necessary for animal survival, we tested the role of 20E signaling in several aspects of larval and pupal energy metabolism.

We first tested the hypothesis that 20E signaling in the fat body is necessary for proper nutrient accumulation during larval development. If blocking 20E signaling in the fat body prevents proper nutrient accumulation, we reasoned that the pupae could starve and die during metamorphosis. If animals were indeed deficient in their ability to accumulate nutrients we expected animals to be smaller in size at the end of larval development. Pupariation marks the end of larval development, therefore experimental animals (*UAS-EcR-DN/cg-Gal4*), sibling controls (*UAS-EcR-DN/CyO*) and parental controls (*cg-Gal4/CyO* and *UAS-EcR-DN*) were collected at pupariation and the dry weights of the animals were determined (Fig. 3). A statistically significant difference among genotypes was found ($F_{3,41} = 6.41$, $P < 0.002$), but this was due to the dry weights of the sibling controls (*UAS-EcR-DN/CyO*), which were greater than one of the parental controls (*cg-Gal4/CyO*) and the experimental animals (*UAS-EcR-DN; cg-Gal4*) (Tukey post-hoc test, $P < 0.01$ for both comparisons). The dry weights of the sibling controls (*UAS-EcR-DN/CyO*), however, did not differ from the dry weights of the other parental control genotype (*UAS-EcR-DN*). A Tukey post-hoc test revealed the dry weights of experimental animals (*UAS-EcR-DN/cg-Gal4*) did not differ from those of either parental control (*cg-Gal4*/CyO and *UAS-EcR-DN*). From these data we concluded that 20E signaling in the fat body does not affect the size of the animal.

3.3. Disruption of 20E signaling in the fat body does not affect energy storage

Although 20E signaling within the fat body did not regulate final larval size, it might be involved in the accumulation of macronutrients that are stored within the fat body. To determine the effect of 20E signaling on the accumulation of macronutrients, we disrupted 20E signaling and measured total protein, glycogen (carbohydrate), and triglycerides at pupariation (Fig. 4). We did not observe a significant difference in glycogen or protein in experimental animals compared to controls ($F_{3,28} = 1.14$, $P > 0.35$ for carbohydrates; $F_{3,28} = 1.31$, $P > 0.29$ for protein). We did, however, detect a significant difference in triglyceride (TG) levels among the groups ($F_{3,28} = 4.25$, $P < 0.02$). A Tukey post-hoc test indicated that this difference was due to the *UAS-EcR-DN* parental control, which had less TG than the other controls (*cg-Gal4/CyO* and *UAS-EcR-DN/CyO*, $P < 0.03$ for both comparisons).

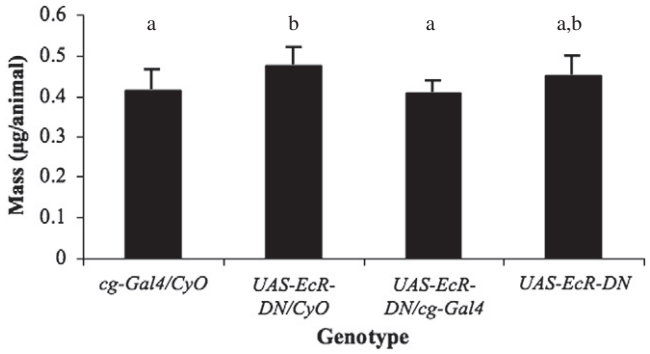

Fig. 3. 20E signaling in the fat body does not determine animal size at pupariation. Animals expressing *EcR-DN* (*UAS-EcR-DN/cg-Gal4*) in the fat body had similar dry weights to control parental control animals (*cg-Gal4/CyO* and *UAS-EcR-DN*), see text for further explanation. Treatments with the same letter above the bar did not differ significantly.

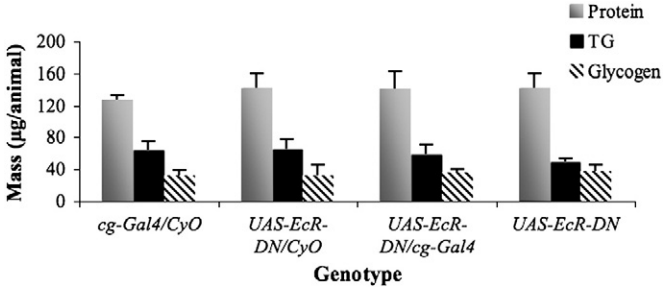

Fig. 4. 20E signaling in the fat body is not required for accumulation of energy stores. Animals were collected at pupariation and protein, triglyceride (TG) and glycogen levels were determined. Experimental animals (*UAS-EcR-DN/cg-Gal4*) accumulated similar amounts of energy stores to controls (*cg-Gal4*, *UAS-EcR-DN/CyO*, and *UAS-EcR-DN*). See text for further explanation.

Experimental animals (*UAS-EcR-DN/cg-Gal4*) did not differ in triglyceride content from any of the controls. From these data we concluded that 20E signaling in the larval fat body was not required for nutrient accumulation.

3.4. Inhibition of 20E signaling in the larval fat body does not affect pupal energy utilization

The results thus far suggest that pupal lethality was not due to defects in larval energy storage. We therefore tested the hypothesis that 20E signaling affects pupal energy consumption. Metabolic rates of holometabolic insects, including *D. melanogaster*, are high at the beginning and end of metamorphosis but low in between, thus forming a U-shaped curve (Wolsky, 1938; Hetz, 2007; Kaiser et al., 2010). This pattern might be an important mechanism for ensuring sufficient energy stores to support metamorphosis. We hypothesized that 20E signaling might be required to reduce metabolism during the middle stages of metamorphosis, and therefore blocking 20E signaling in the fat body would prevent the reduction in metabolic activity. This could lead to rapid consumption of resources resulting in pupal death by starvation. An alternative hypothesis is that 20E signaling in the fat body is necessary for utilization of fat body nutrients during the early or late stages of metamorphosis. In this case, the absence of 20E signaling could result in a decrease in metabolic activity during early or late metamorphosis, thus causing pupal lethality.

We tested whether 20E signaling regulates pupal metabolism using flow-through respirometry. We found that disruption of 20E signaling in the fat body had no effect on pupal metabolic rates (Fig. 5). Metabolic rates initially decreased, then increased at 90 h after puparium formation (APF). Age had a highly significant effect on metabolic rate ($F_{2,58} = 49.3$, $P < 10^{-6}$), but genotype did not ($F_{3,58} = 1.55$, $P > 0.2$), nor was there a significant interaction between age and genotype ($F_{6,58} = 0.95$, $P > 0.4$). From these data we concluded that 20E signaling in the fat body does not regulate pupal metabolic rate.

Our data showed that 20E signaling did not affect the macronutrient content of animals entering metamorphosis (Fig. 4) or pupal metabolism, as indicated by CO_2 release (Fig. 5). These results suggest that pupal lethality was not caused by a restriction in overall metabolism. However, disruption of 20E signaling might alter the fat body's ability to utilize each macronutrient (TG, glycogen, and protein) properly during metamorphosis. Perhaps, for example, disruption of 20E signaling in the fat body results in an inability to utilize proteins during metamorphosis. Such a metabolic defect could feasibly result in developmental defects and pupal lethality. Thus, we tested whether 20E signaling regulates consumption of specific macronutrients by measuring macronutrient content at 90 h APF (Fig. 6). We found no evidence of preferential utilization of protein, glycogen, or TG in experimental animals (*UAS-EcR-DN/cg-Gal4*). Control and experimental animals contained the same amounts of each of the three macronutrients at 90 h APF ($F_{3,24} = 2.96$, 2.79 and 1.87 for TG, glycogen and protein, respectively, $P > 0.05$ for all three macronutrients). Therefore, we concluded that 20E signaling does not affect macronutrient utilization patterns during metamorphosis.

To summarize, we found that 20E signaling in the larval fat body was not necessary for larval nutrient acquisition, size attainment, pupal metabolic activity or pupal macronutrient utilization. Thus, 20E signaling did not affect energy storage or energy consumption, and the pupal lethality associated with disruption of 20E signaling in the fat body was not caused by failure to store nutrients or utilize them correctly.

4. Discussion

Previous studies have implicated 20E signaling in the fat body as a key component in size control. Because the final animal size is determined at puparium formation, growth rate and length of growth period during the larval stage dictate adult animal size (Mirth and Riddiford, 2007). As the primary tissue involved in energy storage, the fat body is likely a key factor in size determination. Colombani et al. (2005) blocked 20E signaling in the fat body and found that this resulted in larger prepupae. In contrast, our data indicate that blocking 20E signaling in the fat body does not alter the size at pupariation (Fig. 3).

The disagreement between our results and those of Colombani et al. (2005) can be explained by differences in how 20E signaling was manipulated. Colombani et al. (2005) used a *Gal4* driver derived from the fat body-specific gene *pumpless* (*ppl-Gal4*) to direct expression of an RNA interference construct specific to the *EcR* transcript (*UAS-EcRi*) (see Tables 1 and 2 for details). In contrast, we employed a different driver, *cg-Gal4* (expressed in hemocytes and fat body) to drive expression of a dominant negative form of the 20E receptor, *UAS-EcR-DN*. The different responders could explain the disparate results. Phenotypes can differ between knock-down via RNA interference versus expression of a dominant–negative allele. However, we found that employing the *cg-Gal4* driver to express *UAS-EcRi* resulted in a phenotype consistent with our results with *UAS-EcR-DN*, i.e., animals pupariated normally with no visible size defects, but exhibited late pupal lethality (N.B., unpublished results). Thus, differences between responders do not explain the differences between the results reported here and by Colombani et al. (2005). Instead, it is likely

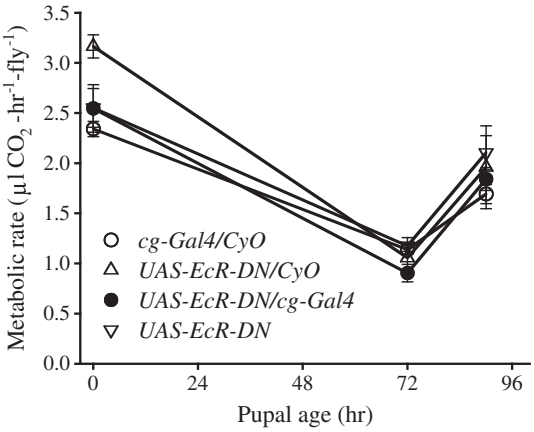

Fig. 5. 20E signaling in the fat body is not required for regulation of metabolic activity during metamorphosis. Metabolic rates of animals at pupariation (0 h APF), 72 h APF, and 90 h APF were determined. Experimental animals (*cg-Gal4/UAS-EcR-DN*) had similar metabolic rates to control animals (*cg-Gal4/CyO*, *UAS-EcR-DN*, and *UAS-EcR-DN/CyO*) at all three time points.

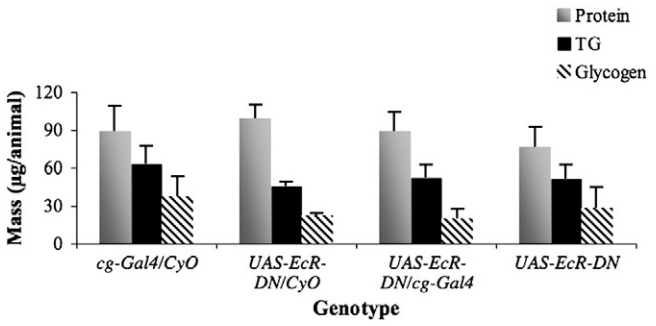

Fig. 6. 20E signaling in the fat body is not required for utilization of energy stores during metamorphosis. Animals were collected at pupariation and incubated at 25 °C for 90 h. Protein, triglyceride (TG) and glycogen levels of the staged animals were determined. Experimental animals (*UAS-EcR-DN/cg-Gal4*) utilized similar amounts of protein, triglyceride (TG) and glycogen to control animals (*cg-Gal4*, *UAS-EcR-DN/CyO*, and *UAS-EcR-DN*).

that the expression of *ppl-Gal4* in tissues other than the fat body (Fig. 1) produces the size defects observed by Colombani et al. (2005). Future studies focused on the other *ppl-Gal4* expressing tissues might delineate the specific tissues in which 20E signaling is required for animal size control.

It is not clear why blocking 20E signaling in the fat body causes pupal lethality. Work by Colombani et al. (2003) suggesting cross-talk between 20E and insulin signaling led us to hypothesize that larval energy storage or energy utilization during metamorphosis might be disrupted in our experimental animals where 20E signaling is disrupted in the fat body. Animals might enter metamorphosis with insufficient energy stores, consume the energy too rapidly and starve, consume it too slowly to produce enough ATP to fuel tissue restructuring, or use the "wrong" type (TG, glycogen, protein) of fuel. Our data do not support these hypotheses. When 20E signaling was blocked in the fat body, larvae pupariated with the same amount and types of energy stores as controls, had normal metabolic rates during metamorphosis, and retained the same energy as controls the end of pupal development (Figs. 4–6). We therefore cannot attribute the pupal lethality to a metabolic defect.

If not a metabolic defect, then why does disruption of 20E signaling in the fat cells kill the pupa? The insect fat body is also an endocrine organ (Hoshizaki, 2005). It is the site for production of growth factors (Kawamura et al., 1999; Martin et al., 2000) and *Drosophila* insulin-like peptides (DILPs) (Okamoto et al., 2009; Slaidina et al., 2009). Recently it has been reported that the insulin-like peptide DILP6 acts as an insulin-like growth factor (IGF) and is required for the growth that occurs post-feeding in *D. melanogaster* (Okamoto et al., 2009; Slaidina et al., 2009). Interestingly, DILP6 expression is highly enriched in the fat body during pupal development, and its expression requires 20E (Okamoto et al., 2009; Slaidina et al., 2009). Okamoto et al. (2009) and Slaidina et al. (2009) propose that DILP6 functions to balance energy allocation during pupal development. In DILP6 mutants, growth of the imaginal (adult) tissues is sacrificed while carbohydrate stores are preserved (Okamoto et al., 2009; Slaidina et al., 2009). Specifically, the DILP6 mutant adult is smaller and is associated with a decrease in cell number and a reduction in the density of wing hair cells (Okamoto et al., 2009). In light of these findings, it is plausible that blocking 20E signaling in the fat body could cause a decline in DILP6 expression during pupal development ultimately resulting in growth defects such as the abdominal bristle phenotype we observed when 20E signaling was blocked in the fat body (Fig. 2).

To summarize, we found that 20E signaling in the larval fat body was not necessary for either larval nutrient acquisition or size attainment, nor did it affect overall pupal metabolism or pupal macronutrient utilization. Thus, our data suggest that the pupal lethality associated with disruption of 20E signaling in the fat body is not caused by a failure to store nutrients or utilize them correctly. Further studies will be required to understand the cross-talk between 20E and insulin signaling in the pupa.

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