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Article

20-hydroxyecdysone Reduces Insect Food Consumption Resulting in Fat Body Lipolysis During Molting and Pupation

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The insect steroid hormone 20-hydroxyecdysone (20E) acts through a specific nuclear receptor complex, ecdysone receptor (EcR) and ultraspiracle (USP). EcR and USP are FXR/LXR and RXR orthologs, respectively, which play critical roles in the regulation of lipid metabolism in mammals. Lipid concentration in *Bombyx* hemolymph and lipase activity in fat body peaked during molting and pupation, suggesting that 20E induces lipolysis at these stages. Differing from their mammalian orthologs, the 20E-bound EcR–USP was not able to directly stimulate fat body lipolysis in both *Bombyx* and *Drosophila*. Instead in *Bombyx*, 20E slowly reduced food consumption and then induced starvation, resulting in fat body lipolysis. Molecular analysis revealed that the evolutionarily conserved adipose triacylglycerol lipase gene *Brummer* was transcriptionally up-regulated by 20E-induced starvation during molting and pupation. To our knowledge, this is the first report demonstrating that the steroid hormone 20E is a critical regulator of lipolysis in insects.

Keywords: 20-hydroxyecdysone, starvation, lipolysis, *Brummer*, EcR, USP, fat body, *Bombyx mori*, *Drosophila melanogaster*

Introduction

Animals living in the wild often encounter periods of time when the food source is limited. To cope, when food is plentiful, animals have evolved a mechanism to store surplus energy as fat in specialized tissues, such as white adipose tissue in mammals and fat body in insects. Conversely, when food is scarce, stored fat is broken down and mobilized as an energy source. In mammals, lipid breakdown (lipolysis) is strictly under hormonal and nutritional regulation (Duncan et al., 2007). The process of lipolysis is mainly catalyzed by two lipases: the hormone-sensitive lipase (HSL) and the adipose triglyceride lipase (ATGL). HSL is positively regulated by catecholamines and the peptide adrenocorticotropic hormone glucagon (Holm et al., 2000), while ATGL accounts for basal and most of HSL-independent lipolytic activity (Zimmerman et al., 2009).

In insects, the fat body serves as an energy storage organ and is equivalent to mammalian adipose tissue and liver. Despite dramatic morphological and physiological differences between insects and mammals, both possess evolutionarily conserved mechanisms for the hormonal and nutritional regulation of lipolysis (Arrese and Soulages, 2009; Liu et al., 2009a, b). Similar to mammalian glucagon in both structure and function (Géminard et al., 2006), the insect neuropeptide adipokinetic hormone (AKH) is considered the major hormone that mobilizes stored lipids in the fat body, especially during flight (Gäde and Auerswald, 2003; Van der Horst, 2003). Direct increases in fat body lipase activity by AKH treatment have been observed in a number of insect species (Arrese and Wells, 1997; Auerswald et al., 2005; Auerswald and Gäde, 2006). Moreover, AKH activates lipolysis when ectopically expressed in *Drosophila* larval fat body, thus producing a lean fly phenotype (Lee and Park, 2004; Grönke et al., 2007). On the other hand, when *Drosophila* AKH-producing cells (corpora cardiaca, CC) are genetically ablated, adult flies are severely obese (Kim and Rulifson, 2004). In line with this observation, AKH receptor (*akhr*) mutant flies lack the ability to mobilize fat and share a similar obesity phenotype to the CC-ablated flies (Grönke et al., 2007; Bharucha et al., 2008). In the tobacco hornworm, *Manduca sexta*, AKH activates cAMP-dependent protein kinase (PKA) via its receptor AKHR, which upon activation phosphorylates a lipid droplet protein Lsd1 and thus enhances lipolysis in the fat body (Patel et al., 2005). A major fat body triacylglycerol

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(TAG) lipase, phospholipase A, accounts for AKH-induced lipolysis in *Manduca* and possibly other insects (Arrese et al., 2006). Meanwhile, orthologs of mammalian ATGL are important in regulating insect lipolysis, especially during starvation. In *Drosophila*, the ATGL gene *Brummer* was identified through genome-wide screening for nutritionally regulated genes (Zinke et al., 2002). Brummer undertakes the basal lipolytic activity, and functions in parallel with AKH to regulate *Drosophila* lipolysis (Grönke et al., 2005). Starvation-induced lipolysis appears to be independent of AKH but largely relies on Brummer (Grönke et al., 2007).

In mammals, ligand-regulated nuclear receptors play important roles in metabolism, development and reproduction. Farnesoid X receptor (FXR) and liver X receptor (LXR) are nuclear receptors associated with lipid metabolism. Each liganded FXR or LXR forms a heterodimer with the retinoid X receptor (RXR) in the nucleus and directly regulate gene transcription. Studies have shown that the plasma concentration of TAG and cholesterol increase in *Fxr*-deficient mice and the transcription level of liver lipogenic genes decreases in *Lxr* null mice (Chawla et al., 2001; Francis et al., 2003; Beaven and Tontonoz, 2006; Lee et al., 2006). In insects, the nuclear ecdysone receptor (EcR) and ultraspiracle (USP) are mammalian FXR/LXR and RXR orthologs, respectively (King-Jones and Thummel, 2005). The active form of molting hormone 20-hydroxyecdysone (20E) is a classical insect steroid hormone and acts through EcR–USP. The 20E–EcR–USP complex induces the 20E-triggered transcriptional cascade, which controls a variety of developmental and physiological events during insect molting and metamorphosis (Riddiford et al., 2003; Palli et al., 2005). Apart from the essential roles played by EcR–USP in development and reproduction, it is still unclear whether the regulatory mechanisms of FXR/LXR and RXR on metabolism are conserved in insects (King-Jones and Thummel, 2005). Microarray analysis of gene expression changes during *Drosophila* metamorphosis revealed that a large number of genes in basic metabolic processes, including glycolysis, the citric acid cycle, oxidative phosphorylation and amino acid, fatty acid and glycogen metabolism, were down-regulated during the larval–pupal transition (White et al., 1999). A recent study identified many genes that not only respond to 20E during the larval–pupal transition, but also rely on EcR for transcriptional regulation (Beckstead et al., 2005). Among them, over 200 are regulated during starvation (Zinke et al., 2002).

We hypothesized that the 20E–EcR–USP complex acts through two different mechanisms to regulate insect metabolism in the fat body: first, it directly induces its gene transcription; second, it acts on other tissues and then indirectly induces its gene transcription. In this study, we demonstrate that 20E activates lipolysis in the larval fat body of the silkworm, *Bombyx mori*, by inducing a condition equivalent to starvation during molting and pupation.

Results

Fat body lipolysis is activated during molting and pupation

To investigate the relationship between 20E and fat body lipid metabolism, hemolymph lipid concentration and fat body lipid content of the silkworm larvae were measured at different developmental stages. Remarkably, hemolymph lipid concentration peaked during each larval molt and at pupation. Conversely, it was undetectable during the feeding period of the third and fourth larval instars. In addition, a small peak was also observed on Days 5–6 of the fifth larval instar, just prior to the larval–pupal transition (Figure 1A). The fat body lipid content was low around the fourth larval molt. During the fifth instar, fat body lipid content increased gradually with feeding, reached a maximum on Day 6, and then decreased during the wandering and pupation stages (Figure 1B). This measurement was verified by BODIPY staining of the dissected fat body. In general, lipid droplets in the fat body were small, numerous and intensively stained during the feeding stages (Figure 1C, E and F) but relatively larger, more limited in number, and less stained during the molting (Figure 1D), wandering (Figure 1G) and pupation stages (Figure 1H). It is important to note that an increase in hemolymph lipid concentration in *Bombyx* during molting and pupation coincides with a decrease in fat body lipid content. In insects, more than 90% of hemolymph lipid is diacylglycerol (DAG), the hydrolysis product of fat body TAG (Arrese and Wells, 1997). As such, the increase in hemolymph lipid concentration observed in molting and pupation should be a direct consequence of lipid mobilization in the fat body, which might be possibly caused by high levels of 20E at these stages.

20E does not affect lipolysis in cultured fat body

It has been well documented that 20E is responsible for the initiation of insect molting (Zitnan et al., 2007). The timing of the developmental changes of lipid levels in the *Bombyx* hemolymph and fat body suggested that 20E induced lipolysis during molting and pupation. Signaling through the action of 20E in the larval fat body was monitored by measuring the mRNA level of the 20E primary response gene *E74A*. As expected, peaks of *E74A* expression were prior to the fourth larval molt and during the prepupal stage (Figure 2A). Consequently, an *in vitro* assay was devised to determine whether 20E had a direct effect on lipid mobilization. Isolated fat body from Day 2 of fifth instar larvae was cultured with varying concentrations of 20E. The concentration of lipid secreted into culture medium (Figure 2B and C) and fat body lipase activity (Figure 2D and E) were measured at 6 and 24 h after hormone treatment, and no significant changes were observed. The data showed that there exists no significant dose–response relationship or time-course effect between 20E and fat body lipid mobilization.

EcR-deficiency has little effects on lipid metabolism in *Drosophila* fat body

As 20E did not directly promote lipolysis in the *Bombyx* fat body, we questioned whether this was a general principle among insects. Using *Drosophila*, 20E was found not to affect lipolysis in cultured *Drosophila* fat body as well (data not shown). Since the *in vitro* assay in *Drosophila* supported the finding in *Bombyx*, *Drosophila* genetic approaches were then used to

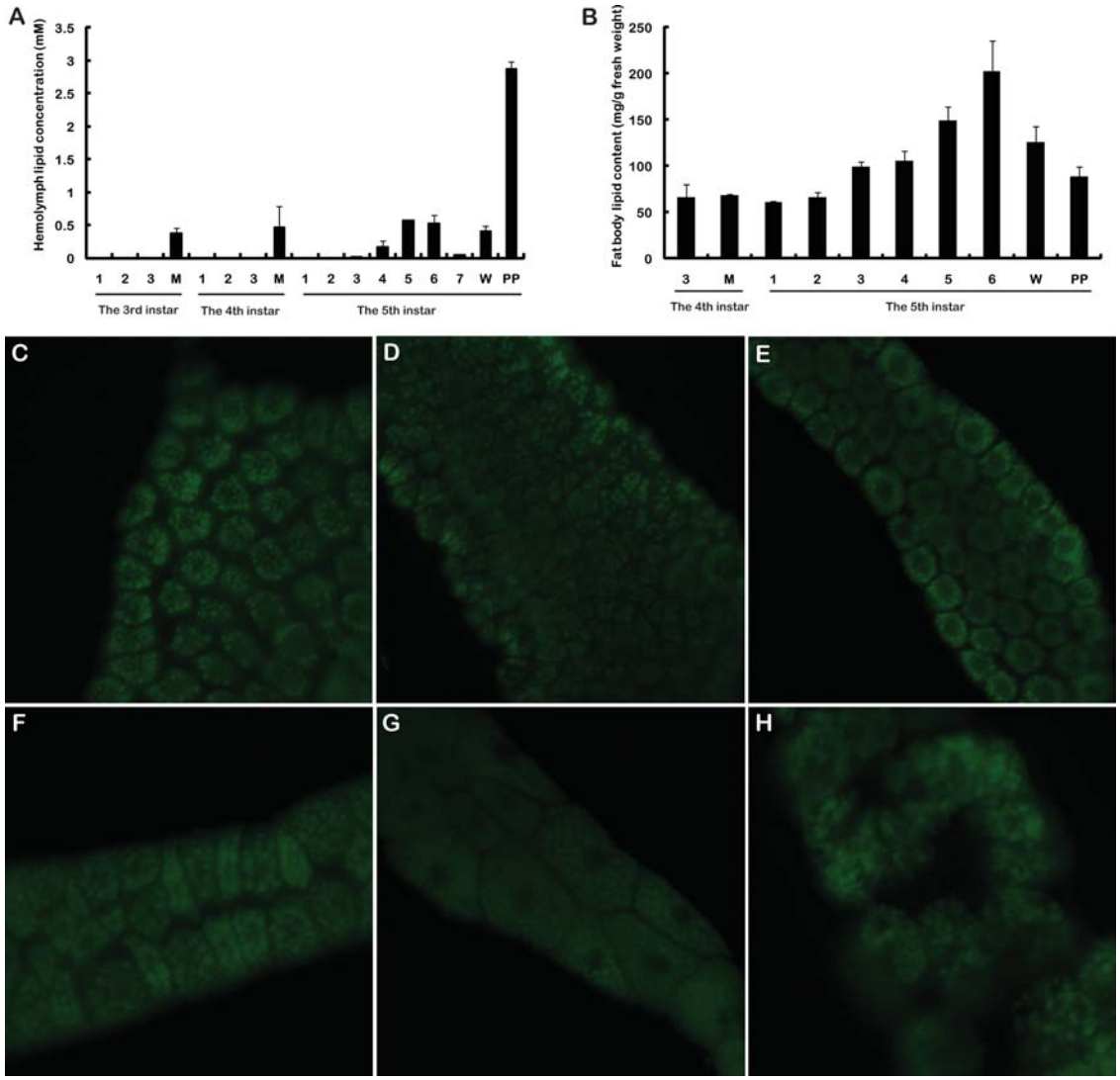

Figure 1 The developmental profiles of hemolymph lipid concentration and fat body lipid content in *Bombyx*. (**A**) Variation in hemolymph lipid concentration at each day during larval development. The fourth larval molt (M), the wandering stage (W), the pupation stage (PP). Error bar represents SD of three independent replicates in this and all subsequent figures. (**B**) Developmental profile of fat body lipid content during larval development. BODIPY stained lipid droplets in dissected larval fat body: (**C**) Day 2 of fourth instar; (**D**) the fourth larval molt; (**E**) Day 2 of fifth instar; (**F**) Day 6 of fifth instar; (**G**) wandering; and (**H**) pupation.

study if the nuclear receptor EcR directly affects fat body lipid metabolism. First, the binary UAS–GAL4 system was used to specifically overexpress a dominantly negative form of *EcR* (EcR^{F654A}) in the larval fat body. Total lipid content of wandering larvae, white prepupae and pupae (24 h after pupation) was measured. No significant difference in lipid content was found between animals bearing the driver control *Lsp2>* and those with fat body overexpressing EcR^{DN} (*Lsp2* > *UAS-*EcR^{DN}) (Figure 3A). Second, we used the FLP-out method to generate fat body cells overexpressing dsRNA against the common region of *EcR*. FLP-out mosaic clones showed that, at the wandering stage, fat body cells overexpressing *EcR*-dsRNA had no significant visual difference in fat storage when compared with neighboring wild-type cells (Figure 3B and B′). These results suggest that, in both *Bombyx* and *Drosophila*, 20E and its receptor complex EcR–USP are not directly involved in fat body lipid metabolism.

Starvation rapidly induces fat body lipolysis

Starvation is an energetic stress condition where lipid mobilization is dramatically promoted. On the basis of the fact that feeding behavior ceased during larval molting and larval–pupal transition, we studied the effect of starvation on lipolysis in *Bombyx* larval fat body. On Day 2 of fifth instar, larvae were starved; hemolymph lipid concentration, fat body lipid content and lipase activity were then measured 6, 12 and 24 h after starvation. The hemolymph lipid concentration increased dramatically in starved larvae up to 70-fold after 24 h of starvation. In feeding larvae, the lipid concentration remained at the same level (Figure 4A). Meanwhile, a significant decrease (~30%) in fat body lipid content was also found in starved larvae after 24 h of starvation (Figure 4B). Importantly, the fat body lipase was activated within 6 h after food removal, and the lipase activity

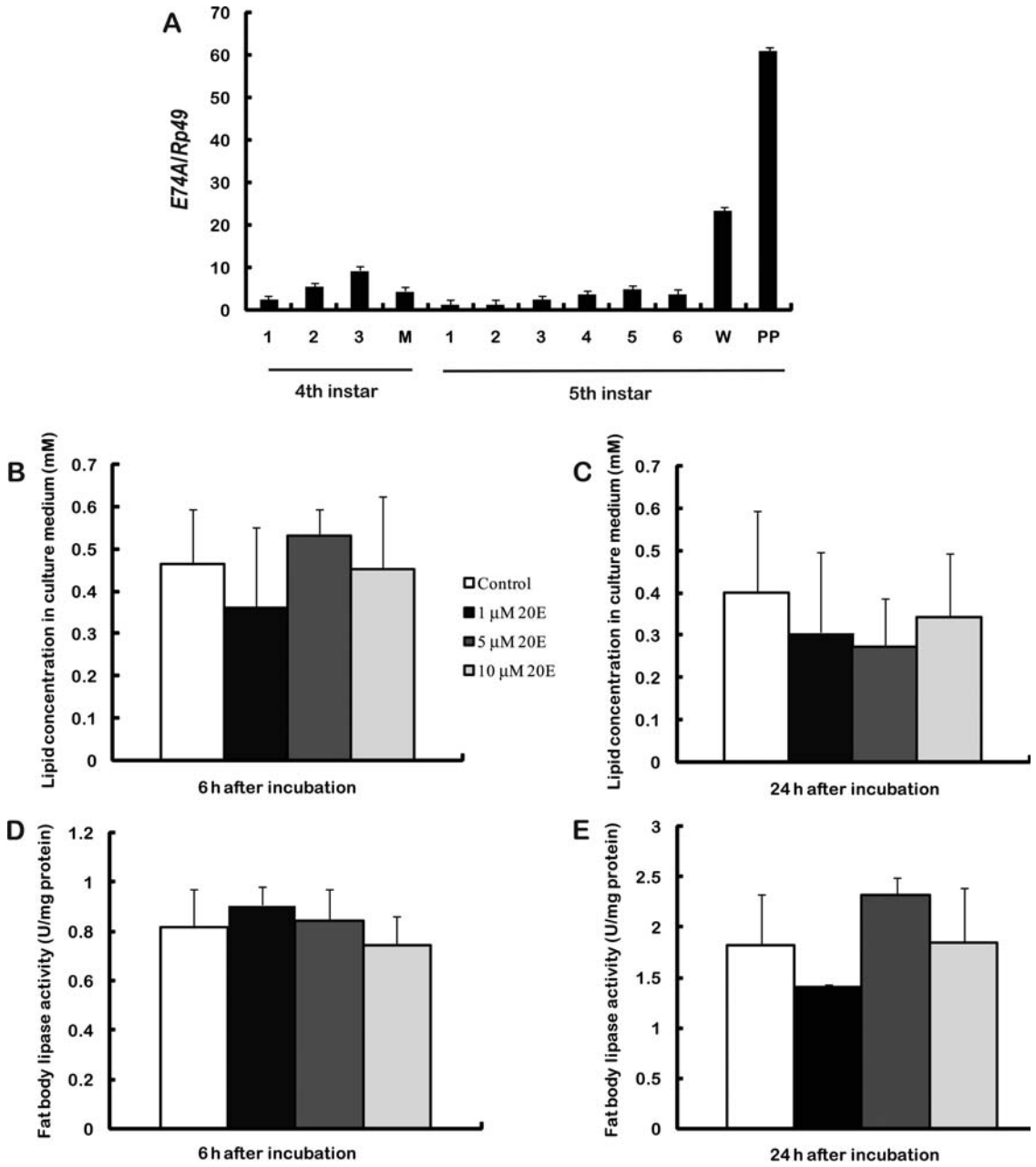

Figure 2 20E does not directly activate lipolysis in cultured fat body. (**A**) 20E primary response gene *E74A* transcripts level in the larval fat body by real-time PCR. *Rp49* is used as an internal control and relative fold change is indicated as *gene*/*Rp49* in this and all subsequent figures. Concentration of lipid released into culture medium after 6 (**B**) and 24 h (**C**). Lipase activity in fat body cultured with different 20E concentrations after 6 (**D**) and 24 h (**E**).

remained high in the starved larvae for the 24 h period assayed (Figure 4C). Similar results were also obtained in *Drosophila* (data not shown). These experiments demonstrated that starvation rapidly induces fat body lipolysis in insects.

20E injection slowly reduces food consumption leading to starvation

Previous studies indicated that 20E inhibited *Bombyx* larval feeding activity when supplied in the food, but this may be due to the presence of a compound(s) that activates a deterrent taste neuron as were noted in many lepidopteran species including *Bombyx* (Tanaka et al., 1994; Marrion-Poll and Descoins, 2002). To bypass oral effects on feeding, 5 μg 20E was directly injected into each *Bombyx* larva on Day 2 of fifth instar and food consumption monitored over time. Approximately 30% decrease in food consumption was observed after 20E injection in the 6–12 h period and food consumption decreased by more than 60% in the following 12–24 h period (Figure 5). Moreover, 24 h after 20E injection, the larvae stopped feeding and wandering behavior initiated. Precocious pupation (metamorphic molting) was never observed by then and never appeared until Days 5–6 of fifth instar. Thus, 20E injection slowly reduces food consumption and then leads to a starvation-like phenotype.

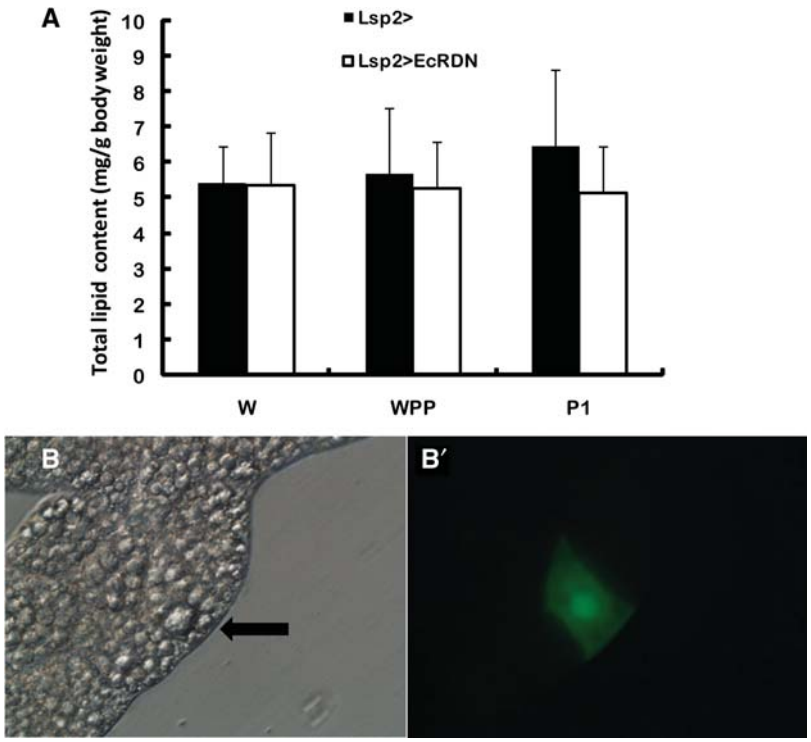

Figure 3 Genetic assays in *Drosophila* demonstrate that EcR does not directly regulate lipid metabolism. (**A**) Measurement of total lipid content in control flies (*Lps2>*) and flies with fat body expressing EcR^{DN} (*Lsp2 > EcRDN*) at wandering (w), white prepupa (wpp) and Day 1 after pupariation (P1). Fat body cell expressing *EcR-dsRNA* generated by Flp-out technique is observed under bright (**B**) (pointed by arrow) or fluorescent field (**B′**) (marked by GFP). No GFP maker is shown in the neighboring wild-type cells.

20E injection slowly induces fat body lipolysis

On the basis of the finding that 20E inhibits feeding behavior and thus induces starvation, we investigated the effect of 20E on fat body lipolysis. On Day 2 of fifth instar, *Bombyx* larvae were injected with 20E as above; the hemolymph lipid concentration, fat body lipid content and lipase activity were then measured 6, 12 and 24 h after 20E injection. In the first 12 h, no significant differences were found in the hemolymph lipid concentration, fat body lipid content and lipase activity between control and 20E injected larvae. Measured at 24 h after 20E injection, hemolymph lipid concentration and fat body lipase activity increased, while fat body lipid content decreased compared with control animals, respectively (Figure 6). These experiments demonstrated that 20E slowly induced *Bombyx* fat body lipolysis. Taken together, we conclude that 20E reduces food consumption and then induces starvation resulting in fat body lipolysis during molting and pupation in *Bombyx*.

Brummer lipase is transcriptionally up-regulated by 20E-induced starvation during molting and pupation

In *Drosophila*, the ATGL gene *Brummer* accounts for the basal and starvation-induced lipolytic activity (Grönke et al., 2005, 2007). Since 20E slowly induces starvation resulting in fat body lipolysis in *Bombyx*, we questioned if a *Brummer* lipase was involved in this process as well. Using a Blastp screen of the *Bombyx* genome, we uncovered a gene with sequence similarity to *Drosophila Brummer*. The corrected cDNA sequence of *Bombyx Brummer* was submitted to GenBank (accession number: GU395971). The amino acids sequences of *Bombyx Brummer* and orthologs from other insect species were aligned (Supplemental Figure S1) and a phylogenetic tree constructed (Figure 7A). The results indicated that the *Bombyx* Brummer lipase was clustered together with *Drosophila* and other insects Brummer lipases, suggesting a conserved function of this protein in *Bombyx*.

The developmental pattern of *Bombyx* fat body *Brummer* transcription showed highest expression levels during molting and pupation (Figure 7B), when hemolymph lipid concentrations were also high (Figure 1A). The tissue distribution pattern of *Bombyx Brummer* transcription was then assessed during the fourth larval molt (Figure 7C) and on Day 2 of fifth instar (Figure 7D). During the non-feeding fourth larval molt, *Brummer* was highly expressed in both fat body and midgut. Differently, on Day 2 of fifth instar when animals are feeding, *Brummer* expression levels dropped to a very low level in the fat body but remained even higher in the midgut. The developmental profile and tissue distribution analyses imply that *Brummer* plays a critical role in fat body lipolysis during molting and pupation in *Bombyx*.

We then determined whether *Brummer* transcription is regulated by starvation and/or 20E in *Bombyx* fat body using Day 2 of fifth instar larvae. Similar to the expression of *Drosophila*

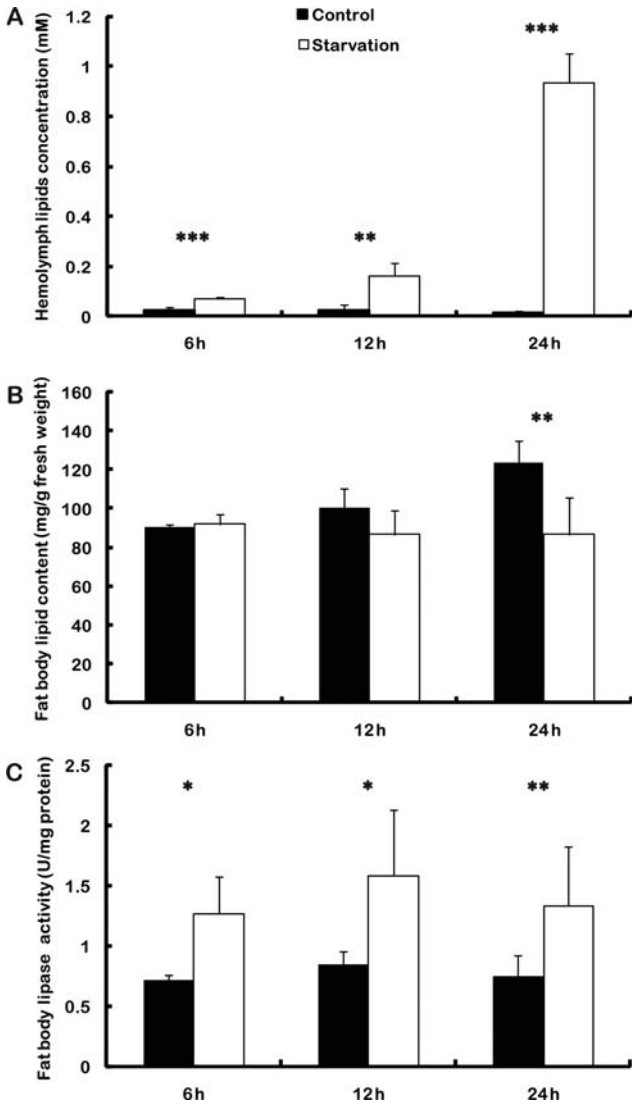

Figure 4 Hemolymph lipid concentration, fat body lipid content and lipase activity of starved *Bombyx* larvae on Day 2 of fifth instar. On Day 2 of fifth instar, larvae are starved; hemolymph lipid concentration (**A**), fat body lipid content (**B**) and lipase activity (**C**) were then measured 6, 12 and 24 h after starvation. Error bars mean (SD). *$0.05 < P < 0.1$; **$0.01 < P < 0.05$; ***$P < 0.01$.

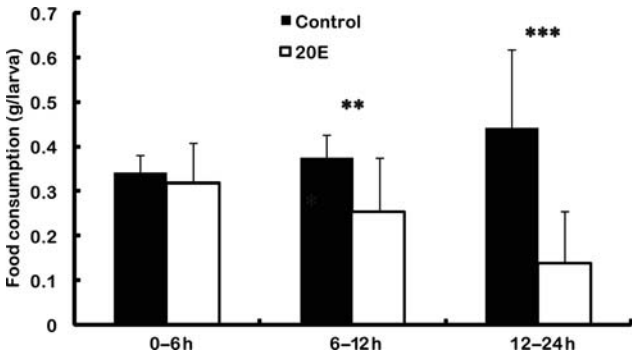

Figure 5 Effect of 20E on feeding activity on *Bombyx* larvae on Day 2 of fifth instar. The food consumption is quantitated as the amount of diet eaten at 0–6, 6–12 or 12–24 h. **$0.01 < P < 0.05$; ***$P < 0.01$.

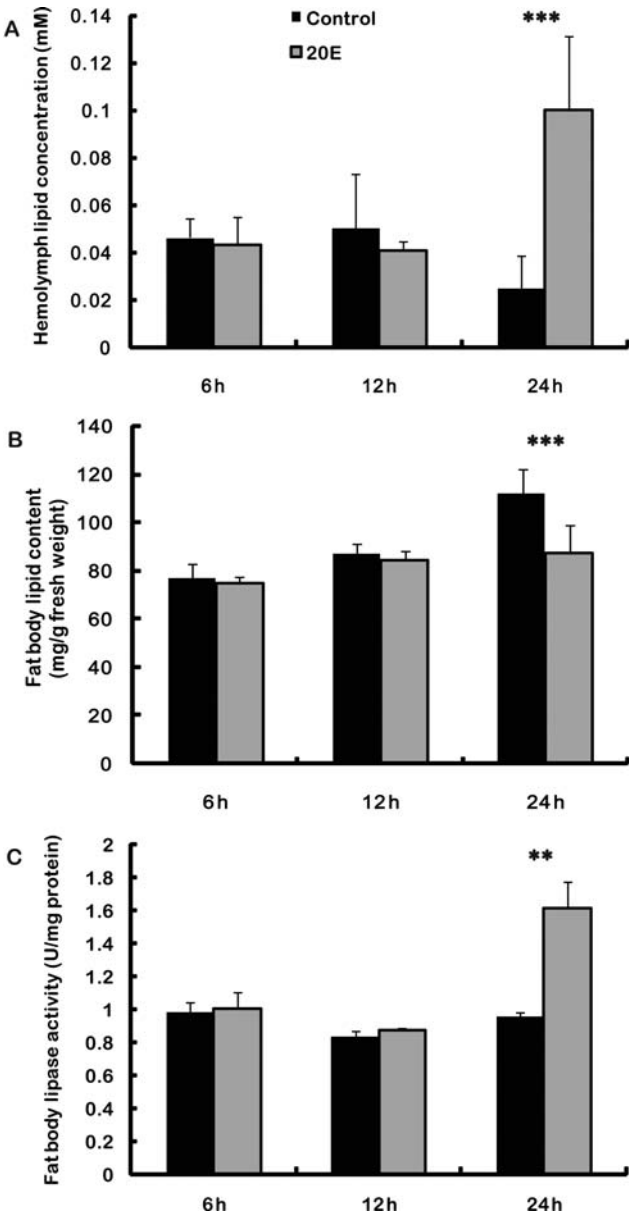

Figure 6 Hemolymph lipid concentration, fat body lipid content and lipase activity of *Bombyx* larvae on Day 2 of fifth instar after 20E injection. Each Day 2 of fifth instar, larva is injected with 5 μg 20E; hemolymph lipid concentration (**A**), fat body lipid content (**B**) and lipase activity (**C**) were then measured 6, 12 and 24 h after starvation. **$0.01 < P < 0.05$; ***$P < 0.01$.

Brummer (Grönke et al., 2005, 2007), the *Bombyx Brummer* mRNA level was quickly up-regulated within 6 h of starvation. High mRNA levels were maintained at 12 and 24 h of starvation (Figure 8A). There was no difference in *Brummer* mRNA level in the fat body between larvae injected with 20E and control solvent within 6 h after 20E injection. *Brummer* transcription started to increase in the following 6 h, and reached a much higher level at 24 h after 20E injection (Figure 8B). This observation was consistent with the data that feeding activity was gradually inhibited by 20E (Figure 5). To monitor 20E action in the larval fat body, the expression pattern of *E74A* (Figure 8C) was measured, showing much earlier expression pattern than

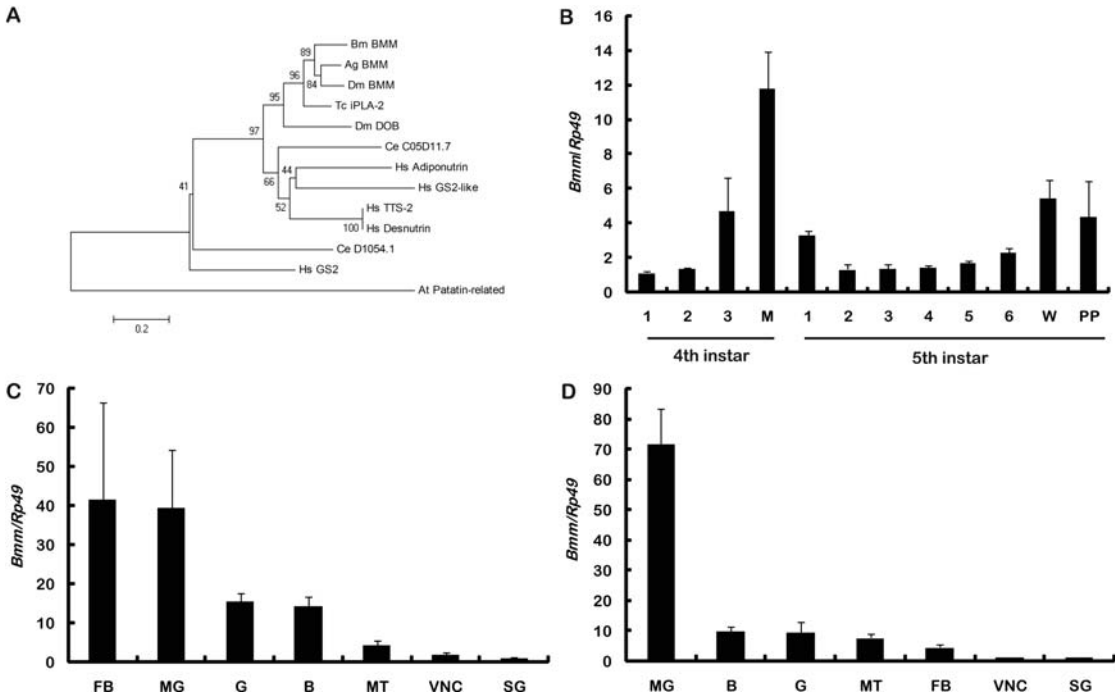

Figure 7 Phylogenetic tree, developmental pattern and tissue distribution of *Bombyx* Brummer. (**A**) Phylogenetic tree of Brummer family members based on the multiple alignment from *Bombyx mori* (Bm), *Anopheles gambiae* (Ag), *Drosophila melanogaster* (Dm), *Tribolium castaneum* (Tc), *Caenorhabditis elegans* (Ce), *Homo sapiens* (Hs) and *Arabidopsis thaliana* (At). (**B**) Developmental pattern of *Brummer (Bmm)* expression in the larval fat body. Tissue distribution of *Bmm* expression at the fourth larval molt (**C**) and on Day 2 of fifth instar (**D**). FB, fat body; MG, midgut; G, gonad; B, brain; MT, Malpighian tubules; VNC, ventral nerve cord; SG, silk gland.

Brummer. These results demonstrated that *Brummer* was transcriptionally up-regulated by 20E-induced starvation during molting and pupation.

Since AKH plays a critical role in insect lipolysis, we questioned if the AKH-regulated phospholipase A1 (Arrese et al., 2006) affects 20E-stimulated *Bombyx* fat body lipolysis. The predicted sequences of *Bombyx* phospholipase A1 in the SilkDB (Gene ID: BGIBMGA001646-TA) shared up to 70% identity to the *Manduca* ortholog in the conserved region. The developmental profile of *Bombyx phospholipase A1* showed a moderate increase during the fourth larval molt, wandering and prepupal stages (Supplemental Figure S2A), but tissue distribution analysis demonstrated that it was highly enriched in the brain and gonads but not in the fat body (Supplemental Figure S2B and C). Moreover, starvation and 20E had no effect on the expression of *Bombyx phospholipase A1* (Supplemental Figure S3). These results suggested that phospholipase A1 plays little, if any, role in 20E-stimulated *Bombyx* fat body lipolysis.

On the basis of the functional importance of the evolutionarily conserved lipase activity, we propose that Brummer accounts for fat body lipolysis during molting and pupation, which is caused by the action of 20E in its capacity to reduce food consumption and then induce starvation (Figure 8D).

Discussion

The developmental profiles of lipid concentration in the *Bombyx* hemolymph and lipase activity in its fat body (Figure 1) suggested that 20E was involved in fat body lipolysis during molting and pupation. In parallel, studies in *Drosophila* have shown that the transcriptional regulation of some starvation-response genes involved in lipid metabolism exhibit an EcR-dependent manner during the larval–pupal transition (Beckstead et al., 2005). Although FXR/LXR and RXR as well as their ligands directly regulate lipolysis in mammals, EcR and 20E did not directly stimulate lipolysis in insects (Figures 2 and 3). It has been suggested that through evolution, insect EcR–USP and 20E might have specialized to transmit critical signals determining developmental and reproductive events and have lost the majority of its regulatory roles in metabolic processes (Hodin and Riddiford, 1998; Thummel, 2001). For the first time, we have provided a physiological and molecular analysis showing that 20E–EcR–USP indirectly induce lipolysis in insects.

Interestingly, recent studies have identified that insect nuclear receptors are also involved in TAG and cholesterol homeostasis. For example, dHNF4, the *Drosophila* ortholog to mammalian HNF4, regulates lipolysis and fatty acids β-oxidation. The *dHNF4* mutants are resistant to diet-induced obesity and have severe defects in lipid mobilization (Palanker et al., 2009). *Drosophila* DHR96, homologous to mammalian PXR and CAR nuclear receptors, is important for midgut lipolysis by regulating the transcription of a gastric lipase *CG5932*. In addition, DHR96 can bind cholesterol and regulate expression of genes that respond to varying levels of cholesterol (Horner et al., 2009; Sieber and Thummel, 2009).

Experiments in this paper clearly showed that 20E injection reduced food consumption and then induced a starvation-like

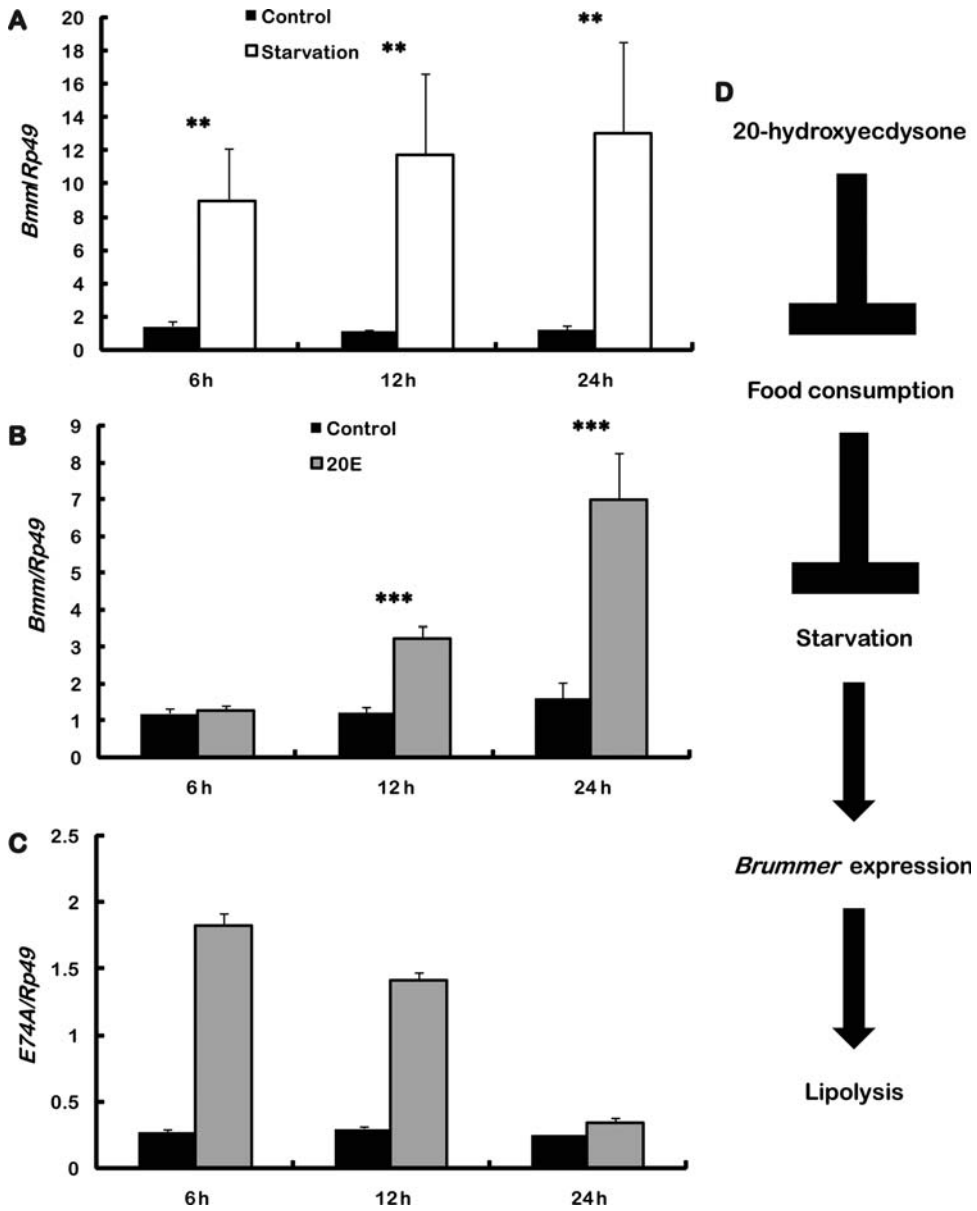

Figure 8 Transcriptional regulation of *Bombyx Brummer* by starvation and 20E. (**A**) Up-regulation of *Bmm* transcription level in starved *Bombyx* larvae compared with well-fed larvae. Up-regulation of *Bmm* transcription level in 20E-injected *Bombyx* larvae compared with control larvae (**B**). The presence of 20E signaling is confirmed by measuring the 20E early response gene *E74A* (**C**). (**D**) A proposed model shows that 20E reduces food consumption and induces starvation resulting in fat body lipolysis. $^{**}0.01 < P < 0.05$; $^{***}P < 0.01$.

phenotype (Figure 5), which appeared slightly earlier than 20E-induced wandering behavior (Dominick and Truman, 1985). Moreover, 20E injection into Day 2 of fifth instar larvae did not induce precocious pupation (metamorphic molting) until Days 5–6, only 1 day ahead of normal pupation. In other words, 20E-induced starvation and wandering behavior were 2–3 days ahead of 20E-induced molting. In *Manduca*, 20E acts directly on the central nervous system, specifically the brain, to initiate wandering behavior (Dominick and Truman, 1986a, b). The key question remained unsolved is whether 20E reduces food consumption in *Bombyx* directly via the central nervous system or through a different target tissue, such as midgut. Recently, it has been shown that neuropeptide F, which is the homolog of the vertebrate neuropeptide Y, is expressed in the brain to promote food intake and regulate body size in *Drosophila* (Lee et al., 2004). Whether 20E and EcR–USP directly regulate the biosynthesis and/or secretion of neuropeptide F is unknown and requires further investigation.

In *Drosophila*, the antagonistic effect of 20E on the insulin/insulin growth factor signaling (IIS) pathway and the target of rapamycin (TOR) pathway has been well documented (Rusten et al., 2004; Colombani et al., 2005). Insulin-like peptides (ILPs), including *Bombyx* Bombyxin, were also found in many insects (Nässel, 2002; Wu and Brown, 2006). Genetic studies in

Drosophila have demonstrated a conserved IIS pathway in regulating growth, metabolism, longevity and reproduction in insects (Tatar et al., 2003; Géminard et al., 2006; Toivonen and Partridge, 2009). Specific inhibition of PI3K activity in *Drosophila* fat body is sufficient to induce mobilization of stored nutrients and appears to phenocopy starvation (Britton et al., 2002; Colombani et al., 2003), and the gene expression and secretion of ILPs also depend on nutritional signals (Ikeya et al., 2002; Géminard et al., 2009). In parallel studies, we have shown that 20E injection slowly and gradually decreased IIS signals in the *Bombyx* fat body (unpublished data). Meanwhile, in *Drosophila*, TOR activity is essential for normal growth. When mutated or inhibited by rapamycin, both cell size and cell cycle are severely reduced, which are similar to events that occur during starvation (Oldham et al., 2000; Zhang et al., 2000). TOR signaling in the fat body is regulated by nutritional cues, especially amino acids, and modulates the local IIS signaling, which enables this tissue to be a sensor that coordinates organism growth with nutrients availability (Colombani et al., 2003). In other parallel studies, we have shown that 20E injection also slowly and gradually decreased TOR signals (unpublished data). Thus, the antagonistic effect between 20E and the IIS-TOR pathway in *Bombyx* is in well agreement with the 20E-induced fat body lipolysis.

Our data have established that 20E indirectly induces fat body lipolysis during molting and pupation in *Bombyx* (Figures 4–6). On the basis of several lines of evidence, we propose that Brummer lipase is responsible for this process. First, *Drosophila* Brummer is known to account for basal and starvation-induced lipolytic activity (Grönke et al., 2005, 2007). Second, phylogenetic analysis showed that *Bombyx* Brummer is highly conserved in evolution (Figure 7A). Third, the tissue distribution and developmental profile indicated that *Bombyx* Brummer is involved in fat body lipolysis during molting and pupation (Figure 7B and C). Finally, starvation rapidly and 20E slowly induced *Brummer* expression in the *Bombyx* fat body (Figure 8A and B). The transcriptional regulation of the ATGL gene *Brummer* by starvation and 20E in *Bombyx* is similar to the results obtained from mammals (Villena et al., 2004), in which *ATGL* expression was directly regulated by FoxO1 (Nakae et al., 2008; Chakrabarti and Kandror, 2009). Since 20E activates FOXO activity in *Drosophila* (Colombani et al., 2005; Mensch et al., 2008), it may be reasonable to assume that 20E induces FOXO activity and thus up-regulates *Brummer* expression during molting and pupation in *Bombyx*.

Many experiments in different insects have shown that starvation-induced lipolysis are independent to AKH but largely rely on Brummer (Grönke et al., 2005, 2007). Moreover, the AKH-regulated phospholipase A1 (Arresr et al., 2006) had little, if any, role in 20E-stimulated fat body lipolysis via reducing food consumption and then inducing starvation (Supplemental Figures 2 and 3). Although AKH could play an important role in fat body lipolysis in *Bombyx*, we assume that AKH is not involved in this process.

Taken together, we propose a model whereby 20E reduces food consumption and induces starvation resulting in fat body lipolysis by Brummer during molting and pupation in *Bombyx* (Figure 8D).

Materials and methods

Silkworm

Bombyx Nistari larvae, a non-diapausing strain, were reared on fresh mulberry leaves at 25°C with a 12 h light:12 h dark photoperiod. Starved larvae were reared under the same conditions without food and placed in tupperware containing a moist paper towel. Ten microliters of either 20E (Sigma, USA) or control solvent were injected into the select larvae through the abdominal leg. 20E was prepared as a stock solution in 100% ethanol and diluted to appropriate concentrations in ddH_2O.

Fly strains and fly genetics

The *Drosophila* strains (1) w^{1118}, (2) *yw; Lsp2-GAL4*, (3) *UAS-EcR*^{F654A} and (4) *yw, hs-FLPase*; *Act-CD2-GAL4; UAS-GFP/TM6B* were obtained from the Bloomington *Drosophila* Stock Center. *UAS-EcR*^{dsRNA} was purchased from the Vienna *Drosophila* RNAi Center. *yw; Lsp2-Gal4* were crossed with *UAS-EcR*^{F654A} to produce *Lsp2* > *UAS-EcR*^{F654A} using the binary UAS-GAL4 system (Brand and Perrimon, 1993). *yw, hs-FLPase; Act-CD2-GAL4; UAS-GFP/TM6B* were crossed with *UAS-EcR*^{dsRNA} and the newly laid eggs were heat shocked for 1 h at 38°C to generate FLP-out mosaic clones (Manfruelli et al., 1999).

Fluorescence microscopy

The fat body was dissected and fixed in 4% formaldehyde/PBS for 30 min, then rinsed in PBS, and finally stained with BODIPY (1:5000 in PBS, Invitrogen, USA) for 5 min. After mounting in 50% glycerol, the BODIPY-stained fat body was visualized using Olympus IX71 inverted fluorescence microscope. In addition, the FLP-out mosaic clones in the fat body were also observed using the same fluorescence microscope.

RNA extraction and real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen). First-strand cDNA was made from 1 μg of RNA primed by oligo$(dT)_{18}$ using MMLV reverse transcriptase (Takara, Japan). Real-time PCR was performed in a 20 μl volume using SYBR Green Master Mix (TOYOBO, Japan) and data were analyzed on the Bio-Rad iQ^{TM}5 Real-Time PCR Detection System (Bio-Rad, USA) using the following procedure: 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec. All assays were repeated three times and normalized to ribosomal protein 49 (*Rp49*) mRNA expression (Liu et al., 2009a, b). Real-time PCR primers are listed (Supplemental Table S1).

Sequence analysis

A Blastp search using *Drosophila* Brummer was used to identify a similar sequence in the *Bombyx* database SilkDB (Xia et al., 2004; Wang et al., 2005). The *Bombyx Brummer* gene was then PCR cloned, sequenced and submitted to GenBank. The protein sequences of *Drosophila melanogaster* (Dm) Brummer and Doppelgänger von Brummer, *Caenorhabditis elegans* (Ce) NP_741196.1 and NP_505749.3, *Anopheles gambiae* (Ag)

predicted Brummer, *Tribolium castaneum* (Tc) predicted iPLA-2, *Homo sapiens* (Hs) Adiponutrin, Desnutrin, GS2, GS2-like, and TTS-2, and *Arabidopsis thaliana* (At) patatin-related were used to do multiple alignments using ClustalW and construct a phylogenetic tree using MEGA 4 (Tamura et al., 2007; Kumar et al., 2008).

Determination of lipid concentration

A small cut was made on the larval abdominal leg and collected hemolymph was centrifuged at 2000*g* for 5 min to remove hemocytes. The fat body dissected from 5 to 10 Day 2 of fifth instar larvae was rinsed in PBS, weighed, then total lipid extracted using 3 ml of chloroform: methanol (v:v, 2:1). Alternatively, identically staged fat body was incubated at 25°C for 6–24 h in 500 μl Grace's insect culture medium (Gibco, USA) containing varying concentrations (0, 5 or 10 μM) 20E or an equivalent amount of control solvent. An aliquot of 10 μl hemolymph, fat body extract or fat body incubated media was used for lipid determination using a TAG assay kit (Rong Sheng Biotech Co., Ltd, Shanghai, China).

Fat body TAG lipase assay

The fat body was dissected in 100 μl ice-cold homogenization buffer (50 mM Tris, 250 mM sucrose, pH 6.9), and homogenized on ice using a glass homogenizer. The homogenate was centrifuged at 16000*g*, 4°C for 10 min. The resultant supernatant was assayed for protein concentration and lipase activity (Auerswald et al., 2005). Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). For the lipase assay, 100 μl of supernatant was added to 1 ml of reaction buffer (20 mM Tris, 500 mM NaCl, pH 7.4) containing 0.5 μCi of [^{3}H]-triolein (PerkinElmer, USA), 37 mM cold triolein and 0.1% Triton X-100. Samples were mixed and then incubated at 37°C for 1 h. After incubation, 100 μl of 1 N NaOH was added, then the mixture extracted using 5 ml chloroform: methanol:benzene (2:2.4:1) by vortexing 10 sec and centrifuging at 2000*g* for 10 min. One millilitre of the aqueous phase was added into 5 ml of liquid scintillation cocktail (PerkinElmer) to quantify radioactivity in a Beckman LS 5600 TD.

Supplementary Data

Supplementary data for this article are available online at http://jmcb.oxfordjournals.org.

Conflict of interest: none declared.

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