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Molecular analysis of nutritional and hormonal regulation of female reproduction in the red flour beetle, *Tribolium castaneum*

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Abstract

Female reproduction includes maturation of oocytes and the synthesis of yolk proteins (vitellogenin, Vg) in the fat body and their deposition into the oocytes. Our recent studies showed that juvenile hormone (JH) regulates Vg synthesis and 20-hydroxyecdysone (20E) regulates oocyte maturation in the red flour beetle (*Tribolium castaneum*). Here, we report on the role of nutritional signaling on vitellogenesis and oogenesis. Comparison of gene expression between fed and starved beetles by microarray analysis showed the up-regulation of genes involved in energy homeostasis and down-regulation of genes involved in egg production in the starved beetles. The RNA interference (RNAi) aided knock-down in the expression of genes involved in insulin and TOR signaling pathways showed that both these signaling pathways play key roles in Vg synthesis and oocyte maturation. Starvation of female beetles resulted in a block in Vg synthesis but not in the progression of primary oocyte development to the resting stage. Feeding after starvation induced Vg synthesis and the progression of primary oocytes from the resting stage to the mature stage. However, in the beetles where JH or 20E synthesis or action was blocked by RNAi, both Vg synthesis and oocyte maturation were affected suggesting that both these hormones (JH and 20E) and nutritional signaling and their cross-talk regulate vitellogenesis and oogenesis.

Keywords

RNAi; vitellogenesis; insulin; TOR; signaling; ovarian maturation; starvation; *Tribolium*

INTRODUCTION

Until recently, most of the studies on female insect reproductive physiology were focused on elucidating the role of 20-hydroxyecdysone (20E) or juvenile hormone (JH) in the regulation of vitellogenesis. Though oogenesis in insects is typically a nutrient-limited process (Wheeler, 1996), little is known about the molecular mechanisms of nutritional signaling in insect reproduction. Nutritional signals can be conveyed by two main signaling pathways: the amino acid signaling pathway mediated through the target of rapamycin (TOR) protein (Hansen et al. 2004) and insulin/insulin-like peptides signaling pathway (IIS) that is conserved in most eukaryotic organisms from yeast to mammals (Garafolo, 2002). The importance of these pathways came into light through the studies on anautogenous mosquito, *Ae. aegypti* where blood meals trigger the initiation of egg production (Attardo et al. 2005; Roy et al, 2007; Hansen et al. 2005, Riehle and Brown, 2002). The functional role

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of some of the proteins involved in these pathways have been reported (Attardo et al. 2003, 2006; Park et al. 2003; Martin et al. 2006, Brown et al. 2008). However, very little is known about the cross-talk between hormones and nutrition signals that regulate reproduction. In *Drosophila melanogaster* the nutritional status of the female's environment affects yolk protein synthesis and egg production (Schwartz et al. 1985; Bownes and Blair, 1986; Bownes and Reid, 1990; Sondergaard et al. 1995; Terashima and Bownes, 2004). Recently, similar studies involving nutritional signaling and hormone production during vitellogenesis have been extended to other insects such as honey bee (Corona et al. 2007) and German cockroach (Maestro et al. 2009). Thus, novel molecular and functional data on signaling molecules and the availability of continuously expanding genome databases shed new light on the molecular mechanisms involved in insect reproduction. We used the red flour beetle, *Tribolium castaneum* as our model system to analyze the complex networks that regulate beetle reproduction.

Previous studies on JH and 20E regulation of *T. castaneum* Vg gene expression showed that both these hormones either directly or indirectly regulate Vg gene expression (Parthasarathy et al., 2010a&b). While JH regulates Vg synthesis in the fat body, 20E regulates oocyte maturation in the ovary. However, Vg synthesis in the fat body is initiated only after oocytes reach to a stage when they can take up Vg. Thus, 20E indirectly regulates Vg synthesis in the fat body. In the current studies, we explored the effect of insulin and amino acid signaling pathways (IIS-TOR) on the Vg gene expression and primary oocyte maturation. The starvation of female beetles resulted in a block in Vg synthesis but not in the progression of primary oocyte development to the resting stage. Feeding after starvation induced the Vg synthesis and the progression of primary oocytes to the vitellogenic stage suggesting that nutritional signaling affects both these processes. Microarray and by RNAi studies showed that nutritional signals play key roles in regulation of vitellogenesis and cross-talk among the nutritional and hormonal signaling is important in the regulation of *T. castaneum* reproduction.

EXPERIMENTAL PROCEDURES

Insect rearing, staging and assays

Strain GA-1 *T. castaneum* beetles were reared on organic wheat flour containing 10% yeast at 30°C under standard conditions (Parthasarathy et al. 2008). The pupae were sexed based on the structural differences of genital papillae according to *Tribolium* rearing protocol (<http://bru.gmpkc.ksu.edu/proj/tribolium/wrangle.asp>). Adult females were staged soon after their emergence; the adults with untanned cuticle (teneral adults) were designated as 0 h and staged thereafter. The staged insects were maintained under similar conditions as mentioned above.

To identify the nutrition signals involved in vitellogenesis, female beetles were either starved for 4 days continuously from 0 day post adult emergence (PAE) or fed on normal diet continuously for 4 days from 0 day PAE. Total RNA isolated from these two groups of beetles was used for microarray analysis performed as described below.

In another experiment, the female beetles were starved for 4 days continuously from 0 day PAE and fed with normal diet after 4 days of starvation. The expression levels of the candidate genes were determined at 0 h, 6 h, 12 h, and 24 h after feeding of starved beetles. For comparison, the expression levels of same set of genes were determined in the beetles continuously fed for 4 days PAE.

To study the cross-talk between hormone and nutrition signals during reproduction, a few representative genes of JH (JHAMT), ecdysteroid (EcR) and IIS-TOR (InR and TOR)

signaling pathways were selected. The female beetles were injected with dsRNA of selected genes or *malE* (control) and starved continuously for 4 days PAE. After 4 days of starvation, the beetles were fed on normal diet for 6 h and Vg2 mRNA levels were determined in these insects.

Microarray analysis

Total RNA was isolated from the whole body of starved and fed female beetles using spin columns (RNeasy, Qiagen, Valencia, CA). The integrity of RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). The labeling of probes, hybridization, washings, and normalization of the data were performed as described in Parthasarathy et al., 2009. The raw data files (.txt) were imported into GeneSpring (GX v. 9.0.1) and the data were normalized and analyzed. Data were transformed to bring any negative value to 0.01. Normalization was performed using a per-chip 50th percentile method that normalizes each chip on its median, allowing comparison among chips. Then a per-gene on median normalization was performed, which normalizes the expression of every gene on its median among samples. The normalized data were subjected to the “t”-test, Bonferroni and Benjamini & Hochberg false discovery rate multiple testing corrections using GeneSpring GX v.9.0.1 software.

Double-stranded RNA (dsRNA) synthesis and injection

The candidate genes for RNAi were selected based on their important roles in IIS-TOR pathways. To study the role of nutritional signaling in vitellogenesis, the genes known to be involved in the IIS-TOR pathways in other organisms such as insulin receptor- InR, Chico, Akt, Foxo, PI3K, Pten, Tsc, RHEB, TOR, S6 kinases and GATA transcription factor (Attardo et al. 2005; Avruch et al. 2006) were selected. In cases where multiple members of a gene family are present (e.g. Foxo and GATA), all the members were tested by RNAi and the only member that showed the maximum effects is shown. *T. castaneum* genome contains insulin receptor homolog (TC010784) and another gene (TC007370) with high sequence similarity to InR; whether this is recently duplicated InR or receptor for other peptides is not known. RNAi of both these genes showed similar effect on female reproduction. Therefore, we only used InR in the current study. The gene identity and primers used to synthesize dsRNA are shown in Table 1. dsRNA were synthesized and injected as described by Parthasarathy et al., 2010a. dsRNA injection of all the candidate genes, except for the dsRNA of genes involved in ecdysteroid biosynthesis or action were injected into day 2 pupae. Since injection of dsRNA of genes involved in ecdysteroid biosynthesis or action blocked pupal-adult metamorphosis, these dsRNAs were injected into day 0 adults. Control adults were injected with dsRNA for *E. coli malE* gene.

cDNA synthesis and Quantitative real-time reverse-transcriptase PCR (qRT-PCR)

Total RNA was extracted from whole body or dissected fat body or ovaries of staged adults and from insects injected with dsRNA using TRI reagent (Molecular Research Center Inc., Cincinnati, OH). cDNA was synthesized and these cDNAs and gene specific primers (Table 1) were used to quantify mRNA levels of selected genes following methods described by Parthasarathy et al. 2010a. Relative expression levels of each gene were quantified using the mRNA levels of ribosomal protein, rp49 as an internal control.

Mating assays

dsRNA of candidate genes were injected into the female insects at appropriate stages as described above. Four days post adult emergence (PAE), the injected virgin beetles were mated with uninjected virgin males on a single pair basis in 24-well plates. After 7 days of rearing, the pair of beetles was removed and the number of eggs laid by each pair was

determined. Also, the number of larvae hatched from the eggs was determined at 14 days after initiation of mating. Female insects injected with *male* dsRNA served as controls.

Light and Electron microscopy

Preparation of tissues, cutting semi- and ultra-thin sections, staining and image capture were performed as described recently (Parthasarathy et al., 2010b).

RESULTS

Microarray analysis

Total RNA samples isolated from all tissues collected on day 4 PAE from the fed and starved beetles were labeled and hybridized to *T. castaneum* custom microarrays. Three biological replicates were included for each treatment. Out of the 15,208 probe sets screened, hybridization to 12,229 probe sets was detected in at least one of the six samples. The spot intensity data for these probe sets were statistically analyzed using GeneSpring software. The fold differences in expression (calculated by dividing the mean value of signal intensities obtained by RNA samples from fed beetles with those obtained by RNA samples from starved beetles) and the significance of difference (p-value from t-test) for 12,229 probe sets are shown as a volcano plot (Fig. 1). When compared to their expression in the fed beetles, the expression levels of 988 and 570 genes were up- and down-regulated respectively in the starved beetles by 2-fold or more with a p value of <0.01. Upon filtering by false discovery rate multiple testing corrections, Benjamini-Hochberg test with medium stringency showed 358 up-regulated genes and 109 down-regulated genes in the starved beetles when compared to their expression in the fed beetles by two-fold or more with a p-value of <0.01. Some of the representative genes in both groups are shown in Table 2. Several genes involved in transporter activity were up-regulated in the starved beetles. Also, enzymes such as cathepsin, lipases, pyruvate carboxylase, and glycoside hydrolases involved in nutrient metabolism were up-regulated in the starved beetles. Importantly, TC005969, a gene involved in transcription repressor activity was up-regulated by 2.5 fold in the starved beetles (Table 2). Among the down-regulated genes in the starved insects, Vg (Vg1 & Vg2) showed more than 100-fold lower expression in the starved beetles when compared to its expression in the fed beetles. Several JH inducible proteins were down-regulated in the starved insects. Enzymes such as fatty acid synthase and trehalose-6-phosphate synthase involved in synthesis of lipids and carbohydrates were down-regulated in the starved beetles (Table 2).

Expression of select genes in the fed and starved beetles

The expression of selected genes identified due to the differences in their expression between the starved and fed beetles in the microarray analysis were tested by qRT-PCR. Among the down-regulated genes, Vg1 and Vg2 showed 923- and 757-fold decrease respectively in the starved beetles when compared to their expression levels in fed beetles. The genes coding for JHIPs (TC007665 and TC014079) showed 8.57- and 6.62-fold lower expression in the starved beetles than in the fed beetles. Also, the insulin receptor (TC007370) showed 7.33-fold decrease in the expression levels in the starved beetles when compared to its expression in the fed beetles. Among the genes up-regulated, the expression levels of TC002633 (Sodium channel activity), TC012787 (uronyl-2-sulfotransferase), TC012760 (sugar transporter), TC016121 (Serine protease), and TC004656 (Lipase) showed 32.1-, 1.7-, 6.0-, 2.98-, and 1.88-fold increase respectively in the starved beetles when compared to their expression levels in the fed beetles (Fig. 2). Thus, qRT-PCR analysis on the expression of these select genes confirmed the data obtained by microarray analysis.

Effect of RNAi

Knock-down efficiency of IIS and TOR pathways genes—The mRNA levels of the genes that codes for proteins known to play important roles in the IIS-TOR pathway were quantified using qRT-PCR in insects injected with *malE* dsRNA (control) and compared with the mRNA levels of these genes in insects injected with respective dsRNA. The knock-down efficiency of these genes ranged between 50–99% (Fig. 3A). Out of 13 genes tested in the IIS-TOR pathways, 8 genes showed around 80–95% knock-down efficiency. The insulin receptors and substrate (InR and Chico) mRNA levels were knocked-down by 50–60%. Among the kinases, Akt, PI3K, and S6K 1 & 2 showed around 88-, 80- and 90% knockdown efficiency. In the TOR pathway, TOR showed around 60% knock-down efficiency, while RHEB 1 & 2 and TSC mRNA levels were reduced by more than 95%.

ISS and TOR regulation of Vg gene expression—Among the genes involved in IIS-TOR signaling pathways, the knock-down in expression of insulin receptor (InR) and substrate (Chico) showed a significant reduction (80–95%) in Vg2 mRNA levels (Fig. 3B). Among the kinases, Akt and PI3K deficiencies showed more effect of around 70–80% reduction in Vg2 mRNA levels than the serine kinases (S6K1 and S6K2) that caused around 50% reduction of Vg2 mRNA levels. The knock-down of the negative regulator of insulin signaling, Pten, did not affect Vg2 gene expression. Among the regulators of TOR signaling pathway, Tsc knock-down alone blocked Vg2 transcription (around 90% less mRNA levels) while RHEB1 & 2 knock-down did not affect Vg2 transcription as the Vg2 mRNA levels were down by only 10–30%. The knock-down of downstream transcriptional factors of IIS-TOR pathway, GATA-1 and Foxo, drastically reduced the Vg2 mRNA levels by more than 90%.

ISS and TOR regulation of egg production—Knock-down in expression of InR, Chico, and TOR rendered beetles sterile and no eggs were produced by these beetles (Fig. 3C). Among the other genes involved in IIS-TOR pathway, knock-down in the expression of Akt, Pten, PI3K, S6K2 severely affected egg production, while knock-down in the expression of Tsc, RHEB1, RHEB2 and S6K1 resulted in 88.1%, 54.8%, 43.3%, and 64.9% reduction in egg production respectively (Fig. 3C). . RNAi of transcription factors, Foxo or GATA-1 was lethal; resulted in the mortality of the injected female beetles beginning on 4th day PAE. Nearly 80–90% of the injected beetles died during 5–6 days PAE in both cases and hence the RNAi animals were not included in the mating bioassays.

Effect of starvation on Vg synthesis and oocyte maturation

For this study, the genes known to be involved in the biosynthesis and action of JH and 20E that play key roles in the beetle vitellogenesis and oocyte maturation, were selected based on our previous studies (Parthasarathy et al. 2010a & b). The starvation did not affect JHAMT gene expression and neither did the feeding of starved beetles as the mRNA levels did not differ significantly among the beetles fed or starved or starved for 96 hr and fed for 24 hr (Fig. 4A). A similar expression pattern was observed for Met as well. Starvation resulted in 2-fold decrease of Kr-h1 mRNA levels, but feeding of starved beetles did not induce the expression of this gene as the mRNA levels of this gene remained lower when compared to the levels in the fed beetles. In contrast, starvation significantly reduced the expression levels of Vg2 mRNA when compared to its levels in the fed beetles. The Vg2 mRNA levels were restored immediately within 6 h after feeding of starved beetles and the levels increased significantly at 12 h and 24 h after feeding of starved beetles (Fig. 4A).

Phantom mRNA levels did not differ significantly among the beetles fed or starved or starved for 96 hr and fed for 24 hr. Shade mRNA levels were 2-fold less in starved beetles but the mRNA levels were not restored upon feeding. Starvation did not significantly affect

EcR mRNA levels, but the feeding after starvation significantly increased the EcR mRNA levels and a peak level of expression was observed at 24 h after feeding. A similar expression pattern was observed for USP, though the starved beetles showed 2-fold less USP mRNA levels when compared to the fed beetles. The expression levels of InR were 4-fold less in the starved beetles when compared to their levels in the fed beetles. InR mRNA levels were restored after 12 h of feeding of starved beetles and increased 10-fold higher 24h after feeding of starved beetles. TOR mRNA levels were 2-fold less in the starved beetles when compared to their levels in the fed beetles. The expression levels of TOR increased by 2-fold at 6 h after feeding of starved beetles and the levels were restored to the levels observed in continuously fed beetles, by 12–24 h after feeding of starved beetles (Fig. 4A).

Effect of IIS and TOR pathways on Vg synthesis in the starved beetles

Vg2 mRNA levels were determined in the starved RNAi beetles by qRT-PCR (Fig. 4B). In the control beetles injected with *malE* dsRNA, Vg2 levels were restored by 6 h after feeding of starved beetles. In JHAMT RNAi beetles, Vg2 mRNA levels were restored partially; 4-fold less when compared to the levels in the starved and fed control beetles. However, in EcR, InR, and TOR RNAi beetles the Vg2 mRNA levels were not restored even after feeding of starved beetles for 6 h suggesting that JH as well as functioning of 20E and IIS-TOR pathways is a prerequisite for Vg gene expression.

Effect of RNAi on oocyte growth and maturation

The temporal patterns of oocyte growth and maturation were described in Parthasarathy et al 2010b. Briefly, initially, the primary oocytes are dormant, prefollicular, located in the posterior portion of the germarium (stage 1), the primary oocytes move to the neck region of the ovariole and these are surrounded by prefollicular tissue (stage 2). The oocytes enlarge and follicles start migrating (stage 3). The oocytes change shape to become spherical, nucleus transforms into germinal vesicle (stage 4). The germinal vesicle moves to the center and the follicle cells organize into columnar epithelium (stage 5). Yolk deposition begins at this stage and there is a rapid increase in oocyte size and patency starts (stage 6). Maximum oocyte size and patency (stage 7). Yolk deposition ceases; follicle deforms, chorion secretion begins (stage 8). In the control beetles, the primary oocytes were well developed in most of the ovarioles and they were at Stage 5. The primary oocyte had well developed follicular epithelial cells and germinal vesicle was located at the center (Fig. 5A, a). Surprisingly, the maturation of the primary oocytes of the ovaries dissected from beetles continuously starved for four days from adult eclosion was not blocked though the overall growth of ovarioles was smaller than the ovarioles of the fed control beetle ovary. The primary oocytes were at Stage 4 with well developed follicular epithelium (Fig. 5A,b). The primary oocyte proceeded to Stage 5 immediately after feeding of starved beetles for 6 h (Fig. 5A, c).

The knock-down in the expression of InR or TOR gene had impaired the maturation of the primary oocyte and the oocyte growth was arrested at Stage 2 (Fig. 5A, d, e). The knockdown expression of Akt, Chico, PI3K or S6k2 gene severely impaired the maturation of the primary oocyte and the oocytes growth was arrested at Stage 2 (Fig. 5A, f-h, l). However, Pten, Tsc, S6k1, RHEB 1 & 2 RNAi did not block the maturation of the primary oocytes and the oocytes were observed at Stage 4 (Fig. 5A, i-k, m & n). The knock-down of Foxo and GATA-1 transcription factors also prevented the ovarian growth and oocyte maturation and the primary oocytes growth was arrested at Stages 1–2 (data not shown).

Observation of the thin sections of ovarioles of starved and fed beetles under an electron microscope showed that the follicle cells of the starved beetles were small, packed with mitochondria but the cellular components such as endoplasmic reticulum and Golgi complex

involved in protein synthesis and secretion were not as many as in cells of fed control beetles. Also, the thickness of the follicular epithelial layer was less than that of the follicular epithelial layer of control beetles (Fig. 5B).

The role of JH, 20E and nutrition on the ovarian growth and oocyte maturation were determined in the starved beetles. The beetles were injected with JHAMT, EcR, USP, InR, TOR, or *male* (control) dsRNA on day 0 PAE and the injected beetles were starved continuously for four days. On the 4th day PAE, the ovaries were dissected and stained with DAPI (Fig. 5C, a-f). In the control beetle ovariole and JHAMT RNAi beetle ovariole, the primary oocyte was enlarged and contained a layer of follicular epithelial cells and the oocytes were observed at Stage 4 (Fig. 5C, a-b). However, in the EcR, USP, InR, and TOR RNAi beetle ovarioles, the primary oocyte growth was arrested at Stage 2 (Fig. 5C, c-f). These data showed that proper functioning of 20E and IIS-TOR signaling pathways is a prerequisite for oocyte maturation.

DISCUSSION

The influence of nutrition on female reproduction especially vitellogenesis mediated by insulin and amino acid signaling pathways have been reported in mosquitoes and a few other insect species (Richard et al. 2005; Wu and Brown, 2006; Shaio, et al. 2008; Arsic and Guerin, 2008; Fronstin and Hatle, 2008; Brown et al. 2008; Maestro et al. 2009). In the present study, we show that the insulin and amino acid signaling pathways regulate female reproduction especially vitellogenesis in the red flour beetle. First, we analyzed the differential gene expression between the starved and fed female beetles using microarrays. The microarray data clearly showed an increase in expression of genes coding for proteins involved in metabolism of stored energy sources and their transport in starved beetles when compared to their expression in the fed beetles. Interestingly, more genes were up-regulated than down-regulated in the starved beetles. Similar observations were reported in the global transcriptome analysis of starvation response in the whole body and head region of *Drosophila melanogaster* (Gronke et al. 2005, Fujikawa et al. 2009).

Among the down-regulated genes, the major protein involved in reproduction, Vg mRNA levels were suppressed by several hundred folds. The enzymes such as fatty acid synthase and trehalose synthase involved in anabolic pathways were also down-regulated in the starved insects. Similar to the data reported in *D. melanogaster* transcriptome analysis, protease inhibitor (serpin) was down-regulated under starved condition. Interestingly, the expression of several genes belonging to the same functional groups changed both in the starved *D. melanogaster* (Gronke et al. 2005, Fujikawa et al. 2009) and *T. castaneum* (present study) suggesting that the regulatory mechanisms that respond to stress are well conserved in these two insects. Strikingly, JH inducible proteins (JHIPs) were up-regulated under starvation condition in *D. melanogaster* (Gronke et al. 2005) but down-regulated in the starved beetles (the current study) this may point to differences in JH regulation of female reproduction between flies and beetles. Overall, our microarray data confirmed by qRT-PCR revealed the up-regulation of genes involved in energy homeostasis and the down-regulation of genes involved in egg production suggesting a trade-off between survival and reproduction.

Our microarray data also showed the importance of nutritional signals in promoting reproduction. Oogenesis in insects is typically a nutrient-regulated process, triggered only if sufficient nourishment is available (Wheeler, 1996). IIS-TOR pathways play major roles in regulating female reproduction in several insects (Tatar, et al. 2001, Hansen, et al. 2004, Wu and brown, 2006, Maestro et al. 2009). The insect fat body is known to be the nutrient sensor organ (Edgar 2006). The requirement of expression of genes coding for proteins

(such as insulin receptor, PI3K, TOR) involved in these pathways in the insect fat body has been confirmed by means of RNAi (Hansen et al. 2005, Roy et al. 2007). The knock-down of these genes by RNAi impaired the Vg gene expression in female mosquitoes (Attardo et al. 2005). The important signal transducing proteins such as the insulin-dependent receptor tyrosine kinase and protein kinase B (Akt/PKB) are present in insect fat body as well as ovaries and their expression levels appear to be regulated during the reproductive cycle (Fullbright et al. 1997; Hebling and Graf, 1998; Riehle and Brown, 2002; 2003). In *D. melanogaster*, mutations in the insulin receptor produce phenotypes showing reduced oogenesis and JH deficiencies (Tatar et al. 2001). These studies suggested the role of IIS-TOR pathway genes in both fat body and ovaries regulating the female reproduction.

We used RNAi in the red flour beetle to analyze the functional roles of proteins involved in IIS-TOR pathways transducing the nutritional signals. We studied the effects of knock-down in the expression of these genes on both Vg synthesis in the fat body and maturation of primary oocytes in the ovary. During the previtellogenic and vitellogenic phase of 1–5 days PAE, mRNA levels of genes coding for proteins involved in both insulin and amino acid signaling were detectable in the whole body, fat body or ovaries by qRT-PCR. The mRNA levels of these genes did not change significantly during 1–5 days PAE (data not shown). However, knock-down in expression of most of these genes by RNAi resulted in a decrease in Vg mRNA levels. Concomitant with this down regulation of Vg mRNA levels, some of these RNAi insects were rendered sterile with no egg production. Since insulin and TOR pathways control many aspects of insect growth, development and reproduction, knockdown in expression of genes coding for some of the key components of these pathways may have affected many events, not just limited to reproduction. Therefore, the observed phenotypes could be direct or indirect effects on reproduction. However, our data are in agreement with the findings in other insect species using insulin receptor/ substrate mutants of *D. melanogaster* (Richard et al. 2005; Tatar et al. 2001) or RNAi effects of insulin/amino acid pathway genes in mosquitoes and cockroaches (Brown et al. 2008; Roy et al. 2007; Maestro et al. 2009). Interestingly, the negative regulators (such as PTEN and TSC) of these pathways (Wu and Brown, 2006; Arsic and Guerin, 2008), did not affect Vg mRNA levels and ovary maturation respectively, while the positive regulators (such as INR, Chico, PI3K, Akt of these pathways (Wu and Brown 2006; Roy et al. 2007; Richard et al. 2005) showed severe effects on Vg gene expression. Moreover, knock-down in the expression of the downstream regulators (such as Foxo and GATA transcription factors) of these pathways also caused down regulation of Vg gene expression and lethal effects. *T. castaneum* genome contains four genes coding for insulin-like peptides (Li et al., 2008). Work is in progress to determine the role of these four ILPs in female reproduction.

Our previous studies showed that knock-down in the expression of genes coding for proteins involved in JH biosynthesis or action (JHAMT, Met, Kr-h1) or 20E biosynthesis or action (Shade, EcR, and USP) blocked Vg synthesis (Parthasarathy et al, 2010a&b). The nutritional environment affects the hormone production in insects (Tatar et al. 2001). Hence, to understand the importance of nutrient inputs, we starved the insect continuously for the entire previtellogenic phase and analyzed the Vg gene expression with or without feeding of normal untreated control or RNAi insects. These experiments showed that: 1) starvation down-regulated the Vg mRNA levels by 1000-fold and also the genes involved in the IIS-TOR pathway (InR and TOR), but did not influence significantly the expression of genes involved in hormone biosynthesis and action (except for Kr-h1, USP); 2) feeding of starved insects up-regulated InR and TOR mRNA levels within 6 h after feeding, concomitant with the restoration of Vg gene expression. EcR and USP gene expression levels but not the expression levels of genes coding for JHAMT, Met, Kr-h1, Phantom, and Shade were up-regulated within 24 h after feeding. These data suggest that nutritional signals are the primary stimuli for initiation of Vg gene expression; 3) knock-down in the expression of

JHAMT, EcR, InR, and TOR levels in the starved insects did not restore the Vg gene transcription even after feeding, indicating the operation of all these regulatory networks at tissue specific levels. The effect of starvation on the hormonal regulation of the *T. castaneum* has not been reported previously. However, the influence of the nutritional milieu on corpus allatum (CA) activity and egg maturation has been widely studied in several insects (Engelmann, 1968). These studies suggest that starved animals have a low protein concentration in the hemolymph which may not permit an activation of CA cells. However, the implanted active CA remained active long enough to cause egg maturation in a certain percentage of the operated animals. This observation on isolated glands affected by the changing internal environment may be the strongest evidence for the theory of direct control of CA by the nutritional milieu. Recently, in insects, where JH plays a major role in vitellogenesis, starvation was shown to cause dramatic reduction of JH production in german cockroach, *Blatella germanica* (Maestro et al, 2001) and lubber grasshopper, *Romalea microptera* (Fei et al. 2005). Also, JH alone was insufficient to stimulate Vg production in this insect. Our data on starvation and feeding on the hormonal regulation and Vg production in *T. castaneum* suggest similar underlying mechanisms of egg maturation.

Taking into the consideration of our parallel studies on these regulatory networks in the vitellogenesis, we conclude that JH is required in the fat body to initiate Vg gene expression (Parthasarathy et al., 2010a); 20E is required for maturation of oocytes in the ovary to signal for initiation of Vg gene expression (Parthasarathy et. al., 2010b); nutritional signals (InR and TOR) are required both in the fat body and ovary for promoting Vg synthesis (current data). Starvation is known to induce a tradeoff between survival and reproduction, where all primary resources are directed towards survival and maintenance and reproduction has to wait till favorable conditions (food) are restored. When met, the machinery resumes normal operation within hours of restoration (Wheeler, 1996). Our microarray and RNAi analyses confirmed the above hypothesis.

Additionally, the starvation of female beetles continuously after emergence did not block the progression of primary oocyte maturation until resting stage. However, the difference in the size of primary oocyte between the fed and starved beetles was prominent. The primary oocytes were smaller with less defined follicular epithelial layers. Concomitantly, the expression of Vg receptors and other three genes were 80–90% less expressed in starved beetle ovaries when compared to their expression in the fed beetle ovaries. This indicates that the female beetle utilizes the stored reserves from the pupal stage for oocyte growth until resting stage. The feeding in the adult beetles induces the terminal differentiation of the oocyte. Upon feeding, the primary oocytes progressed to the vitellogenic stage. This condition is similar to the oocyte maturation observed in *Ae. aegypti* (Nijhout, 1998), where oocyte develops until resting stage and progresses to vitellogenic stage upon blood feeding. However, the progression of primary oocyte to vitellogenic phase did not occur by feeding of starved beetles whose hormone synthesis or action was blocked by RNAi.

Comparison of RNAi effects of individual components of regulatory net work involved in beetle vitellogenesis (Table 3) showed that proteins that are required for oocyte maturation (Shade, ecdysone receptors, most of the IIS-TOR pathway genes) are also required for Vg gene expression suggesting that oocyte maturation to resting stage is a prerequisite for Vg synthesis. It is interesting to note that the egg production was blocked in Pten RNAi insects despite the normal maturation of oocyte and Vg synthesis in these insects. Also, Tsc RNAi insects down regulated Vg synthesis significantly and egg production despite normal oocyte maturation. These observations suggest that some of the genes in the IIS-TOR pathway play critical roles in other physiological functions affecting these processes.

In conjunction with other studies, these data indicate the existence of a functional relationship between the nutritional and endocrine signaling pathways in the regulation of female reproduction. Recent study in cockroach proposed the involvement of stage-specific ovarian factor that stimulates JH synthesis in corpora allata (Elliot et al. 2006). Thus, female beetles must have some mechanisms to restrain ovarian development, and a hormonal control system that is activated under a suitable nutritional milieu becomes ideal.

Taken together, our data suggest that the beetle reproduction is regulated by a myriad of factors, not only metabolic but also hormonal, connected to each other in a complex regulatory network (Fig. 6). Recent studies in other insect species put forth the link between the nutrition and hormones in regulating the female reproductive physiology (Attardo et al. 2005; Corona et al. 2007; Meastro et al. 2009). Though the molecular mechanisms of regulation of Vg gene expression needs further experimentation, this study in conjunction with our recent studies on the hormonal regulation of vitellogenesis and ovarian growth and maturation (Parthasarathy et al., 2010a & b) provide the baseline data on tissue specific physiological roles of ecdysteroids and JH, and involvement of nutritional signaling in the less explored coleopteran insect reproductive processes.

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Fig. 1.

Volcano-plot of differentially-expressed genes identified by microarray analysis. The p-values of the t-test were plotted against the fold-change in gene expression for all genes. The horizontal lines in the plot represent the significance of 0.001 for the t-test, under the assumption that each gene has a unique variance. The vertical bars represent the genes that are a minimum of three-fold up- or down-regulated in starved insects when compared to their expression in the whole body of fed insects at day 4 PAE.

Fig. 2.

Relative expression levels of genes selected from microarray analysis were determined by qRT-PCR. RNA was extracted from the whole body of starved and fed insects at day 4 PAE. Relative expression in comparison to ribosomal protein (rp49) mRNA levels was determined. Mean $\pm$ S.E. of three independent replicates are shown.

Fig. 3A

Fig. 3B

Fig. 3C

Fig 3.

Fig. 3A. Knock-down efficiency of genes by RNAi. dsRNA for IIS-TOR pathway genes (InR, Chico, Akt, Foxo, PI3K, Pten, RHEB 1 & 2, TSC, TOR, S6K1 & 2 and GATA-1) and *male* (control) were injected into 2-day old female pupa. At 4 days after adult emergence, RNA was extracted and the relative expression in comparison to ribosomal protein (rp49) mRNA levels was determined by qRT-PCR. The expression levels of respective genes in control insects were set to 1. Mean $\pm$ S.E. of three independent replicates are shown.

Fig. 3B. Effect of RNAi on the expression of Vg gene (Vg2). dsRNA for IIS-TOR pathway genes (InR, Chico, Akt, Foxo, PI3K, Pten, RHEB 1 & 2, TSC, TOR, S6K1 & 2 and GATA-1) and *male* (control) were injected into 2-day old female pupa. The adults were staged upon emergence. RNA was extracted at day 4 PAE. Relative expressions of Vg2 mRNA levels for individual genes were determined in comparison to ribosomal protein (rp49) mRNA levels by qRT-PCR. The expression ratio of Vg2 mRNA levels in each RNAi insects were calculated by setting the control insect Vg2 mRNA levels as 1.0. Mean $\pm$ S.E. of three independent replicates are shown.

Fig. 3C. Effect of knock-down in the expression of genes involved in IIS-TOR pathways on the number of eggs produced by RNAi females mated with uninjected virgin males. dsRNAs of the above genes were injected as described above. At 4 days after adult emergence, the RNAi females were mated with the virgin males on a single pair basis. The relative per cent eggs produced from each pair with respect to control were shown. The egg produced by female beetles injected with *male* dsRNA served as a control and set as 100%. Mean $\pm$ S.E. of three independent replicates with 10 pairs in each replicate are shown.

Fig 4.

Fig. 4A. Effect of starvation and feeding on the expression of genes involved in JH biosynthesis and action (JHAMT, Met, Kr-h1), 20E biosynthesis and action (Phantom, USP, EcR, USP), IIS-TOR pathway genes (InR and TOR), and Vg gene (Vg2) determined by qRT-PCR. The female beetles were starved continuously for 4 days PAE and fed for 24 h after starvation. The mRNA levels of the above genes were determined at 0, 6, 12 and 24 h after feeding of starved insects. For comparison, the mRNA levels of continuously fed female beetles for 4 days PAE were included. Relative expression in comparison to ribosomal protein (rp49) expression was determined. Mean $\pm$ S.E. of three independent replicates are shown. One-way ANOVA analysis was performed using JMP software (SAS institute). The mean expression levels marked with the same alphabetical letter do not differ significantly at $p=0.05$.

Fig. 4B. mRNA levels of Vg2 in starved and fed RNAi insects. The female beetles were injected with dsRNA for JHAMT, EcR, InR TOR, and *maleE* (control) on day 0 PAE. The injected beetles were starved continuously for 4 days PAE. A set of injected and starved beetles in each group was fed with normal diet for 6 h. RNA was extracted at the end of starvation (4 days PAE) and feeding (4 days starving + 6 h feeding). Relative expression of Vg2 in comparison to ribosomal protein (rp49) expression was determined. Mean $\pm$ S.E. of three independent replicates are shown.

Fig. 5A **Fig. 5B**

Fig. 5C **Fig 5.**

Fig. 5A. Ovarian growth and primary oocyte maturation in the RNAi, starved and fed insects (panels a-p). dsRNA for IIS-TOR pathway genes and *maleE* (control) were injected into 2-day old female pupa. The ovaries were dissected on day 4 PAE in both cases and stained with DAPI. For comparison, the ovaries dissected from insects starved continuously for 4 days or starved for four days and fed for 12 h were included. Each panel represents one of the pair of ovaries of experimental insects and the insert at the bottom shows a single ovariole of the ovary to highlight the stage of the primary oocyte. The ovary in each panel is aligned with nurse cells on top and oocyte at the bottom. The ovariole in each insert are aligned with nurse cells to the right and the primary oocyte to the left. The mature primary oocytes are marked by yellow arrow. Scale Bar: 200 μ m (white); 100 μ m (yellow).

Fig. 5B. Ultrastructure of follicular epithelial cells of continuously fed (a) and continuously starved (b) insect ovary on day 4 PAE. Insert in each panel show a single follicle cell from the epithelium at higher magnification. Bracket denotes the thickness of the follicular epithelium. See text for details. Showing that CH Chromatin, ER Endoplasmic reticulum, GA Golgi apparatus, FC, Follicle cell; FCn, Follicle cell nucleus; FE, Follicular epithelium; FM, Follicular membrane; M, Mitochondria; Oo, Oocyte. Scale Bar: 2 μ m; Insert – 0.5 μ m.

Fig. 5C. Effect of RNAi on the primary oocyte maturation in the starved insects. JHAMT, EcR, USP InR, TOR, and *maleE* (control) dsRNA were injected into female beetles on day 0 PAE and the insects were starved continuously for 4 days PAE. The ovaries were dissected on day 4 PAE and stained with DAPI (a-f). Note that the primary oocyte development was blocked in EcR (panel c), USP (panel d), InR (panel e), TOR (panel f) RNAi insects but not in JHAMT (panel b) and *maleE* (panel a) RNAi insects. Scale Bar: 200 μ m

Fig. 6. Model depicting the involvement of regulatory networks comprising of JH, 20E, and IIS-TOR signaling pathways and their target tissue specific regulation of events during the previtellogenic phase of the female beetle.

Table 1

Primers used in qRT-PCR and to prepare dsRNA

Gene Name	Gene ID	Forward Primer 5'-3'	Reverse Primer 5'-3'
qRT-PCR			
PI3K	TC011996	CTCATGCCTAACGGGATTGT	TCCCAAGGCACTCAATTTTC
Tor	TC005546	GATTTTCGGGAACATGACCAC	TCTCCCTAATGGCAGGTTTG
InR	TC010784	CCTGGATTTCGTTCAACAGGT	GATCGAGTTCACGAAGCACA
Akt	TC007749	CGACTTCACCAAGTGCAAAA	GCCCCCTCATTGTAAACGTA
Chico	TC005892	AAGCTCCAACACGGAGAAGA	ACTGAAAAACGGTCGAATGC
Pten	TC005314	GGCAACATCTTTCCCAATA	TGTTTCGAATCGAGGAGCTT
Foxo	TC001062	CAACGAAGAGGGCAACAAGT	CGCACTGATTTTCCTGGTTT
TSC	TC002959	ATTCGGCCAACACTATCGTC	GCGACTTGCATGTGAATCAT
RHEB1	TC014205	GACTCGTACGACCCGACAAT	TGAATTTTCCCGTTGAGGTC
RHEB2	TC014613	GGCAAGTTCCTGGAGTCGTA	GTACGTCTTCCCGGTGATGT
S6K1	TC011817	AGACGGGAAGCGATAAGGAAAGCA	TCAGCCTTAGTGTGTGCAGTGTCT
S6K2	TC001074	GCCTTTACGCCATGAAGGTGCT	CCACATCCACGAGTATGTTTCGCT
GATA-1	TC010405	AATCGACCCAACCTCTGGACCAACT	TAATTCGACGAGCTTGGCACCCTA
JHIP07665	TC007665	TAGCTGCTCTGACTGAACTGCCA	TGCTAGAAAACAGTTCGCGGTAGT
JHIP14079	TC014079	CACCGTCGTTGATGAATCGAGAGA	TTGCATCAAAGTCCGGCAGCGTTT
Sodium channel	TC002633	ACAGATTCTGGTTACGCTCCCGAA	AGATGTAGTCGGTGTCTGTGCTGT
Uronyl-2-sulfotransferase	TC012787	GCAAGGGCTGAACAATTACAGGCA	AATTGGCGATCGAAGCTCAAAGGG
Sugar Transporter	TC012760	GCGAATCGACGACGAAATTGGTTC	TCTGCCGCAACGGTCCATTAAA
Serine Protease	TC016121	GGAGAACGACATTGGACTTATCCG	CTTACAGTAAGTGGTTCAGACCCC
Lipase	TC004656	AAGAAATCGACTTGCTGGCCGTTG	CGCAAGTTGGTCCACATTTCCGTT
dsRNA			
PI3K	TC011996	ATTGCAGAGGGAGTCAGGAACACA	ATCCAACCCTTCGCTCTTCCACAA
TOR	TC005546	ACGTTTGACTTTGAGGGCCAGAGA	ATCCAACCCTTCGCTCTTCCACAA
INR	TC010784	TCATGTCGTGAGGTTGTTGGGAGT	TTATTCTCTCCACTGCCCGAATGT
AKT	TC007749	TGCAGTGACACCGTCATAGAAA	TGCCCTTGCGATAGTAGTCCGTTT
CHICO	TC005892	TGGTCCGAGTGCTACCTCAAA	AAACAGAGGCGATCGTTGGTTTGG
PTEN	TC005314	ACGATGTGGTGAAGCTCCTCGATT	ACTTCTTCTCCGTGTTGGAGCTT
FOXO	TC001062	AGCCGTCTCCCGACCCCTTAAATA	ACTTGTCTTAAGCAGGGTCTCGT
TSC	TC002959	TGCGAGCGAGAATTTGGAAGACGA	TGCTGGTGATCCCTGAAATCGTCT
RHEB1	TC014205	ACTACGAGCTGACTTTGGTGGACA	ACTCTCGGCTCTTTCCAGGTTTGT
RHEB2	TC014613	TAATCGTGTGAGTCCCGAATGCGT	TTTCCCGTTGAGGTCTGGAGTTT
S6K1	TC011817	ACGTTCTCGCCAAACGAATAACGC	AATAATACGACTCACTATAGGG
S6K2	TC001074	ACTGCGAGGAGGCGATTATTGGA	TAGCCCTGGCGTTTCAACACTTCT
GATA-1	TC010405	TAGGTATCGATGGCCGATACACCGA	TACCTCGTCGATCCGCGTGTATT

Table 2

Genes up- and down-regulated upon starvation for 4 days PAE

Gene ID	Fed - 96 h	Starved - 96 h	Fold change	p-value	Annotations
Down-regulated					
TC013602	3.74	0.01	374.16	0.0035	Vg1
TC010839	1.9	0.01	190.91	0.00422	Vg2
TC007665	2.27	0.32	7.10	0.00201	JHIP
TC014079	1.51	0.49	3.09	0.00254	JHIP
TC000791	0.93	0.36	2.55	0.00876	JHIP
TC007883	1.09	0.52	2.11	0.00243	Trehalose-6-phosphate synthase
TC011471	1.92	0.03	62.41	0.00133	Fatty acid synthase?
TC011522	0.73	0.05	15.43	0.0037	Fatty acid synthase
TC002085	1.57	0.47	3.33	0.00958	Serpin
TC010930	0.90	0.13	7.17	0.00919	serine proteinase
TC015320	1.03	0.29	3.58	0.00464	cytochrome p450
TC009459	163.98	0.16	1016.91	0.0196	ahp12-carrier protein
TC008685	28.06	0.19	149.86	0.0379	alcohol dehydrogenase
TC009867	26.60	0.35	76.28	0.049	activating transcription factor 2
TC006769	1.28	0.01	102.37	0.0027	hexamerin 2 beta
TC007608	1.60	0.26	6.17	0.0277	glucose dehydrogenase
TC000938	0.64	0.14	4.76	0.0402	alpha-amyrase
TC013582	1.60	0.78	2.06	0.038	cathepsin k
TC015806	0.55	0.22	2.45	0.00829	growth differentiation factor
Up-regulated					
Transporter activity					
TC002633	0.14	44.83	321.61	0.00273	Sodium channel activity
TC012787	1.03	4.72	4.57	0.00422	uronyl-2-sulfotransferase
TC006632	0.28	1.16	4.10	0.00667	Na dependent phosphate transporter
TC012760	0.60	4.07	6.81	0.00925	sugar transporter
TC013653	1.00	19.53	19.38	0.00225	glucose transmembrane transporter activity

Gene ID	Fed – 96 h	Starved - 96 h	Fold change	p-value	Annotations
TC014381	0.95	3.25	3.40	0.00554	ATPase activity
TC012592	0.40	1.47	3.68	0.00401	3',5'-cyclic-AMP phosphodiesterase
TC012064	0.79	1.99	2.53	0.00525	glucuronosyltransferase activity
TC009330	0.58	1.68	2.91	0.00217	Na/Ca-exchange protein
Enzymatic activity					
TC014687	0.41	1.18	3.40	0.0078	esterase
TC011002	0.61	2.33	3.86	0.00639	cathepsin L-like proteinase
TC000091	0.65	1.43	2.20	0.00867	glycerol kinase
TC004656	0.78	1.99	2.54	0.00351	lipase
TC016121	0.26	2.40	9.12	0.00161	serine proteinase
TC004553	0.31	1.02	3.28	0.00474	serine-type endopeptidase activity
TC007694	0.666995	2.157539	3.23	0.00571	pyruvate carboxylase
TC009015	0.490899	1.546288	3.15	0.00279	glycoside hydrolases
TC0110386	0.410121	1.114783	2.72	0.00895	protein serine/threonine kinase activity
TC009625	0.449896	2.078377	4.62	0.00415	chitinase 2
TC002542	0.465626	1.082392	2.32	0.00682	cytochrome p450
Others					
TC005969	0.703965	1.717109	2.44	0.00733	transcription repressor activity
TC003109	0.783876	3.030341	3.87	0.00344	cuticle protein
TC005093	0.378914	0.845825	2.23	0.00836	heat shock protein 68

The total RNA isolated from the whole body of the female beetle at 1–4 day post adult emergence (PAE) were used for microarray analysis. The data filtered using Benjamini-Hochberg false discovery rate multiple testing corrections generated by GeneSpring GX v.9.0.1 software were used. The normalized expression levels of selective genes involved in vitellogenesis, JH and 20E synthesis and actions, and IIS-TOR pathways during 1–4 days PAE at different fold changes (1 day PAE Vs 4 day PAE) with p-value of less than 0.05 for each gene are shown for comparison.

Table 3

Comparison of effects of RNAi on the events involved from pre- to post-vitellogenesis

RNAi	Primary oocyte stage	Ovary maturation	Vg transcription	Egg development
<i>malE</i>	5	√	√	√
JHAMT	5	√	X	X
Met	5	√	X	X
Kr	4	√	√	√
Phantom	4	√	√	√
Shade	2	X	X	X
EcR	2	X	X	X
USP	2	X	X	X
InR	2	X	X	X
Akt	2	X	X	X
Chico	2	X	X	X
Pten	4	√	√	X
PI3K	2	X	X	X
Tsc	4	√	X	X
RHEB-1	4	√	√	√
RHEB-2	4	√	√	√
TOR	2	X	X	X
S6K1	4	√	√	√
S6K2	2	X	X	X

The ovarioles (n=30) were observed under 400× magnification. The staging of the primary oocytes was done based on Ullmann (1973). Briefly, Stage 2- oocyte situated, side by side, in neck region of ovariole; Stage 4- Oocyte increase in size, nucleus transforms into a germinal vesicle; Stage 5 – elongation of the oocyte, germinal vesicle central. √ indicates presence of the event, X – indicates absence of the event. The scoring of the events was based on the data from the current study and Parthasarathy et al. 2010a&b.