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**Mechanistic target of rapamycin (mTOR) signaling genes in decapod crustaceans: cloning
and tissue expression of mTOR, Akt, Rheb, and p70 S6 kinase in the green crab,
Carcinus maenas, and blackback land crab, *Gecarcinus lateralis***

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Abstract

Mechanistic target of rapamycin (mTOR) controls global translation of mRNA into protein by phosphorylating p70 S6 kinase (S6K) and eIF4E-binding protein-1. Akt and Rheb, a GTP-binding protein, regulate mTOR protein kinase activity. Molting in crustaceans is regulated by ecdysteroids synthesized by a pair of molting glands, or Y-organs (YOs), located in the cephalothorax. During premolt, the YOs hypertrophy and increase production of ecdysteroids. Rapamycin (1 μ M) inhibited ecdysteroid secretion in *C. maenas* and *G. lateralis* YOs *in vitro*, indicating that ecdysteroidogenesis requires mTOR-dependent protein synthesis. The effects of molting on the expression of four key mTOR signaling genes (mTOR, Akt, Rheb, and S6K) in the YO was investigated. Partial cDNAs encoding green crab (*Carcinus maenas*) mTOR (4031 bp), Akt (855 bp), and S6K (918 bp) were obtained from expressed sequence tags. Identity/similarity of the deduced amino acid sequence of the *C. maenas* cDNAs to human orthologs were 72%/81% for Cm-mTOR, 58%/73% for Cm-Akt, and 77%/88% for Cm-S6K. mTOR, Akt, S6K, and elongation factor 2 (EF2) in *C. maenas* and blackback land crab (*Gecarcinus lateralis*) were expressed in all tissues examined. The two species differed in the effects of molting on gene expression in the YO. In *G. lateralis*, *Gl-mTOR*, *Gl-Akt*, and *Gl-EF2* mRNA levels were increased during premolt. By contrast, molting had no effect on the expression of *Cm-mTOR*, *Cm-Akt*, *Cm-S6K*, *Cm-Rheb*, and *Cm-EF2*. These data suggest that YO activation during premolt involves up regulation of mTOR signaling genes in *G. lateralis*, but is not required in *C. maenas*.

1. Introduction

Mechanistic target of rapamycin (mTOR) controls cellular growth and is up regulated in mammalian cancers (Alayev and Holz, 2013; Cornu et al., 2013; Laplante and Sabatini, 2012; Zoncu et al., 2011). mTOR is a serine/threonine protein kinase member of the phosphoinositide-3-kinase (PI3K)-related kinase (PIKK) family (Laplante and Sabatini, 2012; Yang et al., 2013). It associates with other proteins to form two complexes, designated mTORC1 and mTORC2, that differ in functions (Jewell and Guan, 2013; Magnuson et al., 2012). mTORC1 stimulates protein and lipid synthesis, ribosome biogenesis, and energy metabolism and inhibits autophagy and lysosome biogenesis (Foster, 2013; Laplante and Sabatini, 2012). mTORC2 controls cell division, apoptosis, metabolism, and cytoskeletal organization (Laplante and Sabatini, 2012).

mTORC1 is regulated by various environmental and cellular cues. Stress and hypoxia are inhibitory and growth factors, high energy levels, and amino acids are stimulatory (Laplante and Sabatini, 2012; Roux and Topisirovic, 2012). Two targets of mTORC1 are p70 S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1), which control translation of mRNA (Magnuson et al., 2012; Teleman, 2010). Initiation of mRNA translation is stimulated when phospho-S6K phosphorylates the ribosomal S6 subunit and other translational-control proteins and phospho-4E-BP1 releases eIF4E to bind to the 5' end of an mRNA (Laplante and Sabatini, 2012; Magnuson et al., 2012; Roux and Topisirovic, 2012). Insulin and insulin-like peptides (ILPs) stimulate cell growth by activating a signal transduction cascade involving PI3K, 3'-phosphoinositide-dependent kinase-1 (PDK1), and Akt protein kinases (Laplante and Sabatini, 2012; Teleman, 2010). mTORC1 is directly regulated by Rheb (Ras homolog expressed in brain), a member of the Ras superfamily of GTP-binding proteins. Rheb/GTP activates mTORC1 and is inactivated by Rheb-GTPase activating protein (Rheb-GAP or TSC1/2), which stimulates

the hydrolysis of GTP to GDP. Rheb-GAP is inhibited when phosphorylated by Akt. Thus, insulin and ILP signaling prevents the hydrolysis of GTP by Rheb through the inhibition of Rheb-GAP, which keeps mTORC1 in the active state (Laplante and Sabatini, 2012; Teleman, 2010). Akt and S6K are members of the AGC Ser/Thr kinase family, which also includes PDK1 and protein kinases A, C, and G (Bayascas, 2010; Su and Jacinto, 2011).

Our knowledge of the insulin/mTOR pathway and its role in growth and development in crustaceans is far from complete. ILPs were reported in shrimp and lobster hepatopancreas (Gallardo et al., 2003; Hatt et al., 1997). An insulin-like growth factor expressed by the androgenic gland determines male sexual characteristics in crayfish and prawn (Ventura et al., 2011). Insulin receptor tyrosine kinase and phosphotyrosyl phosphatase are present in crustacean tissues (Chuang and Wang, 1994; Kucharski et al., 1999, 2002; Lin et al., 1993). In the brine shrimp, *Artemia franciscana*, S6K activity increases during pre-emergence development, when protein synthesis is restored after quiescence (Santiago and Sturgill, 2001). Recently we reported the cloning and characterization of cDNAs encoding Akt, mTOR, Rheb, and S6K from the blackback land crab, *Gecarcinus lateralis*, and Rheb from the green crab, *Carcinus maenas*, and the American lobster, *Homarus americanus* (MacLea et al., 2012). An up-regulation of *Gl-Rheb* expression coincides with an increase in protein synthesis in the claw skeletal muscle associated with molting (MacLea et al., 2012). These data indicate that mTOR has the same function in crustaceans as it does in other organisms.

mTOR is crucial for growth, aging, development, reproduction, and metamorphosis in insects (Bjedov et al., 2010; Gibbens et al., 2011; Gu et al., 2012; Mirth and Shingleton, 2012). The ILP/mTOR pathway controls the size and ecdysteroidogenic capacity of the insect molting gland, or prothoracic gland (PG) (Mirth and Shingleton, 2012; Rewitz et al., 2013; Teleman,

2010). Rheb and PI3K stimulate PG growth and protein synthesis (Colombani et al., 2005; Hall et al., 2007; Mirth et al., 2005). In addition, PI3K inhibitors and the mTOR inhibitor rapamycin block prothoracicotropic hormone (PTTH)-induced increases in ecdysteroid secretion in the PG (Gu et al., 2011a, 2012). Like insects, the crustacean molting gland, or Y-organ (YO), hypertrophies and increases in ecdysteroidogenic capacity during premolt (Covi et al., 2012; Lachaise et al., 1993; Skinner, 1985). Cycloheximide, an inhibitor of mRNA translation, suppresses YO ecdysteroid secretion *in vitro* (Mattson and Spaziani, 1986). These data suggest that sustained ecdysteroid synthesis and secretion in insect and crustacean molting glands require mTOR-dependent protein synthesis.

The mTOR pathway is regulated by transcriptional and posttranslational mechanisms. In *G. lateralis*, YO activation alters the levels of hundreds of proteins (Lee and Mykles, 2006). *G. lateralis* and *C. maenas* differ in response to eyestalk ablation (ESA) and multiple limb autotomy (MLA). ESA and MLA induce molting in adult *G. lateralis* (MacLea et al., 2012; Yu et al., 2001). As adult *C. maenas* are refractory to ESA and MLA (Abuhagr et al., 2014), animals undergoing natural molts were studied. *G. lateralis* adults were induced to molt by MLA, which resembles natural molts in this species. In order to examine the role of transcriptional regulation in the YO, cDNAs encoding mTOR, Akt, and S6K from *C. maenas* were characterized and the effects of molting on the expression of *mTOR*, *Rheb*, *Akt*, *S6K*, and *elongation factor 2 (EF2)* were quantified using quantitative PCR (qPCR). The tissue expression of *Akt*, *mTOR*, *S6K*, and *EF2* in both species was determined by endpoint RT-PCR. An *in vitro* YO assay was used to quantify the effects of rapamycin on ecdysteroid biosynthesis.

2. Materials and Methods

2.1. Animals

Adult *G. lateralis* were collected in the Dominican Republic and shipped via commercial air cargo to Colorado, USA. Animals were maintained at 27 °C in 75-90% relative humidity and a 12 h:12 h light:dark cycle (Covi et al., 2010). Molting was induced by multiple leg autotomy (MLA) and animals were kept in individual containers with a quartz sand substrate moistened with 10 ppt Instant Ocean (Covi et al., 2010; Skinner, 1985; Yu et al., 2002). Molt stage was determined by hemolymph ecdysteroid concentration and limb regenerate growth (Covi et al., 2010; Yu et al., 2002). Ecdysteroids were quantified by an enzyme-linked immunoassay (ELISA)(Kingan, 1989). **The ELISA was the same as that described in Abuhagr et al. (2014).** The regeneration (R) index (calculated as the length of the regenerate x 100/carapace width) increases from 0 to ~23 and provides an external measure of the progress of premolt events in *G. lateralis* (Yu et al., 2002).

Adult *C. maenas* were collected from Bodega Harbor, Bodega Bay, California. They were maintained under ambient conditions at about 13 °C in the flow-through seawater system of the Bodega Marine Laboratory. They were fed squid once a week. Green morphs undergoing spontaneous molts during the winter to spring molting season were used for the molting study. Molt stage was determined by hemolymph ecdysteroid concentration and integumentary structure (membranous layer and setal development in maxillae (Moriyasu and Mallet, 1986). Intermolt animals were shipped to Colorado and used for the YO assay, cDNA cloning, and tissue expression analysis. They were kept in aerated 30 ppt Instant Ocean at 20 °C and a 16 h:8 h light:dark cycle and fed cooked chicken liver once a week.

2.2. Effects of rapamycin on Y-organ ecdysteroid secretion

The effect of rapamycin (Selleck Chemicals, Houston, TX), an inhibitor of mTOR, was determined on YOs *in vitro*. The YO assay was the same as that described previously (Covi et al., 2008; Lee et al., 2007). Intermolt *G. lateralis* and *C. maenas* were eyestalk-ablated on Day 0. YO pairs were harvested 2 or 3 days post-ESA. One of each pair was incubated with 1% ethanol in medium; the other was incubated with varying concentrations of rapamycin in medium (5-fold serial dilutions, from 0.04 μM to 1 μM for *C. maenas* and from 0.008 μM to 1 μM for *G. lateralis*) for 30 min. After the 30 min pre-incubation, the culture was replaced with fresh medium with or without rapamycin and incubated for 4.5 h. Samples of the medium (100 μl) were combined with 300 μl methanol and ecdysteroids were quantified with ELISA.

2.3. Cloning of cDNAs encoding Cm-mTOR, Cm-Akt, and Cm-S6K

RT-PCR and RACE were used to clone cDNAs encoding Cm-mTOR, Cm-Akt, and Cm-S6K, based on the sequences of EST clones provided by the Mount Desert Island Biological Laboratory (Towle and Smith, 2006). The ESTs were sequenced by Davis Sequencing (Davis, CA) using vector and sequence-specific primers. All primers were synthesized by IDT (Coralville, IA). Total RNA was isolated from tissues using TRIzol reagent (Life Technologies, Carlsbad, CA) and cDNA was synthesized as described (Covi et al., 2010). The sequences of cDNAs encoding Cm-Rheb and *G. lateralis* Rheb, mTOR, Akt, and S6K are reported in (MacLea et al., 2012). Nested RT-PCR was used to extend the 5' end for Gl-mTOR using mTOR *C. maenas* forward primers with mTOR *G. lateralis* reverse primers to obtain additional Gl-mTOR sequence (Table 1).

Additional Cm-mTOR, Cm-Akt, and Cm-S6K sequences were obtained using nested 3' RACE. The FirstChoice RLM-RACE Kit (Applied Biosystems, Austin, TX) was used according to the manufacturer's instructions. RACE conditions were as follows: 0.4 μ l RACE template cDNA was used in each reaction, with 8 pmol of each primer (Table 1). After denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, lowest annealing temperature of a primer pair (Table 1) for 30 s, and 72 °C for 30 s to 1 min, were completed. Final extension was for 7 min at 72 °C. PCR products were separated by 1% agarose gel electrophoresis, purified using the Gel Extraction Kit (QIAEX II, Qiagen), and sequenced using direct sequencing with sequence-specific primers by Davis Sequencing.

2.4. Tissue expression of *EF2*, *mTOR*, *Akt*, and *S6K* mRNAs

End-point PCR was used to qualitatively assess the distribution of *Cm-EF2* (GenBank GU808334), *Cm-mTOR* (JQ864248), *Cm-Akt* (JQ864249), and *Cm-S6K* (JQ864250) mRNAs in *C. maenas* tissues and *Gl-EF2* (GenBank AY552550), *Gl-mTOR* (HM989973), *Gl-Akt* (HM989974), and *Gl-S6K* (HM989975) mRNAs in *G. lateralis* tissues. Total RNA was purified from tissues harvested from intermolt adult male animals and cDNA was synthesized as described above. Reactions contained 1 μ l template cDNA and 5 pmol each of the appropriate expression primers (Table 2) in GoTaq Green master mix (Promega). After denaturation at 94 °C for 3 min, 30 (for EF2) or 35 (for all other genes) cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, were completed. Final extension was for 7 min at 72 °C. PCR products were separated on a 1% agarose gel containing 40 mM Tris acetate and 2 mM EDTA (pH 8.5). The gels were stained with ethidium bromide and visualized with a UV light source.

2.5. Effects of molting on *mTOR* signaling gene expression in the YO

Molting was induced in *G. lateralis* by multiple leg autotomy. Animals were divided into three premolt stages (early premolt, R index ~10; mid-premolt, R ~15; and late premolt, R ~22) and two postmolt stages (2 days and 10 days postmolt). *C. maenas* green morphs were divided into 4 molt stages: intermolt, early premolt, late premolt and postmolt (2-7 days postecdysis). Hemolymph samples were collected for ecdysteroid ELISA and YOs were harvested for qPCR.

A Light Cycler 480 thermal cycler (Roche Applied Science, Indianapolis, IN) was used to quantify levels of *EF2*, *mTOR*, *Rheb*, *Akt* and *S6K* mRNAs for *G. lateralis* and *C. maenas*. Reactions consisted of 1 μ l first strand cDNA or standard, 5 μ l 2 \times SYBR Green I Master mix (Roche Applied Science), 0.5 μ l each of 10 mM forward and reverse primers (Table 2), and 3 μ l nuclease-free water. PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 20 s, and extensions at 72 °C for 20 s, followed by melting curve analysis of the PCR product. Transcript concentrations were determined with the Light Cycler 480 software (Roche, version 1.5) using a series of dsDNA standards produced by serial dilutions of PCR product for each gene (10 ag/ μ l to 10 ng/ μ l). The absolute amounts of transcript in copy numbers per μ g of total RNA in the cDNA synthesis reaction were calculated based on the standard curve and the calculated molecular weight of the dsDNA products.

2.6. Analyses and software

Multiple sequence alignments were produced with ClustalX version 2.0.12 (Thompson et al., 1997) using deduced amino acid sequences. Illustrator 10 (Adobe Systems, San Jose, CA) was used for constructing/annotating graphs and figures. Statistical analysis was performed using

JMP 5.1.2, 6.0.0, or 8.0.2 (SAS Institute, Cary, NC). Means for different molt stages were compared using analysis of variance. A matched pairs analysis was used to compare the means of the control and rapamycin treatments in the YO assay. Data are represented as mean \pm 1 S.E. and the level of significance was set at $\alpha = 0.05$. qPCR data were log transformed to reduce the variance of the mean.

3. Results

3.1. Effects of rapamycin on *Y*-organ ecdysteroid secretion

Rapamycin, an inhibitor of mTORC1, was a potent inhibitor of YO ecdysteroidogenesis *in vitro*. YOs from *G. lateralis* and *C. maenas* showed a dose-dependent inhibition by rapamycin, with maximum inhibition of ecdysteroid secretion (73% and 85%, respectively) at 1 μ M (Fig. 1).

3.2. Cloning of *C. maenas* mTOR signaling components

cDNAs encoding Cm-mTOR, Cm-Akt, and Cm-S6K were obtained using RT-PCR and 3' RACE and sequence-specific primers based on EST sequences (Towle and Smith, 2006). The characteristics of the three partial cDNAs are summarized in Table 3.

RT-PCR for Cm-mTOR initially yielded two sequences of 1543 and 1624 base pairs (bp). Nested RT-PCR was used to fill the gap between these two sequences and 3' RACE was used to obtain the 3' end of the open reading frame (ORF) and a part of the 3' untranslated region (3'UTR). The DNA and translated amino acid sequence of Cm-mTOR is presented in Figure 2. Multiple alignment of the deduced amino acid sequences of Cm-mTOR and Gl-mTOR with mTOR sequences of *Daphnia pulex* and 2 insect species (*Drosophila melanogaster* and *Aedes aegypti*) identified highly conserved domains, from N-terminus to C-terminus: HEAT repeat, FAT (for FRAP, ATM, and TRRAP), FKBP12-rapamycin-binding (FRB); kinase, and FAT C-

terminus (FATC) domains (Fig. 3). Additional sequence was obtained to extend the 5' end for Gl-mTOR using nested RT-PCR with Cm-mTOR forward primers and Gl-mTOR reverse primers (Table 1). As the human mTOR protein is 2549 amino acids in length, it is estimated that about 43% of the ORF of Gl-mTOR (3705 bp) and about 44% of the ORF of Cm-mTOR (4031 bp) were obtained. The deduced sequences shared high degrees of identity and similarity to the human ortholog at the protein level (Table 3), with even higher identity and similarity to insect orthologs (Fig. 3). The kinase domain included catalytic loop and activation loop motifs (Figs. 2, 3). Amino acids involved in rapamycin binding in the FRB domain (designated with an "r" in Fig. 3) and amino acids involved in ATP binding in the kinase domain (designated with a "#" in Fig. 3) were conserved. The FRB domain contained a phosphatidic acid binding motif (Fig. 3). The sequences of Gl-mTOR and Cm-mTOR were nearly identical, except for a 32-amino acid sequence in Cm-mTOR (amino acids #704-#735) that included a phosphorylation site near the start of the kinase domain (Fig. 3). This divergent sequence did not result from sequencing error, as the chromatograms from the sequencing reactions were unambiguous and the deduced sequence could not have resulted from a shift in the reading frame.

RT-PCR for Cm-Akt yielded a 728-bp product, which contained the pleckstrin homology domain and a portion of the kinase domain. 3' RACE obtained an additional 127 bp of the kinase domain. Attempts to obtain the rest of the open reading frame (ORF), 5' UTR, and 3' UTR by 3' and 5' RACE were unsuccessful. The DNA and translated amino acid sequence of Cm-Akt is presented in Figure 4. Multiple alignments compared the amino acid sequences of Cm-Akt with Akt sequences from *G. lateralis*, *D. pulex*, *D. melanogaster*, and *A. aegypti* (Fig. 5). There were high degrees of identity and similarity in the pleckstrin homology and kinase domains between the orthologs from the 5 arthropod species (Fig. 5) and between Cm-Akt and human Akt (Table

3). The kinase domain included an activation loop motif in the Gl-Akt (Figs. 4, 5). Amino acids involved in ATP binding in the kinase domain (designated with a “#” in Fig. 5) were conserved.

An initial 869-bp cDNA of Cm-S6K was obtained by RT-PCR using primer sets from a multiple sequence alignment of several *C. maenas* ESTs. 3' RACE was used to obtain an additional 49 bp of the ORF; attempts to obtain the rest of the ORF, 5' UTR, and 3' UTR by 3' and 5' RACE were unsuccessful. The cDNA encoded the kinase domain and linker region. The DNA and translated amino acid sequence of Cm-S6K are presented in Figure 6. It showed high degrees of identity and similarity to the human ortholog at the protein level (Table 3). Alignment of the two decapod S6K sequences with Dp-S6K and insect S6Ks showed high sequence identity/similarity (Fig. 7). In the kinase domain, the threonine in the activation loop and amino acids involved in ATP binding were conserved (Fig. 7). In the linker region, the 7 amino acid sequence of the turn motif, including the serine phosphorylation site, was identical between the 5 species (Fig. 7). However, the 6-amino acid sequence of the Cm-S6K hydrophobic motif diverged from that of the other 4 species; a threonine in the phosphorylation site was replaced by a serine in the Cm-S6K (Fig. 7). The sequence C-terminal to the hydrophobic motif contained a 5-amino acid inhibitory sequence followed by a region that varied in amino acid sequence and length (Fig. 7). The Gl-S6K inhibitory sequence differed in two amino acids (ESPRK) from that (RSPRR) in the *D. pulex* and insect sequences (Fig. 7).

3.3. Tissue expression of mTOR signaling genes

Endpoint RT-PCR was used to determine the expression of *EF2*, *mTOR*, *Akt*, and *S6K* in tissues from *G. lateralis* and *C. maenas* (Fig. 8). *EF2* was included as a constitutively expressed gene to assess RNA isolation and cDNA synthesis. All four genes were expressed in all tissues,

including eyestalk ganglia, thoracic ganglion, YO, hepatopancreas, heart, skeletal muscle from the claw and thorax, midgut, hindgut, and testis.

3.4. Effects of molting on expression of *mTOR* signaling genes

G. lateralis were multiple leg autotomized and entered premolt a few weeks later. Molt stage was monitored by measuring the R index. The ecdysteroid titers in the hemolymph were low during early premolt (R index ~10), increased in mid-premolt (R index ~15), and reached a peak in late premolt (R index ~22) (Fig.9A). Ecdysteroid titers were lowest at 2 days and 10 days postmolt, indicating that the YOs had returned to the basal state.

There were significant effects of molting on *Gl-EF2*, *Gl-mTOR*, and *Gl-Akt* expression in the *G. lateralis* YO. *Gl-EF2* mRNA level increased during premolt, with the mean at late premolt (R ~22) about 13-fold higher than the mean at early premolt (R ~10) (Fig. 9B). There was a significant decrease in *Gl-EF2* mRNA levels at 2 and 10 days postmolt to levels that were not significantly different from the *Gl-EF2* level at early premolt (Fig. 9B). *Gl-mTOR* expression was elevated during premolt, with the means at early, mid (R~15), and late premolt not significantly different from each other (Fig. 9C). *Gl-mTOR* mRNA levels decreased during postmolt to a level at 10 days postmolt that was 40-fold lower than that at mid premolt and 42-fold lower than that at late premolt (Fig. 9C). *Gl-Akt* mRNA levels increased during premolt, with the means at mid and late premolt significantly higher than the mean at early premolt (Fig. 9E). There was a significant decrease at 2 days postmolt, with the means at 2 and 10 days postmolt not significantly different from the mean at early premolt (Fig. 9E). There was a trend of increased *Gl-Rheb* and *Gl-S6K* mRNA levels at mid and late premolt and decreased mRNA levels at 10 days postmolt, but the means were not significantly different (Fig. 9D, F).

As adult *C. maenas* are refractory to MLA (Abuhagr et al. 2014), gene expression in the YOs was quantified in *C. maenas* green morphs undergoing natural molts. The hemolymph ecdysteroid titers showed the characteristic pattern over the molt cycle: low levels during intermolt, increasing levels during premolt, and lowest levels during postmolt (Fig. 10A). Expression of *Cm-mTOR*, *Cm-Rheb*, *Cm-Akt*, *Cm-S6K*, and *Cm-EF2* in YOs from intermolt, early premolt, and late premolt animals was quantified. Gene expression at the postmolt stage was not quantified, as the RNA concentrations were too low for cDNA synthesis. Unlike *G. lateralis*, there was no effect of molting on the expression of the five genes (Fig. 10B).

4. Discussion

The mTOR signaling pathway is found in all metazoans and controls cellular growth in response to a variety of environmental cues (Laplante and Sabatini, 2012; Su and Jacinto, 2011; Zoncu et al., 2011). mTORC1 stimulates translation of mRNA via phosphorylation of S6K and 4E-BP1 (Laplante and Sabatini, 2012; Magnuson et al., 2012; Zoncu et al., 2011). Akt is a central component in the mTOR pathway, as it mediates the transduction of extracellular signals to the translational machinery (Roux and Topisirovic, 2012). As members of the AGC protein kinase family, Akt and S6K share structural similarities and mechanisms of activation (Su and Jacinto, 2011). Full activation requires phosphorylation at two sites: the activation loop in the catalytic domain and the hydrophobic motif located C-terminal to the kinase domain (Bayascas, 2010; Magnuson et al., 2012). PDK1 phosphorylates a threonine in the activation loop, but the protein kinases that phosphorylate the hydrophobic domain differ among the different AGC kinase substrates. For example, mTORC1 phosphorylates the hydrophobic motif in S6K, but not in Akt (Bayascas, 2010). By contrast, mTORC2 phosphorylates the hydrophobic motif in Akt,

but not S6K (Bayascas, 2010). mTORC1 is regulated by Rheb, a GTP-binding protein. Akt and Rheb are linked in the pathway by Rheb-GAP, which inactivates mTORC1 by promoting the hydrolysis of the GTP bound to Rheb to GDP. Akt phosphorylates and inactivates Rheb-GAP. Thus, inactivation of Rheb-GAP by Akt leads to the activation of mTORC1 by keeping Rheb in the active state.

mTOR-dependent protein synthesis is involved in PTTH-stimulated ecdysteroidogenesis in the insect PG (Mirth and Shingleton, 2012; Rewitz et al., 2013; Teleman, 2010). Rapamycin inhibits protein synthesis in *Drosophila* S2 cells (Hall et al., 2007). In *Bombyx mori*, rapamycin inhibits PTTH-dependent phosphorylation of S6K and 4E-BP1 and ecdysteroid synthesis and secretion (Gu et al., 2012). In *Manduca sexta*, feeding rapamycin to larvae delays molting and reduces the growth and ecdysteroid production by the PG (Kemirembe et al., 2012). In crustaceans, rapamycin inhibited ecdysteroid secretion in YO from *G. lateralis* and *C. maenas* *in vitro* (Fig. 1). These data indicate that activation of the molting gland requires mTORC1 activity in both insects and crustaceans.

Crustacean tissues express mTOR signaling genes. cDNAs encoding mTOR, Akt, and S6K were characterized from *C. maenas* (Table 3; Figs. 2, 4, and 6) and *G. lateralis* (Table 3; MacLea et al., 2012). In addition, cDNAs encoding Rheb were obtained from *H. americanus*, *C. maenas*, and *G. lateralis* (MacLea et al., 2012). The deduced sequences show high sequence identity and domain organization to orthologs from insects and other species (Table 3; Figs. 3, 5, and 7; MacLea et al., 2012). mTOR, Rheb, Akt, and S6K were expressed in every tissue, indicating that this pathway has essential cellular functions (Fig. 8; MacLea et al., 2012).

The mTOR protein has five domains: a large N-terminal HEAT (Huntington, Elongation Factor 3, a subunit of PP2A and TOR) repeat, FRB (FKBP12 rapamycin binding), FAT

(FRAP, ATM, and TRRAP), kinase, and FATC (FAT C-terminal) domains (Magnuson et al., 2012; Su and Jacinto, 2011; Zoncu et al., 2011). The partial cDNAs of mTOR from *G. lateralis* and *C. maenas* encoded part of the HEAT repeat domain and the FAT, FRB, kinase, and FATC domains (Fig. 2). HEAT repeat units are characteristic of the PIKK family; each unit consists of two antiparallel α -helices and multiple units occur in a series (Perry and Kleckner, 2003). The FAT domain has 28 α -helices arranged in α -helix HEAT-like repeats, forming a solenoid structure that clamps around the kinase domain; it may function as a protein interaction platform (Yang et al., 2013). The kinase domain has a two-lobed structure with catalytic loop and activation loop motifs characteristic of the PIKK family (Fig. 3; Su and Jacinto, 2011; Yang et al., 2013). Phosphorylation of a serine and a threonine, located near the 5' end of the kinase domain, is associated with mTOR activation (Ekim et al., 2011); this site is highly conserved between Gl-mTOR, Dp-mTOR, and insect mTOR (Fig. 3; S710 and T715 in the Gl-mTOR sequence). The Cm-mTOR has a 32-amino acid sequence that includes the phosphorylation site, which diverges from the other arthropod mTOR sequences; the serine and threonine in the phosphorylation site are replaced with valine and aspartate, respectively (Fig. 3, amino acids #728 and #734). However, the 32-amino acid sequence has a potential alternative phosphorylation site that is immediately upstream of the conserved site. The sequence PRSGHRST (Fig. 3, amino acids #704-711) shares features characteristic of phosphorylation sites in various proteins: a serine and a threonine with similar spacing (5-6 amino acids apart), arginines within 2 amino acids of the S706 and T711, and a nearby proline (Rust and Thmpson, 2011; Ubersax and Ferrell, 2007). Rapamycin, when complexed with FKBP12 and other FK506-binding proteins, binds to the FRB domain to inhibit mTORC1 activity; the FRB protrudes from the kinase domain and binding of FKBP12 to the FRB restricts access of substrates to the active

site (Camargo et al., 2012; Marz et al., 2013; Yang et al., 2013). The FRB has a conserved phosphatidic acid binding site and 12 highly conserved amino acids located in and around the rapamycin binding site; 11 of the 12 amino acids are identical at their respective positions in the arthropod sequences (Fig. 3; Foster, 2013; Yang et al., 2013). The FATC domain has an α -helix and a disulfide bonded loop involving two cysteines near the C-terminus (Fig. 3); it is structurally integral to the kinase domain and may stabilize the activation loop (Yang et al., 2013). Reduction of the disulfide bridge increases the flexibility of the FATC region, which destabilizes the protein and reduces mTOR protein level (Dames et al., 2005). Thus, the redox potential may determine mTOR stability in the cell. There is a negative regulatory region located between the kinase and FATC domains; it consists of helix α 9b at the end of the kinase domain, followed by a nonstructured sequence that varies in amino acid sequence and length among species (Fig. 3; Yang et al., 2013). It is thought that helix α 9b controls substrate recruitment to the active site (Yang et al., 2013). Interestingly, the helix α 9b sequence of the Gl-mTOR differs from those in mTORs from *C. maenas*, *D. pulex*, and insects (Fig. 3); the functional significance of this difference is unknown.

Akt is the central mediator of protein synthesis and cell survival in response to growth factors (Fayard et al., 2010; Vasudevan and Garraway, 2011). The protein has four domains: pleckstrin homology, α -helical linker, kinase, and regulatory C-terminal (RC) domains (Fig. 5; Hanada et al., 2004; Vasudevan and Garraway, 2011). The pleckstrin homology domain interacts with the membrane via phosphatidylinositol (3, 4, 5) trisphosphate produced by PI3K. The kinase domain has the activation loop that is characteristic of AGC kinase family members (Hanada et al., 2004). PDK1 phosphorylation of threonine in the activation loop (T320 in Gl-Akt; the Cm-Akt cDNA sequence ended before the activation loop; see Fig. 5) and

phosphorylation of a serine in the hydrophobic motif by mTORC2 or DNA-dependent protein kinase is required for full Akt activity (Fayard et al., 2010; Hanada et al., 2004; Vasudevan and Garraway, 2011). The partial Akt from *G. lateralis* had the pleckstrin homology, helix, and kinase domains and part of the RC domain (Fig. 4). The Cm-Akt cDNA had the pleckstrin homology and helix linker domains and a portion of the kinase domain (Fig. 4). We were unable to extend either sequence to include the hydrophobic motif.

S6K is a major target of mTORC1 and is also a member of the AGC protein kinase family (Fenton and Gout, 2011; Magnuson et al., 2012). S6K consists of acidic N-terminal, kinase, linker, and basic C-terminal tail domains (Magnuson et al., 2012). The deduced amino acid sequences of *G. lateralis* and *C. maenas* S6K shared high identity and similarity with each other and with other arthropod sequences (Fig. 7). The activation loop in the kinase domain contained a threonine residue (T145 in the Cm-S6K and T192 in the Gl-S6K sequences) that is phosphorylated by PDK1 (Fenton and Gout, 2011; Magnuson et al., 2012). The linker domain has two conserved phosphorylation sites that stabilize the active conformation: a serine in the turn motif (S287 in Cm-S6K and S333 in Gl-S6K; Fig. 7) and a threonine in the hydrophobic motif (T351 in Gl-S6K; the Cm-S6K had a serine, S305, at this position; Fig. 7) (Magnuson et al., 2012). The threonine in the hydrophobic motif is phosphorylated by mTORC1 (Fenton and Gout, 2011; Magnuson et al., 2012). S6K is phosphorylated at multiple sites, but, as is the case for Akt, full activation of S6K requires phosphorylation of the sites in the activation loop and hydrophobic motif (Fenton and Gout, 2011; Magnuson et al., 2012). The turn motif may be phosphorylated co-translationally by a constitutive protein kinase (Magnuson et al., 2012). The C-terminal sequences of arthropod S6K vary in length and amino acid sequence, but they do have an inhibitory RSPRR motif that occurs in mammalian S6K (Fig. 7; Magnuson et al., 2012).

The RSPRR motif may function as a binding site for phosphatases (Magnuson et al., 2012). **We were unable to extend the Cm-S6K sequence at the 3' end to include the RSPRR motif.**

mTOR is regulated posttranslationally via PI3K/Akt signaling in insects (Gu et al., 2011a,b, 2012). mTOR can also be regulated transcriptionally. Reduced expression of *Rheb*, *mTOR*, and *S6K* inhibits protein synthesis and growth of *Drosophila* S2 cells, while reduced *TSC2* expression increases protein synthesis and cell growth (Hall et al., 2007). Over-expression of *Rheb*-GAP inhibits PG growth (Layalle et al., 2008). By contrast, over-expression of PI3K, an upstream activator of Akt, stimulates PG growth and protein synthesis in larvae (Colombani et al., 2005; Hall et al., 2007; Layalle et al., 2008; Mirth et al., 2005). Over-expression of *Rheb* increases protein synthesis in *Drosophila* larvae (Hall et al., 2007). PTTH decreases expression of 4E-BP1 and S6K (Gu et al., 2012). In *G. lateralis*, an increase in *Rheb* expression coincides with a molt-associated increase in protein synthesis in skeletal muscle (Covi et al., 2010; MacLea et al., 2012). Molting increased expression of *Gl-mTOR* and *Gl-Akt* (Fig. 9C, E). Levels of *Gl-Rheb* and *Gl-S6K* mRNAs were highest at mid and late premolt, but the means were not significantly different from those at early premolt and at postmolt stages (Fig. 9D, F). Expression of *Gl-EF2* was also increased during premolt (Fig. 9B). EF2 is a constitutively expressed protein that is essential for translation, as it promotes GTP-dependent translocation of the ribosomal during elongation of a polypeptide (Greganova et al., 2011). As it is not a known substrate of mTORC1, the increase in *Gl-EF2* mRNA level suggests that molting also increases the translational machinery of the YO. Thus, YO hypertrophy and increased ecdysteroid synthesis in *G. lateralis* involves the up regulation of mTOR signaling pathway genes and EF2 to stimulate protein synthesis. By contrast, there was no effect of molting on gene expression in *C. maenas* (Fig. 10B), suggesting that mTOR signaling is not regulated by transcriptional mechanisms.

Regardless of the differences in gene expression between the two species, mTORC1 activity is required for sustained ecdysteroidogenesis, as rapamycin inhibited YO ecdysteroid secretion in both species (Fig. 1).

5. Conclusions

cDNAs encoding mTOR, Akt, and S6K were cloned from the green crab, *C. maenas*. The sequences were highly conserved with orthologs from *G. lateralis*, *D. pulex*, insects, and human and were expressed in all tissues examined. Molting processes are triggered by activation of the YO, which hypertrophies to increase ecdysteroid synthesis and secretion during the premolt stage. The mTOR signaling pathway, which controls translation of mRNA into protein, appears to be involved in increased ecdysteroidogenesis in the activated YO, as the mRNA levels of *Gl-mTOR* and *Gl-Akt* were significantly higher during premolt and rapamycin inhibited ecdysteroid secretion by YO from eyestalk-ablated *G. lateralis* and *C. maenas in vitro*. The increase in *Gl-EF2* indicates there was also an up regulation of translational capacity in the *G. lateralis* YO. However, molting had no effect on the expression of *Cm-mTOR*, *Cm-Rheb*, *Cm-Akt*, *Cm-S6K*, and *Cm-EF2*, suggesting that transcriptional up regulation of the four mTOR signaling genes is not necessary for YO activation in *C. maenas*. The relative contributions of transcriptional and posttranscriptional regulation of mTORC1 activity can vary among different species. **It is unlikely that the differences are due to naturally molting animals (*C. maenas*) vs. MLA-induced molting animals (*G. lateralis*), as molting in MLA *G. lateralis* resembles that of intact animals (Covi et al., 2010; Skinner, 1985). MLA stimulates precocious molts, but it usually takes 3-4 weeks before *G. lateralis* enter premolt, during which animals regenerate lost appendages (Skinner, 1985; Yu et al., 2002). The duration of the premolt period (3-4 weeks) is the same**

between intact and MLA animals and MLA-induced *G. lateralis* successfully complete ecdysis at the same rate as intact animals (Covi et al., 2010; Skinner, 1985). Thus, MLA accelerates the decision to molt, but does not shorten the length of the premolt period. Taken together, these data indicate that transcriptional regulation of mTOR-dependent protein synthesis is not necessarily required for increased YO ecdysteroidogenesis during premolt.

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Table 1. Oligonucleotide primers used in the cloning of mTOR signaling genes from *C. maenas* (Cm) and *G. lateralis* (Gl). Abbreviations: Akt, protein kinase B; EF2, elongation factor 2; F, forward; IF, inner forward; IR, inner reverse; mTOR, mechanistic target of rapamycin; OF, outer forward and OR, outer reverse R, reverse; S6K, p70 S6 kinase.

Primer	Sequence (5'-3')	Use	Annealing Temperature
Cm-mTOR OF1	AACGGTGGCATGAGAAGC	Nested PCR	55 °C
Cm-mTOR OR1	AGAGATTACACAGACGCTCC	Nested PCR	
Cm-mTOR IF2	ATGTGCGTGCTCAGATGG	Nested PCR	56 °C
Cm-mTOR IR2	TCTGCACTACAAGGAGGAGG	Nested PCR	
Cm-mTOR F1	CTTGGCAGAGTTCATGGAGC	PCR	57 °C
Cm-mTOR R1	ACAGGTCATGCGATACGTGC	PCR	
Cm-Akt F1	GATGATGCTCAACCTTAACGTG	PCR	53 °C
Cm-Akt R1	CACGTTAAGGTTGAGCATC	PCR	
Cm-S6K F1	GAAGGCGAAAGAAGAGTTTCG	PCR	57 °C
Cm-S6KR1	ACGTCCGTCCCTTGACTCTC	PCR	
Cm-mTOR OF1	ACGAGAGCTGCTACAAGG	Nested PCR	54°C
Cm-mTOR OR1	GTCTCTCCCACTACTACCCTTG	Nested PCR	
Cm-mTOR IF2	GAACCTTGAGGCAATAC	Nested PCR	50 °C
Cm-mTOR IR2	ATCCTGCCTCAAGTCCTC	Nested PCR	
Cm-mTOR 3'F1	AAAGCGTTCTGTGGTGGGTGAG	3'RACE	60 °C
Cm-mTOR 3'F2	TCCTGACCTGCTATTGACACTGCC	3'RACE	60 °C
Cm-Akt 3'F1	ACTCACCCTACTACTACTCGG	3'RACE	60 °C
Cm-Akt 3'F2	TGATGGAGTATGTCAATGGTGGAG	3'RACE	60 °C
Cm-S6K 3'F1	TCTTGGGTAGTTCTCCGTAGACAA	3'RACE	60 °C
Cm-S6K 3'F2	GCGTAAAGATGTGATTATTGAGCG	3'RACE	60 °C
Cm-mTOR OF1	AACGGTGGCATGAGAAGC	Nested PCR	56 °C
Gl-mTOR OR1	CGAGTTGGTAGACAGCGGAAT	Nested PCR	
Cm-mTOR IF2	ATGTGCGTGCTCAGATGG	Nested PCR	57 °C
Gl-mTOR IR2	TGATGAGCAGCGTGTTGACC	Nested PCR	
Cm-mTOR OF1	AGAGATTACACAGACGCTCC	Nested PCR	53 °C
Gl-mTOR OR1	AACCAAACAGTCAGCAGG	Nested PCR	
Cm-mTOR IF2	TCTGCACTACAAGGAGGAGG	Nested PCR	54 °C
Gl-mTOR IR2	GAGATGGAGCGGATGAAC	Nested PCR	

Table 2. Oligonucleotide primers used in the expression analysis of mTOR signaling genes from *G. lateralis* (Gl) and *C. maenas* (Cm). Abbreviations: Akt, protein kinase B; EF2, elongation factor 2; F, forward; mTOR, mechanistic target of rapamycin; R, reverse; Rheb, Ras homolog expressed in brain; S6K, p70 S6 kinase.

Primer	Sequence (5'-3')	Product Size (bp)
Gl-EF2 F1	TTCTATGCCTTTGGCCGTGTCTTCTC	227
Gl-EF2 R1	ATGGTGCCCGTCTTAACCA	
Gl-mTOR F2	AGAAGATCCTGCTGAACATCGAG	159
Gl-mTOR R2	AGGAGGGACTCTTGAACCACAG	
Gl-Rheb F1	TTTGTGGACAGCTATGATCCC	119
Gl-Rheb R1	AAGATGCTATACTCATCCTGACC	
Gl-Akt F2	AACTCAAGTACTCCAGCGATGATG	156
Gl-Akt R1	GGTTGCTACTCTTTTCACGACAGA	
Gl-S6K F2	GGACATGTGAAGCTCACAGACTTT	239
Gl-S6K R1	TTCCCCTTCAGGATCTTCTCTATG	
Cm-EF2 F1	CCATCAAGAGCTCCGACAATGAGCG	278
Cm-EF2 R1	CATTTTCGGCACGGTACTTCTGAGCG	
Cm-mTOR F2	CATCCCTCAAACCTCATGCT	319
Cm-mTOR R2	CACCCACCACAGAACGCTTT	
Cm-Rheb F2	ATGGGCAAAGTCACAGTTCC	281
Cm-Rheb R2	GTCAGGAAGATGGTGGCAAT	
Cm-Akt F1	GTGAAGCAATGCCAGATCCT	259
Cm-Akt R2	CGGGTGTATCATCATCATCG	
Cm-S6K F2	TCTCCGTCATCTGAGCCGCT	258
Cm-S6K R2	GTACATGGCACCCGAGATCC	

Table 3. cDNA clones encoding mTOR signaling genes from *C. maenas* (Cm) and mTOR from *G. lateralis* (Gl). Abbreviations: Akt, protein kinase B; Cm, *C. maenas*; mTOR, mechanistic target of rapamycin; S6K, p70 S6 kinase.

Clone	Accession number	Completeness	Size (bp)	Identity (Similarity) to human ortholog
Cm-mTOR	JQ864248	Partial	4031	72% (81%)
Cm-Akt	JQ864249	Partial	855	58% (73%)
Cm-S6K	JQ864250	Partial	918	77% (88%)
Gl-mTOR	HM989973	Partial	3705	69% (80%)

Figure Legends

Fig. 1. Effects of mTOR complex 1 inhibitor rapamycin on YO ecdysteroidogenesis *in vitro*.

Paired YOs from eyestalk ablated *G. lateralis* (Gl) and *C. maenas* (Cm) were incubated with rapamycin or 1% ethanol for 4.5 h and ecdysteroids secreted into the medium were quantified by ELISA. The secretion with rapamycin was expressed as the % secretion of the paired control. Data presented as mean \pm 1 SE. Gl *P* values and sample size (n) at each rapamycin concentration: 0.008 μ M, *P* = 0.1523 (n = 4); 0.04 μ M, *P* = 0.0239 (n = 6); 0.20 μ M, *P* = 0.0030 (n = 6); and 1 μ M, *P* = 0.0024 (n = 5). Cm *P* values and sample size (n) at each rapamycin concentration: 0.04 μ M, *P* = 0.2211 (n = 6); 0.20 μ M, *P* = 0.0149 (n = 6); and 1 μ M, *P* = 0.0018 (n = 8). Control ecdysteroid secretion of Gl YO was 10.58 ± 0.72 (n = 21) and of Cm YO was 9.17 ± 0.92 (n = 20) in 4.5 h.

Fig. 2. Nucleotide and deduced amino acid sequences of cDNA encoding Cm-mTOR.

The cDNA encoded the 3' end of the ORF and the complete 3'-UTR. Asterisk indicates stop codon. The polyadenylation signal in the 3'-UTR is underlined. Font color indicates different domains, from 5' to 3': red, HEAT repeats; orange, FAT and FATC; green, FRB; and blue, kinase. The catalytic loop and activation loop sequences in the kinase domain are indicated by a double underline and dashed underline, respectively.

Fig. 3. Multiple alignment of deduced amino acid sequences of mTOR proteins in three

crustacean species and two insect species. Abbreviations: Aa, *Aedes aegypti* (AAR97336); Cm, *C. maenas* (JQ864248); Dm, *Drosophila melanogaster* (NP524891); Dp, *Daphnia pulex* (EFX69318); and Gl, *G. lateralis* (HM989973). Amino acid residues that are identical or similar

in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment. The colored solid lines above the sequences indicate the HEAT repeat (red), FAT and FATC (orange), FRB (green), and kinase (blue) domains. In the kinase domain are the catalytic loop (CL) and activation loop (AL). A negative regulatory region extends from helix ka9b (designated by the dotted line under the sequence) at the end of the kinase domain through a variable unstructured sequence to the FATC domain (Yang et al., 2013). The FRB domain has a phosphatidic acid (PA) binding site; the “I” above the sequences indicates location of a serine essential for PA binding (Foster, 2013). Asterisks (*) indicate phosphorylation sites; hashtags (#) indicate amino acids involved in ATP binding; and “r” designates conserved amino acids located around the rapamycin binding site in the FRB domain (Yang et al., 2013).

Fig. 4. Nucleotide and amino acid sequences of cDNA encoding Cm-Akt. The cDNA encoded an incomplete ORF. Font color indicates different domains, from 5' to 3': red, pleckstrin homology; blue, kinase.

Fig. 5. Multiple alignment of deduced amino acid sequences of Akt proteins in three crustacean species and two insect species. Abbreviations: Aa, *A. aegypti* (AAP37655); Cm, *C. maenas* (JQ864249); Dm, *D. melanogaster* (NP732114); Dp, *D. pulex* (EFX86288); and Gl, *G. lateralis* (HM989974). Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment. The colored solid lines above the sequences indicate the pleckstrin homology (red), kinase (blue), and regulatory C-terminal

(green) domains. The kinase domain has the activation loop (AL) and the regulatory C-terminal has the hydrophobic motif (HM). Asterisks (*) indicate phosphorylation sites; hashtags (#) indicate amino acids in the kinase domain involved in ATP binding.

Fig. 6. Nucleotide and amino acid sequences of cDNA encoding Cm-S6K. Partial sequence within the catalytic domain was obtained for *C. maenas*. Blue font color indicates the kinase domain, with the activation loop indicated by the dashed underline. The hydrophobic motif is indicated by the dotted underline; the serine that is phosphorylated is italicized. The putative hydrophobic motif at the end of the sequence is underlined.

Fig. 7. Multiple alignment of deduced amino acid sequences of S6K proteins in three crustacean species and two insect species. Abbreviations: Aa, *A. aegypti* (XP001650653); Cm, *C. maenas* (JQ864250); Dm, *D. melanogaster* (AAC47429); Dp., *D. pulex* (EFX86042); and Gl, *G. lateralis* (HM989975). Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment. The blue solid lines above the sequences indicate the kinase (blue) domain, which has the activation loop (AL). The linker region has the turn motif (TM) and hydrophobic motif (HM) (Magnuson et al., 2012). An inhibitory RSPRR motif is located C-terminal to the HM. Asterisks (*) indicate phosphorylation sites; hashtags (#) indicate amino acids involved in ATP binding.

Fig. 8. Expression of EF2 and mTOR signaling genes in *G. lateralis* (A) and *C. maenas* (B) tissues using endpoint RT-PCR. Elongation factor 2 (EF2) is a constitutively expressed gene

that served as a control for RNA isolation and cDNA synthesis. PCR products after 30 cycles (EF2) or 35 cycles (mTOR, Akt, and S6K) were resolved by agarose gel electrophoresis. Inverted images of ethidium bromide-stained gels are shown. Sizes of expected PCR products are indicated at left. All four genes were expressed in all tissues. Abbreviations, from left to right: EG, eyestalk ganglia; TG, thoracic ganglia; YO, Y-organ; HP, hepatopancreas; H, heart; CM, claw muscle; TM, thoracic muscle; MG, midgut; HG, hindgut; and T, testis.

Fig. 9. Effects of molt induction by MLA on hemolymph ecdysteroid titers and YO expression of *Gl-EF2* and mTOR signaling genes in *G. lateralis*. (A) Hemolymph ecdysteroid levels were quantified by ELISA. *Gl-EF2* (B), *Gl-mTOR* (C), *Gl-Rheb* (D), *Gl-Akt* (E), and *Gl-S6K* (F) mRNA levels at early premolt (10-R), mid premolt (15-R), late premolt (22-R), 2 days postmolt, and 10 days postmolt were quantified by qPCR. Data are presented as mean \pm 1 S.E. (n = 6 for 10-R, 13 for 15-R, 6 for 22-R, 9 for 2 days postmolt, and 4 for 10 days postmolt). Means that were significantly different from each other have the same letter (a, b, and c). There were no significant differences in the means for *Gl-Rheb* and *Gl-S6K*.

Fig. 10. Effects of molting on hemolymph ecdysteroid titers and YO expression of *Cm-EF2* and mTOR signaling genes in *C. maenas*. Data are presented as mean \pm 1 S.E. (intermolt, n = 6; early premolt, n = 12; late premolt, n = 6; and postmolt, n = 8). (A) Hemolymph ecdysteroid levels were quantified by ELISA. Means that were significantly different from each other have the same letter (a, b, and c). (B) *Cm-mTOR*, *Cm-Rheb*, *Cm-Akt*, *Cm-S6K*, and *Cm-EF2* mRNA levels at intermolt, early premolt, and late premolt stages were quantified by qPCR. Postmolt

YOs were not measured, as the RNA concentrations were too low for cDNA synthesis. There were no significant differences in the means for all five genes at all the molt stages.

Figure 1

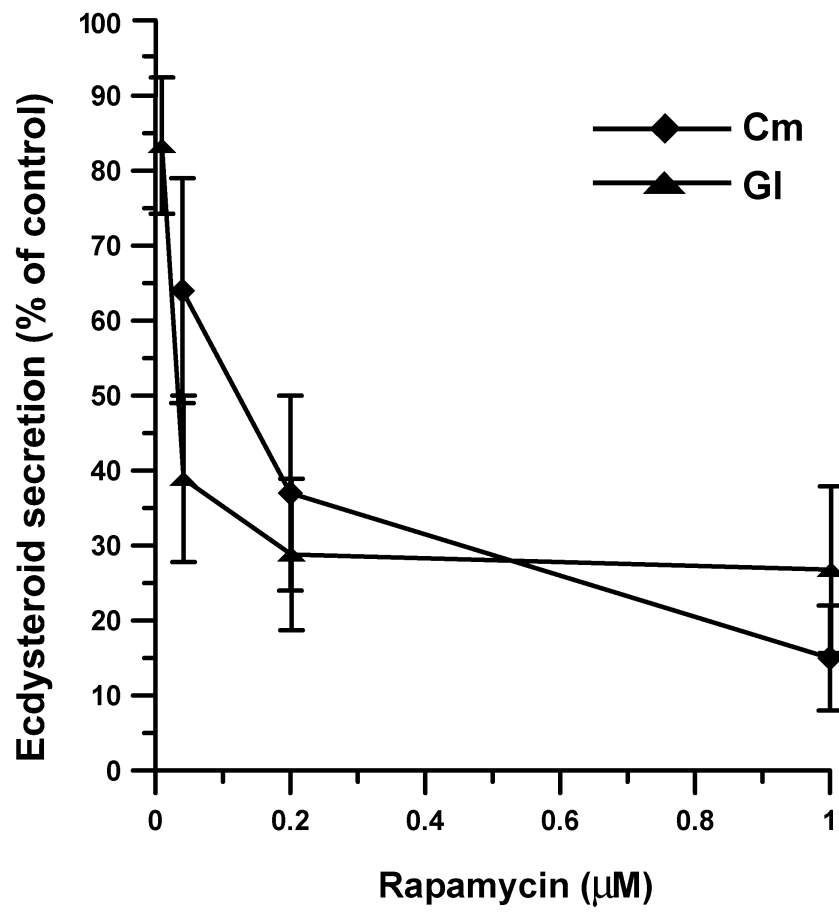


Figure 2

gacataccagagattacacagacgctcctcaacttggcagagttcatggagcattgtgac 60
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aagggtccggttgccttggagctgcagctactcgggaaaaggccatggagtgccggca 120
K G P L P L E L Q L L G E K A M E C R A 40
tatgccaaaggctctgcactacaaggaggaggttccacaaggggcctacctctgaggtc 180
Y A K A L H Y K E E E F H K G P T S E V 60
ttggagcacctcatctccatcaacaacaaactgggacagaaggaggcagctgctggttg 240
L E H L I S I N N K L G Q K E A A A G L 80
ttggaatatgcacgcaagaacaaccgtacagacatgaaggtccaggaacggtggcatgag 300
L E Y A R K N N R T D M K V Q E R W H E 100
aagctgcacgactgggatcaggcactccaagcttactctaccaagctggagactcaacct 360
K L H D W D Q A L Q A Y S T K L E T Q P 120
gatgaccttgcctcgtactgggtcaaatgaggtgtctagaggctctgggagaatggggt 420
D D L A L V L G Q M R C L E A L G E W G 140
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E L Y S V A C D R W M G T M A E D V R A 160
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Q M A R V A S A S A W A M G E W S M M E 180
gagtacagtcgatgcacccaagagacaccaatgagggggctttctaccgtgctgtgctc 600
E Y S R C I P R D T N E G A F Y R A V L 200
tctgtacataaggatcaacatcacatggcccagcagtcacatcgacacagcaagagatta 660
S V H K D Q H H M A Q Q Y I D T A R D L 220
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L D T E L T A M V G E S Y Q R A Y N S M 240
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V A V Q M L A E L E E V I Q Y K L V P E 260
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R R R P I T H I W W E R L Q G C Q R V V 280
gaagactgcaagaagatcctacaggtgcctcctggtgctgtcccctcaggaagacatg 900
E D W Q K I L Q V R S L V L S P Q E D M 300
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R P W L K F A S L C R K S G R L A L S H 320
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K T L V R L L G C D P S L S P T Q P L P 340
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V S H P H V T Y Q Y C K H I Y T Y P D R 360
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R Q E A Y G R L Q K F L Q F L A P A V V 380
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N I L T Y Y T H A K D T D E S C Y K A W 440
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H A Y A Y M N F E A I L F Y K K G F I R 460
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S I S L S D G Y S L Q D T L R L L T V W 480
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F E H G H Q S G V Y E A L V D G L R P S 500
gatatcgacacttggcttcaggtcattcctcagctcatcgcgcgattgacaccctcgc 1560
D I D Q V I P Q L I A R I D T P R 520
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S L V S K L I H Q L L M D I G K H H P Q 540
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A L I Y P L T V A A K S S V P A R S Q A 560
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M V S E E L I R V A I L W H E T W H E G 600
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L E E A S R L Y F G E R N E S G M F R T 620

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L D P L H A M M A R G P Q T L K E M S F 640
 aaccaggcttacgggctgacctgaacgagggcagagtggtgcccgcctaccaacgt 1980
N Q A Y G R D L N E A Q E W C R R Y Q R 660
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S G N V R E L N Q A W D L Y Y H V F R R 680
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I S R T L P Q L T S L E L Q S V S P R L 700
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 L K C P R S G H R S T G F L C S R Y P S 720
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 aaaaaaaaaa 4031

Figure 3

HEAT repeats

Cm-mTOR	: -----DIPEITQTLNLAEFMEHC DKGPLPLELQLLGEKA	: 35
Dm-mTOR	: SCWTELS PDLKNE LTQS LIQALQV TDMPEITQ TILNLAEFMEHC DRDP IPIETKLLGTRA	: 1354
Aa-mTOR	: SCWTDLPDSLKEELSSSLRQALMVPDLPEITQ TILNLAEFMEHCENDALRIDPKILGERA	: 1339
Dp-mTOR	: SCWTELSQQHQNELVKSLEQALRVPDLPEITQ TILNLAEFMEHC EKELCN AELMPKHFIT	: 1371
<hr/>		
G1-mTOR	: -----VLEHLISINNKLOKEAAAAGLLEY--ARKNNRDT	: 32
Cm-mTOR	: MECRAYAKALHYKEEEHKGPTSE--VLEHLISINNKLOKEAAAAGLLEY--ARKNNRDT	: 91
Dm-mTOR	: MACRAYAKALRYKEEE LLREDSQ--VFESLITINNKLOREAAEGLT---RYRNAANE	: 1409
Aa-mTOR	: MECRAYAKALHYKEEELNMKDKDQSVFESLITINNKLOKEAAEGLLEYAMEHRSASEE	: 1399
Dp-mTOR	: KKPSSMSPLLRQDRDLQ---ILRPVVFESLITINNKLOKEAAAAGLLEY--AMKKHEGE	: 1426
<hr/>		
G1-mTOR	: MKVQERWEKLEHWDOALQAYSTKLETQPD-DIALVLGOMRCLEALGEWGE LYSVSCERW	: 91
Cm-mTOR	: MKVQERWEKLEHWDOALQAYSTKLETQPD-DIALVLGOMRCLEALGEWGE LYSVACDRW	: 150
Dm-mTOR	: LNVQGRWEKLEHWDEALEHYERNLKDSS-DIEARLGHOMRCLEALDWSSELNVTKHEW	: 1468
Aa-mTOR	: MKVQVRWEKLEHWSWEKALNLYQDKLESNPG-DIDSR LGOMRCLEALGEWSTLNLTKETW	: 1458
Dp-mTOR	: IRVQERWEKLEHWERALFAYRKESENQQQPELVLGOMRCLEALC EWGOLHLLAETNW	: 1486
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Cm-mTOR	: MGTMAEDVRAQMARVASAWAMGEWSMMEEYSRCIPROTNECAFYR VLSVHKDOHHMA	: 210
Dm-mTOR	: ENFGTEAKSRAGPLAAVAAWCIGDWEAMREYVRCIPEITODGSYYR AVLAVHDDFETA	: 1527
Aa-mTOR	: ESFGTEGQSKAGRLAAA AAWCIGKDWEGMOEFVRFIPEITODGSFYR AVLAVHGEYELA	: 1517
Dp-mTOR	: KQVNVVDVKNRFARMAAAA AAWCIGKWTAMEEYVNFIPKETODCAFYRSVLAHREOYSQA	: 1545
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Cm-mTOR	: QQYIDTARDLLDTELTA MVGESYQRAYNSMVA VQMLAELEEVIOYKLVPERREPIITHLWW	: 270
Dm-mTOR	: QRLIDETRDLLDTELTA MAGESYERAYGAMVCVQMLAELEEVIOYKLIPERREELKTMWW	: 1587
Aa-mTOR	: QTLIDDRDRLDTELTA MAGESYERAYGAMVCVQMLAELEEVIOYKLIPERQETIKMWW	: 1577
Dp-mTOR	: QTLIDSARDLLDTELTA MAGESYQRAYGAMVIVQMLAELEEVIOYKILPERREIRKMWW	: 1605
<hr/>		
G1-mTOR	: ERLOGGQRVVEDWOKILOVRSVLVSPQDMRPLKFA SLCRKSQR LALSHKTLVRLLCD	: 271
Cm-mTOR	: ERLOGGQRVVEDWOKILOVRSVLVSPQDMRPLKFA SLCRKSQR LALSHKTLVRLLCD	: 330
Dm-mTOR	: KRLOGGQRLVEDWRRIIOVHSLVVKPHEDIHTWLKYA SLCRKSQR LALSHKTLVRLLCD	: 1647
Aa-mTOR	: DRLOGGQRLVEDWOKILOVHTLVVHPANDVKT LKFA SLCRKSQDSLKLSEKTLVRLMRYN	: 1637
Dp-mTOR	: QRLLOGGQRIVEDWOKIIQVHSLVISPEDMRTR LKYS SLCRKSQR LALSHKTLVRLLCD	: 1665
<hr/>		
G1-mTOR	: PSLSPSOPLPISHPHVTYQYCKIITYPHRRQIAYGRLOKFLQFLAP-----AVVVV	: 323
Cm-mTOR	: PSLSPTOPLPVSHPHVTYQYCKIITYPDRROIAYGRLOKFLQFLAP-----AVVVV	: 382
Dm-mTOR	: PKLNPNOPLPCNQPOVTYATKYMAANNQL-OBAYEQLTHEVSTYSQ-----ELSSL	: 1698
Aa-mTOR	: PEYDPDHPLEFMQPDISEFAYAKLWAAEG-ERAYNOINRLVADMGI-----EGNFD	: 1688
Dp-mTOR	: PSLNPDHPLEPTLHPHVTYAYSKLWMSNOK-EGAFROHHFVQASLQPSQLSSISTTPVS	: 1724
<hr/>		
G1-mTOR	: GGGNQNGDNKLRKLVSRVYLKLGWYEQHGC-LNEENTANILTYYTHAKDTDETCYKAHW	: 382
Cm-mTOR	: GGGNQNGDNKLRKLVSRVYLKLGWYEQHGC-LNEENTANILTYYTHAKDTDESCYKAHW	: 441
Dm-mTOR	: PPALKQDQ--QRLMARCYLRMATWONKLODSIRPDAIQGALCEFEKATSYPNWKYKAHW	: 1756
Aa-mTOR	: VEE--KDEN--RRLRLARCVMKLGWQONQLOG-LNEQSTKGLACYEKATKHD SNWYKAHW	: 1743
Dp-mTOR	: TPEPDRHVELGKLLARCYLR LGWQEQECLQGC-LNELSTPAVLQYAAATEHDATWYKAHW	: 1783
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G1-mTOR	: AYAYMNFPAILFYK GKMDVKGEAPTPGEDSASGAAAVVTPSKKRSAGDFAVA AVKGFIR	: 442
Cm-mTOR	: AYAYMNFPAILFYK-----KGFIR	: 460
Dm-mTOR	: LWAYMNFKVVQAOKSALDKQPPGASMGMTMGSGL-----DSLMIQRYAVPAVOGFFR	: 1811
Aa-mTOR	: LWAYMNFVVQNKQOEDLIKPNPG-----DKEKCMIRQYAVPAVEGFFR	: 1788
Dp-mTOR	: SWAYMNFPAVLFYKHOGQNTSANQTLIGENTNKG-----LTAQHVSSYTPAVOGFFR	: 1836
<hr/>		
FAT		
G1-mTOR	: SISLSDGNSLQDTRLRLTLVWFHGHQSCVYEAALVDGLKTLQIDTWLQVIPQLIARIDTFR	: 502
Cm-mTOR	: SISLSDGNSLQDTRLRLTLVWFHGHQSCVYEAALVDGLRPSDIDTWLQVIPQLIARIDTFR	: 520
Dm-mTOR	: SISLIKGNLQDTRLRLTLWFDYGNHABVYEAALLSGMKLTEINTWLVQVIPQLIARIDTFR	: 1871
Aa-mTOR	: SINLSHGNSLQDTRLRLTLWFDYQYPPVYEAALVEGMRVTEINTWLVQVIPQLIARIDTFR	: 1848
Dp-mTOR	: SIALSHGSSLQDTRLRLTLWFDYGHWPVYEAALVEGVRTIDVNTWLVQVIPQLIARIDTFR	: 1896
<hr/>		
G1-mTOR	: SLVSKLIHQLLDYGKHHPOALYPLTVAAKSSVAARSQA AEKILKNMREHSANLVQOAM	: 562
Cm-mTOR	: SLVSKLIHQLLDYGKHHPOALYPLTVAAKSSVPARSQA AEKILKNMREHSANLVQOAM	: 580
Dm-mTOR	: QLVGQLIHQLLDYGKHHPOALVYPLTVASKSSASLARNAAFKILDSMRKHSPTLVQOAV	: 1931
Aa-mTOR	: NLVQGLIHQLLNDYGKHHPOALVYPLTVASNSASSARRQA AEKILGSMGEHSANLVQOAI	: 1908
Dp-mTOR	: QLVGRLIHQLLDYGKHHPOALYPLTVASKSALQARHNAAEKILKNMCEHSPVLVQOAV	: 1956

FRB

G1-mTOR : MVSEELIRVAAILWHEHWHEGLEASRLYFGERNESGMFRITLPLHAMMARGPOTLKEMSF : 622
Cm-mTOR : MVSEELIRVAAILWHEHWHEGLEASRLYFGERNESGMFRITLPLHAMMARGPOTLKEMSF : 640
Dm-mTOR : MCSEELIRVAAILWHEHWHEGLEASRLYFGDRNVKGMFEITLPLHAMLERGPOTLKEISF : 1991
Aa-mTOR : MCSEELIRVITLWHEHWHEGLEASRLYFGDRNIKGMFEITLPLHOMLORGPOTLKEISF : 1968
Dp-mTOR : MVSEELIRVAAILWHEHWHEGLEASRLYFGERNVTGMFEITLPLHAMLERGPOTLKEISF : 2016

G1-mTOR : NQAFGRDLNEAQLWCRRYQORSGVRELNQAWDLYYHVFRIRISRTLPQLTSLELCVSPRL : 682
Cm-mTOR : NQAYGRDLNEAQLWCRRYQORSGVRELNQAWDLYYHVFRIRISRTLPQLTSLELCVSPRL : 700
Dm-mTOR : SQAYGRELTEAYWVSRQYKTSVAVMDLDRAWDIYYHVFOKISROLPQLTSLELPYVSPKL : 2051
Aa-mTOR : NQAYGRDLNEAQLWCRHYKNSGNIRDLNCAWDLYYHVFRIRISROLPQLTSLELCVSPKL : 2028
Dp-mTOR : HQAYGRELTEAQDWCRRYKTSLVNVRDLNCAWDLYYHVFRIRISROLPQLTSLELCVSPKL : 2076

PA

G1-mTOR : LQCRDLDLAVPGSYAPGQPVICLSQVQSLQVLSKQRPKLCIRGSNGRNFVFLKKGHE : 742
Cm-mTOR : LKCPRSRHRSTGFLCSRYPSYLSGAVLVVTGIDFKQRPKLCIKGSNGRDFVFLKKGHE : 760
Dm-mTOR : MTCXDLELAVPGSYNPGQELIRTSIKLNLOVITSKQRPKLCIRGSNGKDYMLKKGHE : 2111
Aa-mTOR : LACRDLELAVPGSYAPGQELIRIASIQSNLOVITSKQRPKLCIRGSNGKEYMFLKKGHE : 2088
Dp-mTOR : LLCRDLELAVPGSYVNPQPVIRISQVNSLQVLSKQRPKLCITGSNGKEYMFLKKGHE : 2136

G1-mTOR : DLRODERVMQLFGLVNTLLISNPTFRRNLTIQRYAVIPLSTNSGLIGWVPHCDTLHSLI : 802
Cm-mTOR : DLRODERVMQLFGLVNTLLISNPTFRRNLTIQRYAVIPLSTNSGLIGWVPHCDTLHSLI : 820
Dm-mTOR : DLRODERVMQLFGLVNTLLLDPTFRRNLAIQRYAVIPLSTNSGLIGWVPHCDTLHSLI : 2171
Aa-mTOR : DLRODERVMQLFGLVNTLLLDPTFRRNLTIQRYAVIPLSTNSGLIGWVPHCDTLHSLI : 2148
Dp-mTOR : DLRODERVMQLFGLVNTLLIHDPEFRRNLTIQRYAVIPLSTNSGLIGWVPHCDTLHSLI : 2196

Kinase

G1-mTOR : RDWREKKKILLNTEHRMLRMAQDLDHSLTMQKVEVFEHALPETHGGDLSRLLEWFKSPSS : 862
Cm-mTOR : RDWREKKKILLNTEHRMLRMAQDLDHSLTMQKVEVFEHALPETHGGDLARLLEWFKSPSS : 880
Dm-mTOR : RDYRDKKKMPLNTEHRTMLNFAFDYDHLTMQKVEVFEHALGOTOGDDLAKLLWLKSPSS : 2231
Aa-mTOR : RDYREKKKILLNTEHRMLRMAQDYDHLTMQKVEVFEYALPETHGGDLAKLLWLKSPSS : 2208
Dp-mTOR : RDYREKKKILLNTEHRMLRMAQDYDHLSLMQKVEVFEHALPETHGGDLAKILLWLRSPSS : 2256

G1-mTOR : EVWFDRRINYSR-----SLAVMSMVGYVILGLGDRHPSNMLMDQLSGKILHIDFGDC : 913
Cm-mTOR : EVWFDRRINYSR-----SLAVMSMVGYVILGLGDRHPSNMLMDQLSGKILHIDFGDC : 931
Dm-mTOR : ELWFERRINYSR-----SLAVMSMVGYVILGLGDRHPSNMLMDQLSGKILHIDFGDC : 2282
Aa-mTOR : EVWFDRRINYSR-----SLAVMSMVGYVILGLGDRHPSNMLMDQLSGKILHIDFGDC : 2259
Dp-mTOR : EVWFDRRINYSRHELFFNPINLYTVMSMVGYVILGLGDRHPSNMLMDQLSGKILHIDFGDC : 2316

CL

AL

G1-mTOR : FEVAMMREKFPEKIPFRLTRMLIHAMEVTGIDGTYRMTCESVMALIRANKDSLMAVLEAF : 973
Cm-mTOR : FEVAMMREKFPEKIPFRLTRMLIHAMEVTGIDGTYRMTCESVMALIRANKDSLMAVLEAF : 991
Dm-mTOR : FEVAMTREKFPEKIPFRLTRMLIHAMEVTGIEGTYRRTCESVMVLRANKDSLMAVLEAF : 2342
Aa-mTOR : FEVAMTREKFPEKIPFRLTRMLIHAMEVTGIEGTYRRTCESVMHVLRANKDSLMAVLEAF : 2319
Dp-mTOR : FEVAMTREKFPEKIPFRLTRMLVNAMEVTGIEGTYRSTCESVMSVLRANKDSLMAVLEAF : 2376

G1-mTOR : VPNLLFYWGHRENKHSKGGSGS-----VGADGETGPSAPVSTSAIAPDPSLTPAPAHTL : 1026
Cm-mTOR : VHDELNWRLLMDNTQPKGGRS-----VVGEGEACPSAPASTAIAPDPAITAPAPA--I : 1042
Dm-mTOR : VYDELLNWRLLDVDK-KGNDA-----VAGAGAPGRGGSGMQLSLSNSVEISLPMAKS : 2394
Aa-mTOR : VYDELLNWRLLDVDK-NRISK-----NATVDVSTTESMEEILDLLIN--ARNLRMNEA : 2369
Dp-mTOR : VYDELLNWRLLVDNVANTTTTRRSKSRHESSNSNSGQDVGDSEITANAAAASSSVLNA : 2436

ka9b

FATC

G1-mTOR : TPTSVMGPH--SQAREDDGVSEALNKKAVAIIVHRVRDKLTGRDFC--TEESLDVHROVILL : 1082
Cm-mTOR : TPASVGPQ--SQSREDDGVSEALNKKAVAIIVHRVRDKLTGRDFC--TEEPLDVHROVILL : 1098
Dm-mTOR : KPVDPTLQ--QGG-LHNNVADETNKASQVIKRVKCKLTGTDQF--TEKSVNEQSQVILL : 2449
Aa-mTOR : NGGGDVVD--QGSNCIANPAEATNKKARAIIVDRVKOKLTGKDFN--TVEPVQR--QIDLL : 2423
Dp-mTOR : AVSRSKNETVEAVVNDGQPPEITLNKRALTIIVSRVRDKLTGRDFNETEGTSLSDROVILL : 2496

G1-mTOR : IAHATLHENLCQCYIGWCPFW : 1103
Cm-mTOR : IAHATSHENLCQCYIGWCPFW : 1119
Dm-mTOR : IQAHATNENLCQCYIGWCPFW : 2470
Aa-mTOR : IRAHATNENLCQCYIGWCPFW : 2444
Dp-mTOR : IQAHATSHENLCQCYIGWCPFW : 2517

Figure 4

```
atggatgaggcagcatcccctcccaccctgccattgttaaggaagggtggctcaacaaa 60
M D E A A S P P T P A I V K E G W L N K 20
cgtggggaacacattaataaaactggaggcagcgctacttctttcttcaagaagatggcacc 120
R G E H I K N W R Q R Y F F L Q E D G T 40
cttctgggatttaagacaaaaccagagcatggcttgaagatccacttaacaattttaca 180
L L G F K T K P E H G L E D P L N N F T 60
gtgaagcaatgccagatcctgaaaacagaaagaccgcgccgaacactttcattatccga 240
V K Q C Q I L K T E R P R P N T F I I R 80
ggacttcattggacaactatcattgaaagaacctttaatgctcaatcgctagttagacagg 300
G L H W T T I I E R T F N A Q S A S D R 100
gaatcatggatggaagccatcaaacaggtgtctgagagaatatcagatactcaatcagat 360
E S W M E A I K Q V S E R I S D T Q S D 120
gagtgttttgagatccaggaggcgagtagaatgaaacaattacaactcaactactccagc 420
E C F E I Q E A S R M K Q L Q L N Y S S 140
gatgatgatgatacaccggtttgcgaggcaccaagaagaaaaggaaaattacttttagat 480
D D D D T P G L R G T K K K R K I T L D 160
aactttgaatttcttaaaagtgttgaaaggacgtttgaaaagttatcctgtgtcga 540
N F E F L K V L G K G T F G K V I L C R 180
gaaaaggtcagcaaccatttttacgctattaagatcctgcgtaaagatgtgattattgag 600
E K V S N H F Y A I K I L R K D V I I E 200
cgtgatgaggtggccacacactcacagagaaccgggttctgcaggttagatcatccc 660
R D E V V H T L T E N R V L Q V V D H P 220
ttccttacttacctcaagtattccttccaaaccaacgatcgctctgtttcgtgatggag 720
F L T Y L K Y S F Q T N D R L C F V M E 240
tatgtcaatggtgagaactatTTTTTcatctcaccaggagcgcttcttccctgaagaa 780
Y V N G G E L F F H L T R E R F F P E E 260
cgagccagatTTTtatggagcagaaatatgtcttgcattaggttatttacatgaaaaaaaa 840
R A R F Y G A E I C L A L G Y L H E K K 280
aaaacctatagtgag 855
K T Y S E 285
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Figure 5

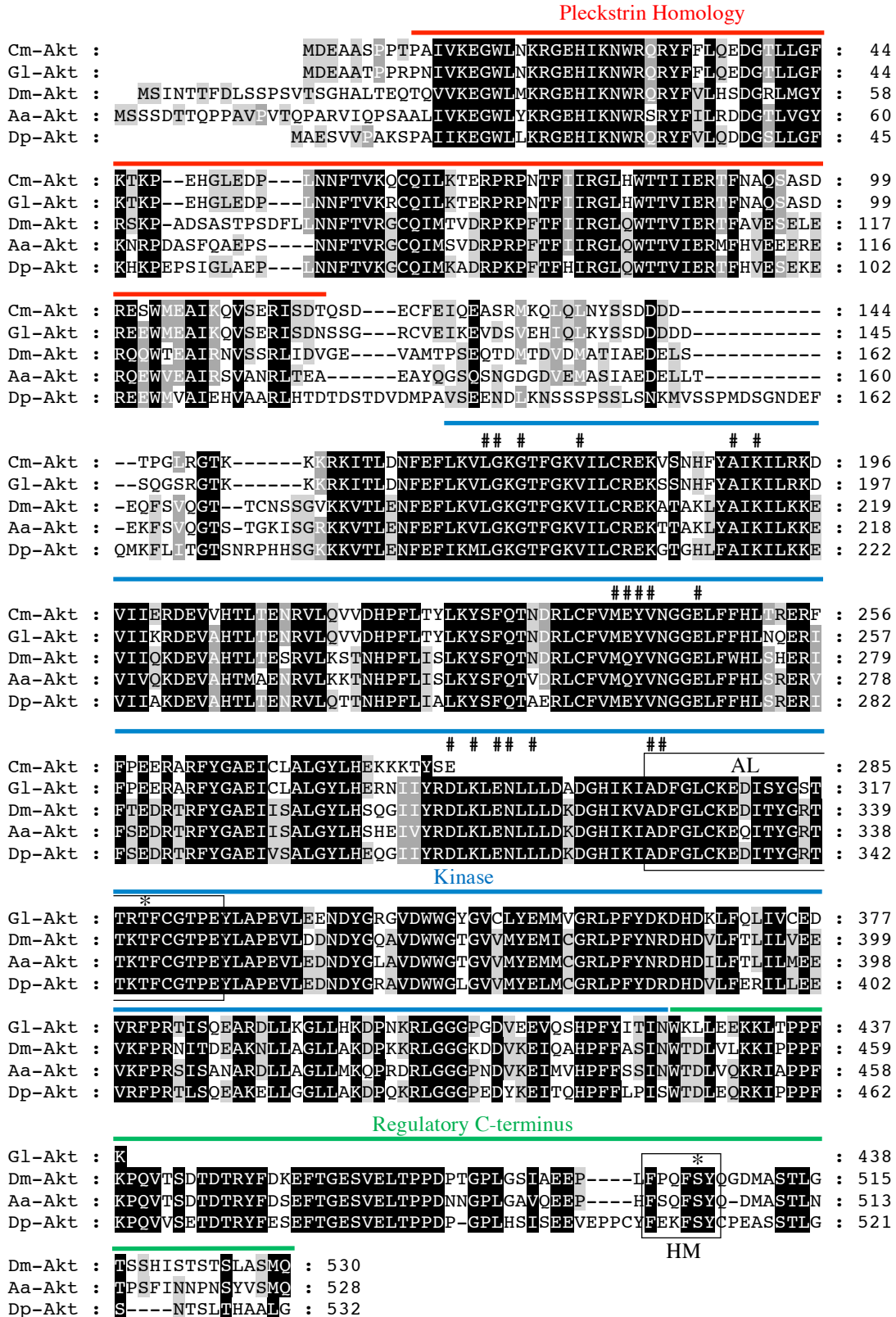


Figure 6

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gctaccatagtagtaacccaaaaggacacagcacacacaaaagctgaaagaaatatacctg 120
A T I V R N Q K D T A H T K A E R N I L 40
gaagctgtgaagcatccattcattgtggatctgggtgatgcatttcaaacgggtggcaag 180
E A V K H P F I V D L V Y A F Q T G G K 60
ttgtacctcatcttgagtagctgtccggtgggtgagcttttcatgcacctggagagagag 240
L Y L I L E Y L S G G E L F M H L E R E 80
ggaatattcatggaggacacagcttgtttttacatatcgaaaatcatactggctctggaa 300
G I F M E D T A C F Y I S E I I L A L E 100
catcttcatctgagggcatctacagagacttgaagccagaaaatatacttctggat 360
H L H S E G I I Y R D L K P E N I L L D 120
tcttatgggcatgtgaagctcacagatthttggattatgcaaagaaaagattcaggatgac 420
S Y G H V K L T D F G L C K E K I O D D 140
tcagtgactcataccttctgtggcaccattgagtacatggcacccgagatcctgacccgc 480
.S.V.T.H.T.F.C.G.T.I.E.Y.M.A.P.E.I.L.T.R 160
accggccatggcaaggcagtgactgggtctcttggagcactcatgtatgacgtgttg 540
T G H G K A V D W W S L G A L M Y D V L 180
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T G A P P F T A E N R K K T I E K I L K 200
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G K L N L P P Y L T P D S R D L I R K L 220
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P F K P V L S G D D D V S Q F D S K F T 280
aaacagacaccagtgactcccctgatgaccacacgctcagtgaaagtccaatatcttg 900
K Q T P...V...D...S...P...D...D H T L S E S A N I L 300
ctgaaaaactcgagccat 918
L K N S S H 306
```

Figure 7

```

G1-S6K :                                     YEQGPDMIEETLQ : 13
Dp-S6K :                                     FNSSPD---VEAVQ : 12
Aa-S6K :          MAGVFDLELH----EEDNIRSDSDDDVIEVDEVDLEPELHINSNLDAEGSETIPL : 50
Dm-S6K :          MADVSDPSELFDELELHDLELQDDKARDSDDDRIELDDVDLEPELCINLHQDTEGQETIQ : 60

##### # # #
Cm-S6K :                                     OKMTGGRRGGGSIFAMKVLKKATIVRN : 26
G1-S6K :          SDSTVNPGRKVRPSDFQLLKVLGKGGYGKVFQVRKMTGGRRGGGKIFAMKVLKKATIVRN : 73
Dp-S6K :          SETTVNPGAEKTGPODFELRRVLGRGGYGKVFQVRKLTGKDSG-HIFAMKVLKKATIVRN : 71
Aa-S6K :          SEETVNPGRMKLGQDFELKKVLGKGGYGKVFQVRKTTGADAN-SYFAMKVLKKASIVRN : 109
Dm-S6K :          CEENVNPGKIKLGPKDFELKKVLGKGGYGKVFQVRKTAGRDAN-KYFAMKVLKKASIVTN : 119

Kinase
# # #
Cm-S6K :          QKDTAHTKAERNILEAVKHPFIVDLVYAFQTGKLYLILEYLSGGELFMHLEREGIFMED : 86
G1-S6K :          QKDTAHTKAERNILEAVKHPFIVDLVYAFQTGKLYLILEYLSGGELFMHLEREGIFMED : 133
Dp-S6K :          QKDTAHTKAERNILEAVKHPFIVDLVYAFQTGKLYLILEYLSGGELFMHLEREGIFMED : 131
Aa-S6K :          QKDTAHTKAERNILEAVRHPFIVDLVYAFQTGKLYLILEYLSGGELFMHLEREGIFLED : 169
Dm-S6K :          QKDTAHTKAERNILEAVKHPFIVDLVYAFQTDGKLYLILEYLSGGELFMHLEREGIFLED : 179

# # # # # # # # # # *
Cm-S6K :          TACFYISEIILALEHLHSEGIYYRDLKPENILLDSYGHVKLTDFGLCKEKIQDDSVTHTF : 146
G1-S6K :          TACFYISEIILALEHLHSEGIYYRDLKPENILLDAFGHVKLTDFGLCKEKIQDDSVTHTF : 193
Dp-S6K :          TASFYLAEIIALEHLHCOGIYYRDLKPENILLDAFGHVKLTDFGLCKESVEDGGVTHTF : 191
Aa-S6K :          TTFYLCEIIALEHLHNLGIYYRDLKPENVLLDAQGHVKLTDFGLCKEHIQEGIVTHTF : 229
Dm-S6K :          TTFYLCEIIIFALEHLHKLGIYYRDLKPENILLDAQGHVKLTDFGLCKEHIQEGIVTHTF : 239

AL
Cm-S6K :          CGTIEYMAPEILTRTGHGKAVDWWSLGALMYDVLTGAPPFTAENRKKTIEKILKQKLNLP : 206
G1-S6K :          CGTIEYMAPEILTRTGHGKAVDWWSLGALMYDMLTGAPPFTAENRKKTIEKILKQKLNLP : 253
Dp-S6K :          CGTIEYMAPEILTRSGHGKAVDWWSLGALMYDMLTGAPPFTAENRKKTIEKILKQKLNLP : 251
Aa-S6K :          CGTIEYMAPEILTRSGHGKAVDWWSLGALMFDMLTGMPPFTADNRKNTIDAILKQKLNIP : 289
Dm-S6K :          CGTIEYMAPEILTRSGHGKAVDWWSLGALMFDMLTGVPPFTAENRKKTIETILKQKLNLP : 299

Cm-S6K :          PYLTPDSRDLIRKLLKROVSORLGSSDDGEPIKRHLFFKLINWDEVINRKLDPPEKPVL : 266
G1-S6K :          PYLTPDARDLIRKLLKROVSORLGSPDGEPIKRHLFFKLINWDEVINRKLDPPEKPVL : 312
Dp-S6K :          PYLTPDARDLIRLLKRGVVSRLGSTVADGEPIRMHFFKTIDWNEVACRRLEPPFKPCL : 311
Aa-S6K :          AYLAADSRDLIRRLMKROVSORLGSPTDGOAVSHSFFKNVNWDDVLARRLDPPIKPVL : 349
Dm-S6K :          AYLTPEARDLVRRLMKROEPORLGSPEDAAAVQIHPFFKHVNWDDVLARRLEPPIKPLL : 359

* * RSPRR
Cm-S6K :          SGDDVSQFDSKFTKQIPVDSPDDHTLSESANILLKNSSH : 306
G1-S6K :          SGDDVSQFDSKFTKQIPVDSPDDHMLSESANMVFEGFTYVAPSVLEEMARPSVVKAESP : 372
Dp-S6K :          ASADDVSQFDTRFTKQIPIDSPDDSTLSESANMVFEGFTYVAPSVLEEMHRPQVIKARSP : 371
Aa-S6K :          RSEDDVSQFDTKFTKQIPVDSPDDSTLSESANLIFOGFTYVAPSVLEEMQOPRVVTARSP : 409
Dm-S6K :          RSEDDVSQFDTRFTKQIPVDSPDDSTLSESANLIFOGFTYVAPSVLEEDMHRANRVPARSP : 419

TM HM
Dp-S6K :          RRGMFTQGHSMTTPF : 386
Aa-S6K :          RRTPRPHGSHHHHHHMGSHSSHHHHGGHGHHRMGGAIGNGVITLEDEQMLSMPRSQAM : 469
Dm-S6K :          RRTPRQLPDSSFRLQFPSANVGANAPAGHARSFAAIRDVCTSHAAASHADICAASIAGAG : 479

Aa-S6K :          PSSHQPQMPHHMFQQQQHQQQQQSQPQQPPQQSAARSTQFVAGPNARHTPAHLQ : 529
Dm-S6K :          RDDGRAGSADGLKAGAIASQPTNPSHPVRSVATQQLHHKLPLPTPKKKKKKKKQKITEI : 539

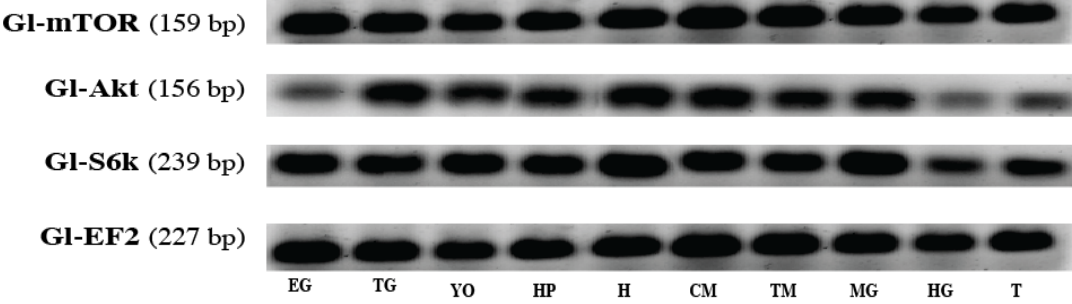
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Dm-S6K :          ALFDVYDYENDYEYDYEADGEDDCATRRKAFVFGYT : 637

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Figure 8

A. *G. lateralis*



B. *C. maenas*

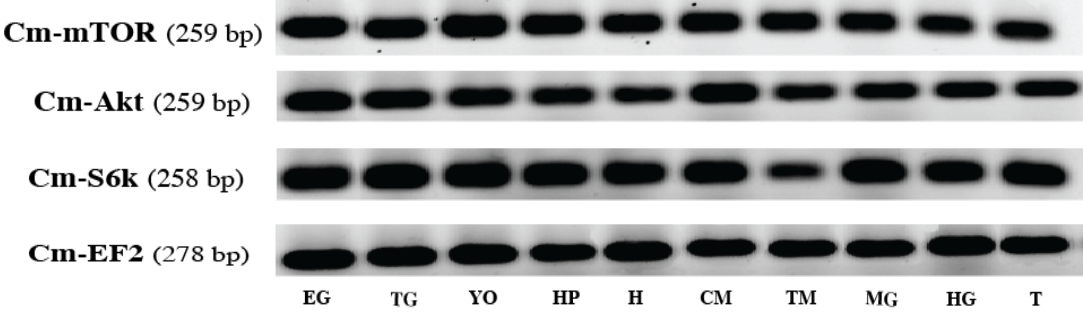
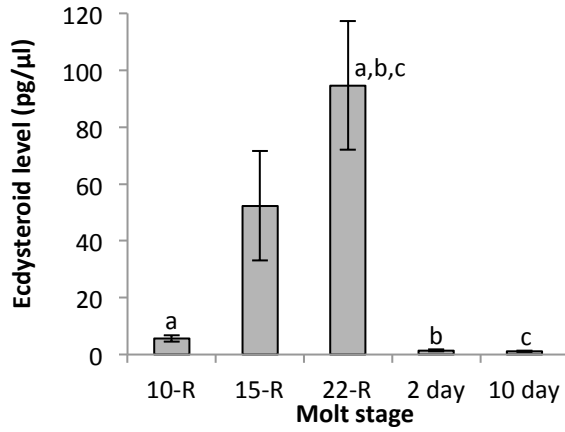
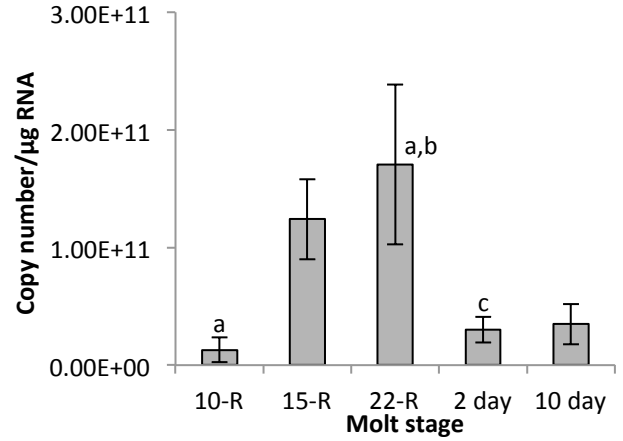


Figure 9

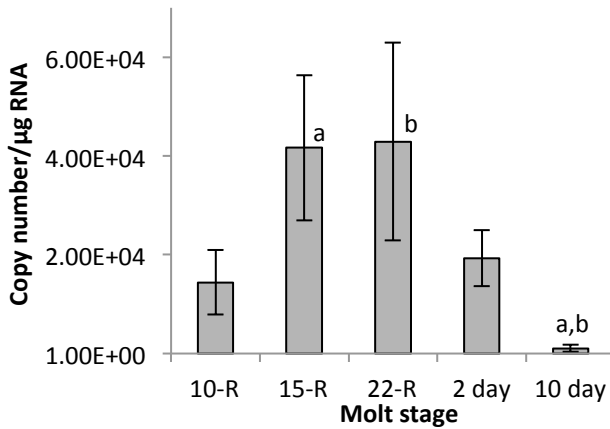
A. Hemolymph ecdysteroid



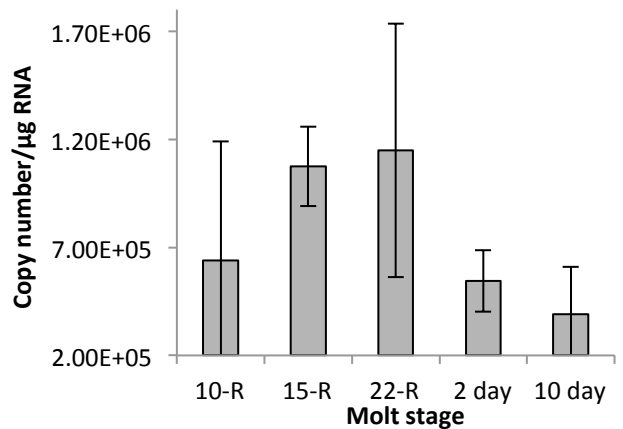
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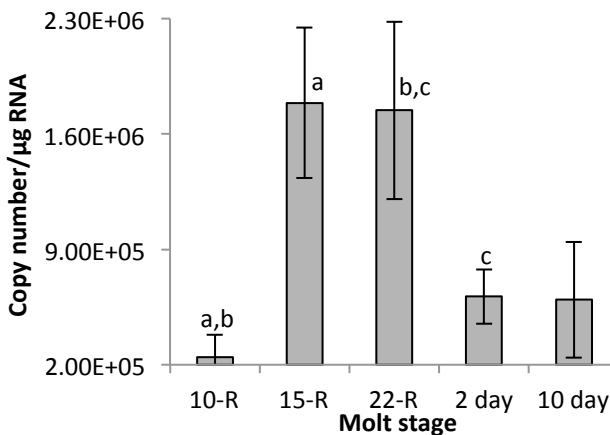
C. GI-mTOR



D. GI-Rheb



E. GI-Akt



F. GI-S6K

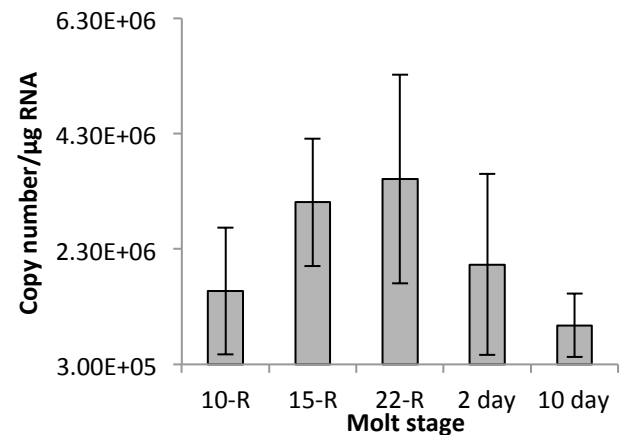
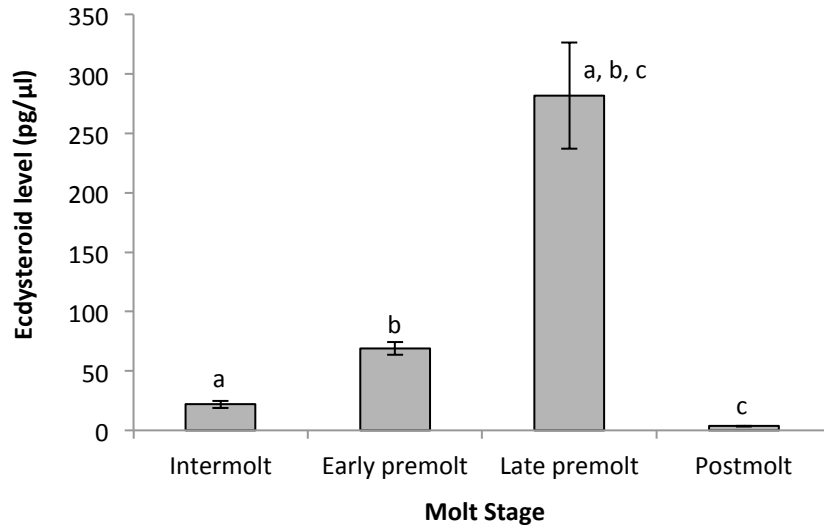


Figure 10

A. Hemolymph ecdysteroid



B. Gene expression

