



Ecdysteroidogenesis and development in *Heliothis virescens* (Lepidoptera: Noctuidae): Focus on PTTH-stimulated pathways

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ABSTRACT

Post-embryonic development and molting in insects are regulated by endocrine changes, including prothoracicotropic hormone (PTTH)-stimulated ecdysone secretion by the prothoracic glands (PGs). In Lepidoptera, two pathways are potentially involved in PTTH-stimulated ecdysteroidogenesis, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/protein kinase B/target of rapamycin (PI3K/Akt/TOR). We investigated the potential roles of both these pathways in *Heliothis virescens* ecdysteroidogenesis. We identified putative proteins belonging to MAPK and PI3K/Akt/TOR signaling cascades, using transcriptomic analyses of PGs from last (fifth) instar larvae. Using western blots, we measured the phosphorylation of 4E-BP and S6K proteins, the main targets of TOR, following the *in vitro* exposure of PGs to brain extract containing PTTH (hereafter referred to as PTTH) and/or the inhibitors of MAPK (U0126), PI3K (LY294002) or TOR (rapamycin). Next, we measured ecdysone production, under the same experimental conditions, by enzyme immunoassay (EIA). We found that in *Heliothis virescens* last instar larvae, both pathways modulated PTTH-stimulated ecdysteroidogenesis. Finally, we analyzed the post-embryonic development of third and fourth instar larvae fed on diet supplemented with rapamycin, in order to better understand the role of the TOR pathway in larval growth. When rapamycin was added to the diet of larvae, the onset of molting was delayed, the growth rate was reduced and abnormally small larvae/pupae with high mortality rates resulted. In larvae fed on diet supplemented with rapamycin, the growth of PGs was suppressed, and ecdysone production and secretion were inhibited. Overall, the *in vivo* and *in vitro* results demonstrated that, similarly to *Bombyx mori*, MAPK and PI3K/Akt/TOR pathways are involved in PTTH signaling-stimulated ecdysteroidogenesis, and indicated the important role of TOR protein in *H. virescens* systemic growth.

1. Introduction

In insects, control of molting and metamorphosis is regulated through the synthesis and secretion of ecdysone by prothoracic glands (PGs) or analogous organs (Gilbert et al., 2002; Iga and Kataoka, 2012). After ecdysone is synthesized and secreted as a precursor (3-

dehydroecdysone in Lepidoptera), it is rapidly converted to ecdysone in the hemolymph and subsequently activated to 20-OH-ecdysone in peripheral tissues, such as fat body or the midgut (Kiriishi et al., 1990; Nation, 2015; Weirich, 1997; Weirich and Bell, 1997). Ecdysteroidogenesis can be triggered by different cellular and environmental stimuli. Several studies showed that the onset of molting may be

Abbreviations: PG, prothoracic gland; BE, brain equivalent; PTTH, prothoracicotropic hormone; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; TOR, target of rapamycin; EIA, enzyme immunoassay; 4E-BP, translational repressor 4E-binding protein; S6K, p70 ribosomal protein S6 kinase; cAMP, cyclic adenosine monophosphate; PBS, phosphate-buffered saline; BLAST, basic local alignment search tool; NCBI, National Center for Biotechnology Information; GO, Gene Ontology; TBS-T, tris-buffered saline-Tween 20; BSA, bovine serum albumin; ECL, enhanced chemo luminescence; TMB, tetramethylbenzidine; OD, optical density; SEM, standard error of mean; ANOVA, analysis of variance

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affected by the concentration of oxygen in tissues (Callier and Nijhout, 2011; Greenlee and Harrison, 2004). Besides, nutrition has direct effects on the size of PGs and their ability to produce and secrete ecdysone, and, consequently, on molting (Kemirembe et al., 2012; Koyama et al., 2013; Mirth et al., 2005). The onset of molting is also influenced by the time required to reach critical weight, namely, the weight at which a larva can pupate without delay even under unfavorable conditions (Nijhout, 1981; Shingleton, 2010). Critical weight is postulated to correspond to a threshold PG size, the achievement of which is associated with the start of a hormonal cascade that ends with the molt (Shingleton, 2010). Therefore, the molting process is closely related to the interplay between the size of the PGs and critical weight. In the PGs, ecdysone production is stimulated by the neuropeptide prothoracicotropic hormone (PTTH) (Huang et al., 2008; McBrayer et al., 2007). PTTH activates a signal transduction cascade by binding to Torso, a tyrosine kinase receptor in the cellular membrane (Rewitz et al., 2009). In *Manduca sexta*, PTTH stimulates ecdysteroidogenesis, which increases the second messengers, Ca²⁺ and cyclic AMP (cAMP), and stimulates signal transduction by mitogen-activated protein kinases (MAPKs) (Rybczynski et al., 2001; Rybczynski and Gilbert, 2003). The MAPK pathway is also involved in ecdysteroidogenesis in the larvae of *Bombyx mori* (Lin and Gu, 2007; Gu et al., 2010) and of *Drosophila melanogaster* (Caldwell et al., 2005). In both these species several studies have shown that the phosphoinositide 3-kinase/protein kinase B/target of rapamycin (PI3K/Akt/TOR) pathway is involved in ecdysteroidogenesis stimulated by PTTH (Layalle et al., 2008; Gu et al., 2012). This pathway in *B. mori* (Gu et al., 2015) is also activated by bombyxin, differently from *M. sexta* (Smith et al., 2014).

The TOR protein is a serine-threonine kinase involved in several conserved anabolic processes, including the biogenesis of lipids, ribosomes, lysosomes and mitochondria (Caron et al., 2010; Laplante and Sabatini, 2013; Liu et al., 2012; Wang and Proud, 2006, 2011). It is also involved in cell cycle, cell migration and the immune system response, and regulates lymphocyte maturation and differentiation (Cobbold, 2013; Kurebayashi et al., 2013; Liu and Parent, 2011; Powell and Delgoffe, 2010; Thomson et al., 2009; Waickman and Powell, 2012; Wang and Proud, 2006). The activity of TOR is modulated by numerous extracellular and intracellular stimuli, including growth factors, nutrients, amino acids, energy levels, oxygen and stress conditions (Ma and Blenis, 2009; Russell et al., 2011). TOR enhances mRNA translation by phosphorylating and inhibiting a repressor, 4E-binding protein (4E-BP), and by phosphorylating and activating p70 ribosomal protein S6 kinase (S6K) (Caron et al., 2010; Dowling et al., 2010; Hay and Sonenberg, 2004; Wang and Proud, 2006). Phosphorylated 4E-BP and S6K are commonly used to evaluate TOR activity *in vivo* and *in vitro*. We analyzed the involvement of MAPK and PI3K/Akt/TOR signaling in ecdysteroidogenesis in *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae), and the specific role of the TOR protein in larval growth and development. The *in vitro* and *in vivo* results allowed us to confirm the involvement of both pathways in PTTH-stimulated ecdysteroidogenesis by PGs in *H. virescens* last (fifth) instar larvae, and suggested that the TOR kinase plays a relevant role in the post-embryonic development of the moth.

2. Materials and methods

2.1. Insect rearing and staging

Heliothis virescens larvae were reared according to Vinson et al. (1973) on a standard artificial diet developed by Vanderzant et al. (1962). Rearing temperature was maintained at 29 ± 1 °C, under a 16:8 light/dark photoperiod and relative humidity 70 ± 5%. Last (fifth) instar larvae were staged according to Webb and Dahlman (1985) and synchronized as reported by Pennacchio et al. (1992).

2.2. Dissection of prothoracic glands

The PGs from 3 to 4 day old last (fifth) instar larvae were dissected in phosphate-buffered saline (PBS) 1X as previously reported (Pennacchio et al., 1998a). Glands were left at 25 °C in 100 µl of Grace's insect medium (Sigma-Aldrich, St. Louis, MO, USA) for 30 min (time of rest) in order to reduce the possibility of their activation by experimental manipulation, as reported for *Manduca sexta* PGs (Bollenbacher et al., 1983; Smith et al., 1986).

2.3. RNA extraction and prothoracic glands *de novo* transcriptome assembly

In order to identify the proteins of the MAPK and PI3K/Akt/TOR pathways expressed in the PGs of *H. virescens* larvae, a *de novo* transcriptome was constructed. Total RNA from 220 PGs explanted from 3 day old last instar larvae was extracted using TRI-Reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. An additional DNase (Turbo DNase, Ambion Inc., Austin, TX, USA) treatment was carried out before the second purification step to remove any contaminating DNA. The DNase enzyme was removed, and the RNA was further purified by the RNeasy MinElute Clean-up Kit (Qiagen, Venio, Netherlands), following the manufacturer's protocol, and eluted in 20 µl of RNA Storage Solution (Ambion Inc., Austin, TX, USA). RNA integrity was verified on an Agilent 2100 Bioanalyzer using RNA nano chips (Agilent Technologies, Palo Alto, CA, USA), and RNA quantity was determined by a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Poly(A) + RNA was isolated from 5 µg total RNA, using the Ambion MicroPoly(A) Purist Kit, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA).

Sequencing was carried out at the Max Planck Genome Center (<http://mpgc.mpiiz.mpg.de/home>) using standard TruSeq procedures on an Illumina HiSeq2500 sequencer, generating approximately 42 million paired-end (2 × 100 bp) reads for each of the tissue samples. Quality control measures, including the filtering of high-quality reads based on the score given in FASTQ files, removal of reads containing primer/adaptor sequences and trimming of read lengths, were carried out using CLC Genomics Workbench v8.1 (<http://www.clcbio.com>). The *de novo* transcriptome assembly was carried out with the same software, selecting the presumed optimal consensus transcriptome as previously described (Vogel et al., 2014). All obtained sequences (contigs) were used to query for a BLASTX (basic local alignment search tool) search (Altschul et al., 1997) in the non-redundant database of the National Center for Biotechnology Information (NCBI), considering all hits with an E-value < 1E-3. The transcriptome was annotated using BLAST, Gene Ontology (GO) and InterProScan searches using BLAST2GO PRO v3.1 (<http://www.blast2go.de>) (Goetz et al., 2008). To optimize the annotation of data, we used GO slim, which uses a subset of the whole GO terms that provides a broader overview of the transcriptome ontology content.

2.4. Protein alignment

To evaluate the sequence similarity among some key proteins belonging to MAPK (p38 MAP kinase and RAF kinase) and PI3K/Akt/TOR (Akt, TOR, S6K and 4E-BP) pathways of *H. virescens*, obtained from *de novo* PG transcriptome assembly, and their orthologs in *Homo sapiens*, *Bombyx mori* and *Manduca sexta*, the alignments of these proteins were performed using the protein BLAST tool (<https://blast.ncbi.nlm.nih.gov>), which also provides the percentage of identity between two sequences. Conserved residues in alignments were highlighted with Multiple Align Show (http://www.bioinformatics.org/sms/multi_align.html).

Sequences of interest belonging to *H. sapiens*, *B. mori* were obtained from the NCBI database. Sequences of interest belonging to *M. sexta*

were obtained from the Manduca Official Gene Set (OGS2) available at https://15k.nal.usda.gov/Manduca_sexta. The translate tool, available at the ExPasy SIB Bioinformatics Resource Portal (<http://web.expasy>), was used to translate nucleotide sequences into their corresponding proteins.

2.5. Extraction of prothoracicotropic hormone

The brain extract containing PTTH (hereafter referred to as PTTH) was prepared by homogenizing brains dissected from an equal number of *H. virescens* 3 day and early 4 day old last instar larvae and stored in ice-cold Grace's insect medium (Sigma-Aldrich, St. Louis, MO, USA). The homogenate was placed in boiling water for 2 min, cooled to 4 °C on ice and centrifuged at 15,000g at 4 °C for 5 min (Pennacchio et al., 1997). Before being used, PTTH extract was diluted in Grace's insect medium to 0.1 brain equivalent/ μ l (BE/ μ l) and either used immediately for the experiments described below or stored at –80 °C.

2.6. Analysis of protein phosphorylation

The involvement of MAPK and PI3K/Akt/TOR pathways in PTTH-stimulated ecdysteroidogenesis was studied under ten different conditions: basal glands incubated in Grace's insect medium (Sigma Aldrich, St. Louis, MO, USA); PGs stimulated with 0.1 BE/ μ l PTTH; incubation with 50 μ M LY294002 (Calbiochem, catalogue number 40204, San Diego, CA, USA), an inhibitor of PI3K; incubation with 50 μ M LY294002 and 0.1 BE/ μ l PTTH; incubation with 1 μ M rapamycin (Calbiochem, catalogue number 553210, San Diego, CA, USA), an inhibitor of TOR; incubation with 1 μ M rapamycin and 0.1 BE/ μ l PTTH; incubation with 10 μ M U0126, a MAPK inhibitor (Calbiochem, catalogue number 662005, San Diego, CA, USA); incubation with 10 μ M U0126 and 0.1 BE/ μ l PTTH; incubation with 50 μ M LY294002 and 10 μ M U0126; incubation with 50 μ M LY294002 and 10 μ M U0126 and challenged with 0.1 BE/ μ l PTTH. The PGs were pre-incubated with each of inhibitors for 30 min, then transferred to fresh medium containing the same dose of inhibitor/inhibitors, with or without PTTH, and then maintained for 3 h at 25 °C. All inhibitors were prepared according to the manufacturer's protocols. For each of these conditions, a pool of 20 PGs was incubated and lysed directly in Laemmli 2X sample buffer (Laemmli, 1970), allowing the activity of proteases and phosphatases to be blocked. The extracted proteins were separated by a 10% polyacrylamide gel electrophoresis and transferred on a Whatman nitrocellulose membrane (Protran, Dassel, Germany). Specific antibodies were used to evaluate the phosphorylation of the two TOR targets: anti-phospho-4E-BP (Cell Signaling, catalogue number 2855S, Danvers, MA, USA) and anti-phospho-S6K (Millipore, catalogue number 04-393, Temecula, CA, USA). To verify that each lane was loaded with the same quantity of proteins, actin was used as an endogenous control using an anti-actin antibody (Abcam, catalogue number 75186, Cambridge, UK). All antibodies were diluted 1:1000 in tris-buffered saline and 0.1% Tween 20 (TBS-T) with 5% bovine serum albumin (BSA), and the incubation was carried out overnight. Membranes were sequentially incubated with each of three antibodies. Goat anti-rabbit conjugated to horseradish peroxidase (Invitrogen, Carlsbad, CA, USA), diluted 1:15000 in TBS-T, was used as a secondary antibody. For detection, enhanced chemo luminescence (ECL) (LiteAB Blot kit - Euroclone, Pavia, Italy) was used and signals were measured with ChemidocTM MP System (Bio-Rad, Milan, Italy).

2.7. In vitro biosynthesis of ecdysone

Following the dissection of PGs, Grace's insect medium was replaced with a fresh medium containing stimulators or inhibitors, as described in the section 2.6. Ecdysone released in the medium was determined by a competitive enzyme immunoassay (EIA). Briefly, EIA was performed in a 96-well microtiter plate and was based on the

competition between ecdysteroids in the analyzed sample and a known amount of peroxidase-labelled conjugated ecdysone (tracer) for binding anti-ecdysone primary antibody (Kingan, 1989). Each sample was diluted 50 times in phosphate buffer for EIA (0.08 M Na₂HPO₄, 0.02 M NaH₂PO₄, 0.15 M NaCl, 0.001 M EDTA, 0.1% BSA). After overnight incubation, the chromogen TMB substrate (Sigma Aldrich, St. Louis, MO, USA) was added and developed a blue color inversely proportional to the concentration of ecdysone. The absorbance was measured at 450 nm, using a multichannel microplate reader (Glomax S.r.l., Bellusco, Italy). The assay was calibrated with 20-hydroxyecdysone (Sigma-Aldrich, St. Louis, MO, USA) as a standard, with a concentration ranging from 10⁻⁷ M to 10^{-11.5} M. All experiments were performed on a single PG, in three technical replicates for each of the eight biological replicates.

2.8. In vivo assay with diet treatment supplemented with rapamycin

To investigate the effects of rapamycin on the onset of molting, larval weight, PG growth and the *in vitro* ecdysone production, bioassays were performed with rapamycin (Calbiochem, San Diego, CA, USA) added to the standard artificial diet (Vanderzant et al., 1962). Two groups of third instar larvae, weighing 9.0 ± 0.5 mg, were selected from the same egg pool and fed on diet supplemented with two concentrations of rapamycin (0.01 mg/g and 0.1 mg/g). Two other groups of fourth instar larvae from the same egg pool, weighing 90.0 ± 5.0 mg, were selected and fed on diet supplemented with rapamycin at the two concentrations previously described. Control larvae, weighing 9.0 ± 0.5 mg, were selected from the same egg pool at the third instar and fed on standard diet, as described in section 2.1. The assay was performed with fifteen individuals for every condition and carried out in triplicate.

2.9. In vitro biosynthetic activity and measurement of prothoracic gland size

The PGs from *H. virescens* 3–4 day old last instar larvae, reared on standard artificial diet (control) and on diet supplemented with rapamycin at 0.01 mg/g and 0.1 mg/g, were isolated, as described in section 2.2. The explanted glands were incubated with 0.1 BE/ μ l PTTH for 3 h after a time of rest of 30 min, and the ecdysone secretion was determined by EIA, as described in section 2.7. Subsequently, the same PGs were fixed in 4% formaldehyde overnight at 4 °C and mounted on Poly-PrepTM slides (Sigma-Aldrich, St. Louis, MO, USA) in 80% glycerol (Sigma-Aldrich, St. Louis, MO, USA). Images of PGs were acquired by a Leica DM LB microscope (Leica, Wetzlar, Germany). For each PG, the total area of explanted tissue was measured in μ m² by a LAS-X Core program for Leica Microsystems microscopes (Leica, Wetzlar, Germany).

2.10. Statistical analysis

Enzyme immunoassay and bioassay data were expressed as the mean ± SEM (standard error of mean) of independent biological replicates and were compared by analysis of variance (ANOVA) and Bonferroni *post-hoc* test using GraphPad Prism 6 software, version for Windows (<http://www.graphpad.com>) (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Transcriptome analysis of *Heliothis virescens* prothoracic glands

The identification in *H. virescens* PGs of proteins belonging to MAPK and PI3K/Akt/TOR pathways, was obtained by performing the next-generation sequencing (RNAseq) of RNA isolated from the PGs on 3 day old last (fifth) instar larvae. The resulting *de novo* reference transcriptome assembly (backbone) contained 34167 contigs, with a N50

contig size of 1885 bp and a maximum contig length of 23686 bp. To identify similarities with known proteins, contigs were translated and searched by BLASTX algorithm (Altschul et al., 1997) against the non-redundant protein database from NCBI with an E-value cut-off of 10^{-3} identifying 19380 contigs (56%) matching entries.

For further functional annotation, all sequences were subjected to Gene Ontology (GO) analysis in Blast2GO. Among the 34167 total contigs, 14825 (43%) shared significant similarity with proteins having assigned molecular functions in the GO database (Altschul et al., 1997). Some of these contigs could be assigned to one or more ontology terms; each contig was then assigned to a set of non-redundant GO terms using GO Slim. Using a combination of the BLAST result descriptions, GO terms, InterPro domains and additional BLAST searches with known insect genes, proteins belonging to the MAPK and PI3K/Akt/TOR pathways were identified in the PG transcriptome, as reported in Supplementary Tables 1 and 2.

3.2. Protein alignments

The percentage of identity of some key proteins belonging to MAPK (p38 MAP kinase and RAF kinase) and PI3K/Akt/TOR pathways (Akt, TOR, S6K and 4E-BP) in *H. virescens* and the corresponding proteins in *Homo sapiens*, *Bombyx mori* and *Manduca sexta*, was analyzed by performing protein sequence alignments. The accession numbers of *H. sapiens*, *B. mori* and *M. sexta* proteins are reported in Supplementary Table 3. Alignments among *H. virescens* and *B. mori*/*M. sexta* proteins showed a very high sequence similarity of up to 96% (Table 1), and the alignments among *H. virescens* and *H. sapiens* proteins still exhibited moderate sequence similarity up to 64% (Table 1). Supplementary Figs. 1–3 report individual protein alignments, highlighting identical and similar amino acids.

3.3. Phosphorylation of 4E-BP and S6K proteins in prothoracic glands

To verify the possible involvement of the PI3K/Akt/TOR pathway in *H. virescens* ecdysteroidogenesis, western blot analyses were performed on PGs previously incubated under different conditions (PTTH, rapamycin, PTTH added with rapamycin). The phosphorylation of the main targets of TOR kinase (Fig. 1a) was detected using antibodies against phospho-4E-BP and phospho-S6K.

The *in vitro* exposure of PGs explanted from 3 day old last instar larvae to PTTH contained in brain extract enhanced the phosphorylation level of both 4E-BP and S6K proteins. No phosphorylation signal was detected in PGs treated with rapamycin, with or without PTTH stimulation (Fig. 1a). In addition to the specific inhibition of TOR, the effects on the phosphorylation of 4E-BP and S6K in PGs explanted from 3 day old last instar larvae, pre-treated *in vitro* with PI3K and MAPK inhibitors (LY294002 and U0126, respectively) and then exposed to PTTH, were also analyzed. The results showed that LY294002 had an inhibitory effect, a finding that supports the involvement of the PI3K signaling pathway in PTTH-stimulated 4E-BP and S6K phosphorylation (Fig. 1b). In contrast, no effects were detected on 4E-BP and S6K phosphorylation when PGs were pre-treated with U0126, supporting

Table 1

Percentage of identity among *H. virescens* proteins (p38 MAP kinase – RAF kinase – Akt – TOR – S6K – 4E-BP) and the corresponding protein sequences from *H. sapiens*, *B. mori*, *M. sexta*. The protein alignments were performed using the protein BLAST tool (<https://blast.ncbi.nlm.nih.gov>).

	p38 MAP kinase	RAF kinase	Akt	TOR	S6K	4E-BP
<i>H. sapiens</i> vs <i>H. virescens</i>	49%	47%	61%	55%	64%	47%
<i>B. mori</i> vs <i>H. virescens</i>	96%	84%	90%	89%	95%	88%
<i>M. sexta</i> vs <i>H. virescens</i>	96%	93%	92%	92%	96%	83%

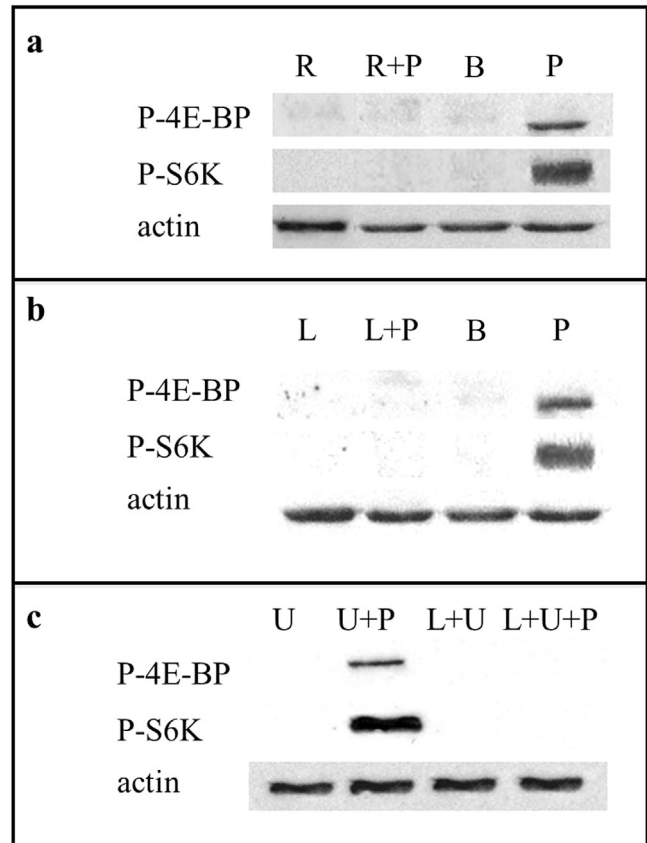


Fig. 1. Phosphorylation of TOR target proteins 4E-BP and S6K in prothoracic glands exposed to PTTH extract (P) and rapamycin (R), LY294002 (L) or U0126 (U). Prothoracic glands (PGs) from 3 day old last (fifth) instar larvae were pre-treated with either (a) 1 μ M rapamycin, (b) 50 μ M LY294002, or (c) 10 μ M U0126 with or without (c) 50 μ M LY294002, for 30 min; then transferred to medium containing the same dose of each inhibitor, with or without 0.1 brain equivalent/ μ l (BE/ μ l) PTTH. Unstimulated PGs (basal, B) were incubated in Grace's insect medium. Incubation was maintained for 3 h in each condition. Glands lysed in Laemmli 2X sample buffer were analyzed by western blot using antibodies against phospho-4E-BP (20 kDa) and against phospho-S6K (70 kDa). Each lane represents the equivalent of 20 PGs. The quantity of loaded proteins was revealed by the endogenous control, α -actin (42 kDa).

the hypothesis that the TOR pathway works independently with respect to MAPK signaling (Fig. 1c).

3.4. *In vitro* effect of MAPK/PI3K pathways inhibitors on PTTH-stimulated ecdysteroidogenesis by prothoracic glands

The *in vitro* biosynthetic activity of PGs explanted from *H. virescens* 3 day old last instar larvae in response to activators or inhibitors is reported in Fig. 2 and Supplementary Table 4.

Ecdysone production was most strongly enhanced by PTTH stimulation in comparison to all other experimental conditions. The levels of ecdysone produced by PGs incubated with inhibitors (rapamycin or LY294002 or U0126 or a combination of LY294002 and U0126) but without PTTH were similar to those of the basal. When PGs were co-incubated with each inhibitor and PTTH, the level of ecdysone production was significantly reduced compared to the level of PTTH-stimulated ecdysone production but was significantly higher than that of the respective basal (Fig. 2a–c).

The combination of two inhibitors, LY294002 and U0126, under PTTH stimulation, provided levels of ecdysone statistically comparable with levels in the basal (Fig. 2d).

Collectively, these results indicate that both MAPK and PI3K/Akt/TOR signaling pathways mediate the PTTH-stimulated ecdysteroidogenesis in *H. virescens* last instar larvae.

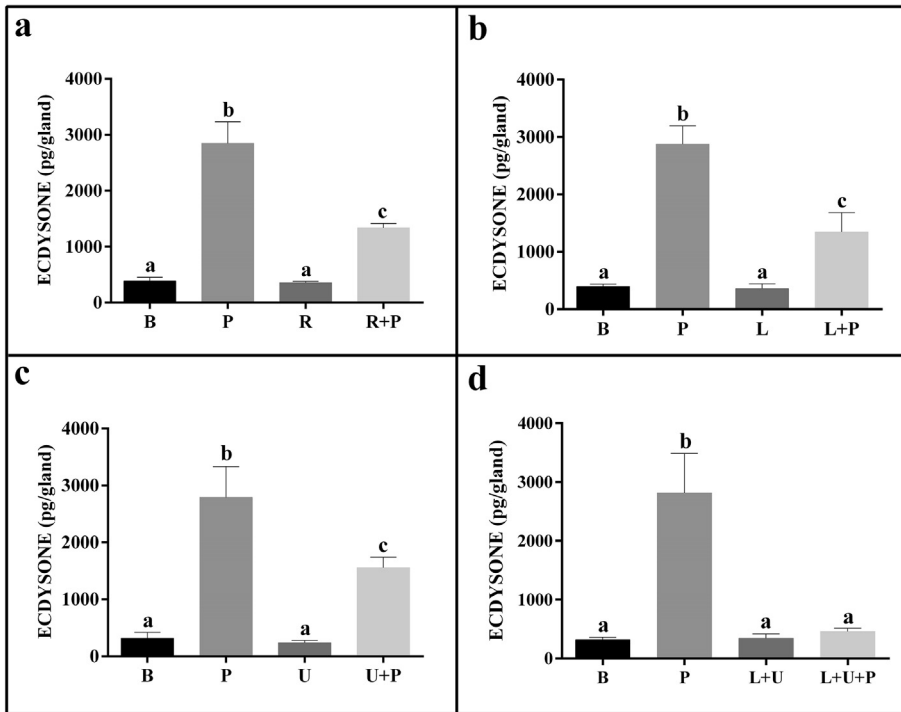


Fig. 2. *In vitro* effects of PTTH (P), rapamycin (R), LY294002 (L) and U0126 (U) on ecdysone synthesis by prothoracic glands. Prothoracic glands (PGs) explanted from 3 day old last (fifth) instar larvae were pre-treated with either (a) 1 μ M rapamycin, (b) 50 μ M LY294002, or (c) 10 μ M U0126, or (d) both 50 μ M LY294002 and 10 μ M U0126, for 30 min; then transferred to medium containing the same dose of each inhibitor, with or without 0.1 brain equivalent/ μ l (BE/ μ l) PTTH. Unstimulated PGs (basal, B) were incubated in Grace's insect medium without addition of inhibitors. Incubation was maintained for 3 h in each condition, after which the ecdysone was determined by enzyme immunoassay (EIA). Data are expressed as the mean \pm SEM of $n = 8$ experiments. Statistical analysis was performed with one-way ANOVA (analysis of variance) and a Bonferroni *post-hoc* test. Different letters indicate significant differences (p value < 0.01).

3.5. *In vivo* effects of rapamycin on larval growth and molting

In order to investigate the *in vivo* effect of rapamycin, *H. virescens* third and fourth instar larvae were reared on diet supplemented with different concentrations of rapamycin. Larval weight, time required to attain pupal stage (Fig. 3) and pupal weight (Fig. 4) were evaluated. Control larvae of *H. virescens* reared, starting from the third instar, on a standard artificial diet (Vanderzant et al., 1962), reached the pupal stage in nine days (Fig. 3). Mortality among control larvae was 7%. The larvae fed on diet supplemented with 0.01 mg/g rapamycin starting from the third instar reached the pupal stage in fifteen days and on the day before pupation weighed less (133.60 ± 2.69 mg) in comparison to controls (297.10 ± 29.71 mg) (Fig. 3a). This difference in weight was also observed in the pupae derived from the same rapamycin-fed larvae: these weighed significantly less (174.40 ± 1.16 mg) compared to controls (281.10 ± 3.82 mg) (Fig. 4) and showed evident morphological alterations (Fig. 5a and b). None of these pupae were able to eclose into viable adults. In addition to the slower growth rate and reduced weight compared to control larvae of *H. virescens*, a high mortality rate was observed starting on the fourth day of rapamycin administration; these larvae had a final mortality rate of 75%.

Similar results were obtained in bioassays performed on third instar larvae fed on diet supplemented with 0.1 mg/g rapamycin. They reached the pupal stage in sixteen days and weighed less (170.57 ± 14.35 mg) than controls (297.10 ± 29.71 mg) on the day before pupation (Fig. 3a). Moreover, their survival rate was 25% and they reached the pupal stage with evident morphological alterations (Fig. 5a and b). The mean pupal weight was 160.30 ± 0.36 mg, lower than that of the control (281.10 ± 3.82 mg) and no adults emerged from these pupae.

The growth curve of fourth instar larvae fed on diet supplemented with rapamycin (0.01 mg/g and 0.1 mg/g, respectively) showed a trend comparable to that of controls (Fig. 3b). Indeed, after a certain body size is achieved, rapamycin seems to no longer exert its strong inhibitory effect as seen for third instar larvae (Fig. 3). Nevertheless, fourth instar larvae rapamycin-fed reached the pupal stage 2–3 days later than controls, and weighed less (134.97 ± 8.72 mg for larvae fed on diet supplemented with 0.01 mg/g rapamycin and

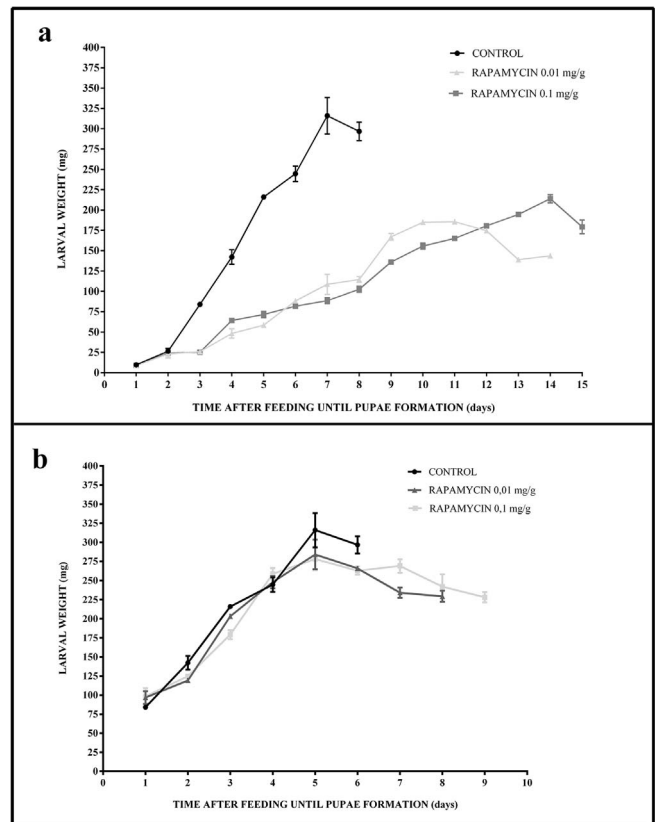


Fig. 3. Effects of different concentrations of rapamycin administered by diet on larval growth and on the onset of molting. Growth curves and onset of molting of larvae fed on standard diet (control) and supplemented diet containing two rapamycin concentrations (0.01 mg/g and 0.1 mg/g) starting from the third (a) or fourth (b) instars. Data are reported as the mean \pm SEM of $n = 15$ larvae, in 3 independent biological replicates.

143.13 ± 15.73 mg for those fed on diet supplemented with 0.1 mg/g rapamycin) than controls (297.10 ± 29.71 mg) (Fig. 3b). The

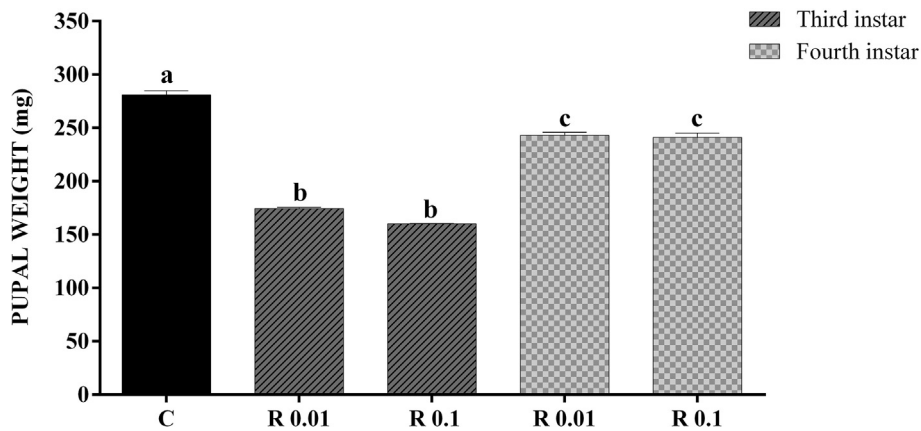


Fig. 4. Effects of different concentrations of rapamycin administered by diet on pupal weight. The bar charts show the weight of pupae derived from control larvae fed on standard diet (C) and supplemented diet containing rapamycin (R) at different concentrations (0.01 mg/g and 0.1 mg/g), administered starting from the third or fourth instars. The experiment was performed starting from 15 individuals. Data are reported as mean \pm SEM of larvae that reached the pupal stage in 3 independent biological replicates. Statistical analysis was performed by one-way ANOVA and with a Bonferroni *post-hoc* test. Different letters indicate significant differences (p value < 0.01).

percentage of mortality in larvae fed on diet supplemented with 0.01 mg/g and 0.1 mg/g rapamycin was 7% and 18%, respectively. Pupae appeared quite similar to controls (Fig. 5c) and weighed less (243.20 ± 2.77 mg for larvae fed on diet supplemented with 0.01 mg/g rapamycin and 241.20 ± 4.11 mg for those fed on diet supplemented with 0.1 mg/g rapamycin) than controls (281.10 ± 3.82 mg) (Fig. 4). Although the pupal weight differences were statistically significant, these differences did not affect adult emergence compared to the controls.

3.6. Effects of rapamycin on growth and biosynthetic activity of prothoracic glands

To further investigate the effects of rapamycin on PG growth and biosynthetic activity, larvae were reared on diet supplemented with 0.01 mg/g and 0.1 mg/g rapamycin, starting from the third or fourth instar. PGs were dissected on day 3 of the last (fifth) instar. PG size, as total cell area in μm^2 (Fig. 6), morphology (Fig. 7) and *in vitro* ecdysone production (Fig. 8a and 9a) were evaluated. The ecdysone biosynthesis was also measured from PGs dissected on day 4 of the fifth instar (Fig. 8b and 9b). When both third and fourth instar larvae were fed on supplemented diet, significant alterations in PG size, morphology and ecdysone production under PTTH stimulation were detected compared to the basal. The size of the PGs from larvae fed on diet supplemented with 0.01 mg/g and 0.1 mg/g rapamycin starting from the third instar ($50587.47 \pm 1458.82 \mu\text{m}^2$ and $37658.80 \pm 3771.92 \mu\text{m}^2$, respectively) (Fig. 6) was significantly smaller than size of the PGs from larvae fed on both concentrations of rapamycin starting from the fourth instar ($109242.70 \pm 4915.23 \mu\text{m}^2$ and $96153.27 \pm 4251.05 \mu\text{m}^2$, respectively) (Fig. 6). In both cases PG size was significantly smaller in comparison to the size of glands in control larvae ($183844.70 \pm 4647.82 \mu\text{m}^2$) (Fig. 6). No significant differences were

seen between the effects of the two rapamycin doses (0.01 and 0.1 mg/g) on PG size.

In addition to their reduced size, PGs from larvae fed on rapamycin starting from the third instar were morphologically altered relative to controls (Fig. 7a–c). In particular, the PGs from third-instar-rapamycin fed larvae appeared wrinkled, creased and fragile, and tended to break during explantation (Fig. 7b and c). Gland morphology from larvae fed on rapamycin starting from the fourth instar was more or less unchanged (Fig. 7d and e) but, as observed during explantation, treated PGs were more fragile than control (Fig. 7a).

Noticeably different degrees of responsiveness to PTTH stimulation were observed, as ecdysone production, measured on day 3 or day 4 of the last instar, was significantly lower in the PGs of larvae fed from third instar on supplemented diet (Fig. 8a and b), in comparison to the PGs of control larvae fed on standard diet (Supplementary Table 5).

The PTTH-stimulated ecdysteroidogenesis in the prothoracic glands of larvae fed on supplemented diet starting from the fourth instar was significantly lower (Fig. 9a and b), in comparison to the PGs of larvae fed on standard diet (Supplementary Table 5).

4. Discussion

The present study investigates the involvement of two cellular signaling pathways, MAPK and PI3K/Akt/TOR, on ecdysteroidogenesis in the prothoracic glands (PGs) of *Heliothis virescens* larvae stimulated by PTTH contained in brain extract, hereafter referred to as PTTH. Moreover, we also examine the role of the TOR protein in *H. virescens* post-embryonic development. Ecdysteroidogenesis, a key process in insect molting, is stimulated by various hormones and dietary components. Among these, the secretion of the neuropeptide prothoracotropic hormone (PTTH) is the most relevant (Gilbert et al., 1997; Marchal et al., 2010). Knowledge of the endocrine system in

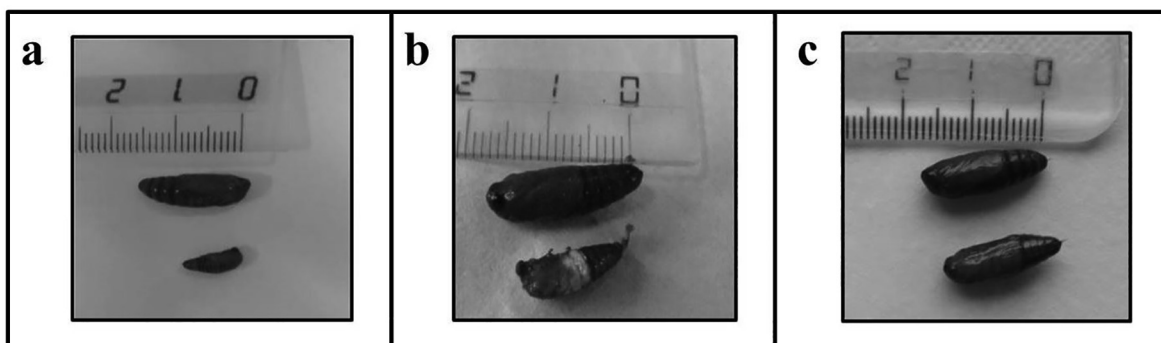


Fig. 5. Effects of different concentrations of rapamycin administered by diet on size and morphology of pupae. Comparison between control pupae (above) and pupae fed on supplemented diet containing rapamycin (below) starting from the third (a–b) or fourth (c) instars. The treated pupae in (a) showed a marked reduction in size while in (b) showed marked cuticle alterations. No evident morphological differences were observed in (c) between treated and control pupae.

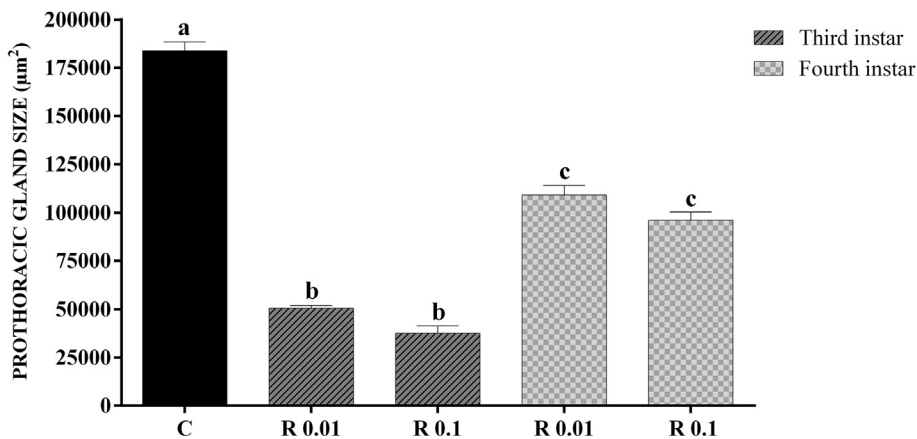


Fig. 6. Effects of different concentrations of rapamycin administered by diet on prothoracic gland size. The bar chart shows the size of the prothoracic glands (PGs) explanted from 3 day old last (fifth) instar larvae fed on standard diet, control (C), and supplemented diet containing 0.01 mg/g and 0.1 mg/g rapamycin (R), starting from third or fourth instars. Data are reported as the mean \pm SEM of $n = 8$ PGs. Statistical analysis was performed by one-way ANOVA and with a Bonferroni *post-hoc* test. The different letters indicate significant differences (p value < 0.01).

Lepidoptera was gained from numerous studies on *Manduca sexta* by Gilbert and colleagues. In addition to the discovery of PTTH-induced events, such as Ca^{2+} influx, cAMP generation, protein kinase A activation, and the upregulation and phosphorylation of some proteins (Gilbert et al., 1996; Rybczynski and Gilbert, 1994; Song and Gilbert, 1995), the MAPK pathway was identified as the main signaling cascade involved in *M. sexta* PTTH-stimulated ecdysteroidogenesis (Rybczynski et al., 2001). Recent studies of PTTH signaling in the PGs of *Bombyx mori* have expanded our understanding of lepidopteran ecdysteroidogenesis. Beside the MAPK pathway, the PI3K/Akt/TOR signaling cascade was also shown to be involved in *B. mori* ecdysteroidogenesis following the stimulation of several factors including PTTH (Lin and Gu, 2007; Gu et al., 2011, 2015). Previous studies of *H. virescens* ecdysteroidogenesis, focused on the relation between the moth and its natural parasitoid, *Toxoneuron nigriceps* (Malva et al., 2004; Pennacchio et al., 1997, 1998a), demonstrated that the signaling transduction process stimulated by PTTH contained in the brain extract was similar

to that reported for *M. sexta*. In fact, in *H. virescens*, PTTH induced an increase of cAMP endocellular levels, activating a biosynthetic pathway that led to the upregulation of protein synthesis and phosphorylation, resulting in the production and secretion of ecdysteroids in PGs (Pennacchio et al., 1997, 1998a). As previously reported (Lin and Gu, 2007; Gu et al., 2011; Rybczynski et al., 2001), both MAPK and PI3K/Akt/TOR pathways are conserved in *M. sexta* and *B. mori*, but their involvement in PTTH-stimulated ecdysteroidogenesis takes completely different forms. In the present work, we evaluated the involvement of the MAPK and PI3K/Akt/TOR pathways on PTTH-stimulated ecdysteroidogenesis in *H. virescens*. We demonstrated that this lepidopteran species is more similar to *B. mori* than to *M. sexta* (Pennacchio et al., 1998b; Kemirembe et al., 2012; Gu et al., 2011).

The analysis of the *de novo* *H. virescens* PG transcriptome and the functional annotation of 34167 contigs allowed us to identify a number of transcripts belonging to both MAPK and PI3K/Akt/TOR pathways. The alignment of some key proteins belonging to MAPK (p38 MAP

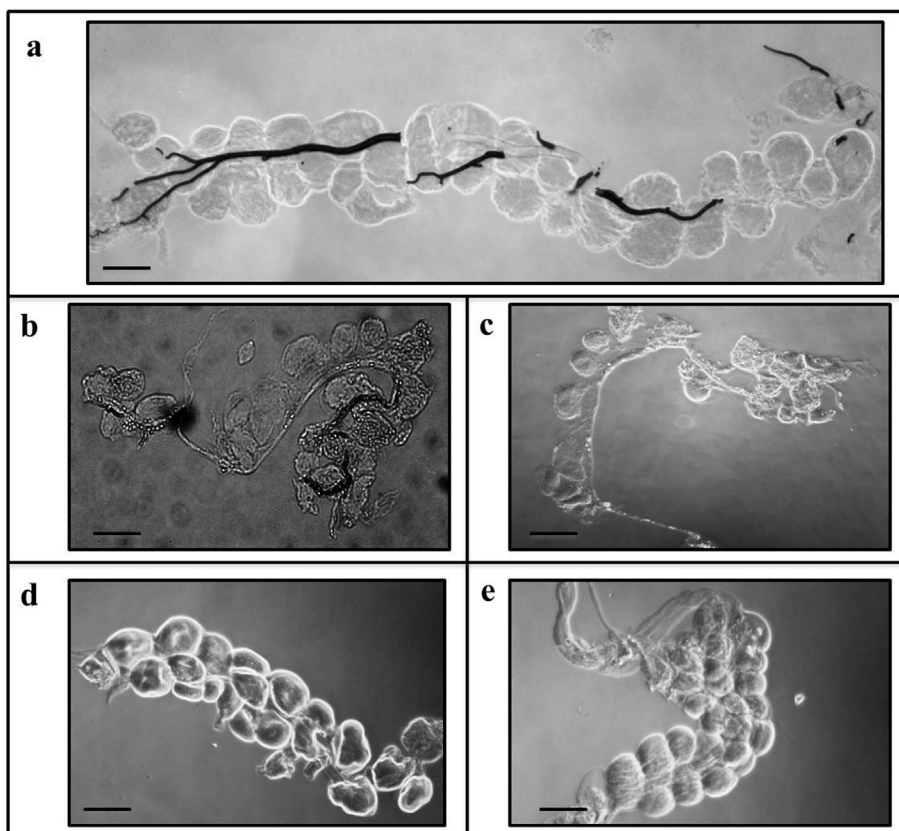


Fig. 7. Effects of different concentrations of rapamycin administered by diet on 3 day old last (fifth) instar larval prothoracic gland morphology. (a) Gland from a larva fed on a standard diet (control); (b) gland from a larva fed on supplemented diet containing rapamycin 0.01 mg/g starting from the third instar; (c) gland from a larva fed on supplemented diet containing rapamycin 0.1 mg/g starting from the third instar; (d) gland from a larva fed on supplemented diet containing rapamycin 0.01 mg/g starting from the fourth instar; (e) gland from a larva fed on supplemented diet containing rapamycin 0.1 mg/g starting from the fourth instar. Scale bars = 300 μ m.

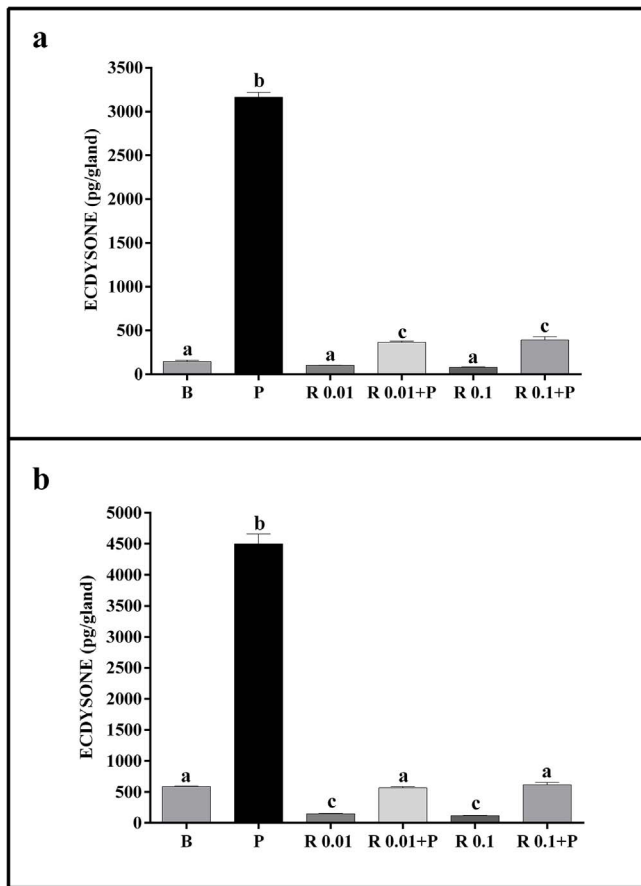


Fig. 8. Effects of rapamycin feeding, starting from the third instar, on last (fifth) instar ecdysone secretion *in vitro*. Larvae were fed on standard basal diet (B), or diet containing 0.01 mg/g or 0.1 mg/g rapamycin (R) from the third instar. On day 3 (a) or day 4 (b) of the last instar, prothoracic glands (PGs) were dissected and challenged with 0.1 BE/ μ l PTTH (P). Ecdysone was determined by enzyme immunoassay (EIA) after 3 h of incubation. Data are reported as the mean \pm SEM of $n = 4$ experiments. Statistical analysis was performed with one-way ANOVA and with a Bonferroni *post-hoc* test. Different letters indicate significant differences (p value < 0.01).

kinase and RAF kinase) and PI3K/Akt/TOR (Akt, TOR, S6K and 4E-BP), using sequences from *H. virescens*, *B. mori* and *M. sexta*, detected a high degree of sequence similarity, confirming that MAPK and PI3K/Akt/TOR pathways are conserved in this restricted set of lepidopteran species. Moreover, the alignment of the same proteins of *H. virescens* compared to *Homo sapiens* revealed a moderate sequence similarity among amino acids, suggesting that these proteins and relative pathways are conserved in phylogenetically distant organisms.

In order to study the possible role of MAPK and PI3K/Akt/TOR pathways in PTTH-stimulated ecdysone biosynthesis in *H. virescens*, PGs were incubated *in vitro* with U0126, a highly selective inhibitor of both MEK1 and MEK2, proteins belonging to the MAPK kinase pathway, LY294002, a specific inhibitor of PI3K, and with rapamycin, the main TOR inhibitor. We confirmed that PTTH stimulates significantly higher levels of the *in vitro* production and secretion of ecdysone by PGs in comparison to levels in non-stimulated PGs (basal), as previously demonstrated in *H. virescens* (Pennacchio et al., 1997), *B. mori* (Gu and Chow 2005, Ishizaki and Suzuki, 1994) and *M. sexta* (Kulesza et al., 1994; Smith et al., 2003). Moreover, in *H. virescens*, the PTTH-stimulated phosphorylation of the 4E-BP and S6K (the targets of TOR) indicates that the PI3K/Akt/TOR pathway is being directly stimulated by the neuropeptide hormone. This result allows us to hypothesize that the PI3K/Akt/TOR pathway could be involved in the production of ecdysone by PTTH-stimulated PGs. In agreement with previous data obtained from *B. mori* (Gu et al., 2011), rapamycin and LY294002

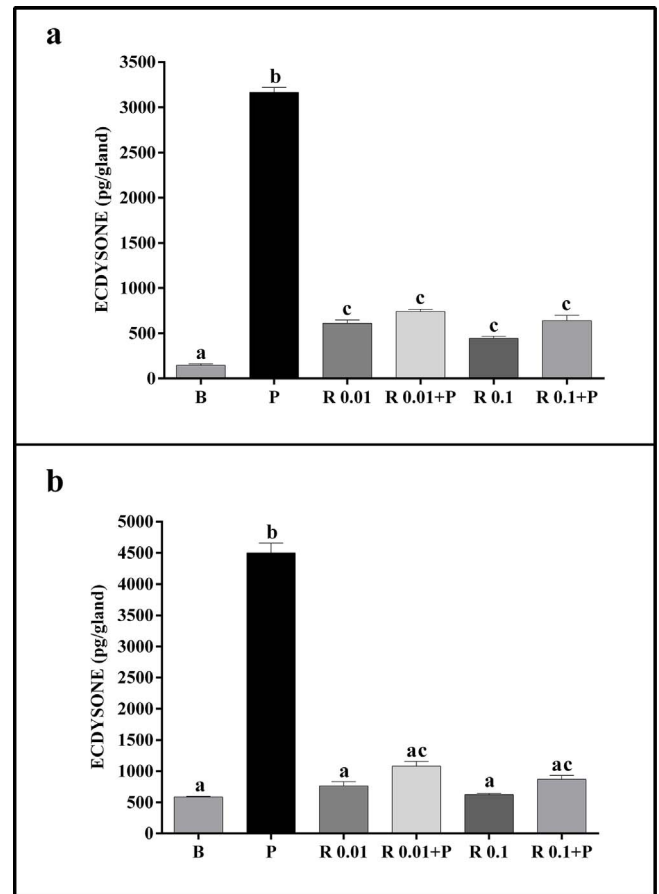


Fig. 9. Effects of rapamycin feeding, starting from the fourth instar, on last (fifth) instar ecdysone secretion *in vitro*. Larvae were fed standard basal diet (B), or diet containing 0.01 mg/g or 0.1 mg/g rapamycin (R) from the fourth instar. On day 3 (a) or day 4 (b) of the last instar, prothoracic glands (PGs) were dissected and challenged with 0.1 BE/ μ l PTTH (P). Ecdysone was determined by enzyme immunoassay (EIA) after 3 h of incubation. Data are reported as the mean \pm SEM of $n = 4$ experiments. Statistical analysis was performed by one-way ANOVA and with a Bonferroni *post-hoc* test. Different letters indicate significant differences (p value < 0.01).

inhibited the phosphorylation of 4E-BP and S6K; rapamycin and LY294002 also significantly, but not totally, reduced ecdysone levels after PTTH stimulation. Such effects confirm the involvement of the PI3K/Akt/TOR pathway in ecdysteroidogenesis in *H. virescens*.

The phosphorylation signals of 4E-BP and S6K in samples incubated with U0126 and PTTH clearly showed that PTTH-stimulated TOR activation is completely independent from the MAPK pathway. To confirm the involvement of the MAPK pathway in ecdysteroidogenesis, ecdysone production was measured *in vitro*, after incubating PGs with the MAPK inhibitor, U0126, and with PTTH. The level of ecdysone production was significantly, but not totally, reduced in comparison to the level in the sample stimulated with PTTH and without the inhibitor; the level was significantly higher than the level in the basal PGs (which was not PTTH-stimulated) with or without the inhibitor. These results allowed us to conclude that in *H. virescens* the MAPK pathway contributes to ecdysteroidogenesis, independently from the PI3K/Akt/TOR pathway.

Collectively our results confirm those obtained from studies on *B. mori*, in which both pathways are involved in PTTH-stimulated ecdysteroidogenesis, and diverge from studies on *M. sexta*, in which only the MAPK cascade seems to be involved. Indeed, although PI3K/Akt/TOR signaling is strictly connected to nutrient-dependent growth and development in *M. sexta* larvae (Hatem et al., 2015; Kemirembe et al., 2012), there is no strong evidence that this pathway is involved in ecdysteroidogenesis stimulated by PTTH or other factors (Smith et al.,

2014).

Although further investigation is needed to deepen our understanding regarding the contribution of each pathway and the specific role of the brain extract components, we can speculate that multiple transduction signals, involving both pathways, are triggered by PTH (Gu et al., 2010, 2011, 2012). Anyway, our results cannot exclude the possible involvement of other factors contained in the brain extract, such as the bombyxin which, in *B. mori*, contributes to the ecdysteroidogenesis, through the PI3K/Akt/TOR pathway, even if the PTH stimulation is much more powerful than stimulation by bombyxin (Gu et al., 2015).

In addition to *in vitro* tests, *in vivo* assays were carried out, in order to deepen the role of the TOR protein in post-embryonic development of our insect model. We fed third and fourth instar *H. virescens* larvae on diet supplemented with rapamycin at two different concentrations (0.01 mg/g and 0.1 mg/g). Results showed that the administration of rapamycin affected larval growth, molting, time to attain metamorphosis, weight and mortality, pupal weight and formation, size of PGs, morphology and responsiveness to PTH stimulus. Rapamycin had a general effect on larval growth and on PGs, with different impacts depending on the concentration of drug and the larval stage at which the supplement was administered. The strongest effect was observed when rapamycin was administered to third instar larvae; in contrast, when rapamycin was administered to fourth instar *H. virescens* larvae, its effect was limited. In *M. sexta* larvae (Kemirembe et al., 2012), rapamycin regulates the onset of molting in a concentration-dependent way, but *M. sexta* larvae fed on diet supplemented with rapamycin at the same concentration used in our bioassay (0.1 mg/g) pupated just one day later than controls, whereas *H. virescens* fourth instar larvae needed three days more than controls. Moreover, whereas the administration of rapamycin in *H. virescens* led to an increase of mortality in both instars (third and fourth), the literature contains no information about mortality of *M. sexta*.

The different effects of rapamycin could be due to the different body size of the two species. The administration of rapamycin to the tiny *H. virescens* third instar larvae might have jeopardized their ability to grow and molt, and, under the negative modulation of the TOR pathway, diminished the ability of cells to survive. We speculate that rapamycin has a systemic modulatory effect, influencing the development of all tissues, including the PGs. In general, the ability of a holometabolous insect to pupate is closely related to its attainment of the weight at which a larva can pupate without delay and to the size of the PGs and the level of ecdysone they produce (Shingleton, 2010). The post-embryonic development of the lepidopteran body is connected to the growth of the PGs. In our bioassay, the body weight and PG size of larvae fed on diet supplemented with rapamycin starting from the third instar were both very small compared to the body weight and PG size of control larvae; in fourth instar rapamycin-fed larvae the PGs were smaller than the PGs in controls, while body size remained similar. This effect is similar to what has been described in *M. sexta* larvae, where rapamycin suppressed PG growth relative to the whole body growth (Kemirembe et al., 2012). Moreover, when the third and fourth instar larvae of *H. virescens* were fed on diet supplemented with rapamycin, the *in vitro* production of ecdysone by PGs was dramatically suppressed, despite PTH stimulation, compared to PGs from control larvae. These results suggest both that in *H. virescens*, ecdysteroidogenesis is partially dependent on PI3K/Akt/TOR pathway and that the PGs of *H. virescens* larvae need to attain a threshold size in order to obtain adequate ecdysone levels.

We observed that even if fourth instar rapamycin-fed larvae needed more time than controls to reach the pupal stage, they pupate with slightly reduced weight compared to control. Unlike fourth instar larvae, third instar larvae fed on diet supplemented with rapamycin were much smaller than controls, had a high mortality rate, showed critical alterations of PG morphology and had levels of ecdysone production that were insufficient to trigger the molt.

The *in vivo* data support the results obtained *in vitro* and confirm the involvement of the TOR pathway in larval growth and development. Our results underline the two roles of the TOR kinase: a specific role in PGs, promoting ecdysteroidogenesis as a component of one of the pathways involved in the PTH signaling transduction cascade, and a general role, promoting the growth and development of the whole organism. The inhibition of TOR activity by rapamycin administration reduces the overall development of larval body and organs, including PGs, which are unable to mature and produce an adequate level of ecdysone. Moreover, rapamycin can inhibit the translation of ecdysteroidogenic genes, further weakening the activity of PGs. The evidence of the involvement of MAPK and PI3K/Akt/TOR pathways in PTH-stimulated ecdysteroidogenesis and in larval growth suggests a parallelism between events in developing mammalian cells and in similar processes in other biological systems. In fact, in mammals, the potential targets of MAPK phosphorylation include transcription factors, steroidogenic signaling pathways, growth factor receptors, kinases and proteins involved in regulating translation (Lake et al., 2016; Manna and Stocco 2011; Roux and Blenis, 2004); the PI3K/Akt/TOR pathway promotes cell-growth-stimulating proteins that activate catabolic and anabolic processes, such as the biosynthesis of proteins, lipids and other cellular components (Caron et al., 2010; Laplante and Sabatini, 2013; Lien et al., 2017; Raught et al., 2001). In mammals, protein synthesis is regulated by numerous extracellular and intracellular stimuli and activates several anabolic processes; in insects, ecdysone controls and regulates embryonic development, molting and metamorphosis (Nagakawa and Sonobe, 2016). PTH could therefore represent one of the stimuli that trigger the cascade of cell signaling involving proteins belonging to MAPK and/or PI3K/Akt/TOR pathways; subsequently, these proteins activate the translation of genes related to ecdysteroidogenesis.

The study of the mechanisms underlying the post-embryonic development of our model insect could help to clarify the endocrinology of Lepidoptera. Surprisingly, although insects of the same order share the same pathways and the same proteins their involvement within the same cellular process can be regulated differently. In addition, the in-depth study of insect biology at a molecular level offers useful information about the physiology of worldwide pests such as *H. virescens*, and their complex interactions with the environment, including other organisms. This background information could be used to develop innovative and eco-friendly strategies for pest control.

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Conflict of interest

The authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2018.02.008>.

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