Article

# STUDY ON ECDYSTEROID LEVELS AND GENE EXPRESSION OF ENZYMES RELATED TO ECDYSTEROID BIOSYNTHESIS IN THE LARVAL TESTIS OF *Spodoptera littoralis*

Masatoshi Iga
*Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium*

Catherine Blais
*UMR 1272, Physiologie de l'insecte, Signalisation et Communication, UPMC Université Paris 6, 75005 Paris, France*

Guy Smagghe
*Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium*

*We investigated here the ecdysteroid titers and the expression of six genes coding for known enzymes of the ecdysteroid biosynthesis in the testes of last instar larvae of the pest cotton leafworm, Spodoptera littoralis. We showed that the timing of the ecdysteroid profile was the same in testes and in hemolymph, with a small peak at day 2 and a large one at day 4 after ecdysis. Ecdysone and 20-hydroxyecdysone (20E) were detected in both tissues. 20E was the major ecdysteroid in testes and in hemolymph from day 4. Interestingly, the gene expression of the steroidogenetic enzymes, Neverland, and the five cytochrome P450 enzymes encoded by the Halloween genes was confirmed in the testes, and varied during the instar. However, from the data obtained so far, we cannot conclude that the*

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Present address of Masatoshi Iga: Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba, Japan
Correspondence to: Guy Smagghe, Department of Crop Protection, Ghent University, Coupure Links 653, 9000 Ghent, Belgium. E-mail: guy.smagghe@ugent.be

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*measured ecdysteroids in the testes result from the activity of the genes under study. Indeed, it is suggested that the ecdysone produced centrally in the prothoracic glands, could have been transformed into 20E in the testes, where Sl-shade is well expressed.* © *2012 Wiley Periodicals, Inc.*

**Keywords:** ecdysteroids; testes; Neverland; cytochrome P450 enzyme; Halloween genes; *Spodoptera*

## INTRODUCTION

Ecdysteroids, the molting hormones of insects, are important for controlling their growth, development, and reproduction. Classically, during postembryonic development, ecdysone (E) is predominantly synthesized in the prothoracic glands (PG), then secreted into the hemolymph. The secreted E is converted into 20-hydroxyecdysone (20E) in the peripheral tissues. The machinery of ecdysteroid biosynthesis is studied thoroughly in model insects such as *Drosophila melanogaster* and *Bombyx mori*, and seven important genes related to ecdysteroid biosynthesis have been identified to date, that is, Neverland (Nvd), nonmolting glossy/shroud (Nm-g/Sro), and the five well-known Halloween genes (Chávez et al., 2000; Warren et al., 2002, 2004; Petryk et al., 2003; Niwa et al., 2004, 2005; Maeda et al., 2008; reviewed in Brown et al., 2009).

In insects, ecdysteroids are synthesized from dietary cholesterol (C) or phytosterols. The initial step of 20E biosynthesis, from C to 7-dehydrocholesterol (7dC), is mediated by an oxygenase-like protein, Nvd (Yoshiyama et al., 2006, 2011). The next steps leading to 2,22,25-trideoxyecdysone (ketodiol) have not been fully understood and remain as the "Black Box." Recently two genes believed to be involved in these steps have been identified. A cytochrome P450 (CYP) enzyme, CYP307A1/A2 (Spook: Spo/Spookier: Spok), and a short-chain dehydrogenase/reductase, Nm-g/Sro may act as tissue- and stage-specific components in the part of the Black Box, but the details of biochemical function and their substrates have not been understood yet (Namiki et al., 2005; Ono et al., 2006; Niwa et al., 2010).

On the other hand, the last four hydroxylation steps from ketodiol to 20E have been studied thoroughly in *D. melanogaster* and *B. mori*. These steps are catalyzed by the four CYP enzymes, encoded by the Halloween genes: CYP306A1 (Phantom: Phm), CYP302A1 (Disembodied: Dib), CYP315A1 (Shadow: Sad), CYP314A1 (Shade: Shd). The orthologs of the corresponding genes had been identified in other insects such as *Aedes aegypti* (Sieglaff et al., 2005), *Manduca sexta* (Rewitz et al., 2006a,b), *Spodoptera littoralis* (Iga and Smagghe, 2010), *Acyrthosiphon pisum* (Christiaens et al., 2010), and noninsect Arthropods as *Daphnia pulex* (Rewitz and Gilbert, 2008).

As reviewed by Brown et al. (2009), the PGs are not the only tissue that can synthesize ecdysteroids. In the female gonad, ovary, biosynthesis of ecdysteroids has been reported during larval, pupal, and adult stages in many species. The molecular aspects of ovary ecdysteroid biosynthesis have been accumulated in mosquitoes and *B. mori*, and they demonstrated the similarity of the machinery to that in the PG (Sieglaff et al., 2005; Ito et al., 2008; Brown et al., 2009). Furthermore, ecdysteroids have also been reported in the male gonad, testis, in many lepidopteran insects, including *Heliothis virescens* (Loeb et al., 1982, 1984), *Lymantria dispar* (Loeb et al., 1988), *Ostrinia nubilalis* (Gelman et al., 1989), *Mamestra brassicae* (Shimizu et al., 1985), *S. littoralis* (Jarvis et al., 1994; Polanska

et al., 2009), and *B. mori* (Fugo et al, 1996), and this also during the larval, pupal, or adult stages. In addition, ecdysteroids were detected in the testis of Diptera such as *D. melanogaster* (Bownes et al., 1984), *Calliphora vicina* (Koolman et al., 1979), Orthoptera with *Gryllus bimaculatus* (Hoffmann and Behrens, 1982), and Hemiptera with *Rhodnius prolixus* (Vafopoulou and Steel, 2005). In vitro and immunocytological studies in *H. virescens* and *L. dispar* suggested that ecdysteroids were synthesized and/or present in the sheaths of testes (Loeb, 1986; Loeb et al., 1988). However, the pathway of ecdysteroid biosynthesis and the function of synthesized ecdysteroids in testes have not been fully understood yet.

In this article, we investigated and compared the titer of ecdysteroids and their nature in the testes and hemolymph during the last (sixth) larval instar (L6). Furthermore, we identified the homolog of *nvd* in *S. littoralis* and studied the tissue specificity. In addition, the expression profiles of five Halloween genes and *nvd* were investigated in the whole testes and their sheaths during L6. We finally discussed the interpretation that the testes are possible alternative sites of de novo ecdysteroid biosynthesis and/or that they only convert E absorbed from hemolymph into 20E, as other peripheral tissues.

## MATERIALS AND METHODS

### *Animals*

Larvae of the cotton leafworm, *S. littoralis*, were reared on an artificial diet under standard conditions of 24 ± 2°C, 65 ± 5% relative humidity, and a 16:8 light:dark photoperiod (Smagghe et al., 2002). In our rearing conditions, the sixth instar lasts 5.5 ± 0.5 days. The first feeding day was designated as day 0 of the sixth instar (L6D0).

### *Ecdysteroid Extraction and Measurement*

Hemolymph of L6 larvae was collected by cutting a proleg by scissors, and centrifuged at 18,000 × *g* for 10 min. The supernatant was used for ecdysteroid extraction. Pure methanol (2.5 times volume) was added to the supernatant, vortexed and centrifuged at 18,000 × *g* for 10 min. After collecting the supernatant, the pellet was rinsed with 70% methanol, vortexed and centrifuged at 18,000 × *g* for 10 min. Testes of L6 larvae were collected and homogenized in 300 μl of 70% methanol in a polypropylene tube, using a plastic pestle. The samples were vortexed and centrifuged at 18,000 × *g* for 10 min. After collecting the supernatant, the pellet was resuspended for a second extraction. For ecdysteroid quantification by enzyme immunoassay (EIA), the combined extracts were evaporated and dissolved in EIA sample buffer (80 mM $K_2HPO_4$, 20 mM $KH_2PO_4$, 0.4 M NaCl, 1.25 mM EDTA, 0.1% BSA, 0.01% thimerosal, pH 7.4).

For further high-pressure liquid chromatography (HPLC) analyses, the samples were more purified. After the methanol extraction, the methanol phase was evaporated and a partition with chloroform/water (1:2, v/v) was performed twice. The aqueous phase was purified on a $C_{18}$ Sep-Pak cartridge (Lafont et al., 1982): a polar fraction was first eluted with 5 mL of 30% methanol and free ecdysteroids were then eluted with 5 mL of absolute methanol. Only this fraction was further analyzed by HPLC: methanol was evaporated and the samples were redissolved in HPLC mobile phase. Ecdysteroids were separated by normal-phase HPLC using a silica column (250 mm × 4.6 mm, Hypersil, AIT Chromato Le Mesnil Le Roi, France) and dichloromethane/propan-2-ol/water (125:30:1.5, v/v/v)

as solvent, at a flow rate of 1 mL $min^{-1}$, on a Beckman apparatus (System Gold; Fullerton, CA), with UV detection at 250 nm. Fractions were collected every 0.7 min and evaporated until dry.

Ecdysteroids present in hemolymph and testes extracts and in the HPLC fractions were quantified by EIA adapted from the method described by Porcheron et al. (1989), using 2-succinyl-20E coupled to peroxidase as a tracer and a rabbit polyclonal anti-E antiserum L2 (generous gift from Dr. J.-P. Delbecque, Bordeaux, France, and Dr. M. De Reggi, Marseille, France). The antiserum L2 displayed the highest affinity toward E (23 fmol giving 50% maximum binding), and it recognized 20E 4.3 times less than E. Dried samples were resuspended in EIA sample buffer. In routine experiments, calibration curves were generated with E (500–3.8 fmol/tube) and results are given as E equivalents. HPLC coupled to EIA allows us to detect immunoreactive peaks at precise retention times, corresponding eventually to known ecdysteroids, as the references 20E (generous gift from Dr. Juraj Harmatha, Prague, Czech Republic) and E (Sigma, St Louis, MO).

### *Total RNA Isolation and cDNA Synthesis*

PG, fat body, testes, Malpighian tubules, midgut, brain, and epidermis were dissected from L6 and the total RNA was isolated using High Pure RNA Tissue Kit (Roche, Indianapolis, IN) or TRI REAGENT (Sigma) according to the kit protocol. For the preparation of testis sheath, the testis was cut parallel to the antero-posterior axis with a scalpel, and the content was blown apart using a glass capillary in the insect ringer solution under a binocular. The testis sheath appeared clear of adhering tissue. The total RNA was quantified with a NanoDrop 1000 Spectrophotometer (Thermo scientific, Wilmington, DE) and cDNA was synthesized from 0.5 to 1 μg total RNA with anchored oligo-$dT_{12}$ [5′-(T)12(A/C/G)(A/C/G/T)-3′] and the reverse transcriptase Superscript II (Invitrogen, Carlsbad, CA).

### *Identification of Nvd in* S. littoralis

The primary fragment of *S. littoralis Neverland* (*Sl-nvd*) was amplified from the cDNA of PG in L6 by RT-PCR using degenerate primers with recombinant Taq DNA polymerase (Invitrogen). The degenerate primers were designed from highly conserved regions of amino acid sequences between *B. mori* (GenBank accession no. AB232986) and *D. melanogaster* (GenBank accession no. AB232987). The used degenerate primer sequences were F: 5′-CCNCCNTAYCCNAAYGGNTGGT-3′ and R: 5′-GCNCCRTTYTCNGGDATYTCYTG-3′. The PCR product was purified by Cycle Pure kit (Omega Bio-Tek, Norcross, GA). The purified PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) for sequencing. Positive clones were verified by colony PCR and several of these clones were sequenced. Gene specific primers (GSPs) were designed in the partial sequence and 5′- and 3′-ends were amplified by rapid amplification of cDNA ends (RACE) by SMART-RACE cDNA Amplification Kit (Clontech, Mountain View, CA) according to the kit instructions. The used GSPs were 5′RACE: 5′-GCATTCGATACAGGAGCCGCTGACG-3′and 3′RACE: 5′-GACGGCGCAGTGTGGATCTGGTATGA-3′. The amplified products were subjected to gel electrophoresis, purified, cloned into the pGEM-T vector, and several of the longest cloned PCR products were sequenced.

***Table 1. Oligonucleotide Primers Used for RT-PCR***

| *Gene* | *Forward primer (5′-3′)* | *Reverse primer (5′-3′)* | *Amplicon (bp)* |
|---|---|---|---|
| *Neverland* | TGCTTCGATGTTGTGTTATCTGTG | ATTCGGAAATGGTGGTGGAG | 528 |
| *Spook* | ACAAACTCTCAAACTGGTCTTCCG | CTGTAGCTTCTGTGTATGGTATGCTG | 351 |
| *Phantom* | ACACGCGGTCAAGCTCAAA | TGTGGCAGTCTGGAACCGT | 482 |
| *disembodied* | TCAAAAGAATTTCACCAGCCC | TCAACCATCACTCCAATGACG | 483 |
| *Shadow* | GAATGGTTAGAAAATCGAAGAGTTGT | GGTTGTTTATTATTTATCATCTCCTCG | 428 |
| *Shade* | ATGTTCAGGCGTTACGGGTT | GTCTCGTTGGGCTCTGAAGTG | 501 |
| *Ribosomal protein S3* | TTCAAGGCGGAACTCAACG | AACCACGGGCTCCAGATTC | 352 |

### *Phylogenetic Analysis of Nvd*

The deduced amino acid sequence of *Sl*-Nvd was aligned by MUSCLE (Edgar, 2004) with *A. aegypti* (GenBank accession no. XP_001655171), *Culex quinquefasciatus* (GenBank accession no. XP_001848026), and *Caenorhabditis elegans* (GenBank accession no. NP_505629) in addition to *B. mori* and *D. melanogaster*. The phylogenetic tree was generated by neighbor-joining method using ClustalX with a bootstrap value of 1000 trials for each branch position, exclude the gap position.

### *Semi-Quantitative RT-PCR*

The primer sets used for making the expression profile of *Sl-nvd* and Halloween genes are shown in Table 1. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The intensity of the bands was measured by Image J program (U.S. National Institutes of Health, Bethesda, MD). The result was normalized by the expression level of an internal standard gene, *Ribosomal protein S3* (*RpS3*). At the same time of cDNA preparation, without reverse transcriptase, samples (non-RT templates) were prepared for evaluating the genomic DNA contamination. All the PCR results using non-RT templates showed no amplification (data not shown).

## RESULTS

### *Titer and Nature of Ecdysteroids in the Testes*

Ecdysteroid concentrations in the testes were measured by EIA during the last larval instar (0–5 days after ecdysis). As depicted in Figure 1, the ecdysteroid titer showed two peaks, namely a small one at L6D2 (64 h after ecdysis, Fig. 1B) and a larger one at L6D4 (104 h after ecdysis, Fig. 1A). This pattern is parallel to that observed in hemolymph (Fig. 1; Iga and Smagghe, 2010). The nature of free ecdysteroids in testes and hemolymph was investigated at L6D1, L6D2, and L6D4 by HPLC coupled to EIA. At L6D1, the pattern of ecdysteroids in the two tissues was very similar (Fig. 2A), with a major immunoreactive peak, corresponding to 20E retention time, and a minor one, corresponding to E retention time. At L6D2, both ecdysteroids were detected in the hemolymph and testes, but their relative proportions were different (Fig. 2B). The major immunoreactive compound in the testes appeared as 20E, with a very low level of E, whereas a large peak at retention time of E was observed in the hemolymph. Then, taking into account the cross reactivity

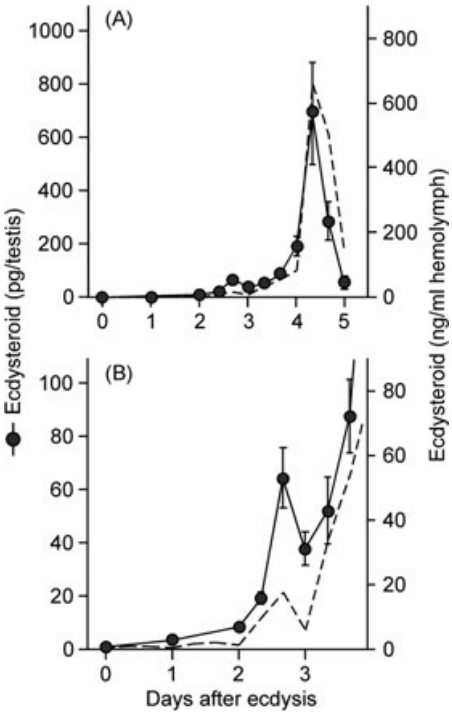


**Figure 1.** Titers of ecdysteroids in testes and hemolymph of L6. The titers were quantified by enzyme immunoassay (EIA), and expressed as E equivalents. (**A**) The left ordinate is for the titer of ecdysteroids in testes (closed circles) and the right ordinate is for that in hemolymph (dashed line). The titer of ecdysteroids in hemolymph is from Iga and Smagghe (2010). (**B**) Ten times enlarged graph of (**A**) from L6D0 to L6D3. The titer of ecdysteroids was measured every 8 h from L6D2 (48 h) to L6D5 (120 h). Each datum point represents the mean ± SEM ($n = 11$–32).

of the antibody L2 recognizing E 4.3 times better than 20E, the ratio of 20E:E is about 3.6:1 in the hemolymph, while the ratio is about 56:1 in the testes at L6D2. At L6D4, hemolymph and testes contained both putative E and 20E. However, the ratio of 20E:E was different with 14:1 and 25:1 in the hemolymph and testes, respectively (Fig. 2C).

### *Characterization of Nvd*

A primary fragment of *Sl-nvd* was amplified by RT-PCR using a degenerate primer set designed from the conserved region of Nvd amino acid sequences between *B. mori* and *D. melanogaster*. The obtained partial sequence was processed for BLAST search and this confirmed the similarity to other species. To obtain the entire sequence of *Sl-nvd*, 5′ and 3′ RACE were performed, and got the whole sequence (2,405 bp, encoding 451 aa, GenBank accession no. GU391576). The conserved motifs of Nvd, a Rieske [2Fe-2S] center binding motif, and a nonheme iron-binding motif were well conserved in *Sl*-Nvd (Fig. 3A).

Phylogenetic analysis was held using the deduced amino acid sequences of Nvd in *A. aegypti*, *C. quinquefasciatus*, *C. elegans*, in addition to *B. mori* and *D. melanogaster*. The phylogenetic tree clearly showed three individual clusters of Diptera, Lepidoptera, and Nematoda (Fig. 3B). The *Sl*-Nvd showed highest identity with *B. mori* (62.5%). On the other hand, the identity between *S. littoralis* and Diptera was relatively low, and it showed

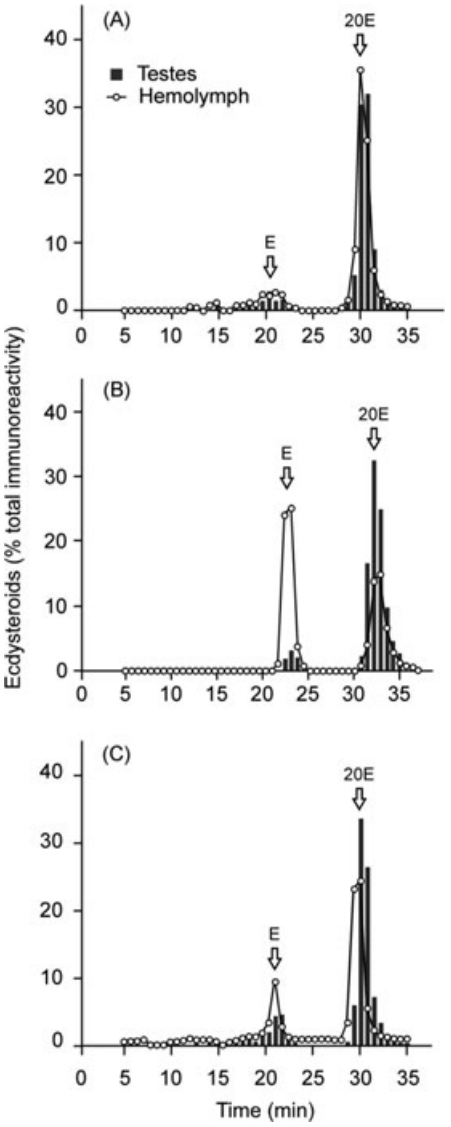


**Figure 2.** NP-HPLC-EIA (where NP-HPLC is normal-phase HPLC and EIA is enzyme immunoassay) analysis of ecdysteroids in testes and hemolymph. Results are expressed in percent of total immunoreactivity (measured in E equivalents) per 0.7-min fraction. (**A**) L6D1, (**B**) L6D2, and (**C**) L6D4. Gray bars and opened circles indicate testes and hemolymph ecdysteroids, respectively. Arrows indicate the retention time of E and 20E standards.

34.8% identity with *Drosophila*, 39.7% with *Aedes*, and 40% with *Culex*. Furthermore, the homologous protein of Nvd in *C. elegans*, an abnormal dauer formation family member 36 (DAF-36), showed 31.5% identity with *Sl*-Nvd.

The tissue distribution of *Sl-nvd* was evaluated in the selected tissues (PG, fat body, testes, Malpighian tubules, midgut, brain, and epidermis) at L6D1 by semiquantitative RT-PCR. *Sl-nvd* was expressed predominantly in the PG, while smaller amounts of *Sl-nvd* were expressed in the midgut and the testes (Fig. 3C).

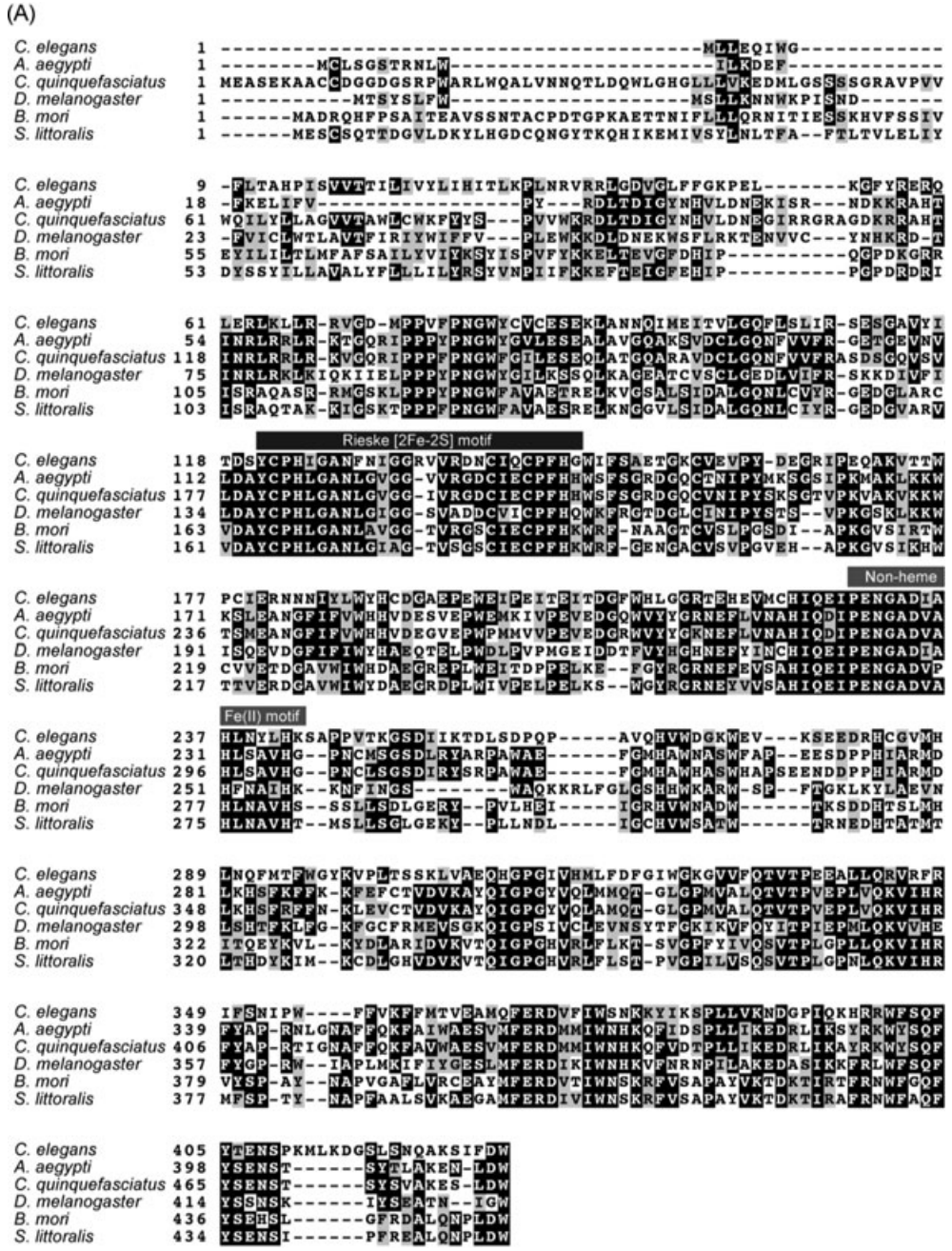


**Figure 3.** Characterization of *Sl*-Nvd. **(A)** Amino acid sequence alignment of *Sl*-Nvd. Residues in black are identities and in gray are similarities. The conserved motifs, that is, a Rieske [2Fe-2S] center binding motif (C-X-H-$X_{16–17}$-C-$X_2$-H) and a nonheme iron-binding motif (E/D-$X_3$-D-$X_2$-H-$X_4$-H) are shown in the figure. (B) Phylogenetic tree of Nvd. The indicated numbers are bootstrap value as percentage of a 1,000 replicates and the scale bar indicates 0.05 change per residue. The bootstrap values higher than 50% were indicated. **(C)** Tissue distribution of *Sl-nvd*. The expression of *Sl-nvd* and of an internal standard gene, *RpS3*, was evaluated by semiquantitative RT-PCR. The gene expression was measured in prothoracic glands (PG), fat body (FB), testes (TE), Malpighian tubules (MT), midgut (MG), brain (BR), and epidermis (EP) at L6D1.

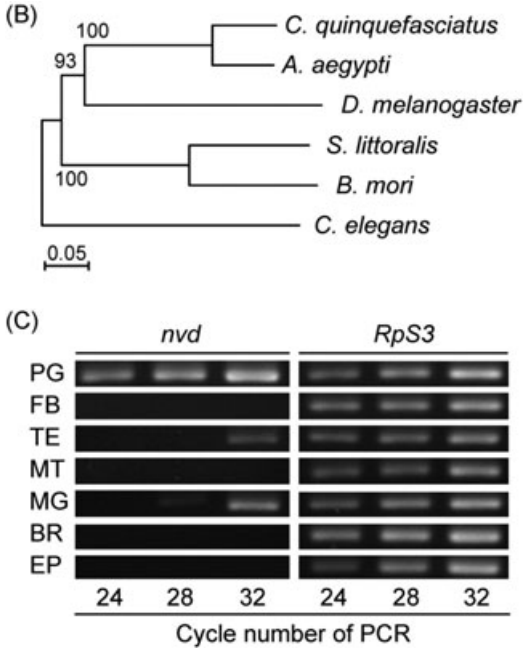


**Figure 3.** *Continued.*

### *Gene Expression Profile of Ecdysteroid Biosynthesis-Related Enzymes in the Testes*

The expression of *Sl-nvd*, *Sl-spo*, *Sl-phm*, *Sl-dib*, *Sl-sad*, and *Sl-shd* in the whole testes and the sheaths of testes was measured by semiquantitative RT-PCR during L6 (Fig. 4). The expression pattern of all these genes showed a similar tendency both in whole testes and sheaths of testes. The expression of *Sl-nvd*, *Sl-spo*, *Sl-phm*, and *Sl-sad* gradually decreased in the course of the instar. On the other hand, the expression of *Sl-dib* and *Sl-shd* showed correlations with ecdysteroid concentrations in testes. The expression of *Sl-dib* clearly peaked at L6D3, and is then maintained at a high level during the large ecdysteroid peak at L6D4. The expression of *Sl-shd* was the most dominant compared to the other five genes (detection after 26 PCR cycles). It gradually increased until L6D2, in correlation with the first small ecdysteroid peak, and decreased at L6D3 and again showed a high expression at L6D5.

## DISCUSSION

The testis ecdysteroid titer in the last-instar larvae (L6) of *S. littoralis* showed two clear peaks: a small one at 64 h (L6D2) and a large one at 104 h (L6D4) after the ecdysis. This result was in agreement with a previously described profile of ecdysteroids in the testes of *S. littoralis* (Jarvis et al., 1994). The timing of both peaks of ecdysteroids was the same in testes and hemolymph (Fig. 1). This was not the case in a previous report (Jarvis et al., 1994), where a large peak in testes occurred 8 h before that in the hemolymph. This difference could be due to a difference of rearing conditions of the insects, and/or, in our case, to the fact that ecdysteroid titers were measured in hemolymph previously

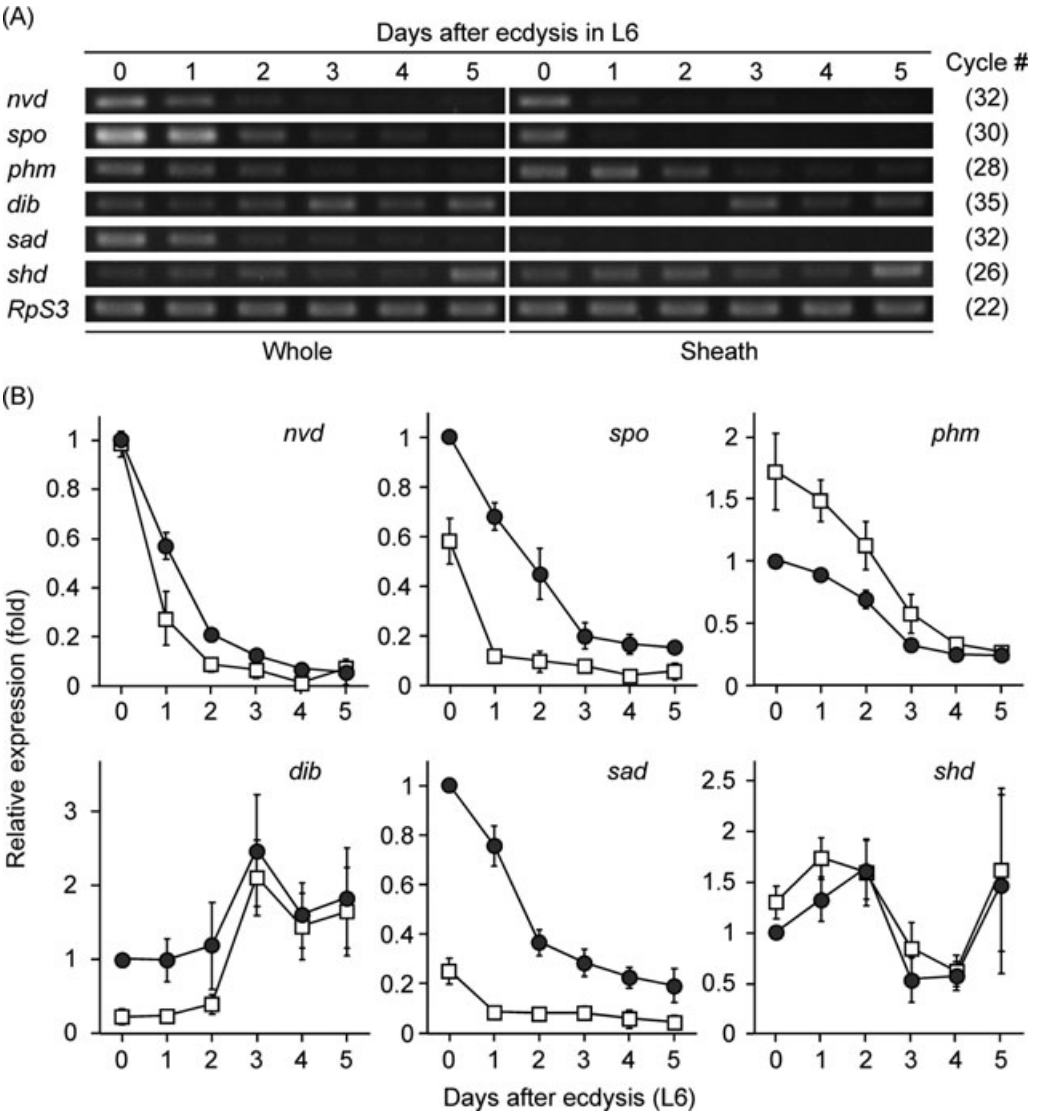


**Figure 4.** Relative gene expression profile of ecdysteroid biosynthesis-related enzymes in testes. The cDNA of whole testes and sheaths of testes was prepared from the same insects. (**A**) Expression profile of *Sl-nvd*, *Sl-spo*, *Sl-phm*, *Sl-dib*, *Sl-sad*, and *Sl-shd* in the whole testes and sheaths of testes during L6. (**B**) Triplicate densitometry values were obtained from independent samples for each time point and normalized with *RpS3* expression level. The normalized expression level of whole testes at L6D0 set as 1. Closed circles indicate the gene expression level of whole testes and opened squares indicate that of testis sheaths. Each datum point represents the mean ± SEM ($n = 3$).

(Iga and Smagghe, 2010), in different animals. To date, the function and importance of ecdysteroids detected in the testes of insect larvae are not fully understood. The large peak of ecdysteroids in the testes may be important for regulating the rate of division and differentiation of the germ cells related to the larval–pupal development (Dumser, 1980). On the other hand, the first small peak of ecdysteroids in the testes may have some important roles for development/maturation of the testes, like the commitment of pupal

differentiation in epidermal cells induced by the first release of E (Riddiford, 1978), but the details are unclear.

The nature of ecdysteroids in testes and hemolymph was measured at three characteristic time points during L6: at the period of basal level (L6D1), at the small peak (L6D2), and at the large peak (L6D4) of ecdysteroids. In all the cases, we detected two ecdysteroids, which comigrated with E and 20E references. These two ecdysteroids were also the major ones in a previous report in *S. littoralis* (Jarvis et al., 1994). The relative proportion of both ecdysteroids varied with the tissue and the day of the instar. At L6D1, the nature of ecdysteroids was very similar in the testes and hemolymph: 20E was the major compound, with only a small amount of E (Fig. 2A). On the other hand, the relative proportions of 20E and E were clearly different in the testes and hemolymph at L6D2 and L6D4 (Fig. 2 B and C). At L6D2, the ratio of 20E:E is very high in the testes (56:1) compared to that in the hemolymph (3.6:1), with the ratios corrected for the cross-reactivity of 20E to L2 antibody. At L6D4, the ratio of 20E:E is 14:1 and 25:1 in the hemolymph and testes, respectively. The different relative compositions of ecdysteroids in the two tissues have also been reported previously in lepidopteran larvae of *O. nubilalis* (Gelman et al., 1988) and *S. littoralis* (Jarvis et al., 1994), with a higher ratio of 20E:E in the hemolymph than in the testes in this last species. The fact that we detected more 20E than E in the testes compared to the hemolymph can be explained since E is released by the PG, circulates in the hemolymph and then is converted to 20E in peripheral target tissues. A plausible explanation is therefore that E from the hemolymph is converted to 20E in the testes by Shade or that circulating 20E is accumulating in the testes. This hypothesis also agrees with Figure 4 showing that *shade* is the most highly expressed gene in the testes of all the Halloween genes (detection after 26 PCR cycles). However, so far we have no firm conclusion whether the latter process of conversion of E into 20E can fully explain the differences in concentration between E and 20E between testes and hemolymph. On the other hand, the fact that the testes content of ecdysteroids fluctuates in precise synchrony with the hemolymph ecdysteroid titer (Fig. 1) is equally consistent with the present interpretation that the testes could have passively absorbed ecdysteroids from the hemolymph rather than engaging in ecdysteroid biosynthesis. In addition, it is also plausible that a part of the free E and 20E found in the testes could have been formed by hydrolysis of ecdysteroid conjugates. Indeed, the gonads (both testes and ovaries) of other insect species can contain E and 20E and also various conjugates and other free ecdysteroids (reviewed in Brown et al., 2009). However, to date there are no data available on the impact (of the pathway) of deconjugation versus the amounts of E/20E that are derived from cholesterol.

In the context of the puzzling difference in the ratios of the concentrations of the ecdysteroids in hemolymph and testes, it should also be remarked that Loeb et al. (1987, 1988) and Meola et al. (1998) have shown that there are at least two unique ecdysteroidogenic peptides which differ in immunological specificity and biochemical composition, and that originate in different, specific groups of cells in the insect brain. One of the peptides, testis ecdysiotropin, appears to induce ecdysteroid synthesis by testis sheaths. Therefore, the difference in ecdysteroid ratios may also be explained by differences in receptors or receptor concentrations for the stimulating neuropeptides in ecdysteroid-producing tissues, perhaps controlling the "black box" enzymes. It might therefore be of interest to follow this lead in future research.

In *S. littoralis*, we have previously identified the five Halloween enzymes mediating the biosynthesis of 20E, and all of them are well conserved in insects (Rewitz et al., 2007; Iga and Smagghe, 2010). In addition to these enzymes, here we identified a Rieske-domain protein, Nvd (in *S. littoralis*), which catalyzes the initial conversion step from C to 7dC

(Yoshiyama et al., 2006, 2011). The amino acid sequence and the characteristic domains were well conserved in *Sl*-Nvd, and it was predominantly expressed in PG as in the cases of *B. mori* and *D. melanogaster* (Fig. 3). *Phm*, *dib*, *sad*, and *shd*, whose products catalyze the last four hydroxylation steps of ecdysteroid biosynthesis, were all expressed in the testes of *S. littoralis* at L6 (Fig. 4). This is consistent with a previous report showing that the in vitro transformation of radioactive ketodiol, an ecdysteroid intermediate, into 20E could take place in the testes (Jarvis et al., 1994). On the other hand, the conversion of C into ecdysteroids has been unsuccessful to date, possibly due to the storage of C in testes and/or permeability problem of C and 7dC. As we have shown that *Sl-nvd* and *Sl-spo*, that are involved in some conversion steps from C to ketodiol (reviewed in Brown et al., 2009), were expressed in the testes, this could suggest that the conversion of C into ketodiol takes place in the testes. However, it should be noticed here that, first, the expression profiles of *Sl-nvd*, *Sl-spo*, *Sl-phm*, and *Sl-sad* did not correlate with the ecdysteroid titer in the testes (Figs. 1 and 4), and it was clear that their expression pattern in the whole testes was relatively similar to that in the testis sheaths (Fig. 4). Second, we remark that several tissues including the peripheral tissues as midgut and fat body have been shown to convert the ketodiol into 20E (reviewed in Brown et al., 2009). The reason is probably that *phm*, *dib*, and *sad* expression can be detected at low levels in different tissues in addition to the PG. Thus, the current data do not support that the testes have a specific role in de novo ecdysteroidogenesis. Besides, further biochemical data would be required to provide firm evidence that the conversion of cholesterol into 20E can be mediated by testes. As a consequence, we can agree with the hypothesis that it is more likely that the "true" ecdysteroidogenic tissue is expressing specifically the possible rate-limiting enzymes which are believed to be in the black box reaction (as reviewed by Brown et al., 2009). Hence, we want to remark that although the current molecular tools are very useful, however, earlier attempts for specific silencing by RNAi of Halloween genes were not successful in *S. littoralis* larvae (Iga and Smagghe, 2010; own unpublished data; Terenius et al., 2011). As a consequence, this hampers to determine in detail the physiological role of the different Halloween genes in the testis and ecdysteroidogenesis.

The expression pattern of *Sl-dib* differed from the other Halloween genes *Sl-spo*, *Sl-phm*, and *Sl-sad*, with the highest value in the middle of the stage (Fig. 4), which is just after the small peak of testis ecdysteroids (at L6D3). We believe that *Sl*-Dib could be important for regulating the large peak of ecdysteroids at L6D4. This hypothesis is consistent with previous studies on in vitro conversion of radiolabeled precursors by *S. littoralis* testes (Jarvis et al., 1994). The latter authors suggested there could be a control step late in the ecdysteroid biosynthetic pathway, leading to an accumulation of 2,22-dideoxyecdysone, which is the substrate of Dib. Following the increase of *dib* expression at L6D3, there could be an increase of the enzyme Dib activity, and a completion of the ecdysteroid biosynthetic pathway in testes, when the large peak of ecdysteroids was detected. The latter observations could support potential ecdysteroid biosynthesis in the testes of *Spodoptera*. Concerning the expression level of *Sl-shd*, governing the conversion of E into 20E, it was of interest that its expression levels in the testes were the highest as compared to all other Halloween genes. In addition, its expression in the sheaths of testes was similar to that in the whole testes. The expression level of *Sl-shd* was high at L6D2 (Fig. 4), in correlation with the small peak of ecdysteroids in the testes as seen in the current study (Fig. 1). For the second peak of *Sl-shd* expression at L6D5, it can be hypothesized that this could be related to the forthcoming pupation or the increase of ecdysteroid titer after the pupation. As discussed above, we believe here it is plausible that E from the hemolymph as produced by the PG is converted to 20E in the testes by high levels of Shade enzyme resulting from

these expression peaks. In addition, because the predominantly expressed region in the testes was different among the evaluated genes, transport of 20E intermediates and/or the enzymes between the sheath and content of testes was suggested. However, the current data do not allow to conclude that the corresponding enzymes are located preferentially in the sheaths which has been reported in the past by Loeb (1986) and Loeb et al. (1988) in *Heliothis*, another lepidopteran species.

In conclusion, it was of interest that the present data could confirm the gene expression of six ecdysteroid biosynthesis-related enzymes in *S. littoralis* testes. Therefore, these data may suggest that de novo biosynthesis of ecdysteroids could have taken place in the larval testes of *S. littoralis*. However, although the search for ecdysteroidogenic tissues is compelling, the data obtained so far prevent direct claim that the measured ecdysteroids in the testes resulted from the activity of the genes under study, so not supporting that the male gonads, testes, are a site of ecdysteroidogenesis. Therefore, it is suggested that circulating E synthesized in the PG could be absorbed and then transformed into 20E in the testes, where *Sl-shade* is well expressed.

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