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# Stage-dependent expression of *Chelonus inanitus* polydnavirus genes in the host and the parasitoid

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**Abstract**

*Chelonus inanitus* (Braconidae) is a solitary egg–larval parasitoid of *Spodoptera littoralis* (Noctuidae). Along with the egg it also injects polydnaviruses (CiV) and venom, which are prerequisites for successful parasitoid development. CiV protects the parasitoid from encapsulation by the host's immune system and induces a developmental arrest in the prepupal stage. The polydnavirus genome consists of several double-stranded circular DNA segments. Proviral DNA is integrated in the wasp's genome and virus replication is restricted to the wasp's ovary. Here, the analysis of eight CiV genes located on five different segments revealed four patterns of expression in the course of parasitization: early, late, persistent but variable, and early and late. The comparison between parasitized and CiV/venom only containing hosts indicated that the presence of the parasitoid larva modulates transcript levels. Haemocytes, fat body and nervous tissue contained viral transcripts, values being highest in haemocytes. Small amounts of CiV transcripts were also observed in parasitoid larvae and pupae, suggesting transcription from the proviral integrated form of viral DNA. This is the first comparative analysis of the expression patterns of several viral genes in both parasitized and CiV/venom only containing hosts over the entire period of parasitization, and it reveals intricate interactions between the parasitoid, the polydnavirus and the host.


*Keywords:* Parasitic wasp; Polydnavirus; Parasitoid–host interaction

## 1. Introduction

Successful parasitism by many endoparasitic wasps depends on the supporting action of symbiotic polydnaviruses (reviewed in Webb, 1998; Drezen et al., 2003). These unique viruses are found in some subfamilies of ichneumonid and braconid wasps (reviewed in Whitfield and Asgari, 2003) and are referred to as ichnoviruses and bracoviruses. They are produced in the wasp's ovary and are injected together with the egg(s) into the host at parasitization where they play an important role in protecting the parasitoid from the host's immune system and in manipulating host development (reviewed in Turnbull and Webb, 2002; Kroemer and Webb, 2004). The polydnavirus genome consists of various segments of double-stranded circular DNA. For the bracovirus of *Chelonus inanitus* (CiV) it was shown that individual segments are singly encapsidated (Albrecht et al., 1994), while for ichnoviruses it is assumed that the entire genome is packaged in a single virion (Webb, 1998). In its proviral form, the poyldnavirus genome is integrated into the wasp's genome and transmitted vertically through the germ line (Stoltz, 1990). In bracoviruses the segments appear to be clustered (Belle et al., 2002; Wyder et al., 2002), while in ichnoviruses they seem to be dispersed (Fleming and Summers, 1991). Viral DNA replication and virion formation take place in the calyx cells of the ovary from

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where they are released by budding in the case of ichnoviruses (Volkoff et al., 1995) and by cell lysis in the case of bracoviruses (Wyler and Lanzrein 2003). In the parasitized host, there is no replication of viral DNA (reviewed in Turnbull and Webb, 2002).

Transcription of polydnaviral genes has been documented in hosts of several parasitoids, but very little is known on the transcription of viral genes in the parasitoid. Most data on viral transcription have been obtained from hosts of larval parasitoids where the wasp oviposits into larval stage hosts. In these, the host's immune system has to be compromised immediately after parasitization to prevent encapsulation of the parasitoid egg. Several polydnavirus genes have been shown to be involved in protecting the parasitoid from the host's immune system (reviewed in Webb, 1998; Turnbull and Webb, 2002). Transcript quantities of such viral genes are high shortly after parasitization and then persist or decline. Haemocytes and fat body are the most heavily infected tissues (Stoltz and Vinson, 1979; Strand et al., 1992; Hayakawa et al., 1994; Wyder et al., 2003) and are a major site of viral transcription (Kroemer and Webb, 2002). The knowledge on viral genes being involved in other aspects of host regulation such as effects on host metabolism and host development is still very limited.

We are studying the effects of the egg–larval parasitoid *C. inanitus* and its polydnavirus (CiV) on one of its natural hosts *Spodoptera littoralis*. In egg–larval parasitoids, the wasp oviposits into the egg stage of the host which is believed not to be immune competent (Salt, 1968) and thus the immediate protection of the parasitoid egg may be less important. Later on, however, once the host has become a larva, inhibition of encapsulation of the parasitoid larva by polydnaviruses appears to be essential (Pfister-Wilhelm and Lanzrein, 1996; Stettler et al., 1998). Parasitization by *C. inanitus* has a great influence on the development of *S. littoralis* as metamorphosis is induced precociously in the fifth instar followed by a developmental arrest in the prepupal stage (Grossniklaus-Bürgin et al., 1994). CiV, synergized by venom, was shown to be responsible for the developmental arrest in the prepupal stage (Soller and Lanzrein, 1996) and to cause an inhibition of the prothoracic gland and a reduction of haemolymph ecdysteroids at the stage of pupal cell formation (Grossniklaus-Bürgin et al., 1998; Lanzrein et al., 2001). For the precocious onset of metamorphosis, the parasitoid larva, in the presence of CiV/venom, has been shown to be responsible and to cause a premature decline in juvenile hormone (Pfister-Wilhelm and Lanzrein, 1996; Steiner et al., 1999; Lanzrein et al., 2001). Northern dot blots with total CiV DNA as probe revealed that, in contrast to the situation in larval parasitoids, viral transcript levels were low in the early phase of parasitization and increased in the last larval instar (Johner et al., 1999). The first two cloned and analyzed CiV genes (14g1 and 14g2) were found to be upregulated in the stage of digging and pupal cell formation and it was proposed that they might play a role in inducing the developmental arrest of the host in the prepupal stage (Johner and Lanzrein, 2002). The genome of CiV consists of at least 12 segments with sizes between 7 and 31 kbp (Albrecht et al., 1994; Wyder et al., 2002). Five segments (CiV12, CiV14, CiV14.5, CiV16.8 and CiV21) have been fully sequenced up to now and this led to the identification of eight genes (Wyder et al., 2002, and EMBL accession no. AJ627175).

Here we present data on relative transcripts levels of these CiV genes in the entire course of parasitization. To analyze a putative effect of the developing parasitoid larva on transcription of viral genes, relative transcript quantities were also measured in host eggs and larvae containing only CiV/venom but no parasitoid larva. To find out whether CiV genes expressed in the host are also transcribed from the integrated proviral form, the parasitoid larva and pupa were also analyzed. In addition, relative transcript quantities of four CiV genes were compared between haemolymph, fat body and nervous tissue. In situ hybridization was performed to investigate the presence of viral transcripts in plasmatocytes and granular cells.

## 2. Methods

### 2.1. Insects and X-ray irradiation of wasps

*C. inanitus* (Braconidae) is a solitary egg–larval parasitoid which was reared on its natural host *S. littoralis* (Noctuidae). Details about the biology and rearing of parasitoid and host are given in Grossniklaus-Bürgin et al. (1994). The degree of parasitization was always verified by dissection of some parasitized eggs; and eggs and larvae were only used for RNA isolation when parasitization was above 90%. To study the effects of polydnavirus/venom in the absence of a developing parasitoid larva, female wasps were irradiated with X-rays (146 Gy ± 10%) as described in Soller and Lanzrein (1996). This treatment lethally damages the parasitoid eggs. Host eggs and larvae developing from eggs parasitized with X-ray-irradiated wasps are designated as "X-ray parasitized".

### 2.2. Collection of insect material and RNA isolation

Nonparasitized, parasitized and X-ray parasititzed eggs and larvae of *S. littoralis* were frozen at −20 °C for at least 2 h and no longer than 2 weeks. RNA was isolated as described in Johner et al. (1999) and Johner and Lanzrein (2002): for eggs and L1 to L4, approx.

100 mg (from at least three larvae) was homogenized in 0.45 ml lysis buffer RLT (Qiagen) plus 145 mM β-mercaptoethanol, and for L5 and L6 volumes were adapted and either the RNeasy Plant mini-Kit or Midi Kit (Qiagen) was used. For isolation of RNA from haemolymph, six larvae were bled as described in Johner et al. (1999) and the haemolymph was taken up in 0.45 ml lysis buffer as above. For collection of fat body and nervous tissue, 10–15 larvae were anaesthetized on ice and tissues were dissected with sterile forceps in diethyl pyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS). First, nervous tissue (brain and ventral nerve cord) was removed and washed in DEPC-treated PBS and then pieces of fat body were removed and washed. After no longer than 5 min washing, the tissues were transferred to sterile ice-chilled Eppendorf tubes containing 0.45 ml lysis buffer (see above) and vortexed vigorously. Isolation of RNA from *C. inanitus* was either from five to eight larvae dissected out of their L5 hosts and washed in DEPC-treated PBS, or from four external L3 or from three pupae. Homogenization was always done in 0.45 ml lysis buffer as above. After homogenization, a proteinase K digestion was performed as described in Johner and Lanzrein (2002). The RNA isolation including an on-column DNAse digestion (RNase-free DNAse set, Qiagen) was performed according to the manufacturer's protocol. RNA was eluted from the column with 100 µl (Mini-Kit) or 300 µl (Midi-Kit) of RNase-free water. A second digestion with DNase I was carried out and the samples were then extracted with acidic phenol, and RNA was precipitated as described (Johner et al., 1999). RNA concentration was measured with a spectrophotometer, and RNA quality was checked by running an aliquot on a 1% agarose gel in TBE (Tris 10.8 g/l, boric acid 5.5 g/l, $Na_2EDTA$ 20 mM, pH 8.0).

### 2.3. Real-time PCR

Reverse transcription was carried out with 5 µg of total RNA, 90 ng oligo$(dT)_{12-18}$ primers and either 100 U of Superscript II reverse transcriptase (Gibco) or 50 U StrataScript Reverse Transcriptase (Stratagene, 50), 1 × first-strand buffer (Stratagene), 40 U RNAsin (Promega) and dNTPs mix (Roche, 400 nmol each). For the subsequent real-time PCR, primers were designed with Primer Express software (prelease version 1.0b6, Applied Biosystem). The primers used for the cloned or predicted CiV genes analyzed were the following. On segment CiV12, two genes were analyzed and the primers are CiV12g1U/CiV12g1L and CiV12g2U/CiV12g2L (EMBL accession no. Z58828). Both produce an amplicon of 50 bp. On segment CiV14, one new gene was analyzed (14g3) and the primers are CiV14g3U/CiV14g3L (EMBL accession no. AJ278677), yielding an amplicon of 56 bp; furthermore, additional stages were analyzed for genes 14g1 and 14g2 with already described primers (Johner and Lanzrein, 2002). On segment CiV14.5, one gene was analyzed and the primers are CiV14.5g1U/CiV14.5g1L (EMBL accession no. AJ319654), yielding an amplicon of 56 bp. On segment CiV16.8, one gene was analyzed and the primers are CiV16.8g1U/CiV16.8g1L (EMBL accession no. Z31378), yielding an amplicon of 50 bp. On segment CiV21, one gene was analyzed and the primers are CiV21g1U/CiV21g1L (EMBL accession no. AJ627175), yielding an amplicon of 55 bp.

The PCR was performed in 96-well optical reaction plates (Applied Biosystems) with the SYBR Green I Reaction System (Eurogentec): an appropriate amount of cDNA (corresponding to 100 ng total RNA = 1/50 of the reverse transcription reaction) was amplified in a volume of 30 µl containing 200 µM of each dNTP, 3.5 mM $MgCl_2$, 0.75 U Hot GoldStar enzyme, 200 nM of each primer and 0.9 µl of a 1/2000 dilution of SYBR Green I stock. The PCR was carried out on a ABI PRISM 5700 Sequence Detection System (Applied Biosystems) with the GeneAmp 5700 SDS software version 1.3, using the following thermal profile: 10 min 95 °C (initial step), 15 s 95 °C (denaturation), 1 min 60 °C (annealing and elongation). After 40 cycles, a melting curve was calculated by slowly heating from 60 to 95 °C, to check the specificity of amplification. According to Liu and Saint (2002), the primer efficiency was determined from the slope of the curve and this was done for each primer combination on every microplate. On each plate, a reference cDNA was amplified and the initial amount of cDNA template was expressed as a percentage of the reference cDNA. As reference cDNA, a stage displaying high transcript levels for a particular gene was chosen. For each gene, 2–4 measurements were made with each cDNA, and 2–4 cDNAs made of independent RNA isolations were used. Values are expressed as mean percentages + standard error from all measurements of a particular stage. Normalization with actin was not applicable as actin values were similar only in the various larval stages, but much lower and variable in embryos (data not shown) and towards metamorphosis (Johner and Lanzrein, 2002). In default of a reference gene for normalization, various control experiments were carried out. To check the efficiency of the reverse transcription, the plant mRNA rubisco (SpotReportTM *A. thaliana* mRNA Spike 3, Stratagene) was added as external standard to RNAs from various stages. Template amounts of 1 pg per µg total RNA were found to be sufficient to obtain good amplification. Thus, in each of these control reactions 5 pg rubisco mRNA were added to the 5 µg total RNA. The results revealed that the efficiency of the reverse transcription reactions was comparable between the RNAs of various stages of host and parasitoid. In another control approach, the quantity of ribosomal

RNA (18S) was measured in a set of cDNAs where random hexamers were used as primers instead of oligo(dT) primers. Similar values were obtained for various stages of the host and the parasitoid. Taken together, these observations indicated that the various RNA isolations are of comparable quality. To allow comparisons between genes, the various CiV genes were analyzed with the same set of cDNAs and corresponding references but with gene-specific primers.

### 2.4. In situ hybridization with haemocytes

Probes were labelled with digoxigenin (DIG) by the use of the PCR DIG Labeling mix (Roche). Plasmid DNA (30 pg), 200 nM of each primer and 2.5 U Taq Polymerase per 50 µl reaction were used. For 14g1 the *Not*I-cut clone λ6/5.11 of CiV14 with primers (5′-CCTACAGCTATCGCATTTTATGA-3′) and (5′-CCAAGACGTTTTGACACCCATC-3′) was used as template, and for 14g2 the *Not*I-cut clone λ6/6.5 with primers (5′-GGCTAAATCCGACGAAG-3′) and (5′-ATTTGTAAAAGCGTTTCAGAC-3′) was used as template. Thirty-five cycles were performed at 94 and 52 °C for 1 min each, and at 68 °C for 1.5 min. After removal of free nucleotides with the QIA Quick PCR Purification Kit (Qiagen), products were analyzed on a 1% agarose gel and the amount of DNA containing DIG-11-dUTP was determined by dotting several dilutions on a nylon membrane and detecting it as described below. The spot intensity was compared with a DIG-labelled standard DNA, and probes were used only if the concentration of labelled DNA was at least 10 ng/µl.

All buffers used were treated with DEPC. For collection of haemolymph, larvae were anaesthetized on ice. The caudal disk was cut off and the haemolymph was bled directly on a poly-L-lysine-treated round cover slip with a diameter of 10 mm. Haemocytes were allowed to settle for 10 min at 4 °C. The fluid was then sucked off and the adhering haemocytes were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Samples were then washed three times for 5 min with DEPC-treated PBS. For permeabilization, haemocytes were exposed to microwaves (Bull and Harnden, 1999). Microwave treatment was done five times for 2 min at 800 W. After two washes with DEPC-treated PBS, cover slips were placed into a 24-well cell culture plate. Prehybridization was done with 30 µl hybridization solution (50% formamide, 10% dextran sulphate, 2 × SSC, 50 mM sodium phosphate buffer, 250 ng/µl salmon sperm DNA) without probe at 42 °C for at least 2 h in a box saturated with 2 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7). For hybridization, cover slips were placed upside down on 10 µl hybridization solution with DIG-labelled probe (6 ng/µl). Hybridization was carried out in a box saturated with 2 × SSC for 36 h at 42 °C. After hybridization, slides were placed back into the 24-well plate. Cells were washed first with 50% formamide in 2 × SSC (three times for 5 min at 42 °C), then with 2 × SSC (three times for 5 min at 42 °C) and finally with 4 × SSC, 0.1% Tween 20 (once for 5 min at 42 °C). For detection, samples were first equilibrated with 300 µl DIG-1 buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5). Blocking was for 30 min in 300 µl DIG-2 buffer (DIG-1 buffer containing 1% blocking reagent from Roche) at room temperature. After blocking, 300 µl of a sheep anti-digoxigenin antibody conjugated with alkaline phosphatase (Anti-Digoxigenin–AP, Fab fragments, Roche) diluted 1:500 in DIG-3 buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) was added to each well. Incubation was either for 2 h at room temperature or overnight at 4 °C. The antibody solution was then removed and samples were washed twice with 300 µl DIG-1 buffer for 15 min at room temperature. Then 300 µl DIG-3 buffer was added for equilibration. Staining was carried out as described in Wyder et al. (2003) and the extent of staining was regularly checked under the microscope and was stopped after 5–26 h by two washes with TE (10 mM Tris, 1 mM $Na_2EDTA$, pH 8). Coverslips were mounted with GLYCERGEL™ (DAKO) on Superfrost slides. Observation was done with an Eclipse E600 (Nikon) microscope connected to a digital camera (DXM1200, Nikon). Pictures of the experimental samples and controls were taken under exactly the same conditions.

## 3. Results

### 3.1. Viral transcripts in the course of parasitization and X-ray parasitization

Nonparasitized and X-ray parasitized larvae pass through six larval instars (L1–L6) but the latter become developmentally arrested in the prepupal stage, as do larvae developing from eggs injected with calyx fluid/venom (Soller and Lanzrein, 1996). Parasitized larvae enter metamorphosis precociously in L5 and the parasitoid L3 emerges from the precocious prepupa and consumes the host remains (Grossniklaus-Bürgin et al., 1994). The last larval instar was subdivided into early and late feeding, digging, pupal cell formation early and pupal cell formation late as described by Grossniklaus-Bürgin et al. (1998). To investigate the expression of six newly identified CiV genes during parasitization, transcript levels were measured using real-time PCR. mRNA was isolated from eggs 6 h post-parasitization (p.p.) until L5 at the late pupal cell formation stage, i.e. immediately before egression of the parasitoid larva. To find out whether the presence of a parasitoid influences viral transcript levels, X-ray

parasitized hosts, where no parasitoid develops, were also analyzed (eggs 6 h p.p. until L6 at the late pupal cell formation stage). Relative transcript levels of two early expressed viral genes are shown in Fig. 1. Levels of 14.5g1 increased rapidly after parasitization, reached highest levels in eggs 24 h (parasitized) or 48 h (X-ray parasitized) p.p. and dropped to very low levels after L2. 21g1 transcripts also increased shortly after parasitization but were most abundant in L1 in both parasitized and X-ray parasitized hosts; the decrease was then more rapid in X-ray parasitized larvae. 14g3 (Fig. 2) displayed a peak in transcript levels in parasitized and X-ray parasitized eggs 48 h p.p. and increased again in the final phase of parasitization and X-ray parasitization. Transcripts of 12g1 (Fig. 2) were most abundant in X-ray parasitized eggs 48 h p.p.; they were less abundant in early larval instars and increased in the early pupal cell formation stage in X-ray parasitized hosts. In parasitized hosts, 12g1 levels were less variable. 12g2 offers an entirely different picture as it was upregulated only in the final phase of parasitization and to a much higher extent in X-ray parasitized hosts (Fig. 3). Still another pattern was seen with 16.8g1: transcripts were present from the egg stage up to the onset of metamorphosis and dropped before the developmental arrest (Fig. 4). For this gene, transcript quantities were higher in parasitized than in X-ray parasitized larvae in the penultimate and early last stadium.

An overview of the variation in transcripts in the course of parasitization is given in Fig. 5 for all eight CiV genes identified up to now. Transcript levels are presented relative to the maximum for each gene, and genes are grouped per segment. For two genes (12g1 and 16.8g1) transcripts were seen throughout parasitization but maximal levels were seen at different stages (Fig. 5a,h). Transcripts of three genes appeared only in the last instar, with peaks in the digging stage for 14g1 and 14g2 (Fig. 5c,d) and in the late cell formation stage for 12g2 (Fig. 5b). Two genes were only expressed in the beginning of parasitization, 14.5g1 having highest transcript levels in the egg 24 h p.p. and 21g1 in L1 (Fig. 5f,g). 14g3 is particular with a short peak of transcripts in eggs 48 h p.p. and an increase in the last instar. It was then attempted to estimate the quantity of the various viral transcripts. For each gene, actin levels of particular larval stages were measured on the same plate, namely from X-ray parasitized L6 at early cell formation for 12g1, 12g2 and 14g3, from parasitized L5 feeding for 14g1 and 14g2, from X-ray parasitized L1 for 14.5g1 and 16.8g1 and from parasitized L1 for 21g1. With this value, the ratio of actin to the maximum of each gene in parasitized hosts was calculated. Amplicons are between 50 and 56 bp and are thus unlikely to form higher secondary structures influencing fluorescence properties. Furthermore, melting curves showed no unspecific amplicons. Thus, differences in fluorescence of amplicons and specificities of primers seem to be neglectable. Defining the value of 12g1 in L1 as 100%, the following approximate values were then calculated for the various genes in their maximum: 100% for 14g1, 500% for 12g2 and 14g2, 10% for 14.5g1, 21g1 and 16.8g1, and 0.01% for 14g3. Thus, the late expressed genes reach much higher levels of transcripts than those expressed in the early phase of parasitism. The quantity of the most abundant transcripts (12g2 and 14g2) in their maximum was also calculated relative to actin and was found to be approx. 50 times lower than actin.

### 3.2. Viral transcripts in host and parasitoid

To investigate whether viral transcripts are also found in parasitoid larvae, transcript levels were measured in L2 parasitoids and their L5 hosts. In addition, external L3 parasitoids and male and female pupae were analyzed; in females, a distinction was made between young and old pupae, i.e., respectively, before and after excision of viral DNA (Gruber et al., 1996; Wyder et al., 2002). The data show that for 12g2 and 14g2, i.e. genes with high expression in the host, no transcripts were seen in the parasitoid larvae and pupae (Fig. 6b,e). For genes with medium transcript levels in the host, like 14g1 and 12g1, some transcripts were detected in the parasitoid whereby levels in the parasitoid larvae were slightly higher for 14g1 than for 12g1 (Fig. 6a,d). For 16.8g1, a gene with low transcript amounts in the host, relatively high levels, compared to the host, were observed in male and old female pupae (Fig. 6f). For 14g3, a gene with very low transcript levels in the host, relatively high quantities were seen in late L2 parasitoids and female old pupae (Fig. 6c). Altogether, transcript levels in the parasitoid were lower or much lower than those in the host. Nevertheless, the data of parasitoid larvae and male and young female pupae suggest transcription from the proviral integrated form of viral DNA.

### 3.3. Distribution of viral transcripts in various host tissues

To investigate the distribution of viral transcripts in various host tissues, RNA was isolated from haemolymph, fat body and nervous tissue of parasitized L5 at the digging stage, and transcript levels of 4 CiV genes were measured with real-time PCR (Fig. 7). The majority of transcripts were always found in haemolymph, but transcripts of 12g1 were almost equally abundant in fat body. Transcripts of 12g2, 14g1 and 14g2 were less abundant in the fat body. The proportion of transcripts in nervous tissue was similar to that in fat body for 12g2 and 14g1, lower for 12g1 and much lower for 14g2. Thus, the tissue distribution of viral transcripts

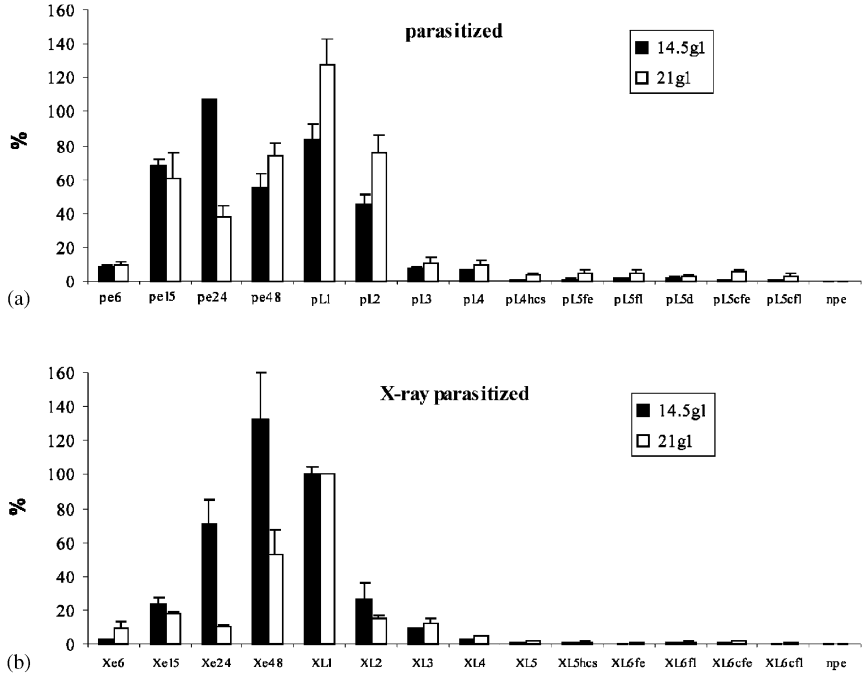


Fig. 1. Relative quantities of 14.5g1 and 21g1 transcripts in parasitized (a) and X-ray parasitized (b) *S. littoralis*. Data are means ± SE of 4–9 measurements per stage. All data are given relative to the reference value of XL1. p: parasitized; X-ray: X-ray parasitized; e6–e48: eggs 6–48 h p.p.; L1–L6: first to sixth larval instar; hcs: head capsule slippage stage; fe: feeding early; fl: feeding late; d: digging; cfe: cell formation early; cfl: cell formation late; npe: nonparasitized eggs.

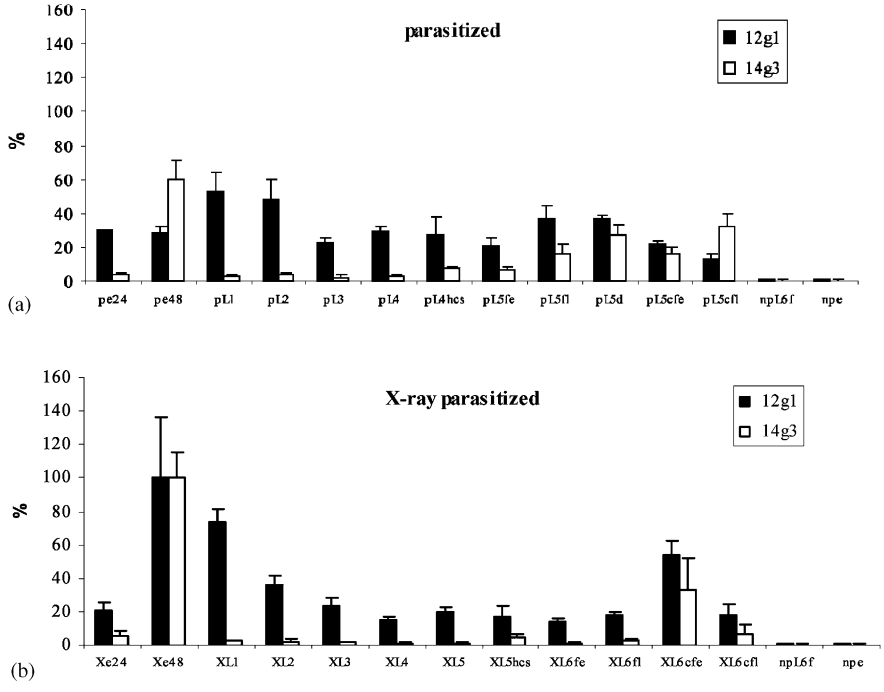


Fig. 2. Relative quantities of 12g1 and 14g3 transcripts in parasitized (a) and X-ray parasitized (b) *S. littoralis*. Data are means ± SE of 4–9 measurements per stage. All data are given relative to the reference value Xe48. npL6f: nonparasitized feeding L6; for all other abbreviations see Fig. 1.

is to some extent gene specific. We also analyzed whether most haemocytes contain transcripts or only a portion of them. Only granular cells and plasmatocytes adhere to glass and thus only these two cell types could be analyzed by in situ hybridization. Figs. 8a and c show that all haemocytes contained transcripts of either 14g1

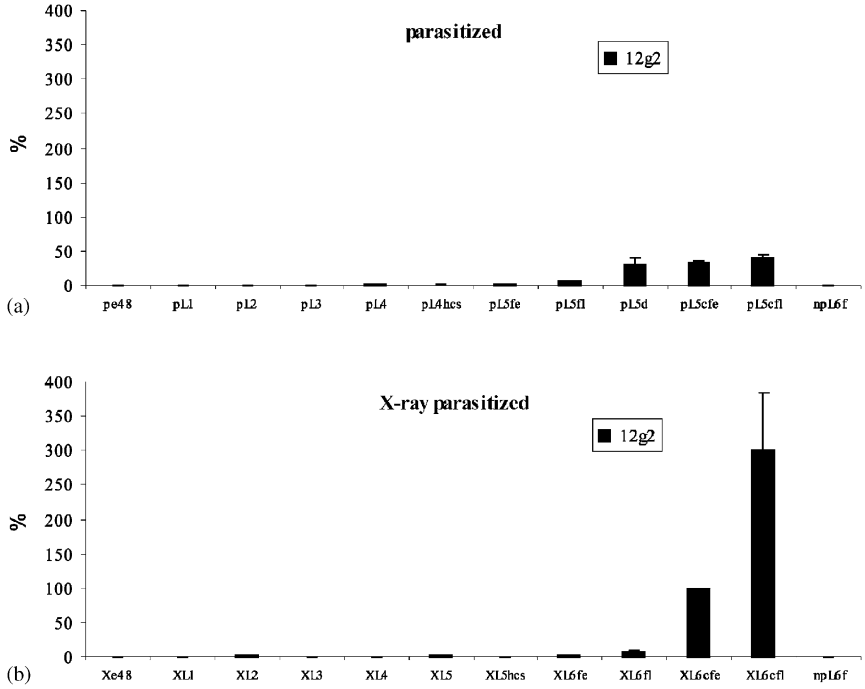


Fig. 3. Relative quantities of 12g2 transcripts in parasitized (a) and X-ray parasitized (b) *S. littoralis*. Data are means ± SE of 4–9 measurements per stage. All data are given relative to the reference value XL6cfe. Abbreviations as in Fig. 1.

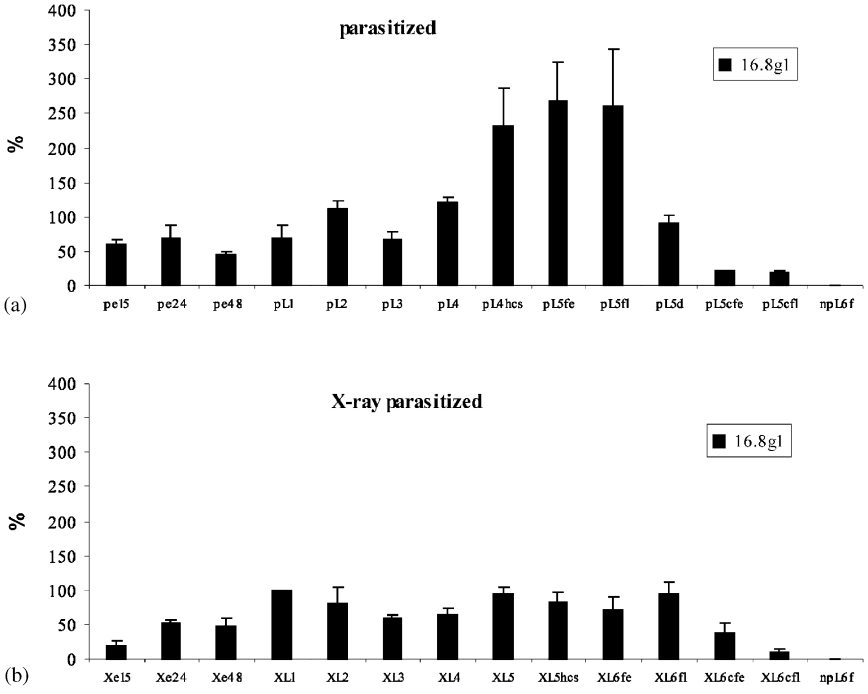


Fig. 4. Relative quantities of 16.8g1 transcripts in parasitized (a) and X-ray parasitized (b) *S. littoralis*. Data are means ± SE of 4–9 measurements per stage. All data are given relative to the reference value XL1: Abbreviations as in Fig. 1.

or 14g2, while no signals were seen with haemocytes of nonparasitized larvae (Fig. 8b,d). The higher magnification of a granular cell and a plasmatocyte (Fig. 8e,f) revealed that transcripts are located in the cytoplasm. The same results were obtained with 12g1 and 12g2 probes (data not shown).

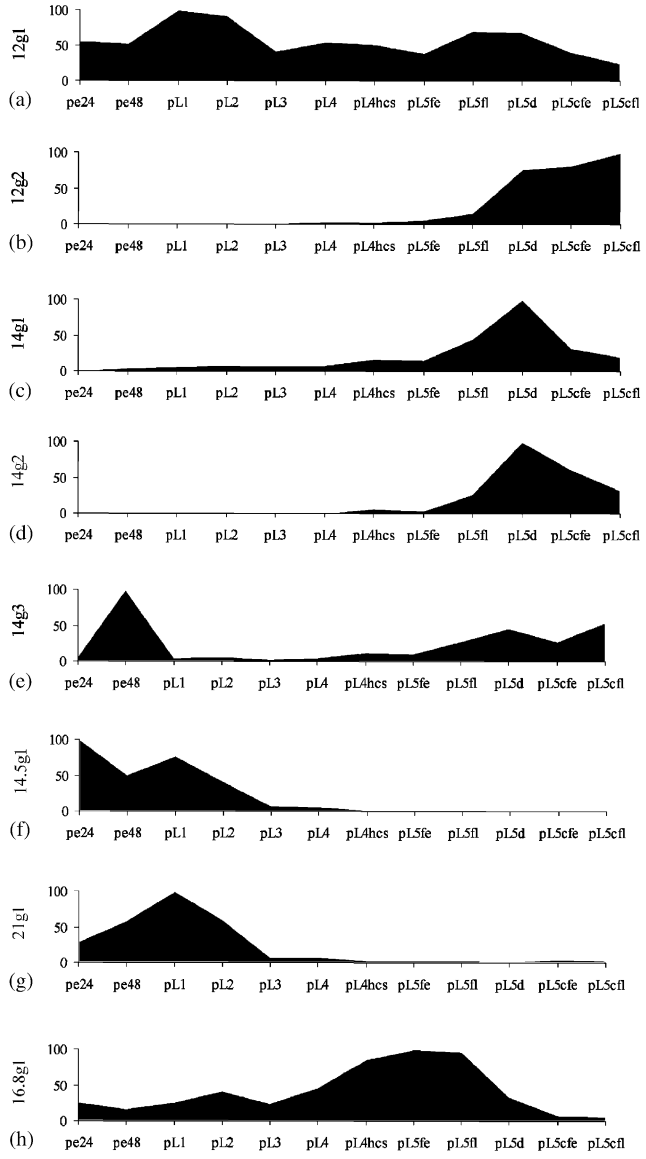


Fig. 5. Overview of relative transcript levels of the 8 CiV genes identified until now throughout development of parasitized *S. littoralis*. For each gene, values are presented relative to the maximal level. Data for 14g1 and 14g2 from L1 to L5fe are from Johner and Lanzrein (2002) and were complemented with egg stages and several last instar stages. For 12g2, 14g1 and 14g2 the stage pe24 was not measured. Abbreviations are as in Fig. 1.

## 4. Discussion

So far, eight CiV genes located on five different fully sequenced segments have been analyzed (Figs. 1–5). On each segment, one to three genes were found for which expression could be documented, but possibly additional genes exist which were not identified yet. As the CiV genome consists of at least 12 segments (Albrecht et al., 1994; Wyder et al., 2002), one can extrapolate the existence of at least 20 to 35 CiV genes, which would mean that we have analyzed up to now around one-third of the CiV genes. Although the data shown here do not allow conclusions on absolute transcript quantities, the relative transcript levels in the course of parasitization and X-ray parasitization revealed the existence of four patterns of expression: early (14.5g1, 21g1), late (12g2, 14g1, 14g2), persistent but variable (12g1, 16.8g1) and early and late (14g3); thus, expression patterns are not segment specific. 14g3, 14.5g1, 16.8g1 and 21g1 have no sequence similarities to other CiV genes, while 12g1 and 12g2 have high similarities to 14g1 and 14g2, respectively (Wyder et al., 2002). Nevertheless, the expression pattern of 12g1 was different from that of 14g1. Up to now, no gene families could be assigned in CiV; for the fully sequenced CsIV, around 35 genes were reported belonging to three gene families (Turnbull and Webb, 2002) and the existence of gene families has been documented also in other polydnaviruses (Kroemer and Webb, 2004). Here we show the first comparative analysis by real-time PCR of the expression of several polydnavirus genes covering the entire period of parasitization, and it is the single one for an egg–larval parasitoid. All other information on expression of viral genes in the host stems from studies with larval parasitoids. In these, high amounts of viral transcripts were observed either in the very beginning of parasitization only (Asgari et al., 1996; Glatz et al., 2003; Le et al., 2003; Yin et al., 2003) or from the beginning up to a few days p.p. (Blissard et al., 1989; Strand et al., 1992; Béliveau et al., 2003; Chen et al., 2003). For several of these early expressed genes, a role in abrogating the host's immune response has been demonstrated (reviewed in Turnbull and Webb, 2002). Thus, CiV is the only polydnavirus analyzed up to now in which some genes are upregulated only at the end of parasitization or have maxima in the initial and final phase of parasitization.

Small amounts of viral transcripts were also found in parasitoid larvae and pupae whereby relative quantities were always lower in the parasitoid than maximal values found in the host (Fig. 6). These observations indicate transcription from the proviral integrated form as in *C. inanitus* excised and circular viral DNA is only seen in ovaries and not in other female or male tissues (Gruber et al., 1996). Small quantities of viral transcripts were already observed in an earlier study in male and female pupae for ORF2 on CiV12 (Johner et al., 1999) what is part of 12g1. Whether the small although variable amounts of viral transcripts found in larval and pupal *C. inanitus* have a biological significance is unclear. It is the first analysis of this type and only for one ichnovirus there was indication for the presence of viral transcripts in old pupae (Fleming et al., 1983).

Transcript levels in the host appear to be largely dependent on the host milieu as they were similar in parasitized and X-ray parasitized hosts from the egg to the fourth stadium followed by comparable changes in

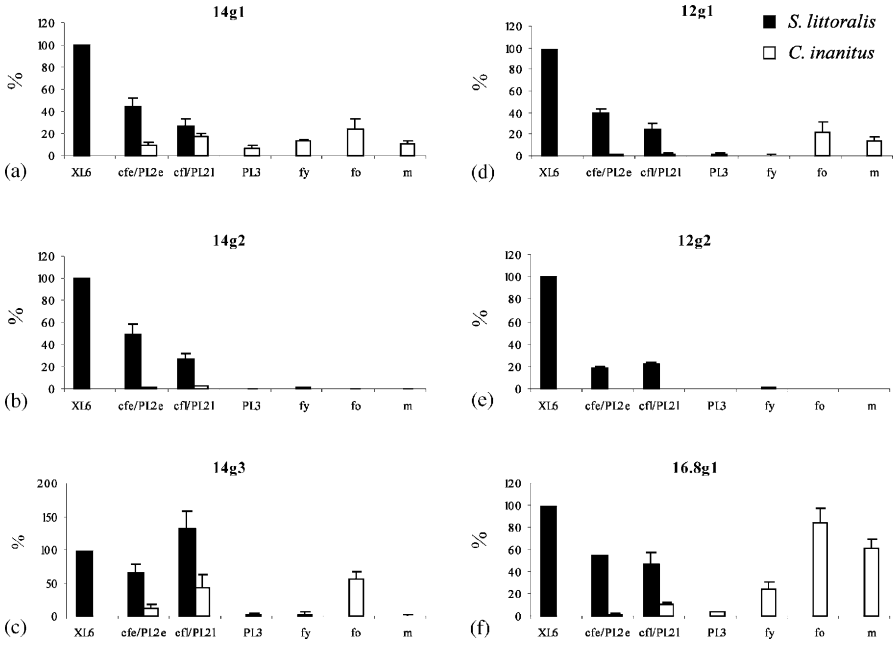


Fig. 6. Relative transcript levels of CiV genes 14g1, 14g2, 14g3, 12g1, 12g2 and 16.8g1 in parasitoid larvae and pupae in relation to the L5 host. Data are means ± SE of 4–9 measurements. Values are given relative to the reference of XL6 (X-ray parasitized larvae at early cell formation stage). cfe/PL2e: cell formation early hosts and their early second instar parasitoid larvae; cfl/PL2l: cell formation late hosts and their late second instar parasitoid larvae; PL3: third instar parasitoid larvae having consumed the host; fy: female young pupae of stages 1–2 according to Albrecht et al. (1994); fo: female old pupae of stages 3b-6 according to Albrecht et al. (1994); m: male pupae of mixed stages.

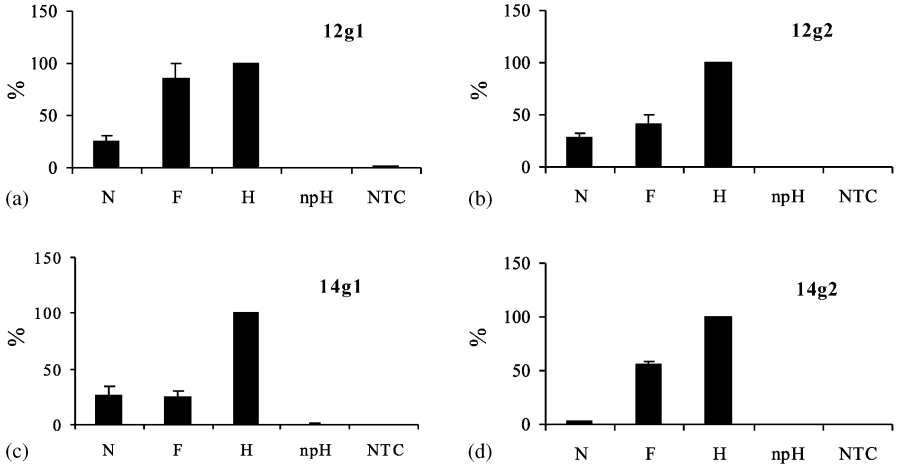


Fig. 7. Relative transcript levels of CiV genes 12g1, 12g2, 14g1 and 14g2 in haemolymph (H), fat body (F) and nervous tissue (N) of L5 parasitized hosts at digging stage. Data are means ± SE of 6–12 measurements and are expressed relative to the value in haemolymph. npH: haemolymph of nonparasitized L6; NTC: non template control.

the last stadium, i.e. the fifth for parasitized larvae and the sixth for X-ray parasitized larvae (Figs. 1–5 and Johner and Lanzrein, 2002). Changes in juvenile hormone and ecdysteroids are very similar in early last instar parasitized and X-ray parasitized larvae (Lanzrein et al., 2001) and might play a role in transcription of viral genes being upregulated after the onset of metamorphosis such as 12g2, 14g1 and 14g2. 12g1 is particular as its transcripts are found throughout parasitization with maxima in the early and late phases of parasitization (Fig. 2). In an earlier investigation, calyx fluid/venom had been injected into fifth instar nonparasitized *S. littoralis* and 24 h later transcripts corresponding to 12g1 were found (Johner et al., 1999). This shows that transcription of this gene was initiated in a stage when it is normally transcribed even if the virus entered the host at a nonphysiological stage. The presence of a parasitoid embryo or larva seems to

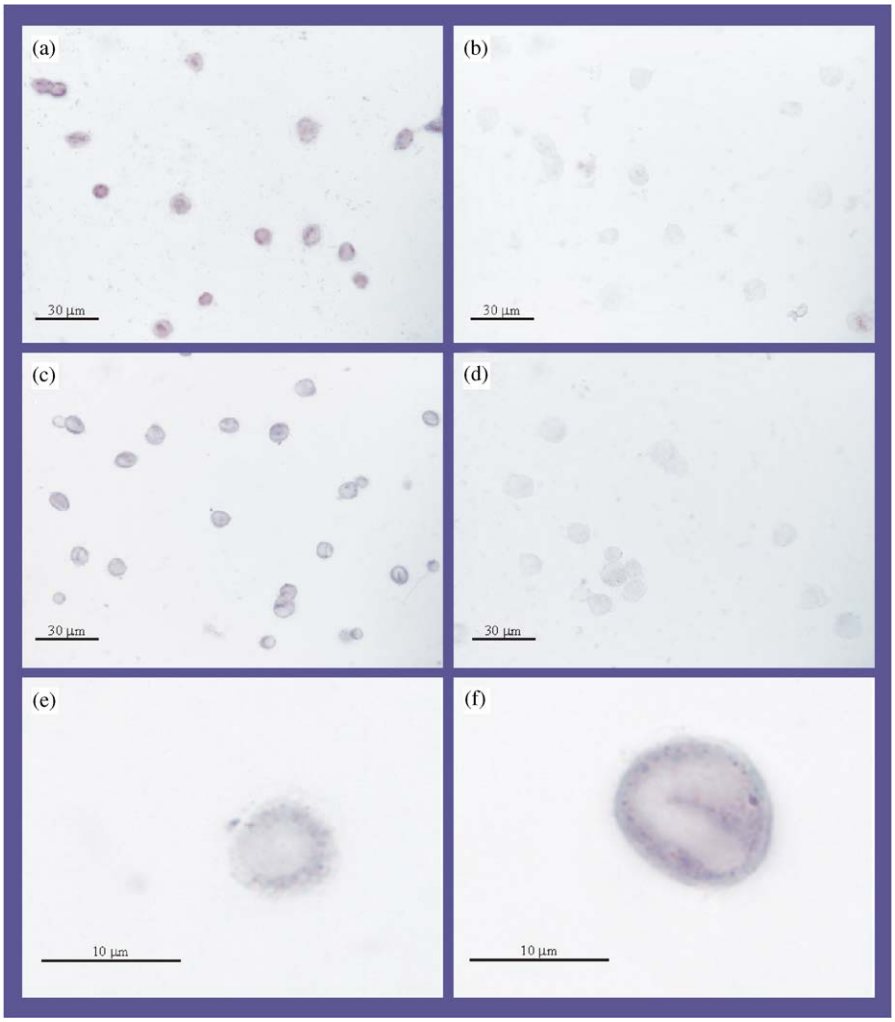


Fig. 8. Analysis of 14g1 (a,b) and 14g2 (c,d,e,f) transcripts by in situ hybridization in haemocytes of parasitized L5 (a,c,e,f) and nonparasitized L6 (b,d = negative control). At the lower magnification, plasmatocytes and granular cells cannot be distinguished but all cells contain transcripts. The higher magnification shows a granular cell (e) with a roundish nucleus and a plasmatocyte (f) with the typical lobulated nucleus.

modulate the quantity of viral transcripts to some extent as can be seen from the comparative analysis of parasitized and X-ray parasitized larvae (Figs. 1–4). 14.5g1 and 21g1 transcripts increased earlier in parasitized than in X-ray parasitized eggs, and 12g1 variations were less pronounced in the course of development in parasitized larvae. 16.8g1 was higher in penultimate and early last instar parasitized larvae, and 12g2 reached much lower levels in parasitized hosts. Differences in transcript levels between parasitized larvae and larvae containing virus alone have been analyzed in only few other cases. After injection of *Cotesia congregata* bracovirus into *Manduca sexta* larvae, EP1 expression was observed (Harwood et al., 1994) but no transcripts of the CrV1 homolog were found (Le et al., 2003). In hosts of the ichneumonid *Tranosema rostrale*, transcript quantities of TrV4 were higher but then disappeared more rapidly after injection of virus than after parasitization (Béliveau et al., 2003). Thus, transcript quantities of some polydnaviral genes appear to be influenced by the presence of parasitoid-associated factors.

Highest levels of viral transcripts were seen in haemocytes (Fig. 7), as also observed in several other systems (Strand, 1994; Teramato and Tanaka, 2003; Falabella et al., 2003; Chen et al., 2003). Dependent on the gene, comparable or somewhat lower values were seen in the fat body and still lower values in nervous tissue (Fig. 7). A similar distribution was seen for CiV DNA with haemocytes and fat body containing each 30–40% and nervous tissue approx. 10% of the total viral DNA load (Wyder et al., 2003). All plasmatocytes and granular cells, i.e. the two haemocyte types known to be involved in capsule formation (Lavine and Strand, 2002), contained transcripts of the four late expressed genes analyzed (Fig. 8 and data not shown). However, haemocyte ultrastructure, spreading and general encapsulation activity are not affected by CiV at this stage (Stettler et al., 1998). Thus, the presence of transcripts in haemocytes is not necessarily linked with a role in

preventing encapsulation of the parasitoid. Furthermore, transcripts of the same genes were also seen in fat body and nervous tissue (Fig. 7). The late expressed genes might rather play a role in inducing the CiV-dependent developmental arrest in the prepupal stage, as work in progress with RNAi suggests. No functional role can be assigned yet to the other viral genes described here; they have no similarity to known genes and it is hoped that RNAi approaches will help to elucidate their role in parasitization.

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