

# Cloning, characterization and analysis by RNA interference of various genes of the *Chelonus inanitus* polydnavirus

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Successful parasitism of some endoparasitic wasps depends on an obligately symbiotic association with polydnaviruses. These unique viruses have a segmented genome consisting of circles of double-stranded (ds) DNA and do not replicate in the parasitized host. They are produced in the wasp's ovary and injected into the host along with the egg. *Chelonus inanitus* is an egg–larval parasitoid; its polydnavirus (CiV) has been shown to protect the parasitoid larva from the host's immune system and to induce developmental arrest in the prepupal stage. The genome of CiV consists of at least 10–12 segments and five have been sequenced up to now. Here, the complete (CiV12g2) or partial (CiV12g1, CiV16.8g1) cloning of three new CiV genes is reported. All three occur only on one viral segment and have no similarity to other known polydnavirus genes, with the exception of a high similarity of CiV12g1 to CiV14g1 and CiV12g2 to CiV14g2. Furthermore, the first attempt of *in vivo* application of RNA interference to study the function of polydnavirus genes is shown. Injection of dsRNA of two late- and one early- and late-expressed CiV genes into CiV/venom-containing host eggs partially rescued last-instar larvae from developmental arrest. Injection of the same dsRNAs into parasitized eggs partially reduced parasitoid survival, mainly by preventing the successful emergence of the parasitoid from the host. These viral genes thus seem to be involved in inducing developmental arrest and in keeping the cuticle soft, which appears to be necessary for parasitoid emergence and host feeding.

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## INTRODUCTION

Polydnaviruses are a unique type of virus that is found in obligately symbiotic association with endoparasitic wasps in the families Braconidae and Ichneumonidae, as the genera *Bracovirus* and *Ichnovirus*, respectively (reviewed by Turnbull & Webb, 2002; Kroemer & Webb, 2004). It is assumed that the two genera evolved independently (Whitfield & Asgari, 2003), but their life cycles and genome organizations are similar. Polydnaviruses have a segmented genome of double-stranded (ds) circular DNA; they replicate from a proviral integrated form in the calyx cells of the wasp's ovary and are injected along with the parasitoid egg(s) into the host. In the parasitized host, they do not replicate, but expression of viral genes has been documented in several systems (reviewed by Turnbull & Webb, 2002). They play an essential role in protecting the parasitoid from the host's immune system (Schmidt *et al.*, 2001) and they also often manipulate host development by inhibiting pupation (reviewed by Lawrence & Lanzrein, 1993; Turnbull & Webb, 2002). Sequence analyses revealed that

the genomes of these viral mutualists are largely non-coding, but maintain genes and gene families that are unrelated to other viral genes (reviewed by Kroemer & Webb, 2004). In ichnoviruses, segment nesting has been observed: nested segments exist as single-copy proviral segments, but hybridize to multiple episomal DNA segments with viral genomic DNA (reviewed by Turnbull & Webb, 2002). The majority of information has been obtained with polydnaviruses of larval parasitoids, where the wasp oviposits into the larval stages of the host. In these, the host's immune system has to be compromised immediately after parasitization to prevent encapsulation of the parasitoid egg. In several hosts of larval parasitoids, viral genes were seen to be expressed from the beginning of parasitization onwards or only in the beginning and, for some of them, a role in abrogating the host's immune response has been documented (reviewed by Kroemer & Webb, 2004). Much less information is available on viral genes that are putatively involved in developmental alterations of the host.

We are studying the bracovirus of the egg–larval parasitoid *Chelonus inanitus* (CiV) and its role in parasitism of one of its natural hosts, *Spodoptera littoralis*. The genome of CiV

The GenBank/EMBL/DDBJ accession numbers for the sequences described in this paper are AJ278677, Z58828 and Z31378.

consists of at least 10–12 segments with sizes between 7 and 31 kb, which appear to be singly encapsidated (Albrecht *et al.*, 1994). Five segments (CiV12, CiV14, CiV14.5, CiV16.8 and CiV21) have been fully sequenced up to now (Wyder *et al.*, 2002; GenBank accession no. AJ627175) and all of them appear to contain at least one gene. The wasp oviposits into the egg stage of the host and it was found that viral DNA then enters the cells of the host embryo within the first day after parasitization (Wyder *et al.*, 2003). CiV prevents encapsulation of the parasitoid (Pfister-Wilhelm & Lanzrein, 1996; Stettler *et al.*, 1998) and, along with the parasitoid larva, greatly influences development of the host. 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 seen to be responsible for the developmental arrest (Soller & Lanzrein, 1996), whilst for the precocious onset of metamorphosis, the parasitoid larva, in the presence of CiV/venom, has been shown to be responsible (Pfister-Wilhelm & Lanzrein, 1996). Analysis of expression of eight CiV genes in the course of parasitization revealed four patterns, namely early, late, persistent and early and late, whereby highest amounts of transcripts were seen for the late-expressed genes (Bonvin *et al.*, 2004). Here, we describe the partial or complete cloning and analysis of three CiV genes that had been predicted from sequencing segments CiV12 and CiV16.8. Furthermore, we report on the use of RNA interference (RNAi) to analyse the function of late-expressed viral genes in parasitized and in CiV/venom only-containing hosts.

## METHODS

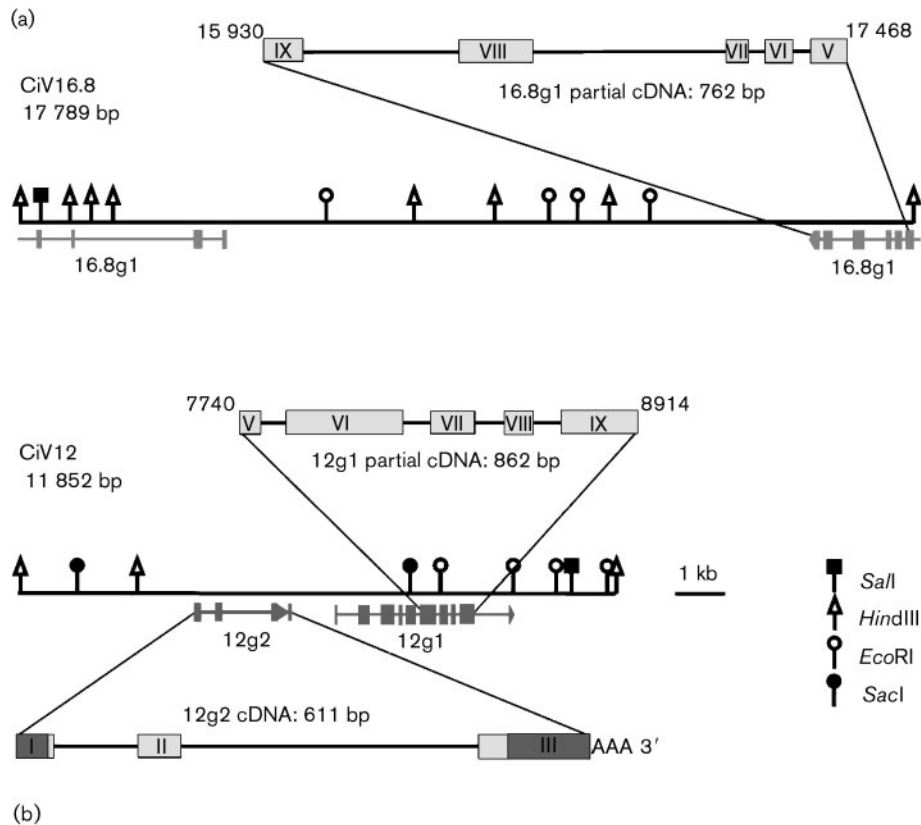
**Insects, parasitization and X-ray irradiation of wasps.** *C. inanitus* (Braconidae) is a solitary egg–larval parasitoid that was reared on one of its natural hosts, *S. littoralis* (Noctuidae). For parasitization, 27–32-h-old eggs were used. The parasitoid larva hatches approximately 16 h later and then remains in the first instar until the host has moulted into its fifth (precocious last) instar. In the second instar, it drinks haemolymph of the host and approximately 2 days later, it moults into the third instar, emerges from the host and eats it up. Details about the biology and rearing of parasitoid and host were given previously (Grossniklaus-Bürgin *et al.*, 1994). To study the effect of CiV/venom in the absence of a developing parasitoid larva, female wasps were irradiated with X-rays (146 Gy  $\pm$  10%) as described previously; this treatment lethally damages parasitoid eggs, but does not affect the polydnvirus (Soller & Lanzrein, 1996). Host eggs and larvae developing from eggs parasitized with X-ray-irradiated wasps are designated 'X-ray-parasitized'. Non-parasitized and X-ray-parasitized larvae pass through six larval instars, but the latter become developmentally arrested in the prepupal stage, as do larvae developing from eggs injected with CiV/venom (Soller & Lanzrein, 1996). X-ray irradiation of wasps (24–96 Gy) has recently also been used in another parasitoid–host system to study the role of the associated bracovirus (Tillinger *et al.*, 2004).

**Screening and cloning.** Two existing cDNA libraries in  $\lambda$ gt11, one from fifth-instar parasitized larvae and the other from sixth-instar X-ray-parasitized larvae at the early cell-formation stage, were used (Johner & Lanzrein, 2002). Production of digoxigenin-labelled probes with primers on predicted exons of 12g2, library screening,

cDNA cloning into pBluescript and sequencing were done as described previously (Johner & Lanzrein, 2002). For partial cloning of 12g1, primers 12g1forw (5'-GAGTCCATGCCGAATGTAC-3') and 12g1rev (5'-CTTCTTGACAGCGACGAAC-3') were set to amplify the middle region of 12g1, as predicted with FGENESH 1.0 and *Drosophila* settings. For partial cloning of 16.8g1, primers 16.8g1forw (5'-CGAAACTTCTTCCAGATCCAG-3') and 16.8g1rev (5'-CCGGACGCCAGTAATAAAG-3') were set to amplify the middle region of 16.8g1, as predicted with FGENESH 1.0 and *Drosophila* settings. For both genes, the reaction was carried out with 250 ng cDNA of X-ray-parasitized first-instar larvae and a *Taq* core kit (Qiagen). Gel-purified PCR products were cloned into a pCR II TOPO vector (Invitrogen) and sequencing was done on an ABI 3000 sequencer (Applied Biosystems).

**Southern and Northern blot analyses.** For Southern blots, calyx fluid was collected and purified as described previously (Albrecht *et al.*, 1994). Undigested and *Hind*III-digested calyx DNA (1–3  $\mu$ g) was separated on a 0.8% agarose gel by field-inversion gel electrophoresis (FIGE), blotted and hybridized as described previously (Wyder *et al.*, 2002). High-stringency washes were done in 0.2  $\times$  SSC [ $1 \times$  SSC is 0.15 M NaCl, 15 mM sodium citrate (pH 7.0)], 0.1% SDS, at 65 °C twice for 15 min. cDNAs of 12g1, 12g2 and 16.8g1 were used as probes after two gel-purification steps and labelling with Ready-to-Go DNA-labelling beads (Amersham Biosciences) in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP. Northern blots were performed as described previously (Johner & Lanzrein, 2002). As a probe, 12g2 cDNA radiolabelled as described above was used. As a control, a fragment of *S. littoralis* actin (GenBank accession no. Z46873) was used.

**Preparation of dsRNA.** To synthesize both sense and antisense RNAs from CiV genes, two clones containing the insert in opposite directions served as template, or a single clone was transcribed with two RNA polymerases. The following plasmids, which were first linearized, were used. On CiV14, cloning of 14g1 and 14g2 has been reported (Johner & Lanzrein, 2002); clone 6.5.1.#16 contains the 5' end of 14g1 cDNA (1555 bp) and was transcribed with either T7 or T3 RNA polymerase. Clones 6.5.1.#17 and  $\lambda$ 7.3#5 contain the full 14g2 cDNA in opposite directions (615 bp). On CiV12, 12g1 was partially and 12g2 fully cloned (Fig. 1). In the case of 12g1, clones 12g1#2 and 12g1#4 contain the middle part of the ORF in opposite directions (862 bp) and, in the case of 12g2, clones 12g2#11 and 12g2Z1T7 contain the insert in opposite directions. T7 RNA polymerase was used with all of these clones. For the partially cloned 16.8g1 (Fig. 1), clone Ro#7 (762 bp) was used with either T7 or SP6 RNA polymerase. RNA strands were synthesized in a 100  $\mu$ l reaction volume, containing 5  $\mu$ g plasmid DNA, 5  $\times$  HEPES/KOH/DDT [ $1 \times$  HEPES/KOH/DDT is 1 M HEPES/KOH (pH 7.5), 0.5 M dithiothreitol], 5 mM each NTP, 100  $\mu$ g BSA ml<sup>-1</sup>, 20 U RNasin and 300 U RNA polymerase. For T3 polymerase, 10  $\times$  transcription buffer (Boehringer Mannheim) was added and for T7 polymerase, 5  $\times$  MgCl<sub>2</sub>/spermidine buffer (1  $\times$  MgCl<sub>2</sub>/spermidine buffer is 1 M MgCl<sub>2</sub>, 1 M spermidine) was used. After 2 h incubation at 37 °C, an additional 300 U polymerase was added and the mix was incubated for another 2 h. Thereafter, 200  $\mu$ l diethyl pyrocarbonate (DEPC) H<sub>2</sub>O was added and the solution was centrifuged briefly. The RNA in the supernatant was purified by acidic phenol extraction and ethanol precipitation in the presence of 100  $\mu$ g glycogen. The pellet was diluted in DEPC H<sub>2</sub>O and the yield was measured in a spectrophotometer. Quality of the transcripts was checked on a denaturing gel. For annealing of the complementary strands, approximately 1  $\mu$ g of each was dissolved in 10 mM Tris and 1 mM EDTA, pH 8, in a 50  $\mu$ l reaction volume and the tube was placed in a PCR machine (Mastercycler gradient; Eppendorf). At first, the temperature was set for 10 min at 95 °C, then it was reduced by 0.5 °C every 2 min until 70 °C was reached, followed by cooling down to room



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TCAGTCTCTTGTGGCACACCAATTCATTTCAGTCTGTGTACTTGGGACCTATTGCACGGTCGTATGTAATTTATCCTGCAGTTTGAG      87
AAAAAATATTC AAC..... 101
ATG GCC AAA AAT GAA AAG GAA GTG AGT GGA CCA AGT GGT AAA GCC AAC ACT ACG AAT AGC AAA AAC      167
M A K N E K E V S G P S G K A N T T N S K N 22
AAT GCC AGT GCT AAG AAG AGT CCT CAG TCT GAA GGA TTG AAT CCT AAC AAT ATG TTC TGG GCT ATA      233
N A S A K K S P Q S E G L N P N N M F W A I 44
AAA TAT AAA GAT GAC ATC CCC GAA AAG CTC GTC ATC CTG CAT TTT GAT GAG TCC ATG ATG AAG GCG      299
K Y K D D I P E K L V I L H F D E S M M K A 66
ATG GAA CTG AAA TCT GAA AAA CTC CAC AAA CAT GTC ACA GGA TAA ..... 344
M E L K S E K L H K H V T G * 80
TTAAAACAACGATTTTTTATAAATTTCTACATCATATTAATTACTTATGTACTCCCAAGGGTAGTGCAGGTAATGATTGAGTGGGT      431
TCCAGTGCCAAAATTAATAATTAGATCTCGTGTGCGAATTAATAGGATCTAATGTTCAAAATACTCTTAAAGTCTTGCAAGTAAGAA      518
TCAGGGTAGTATTTTTTTGATTTTAGTTATTAAGTTGTCAAATTAATATTACATGTAAGGTTTGAATAAAGACAAATTTTAGA      605
TTGTxxAAAAAAAAAAAAAAAAAAAAAAAAA 636

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**Fig. 1.** (a) Cloned cDNAs on viral segments CiV16.8 (GenBank accession no. Z31378) and CiV12 (accession no. Z58828). Exons are represented in boxes and are numbered with Roman numerals. Light-grey regions indicate the predicted ORFs for hypothetical peptides. (b) Nucleotide and predicted amino acid sequence of 12g2. Start and stop codons are shown in bold and italic characters and exon-exon borders are indicated with small arrows. Potential *N*-glycosylation sites are underlined and potential protein kinase C phosphorylation sites are shown in italic. The 'xx' in the 3' UTR upstream of the poly(A) tail indicates two nucleotides that could not be identified (GC, AG or GT).

temperature. The annealed RNA was analysed on a gel (1% agarose/TBE) and was only used for injection when most of the RNA was of the expected length. Before injection, the dsRNA in TE was diluted with DEPC H<sub>2</sub>O. Initially, various concentrations were tested (0.1, 1 and 10  $\mu$ M), but 0.2  $\mu$ M was used most often.

**Injection of dsRNA and evaluation of effects.** *S. littoralis* egg clutches layed on paper were incubated at 20 °C until they were 27–32 h old. Then, they were brushed until only a single layer remained on the paper, disinfected in 1.5% formaldehyde, rinsed in water and offered to three to four normal or X-ray-irradiated females. The degree of parasitization was checked regularly by dissecting some eggs and a clutch was not used until at least one

parasitoid egg was found per host egg. Afterwards, the parasitized egg clutch was divided into three parts, which were fixed with Scotch tape onto a glass slide: one was for dsRNA, one for controls (ssRNA or TE) and one was left non-injected. Approximately 7 nl was injected per egg with a microcapillary and the use of a micro-manipulator and an injection apparatus [for details of injections, see Soller & Lanzrein (1996)]. Eggs and larvae were reared as described above and observed regularly. Rescue effects in X-ray-parasitized larvae were scored with a prepupa point (pp) system developed by Soller & Lanzrein (1996) and effects in parasitized larvae and on parasitoid development were scored with a development-failure point (dfp) system, as described in Table 1 and Fig. 6. For statistical analyses, classes of scoring points were defined and a  $\chi^2$  test was used.

**Table 1.** System for scoring developmental defects of parasitoid and host

Categories II–V refer to lethal phenotypes, whereby parasitoids died at increasingly early stages.

Category	Stage of parasitoid/host	Score (dfp)	Characteristics
I	Wasp imago	0	Non-affected adult phenotype
II	Adult or pupal wasp (in a cocoon)	1*	Fully developed wasp, but not eclosed (hind gut still attached to the meconium)
		2*	Intermediate pupal stages (3–5)† or adult wasp with some abnormalities (atrophy of the wings, head etc.)
		3*	Pupal stage 1† with achieved cocoon spinning
III	Naked parasitoid pupa	4*	Pupal stage 1† (or larval–pupal intermediate) with unsuccessful cocoon spinning, eyes slightly pigmented
		5	Waist visible, meconium excreted
		6*	Oval shape, eyes barely visible, mandibles brownish
IV	Naked parasitoid PL3 larva Host larva (fifth instar, cfl‡ stage) with internal L2 parasitoid	7*	Parasitoid larva partially emerging from the host, head sticking out
		8	Parasitoid forming a bulge under the host cuticula
		9*	Strongly contracted host, parasitoid not visible
		10	Slightly contracted host
V	Stationary host prepupa	11*	L6 mini prepupa§, body length between 6 and 14 mm, head capsule width 1.9–2.3 mm
		12*	L6 prepupa (15 mm) scoring 12 pp
		13*	L6 prepupa scoring 9 pp or less

\*A picture is shown in Fig. 6.

†For assignment of pupal stages, see Albrecht *et al.* (1994).

‡cfl, Cell formation late.

§For a description of L6 mini phenotypes, see Pfister-Wilhelm & Lanzrein (1996).

||For a classification of X-ray phenotypes, see Soller & Lanzrein (1996).

## RESULTS

### Cloning and analysis of CiV genes 16.8g1, 12g1 and 12g2

Firstly, the two cDNA libraries of parasitized fifth-instar and X-ray-parasitized sixth-instar larvae at early cell-formation stage were analysed for the presence of 16.8g1, 12g1 and 12g2 with primers on predicted exons (Wyder *et al.*, 2002). A product of the expected size was found only for 12g2 with the library from X-ray-parasitized larvae. Thus, partial cloning was attempted for 16.8g1 and 12g1. Partial 16.8g1 and partial 12g1 were RT-PCR-amplified with primers on predicted exons (Wyder *et al.*, 2002) from RNA of X-ray-parasitized first-instar larvae, a stage when these genes are expressed (Bonvin *et al.*, 2004). For 16.8g1, two bands were visible on an agarose gel and were cloned into the TOPO vector. Sequencing of the inserts and comparison with genomic CiV16.8 revealed that both localized on CiV16.8 and that they were identical, with the exception of an additional exon of 244 bp in the long product. Interestingly, skipping of the alternatively spliced exon in the short splice variant leads to a frameshift that results in a premature stop codon downstream. The relative amounts of the two splice variants were determined with RT-PCR, using primers spanning the skipped exon: the long splice variant was seen to be much more abundant (data not

shown) and, thus, only the long variant is presented in Fig. 1(a). The cDNA sequence of partial 16.8g1 was very similar to the prediction by FGENESH 1.0 on the genomic sequence: seven out of eight splice sites were predicted correctly. The predicted entire 16.8g1 has ten exons, encoding a 447 aa protein (GenBank accession no. Z31378). Unfortunately, several attempts to clone the 3' end by using rapid amplification of cDNA ends failed. Database searches revealed no similarity to known genes.

Sequencing of 12g1, comparison with genomic CiV12 and predictions with FGENESH revealed that all four splice sites were predicted correctly, again illustrating the high accuracy of FGENESH 1.0. The sequence of the partial cDNA thus reflects the predicted ORF1 on CiV12 and encompasses positions 7740–8914 on the genomic clone (Fig. 1; GenBank accession no. Z58828). Only at position 630 of the cDNA (position 8682 of the genomic clone), we found an A, whereas genomic clone 1G10 has a T. This mutation possibly occurred in the cloning process. Database searches revealed that 12g1 has high similarity to 14g1, but not to other known proteins.

12g2 is expressed very late in parasitization (Bonvin *et al.*, 2004) and this gene could be cloned from the cDNA library of last-instar X-ray-parasitized larvae (Fig. 1a, b). The two sequenced clones were identical, with the exception of the

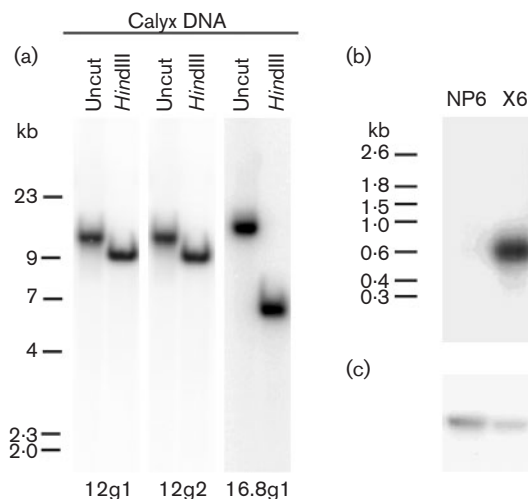
5' untranslated region (UTR), where one clone was 2 bp longer (611 and 609 bp). Comparison with the genomic clone CiV12 and the FGENESH predictions revealed that all three exons and both introns had been predicted correctly. A polyadenylation signal (AATAAA) was found 19 nt upstream of the poly(A) tail (Fig. 1b). 12g2 cDNA contains one putative ORF of 243 bp, encoding a 80 aa putative peptide. Two stop codons were found in-frame in the 5' UTR, indicating that the 5' end is complete. The hypothetical peptide contains two potential *N*-glycosylation sites and three potential protein kinase C phosphorylation sites (Fig. 2b). The peptide is predicted to be localized in the nucleus. No significant similarity to known proteins was found, with the exception of CiV14g2 putative protein (Johner & Lanzrein, 2002), to which 57 % identity and 76 % similarity were found.

To investigate whether 12g1, 12g2 and 16.8g1 occur only on their respective segments and whether closely related genes exist on other segments, Southern blot analysis was carried out. Either undigested or *Hind*III-digested calyx DNA was separated by FIGE and cDNAs of the three genes were used as probes. Fig. 2(a) shows that all three genes are only present on their respective segments; also, the *Hind*III fragments were all of the expected size. To analyse whether the 12g2 cDNA was full-length, a Northern blot was made with RNA from X-ray-parasitized sixth-instar larvae at the pupal cell-formation stage, when

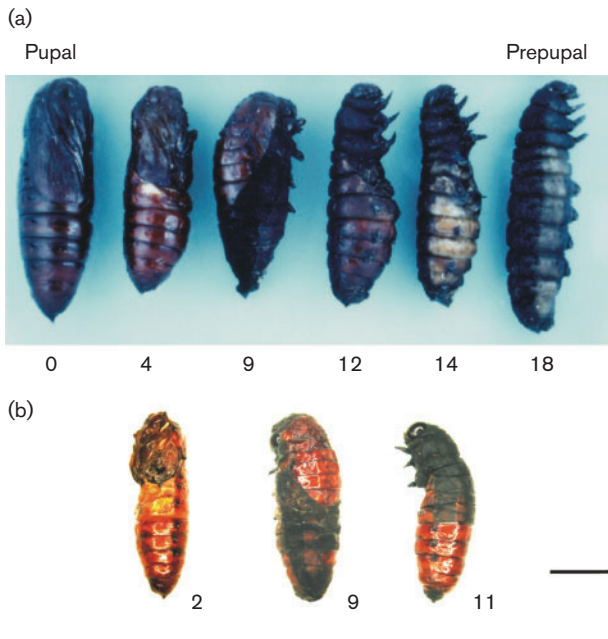
transcript quantities are high (Bonvin *et al.*, 2004). Non-parasitized sixth-instar larvae served as a negative control. Fig. 2(b) shows a strong signal at approximately 600 nt, indicating that the 12g2 cDNA is full-length. Rehybridization of the stripped membrane with a fragment of *S. littoralis* actin (Fig. 2c) indicated that comparable amounts of RNA were present in both lanes.

### RNAi with X-ray-parasitized *S. littoralis*

For the CiV genes 14g1 and 14g2, it had been shown that they are upregulated in last-instar X-ray-parasitized larvae when developmental arrest is induced (Johner & Lanzrein, 2002). Here, it was attempted to rescue X-ray-parasitized larvae from developmental arrest by silencing these two genes. *S. littoralis* eggs were parasitized with X-ray-irradiated wasps and thereafter, single-stranded (ss) RNA or TE (controls) or dsRNA of 14g1 and 14g2 alone or in combination was injected; a portion of the eggs was left uninjected. The larvae were observed and, after pupation or developmental arrest, the resulting phenotypes were scored by using the pp system of Soller & Lanzrein (1996). Fig. 3(a) shows pictures of a normal pupa (0 pp) and several arrested larval-pupal intermediates, as observed after injection of calyx fluid/venom; after X-ray parasitization, the majority score 12 pp and about 20 % score 14 pp (Soller & Lanzrein, 1996). Fig. 3(b) shows typical examples of X-ray-parasitized larvae being partially rescued by RNAi with 14g1 and 14g2: a deformed pupa scoring 2 pp or larval-pupal intermediates scoring 9 or 11 pp. Injections of various concentrations from 0.1 to 10  $\mu$ M gave comparable effects and thus results of the scores of all injections and controls are shown in Fig. 4. After control injections or no injection, all larvae became developmentally arrested and scored mainly 12–14 pp (Fig. 4a). When 14g1 dsRNA was injected, a small percentage of the larvae were rescued almost fully (0–4 pp) and approximately 10 % were partially rescued and scored 9 pp (Fig. 4b). These larval-pupal intermediates had larval legs and their abdomen and the ventral part of the body was covered with the partially ecdysed larval cuticle; however, the dorsal part of thorax and head displayed a pupa-like cuticle (Fig. 3b). When 14g2 dsRNA was injected, only very few scored 0–3 pp, but around 30 % were partially rescued and scored 11 pp (Fig. 4c); these larval-pupal intermediates were more pupa-like in the abdomen than X-ray-parasitized larvae (Fig. 3b). Thus, 14g1 and 14g2 dsRNA injection led to slightly different phenotypes. When 14g1 and 14g2 dsRNAs were injected together, a large portion scored 9–11 pp and some scored 1–3 pp (Fig. 4d). For statistical evaluation, three classes of pp scores were defined: 12–17 pp (no rescue effect), 6–11 pp (intermediate rescue effect) and 1–5 pp (strong rescue effect). Normal pupae scoring 0 pp were not included in this presentation, as it is possible that single eggs were not parasitized and these would then erroneously be valued as a strong rescue effect. Prepupae scoring 18 pp were also not included, as they are not the result of X-ray parasitization, but rather of sickness. Statistical analyses by a  $\chi^2$  test



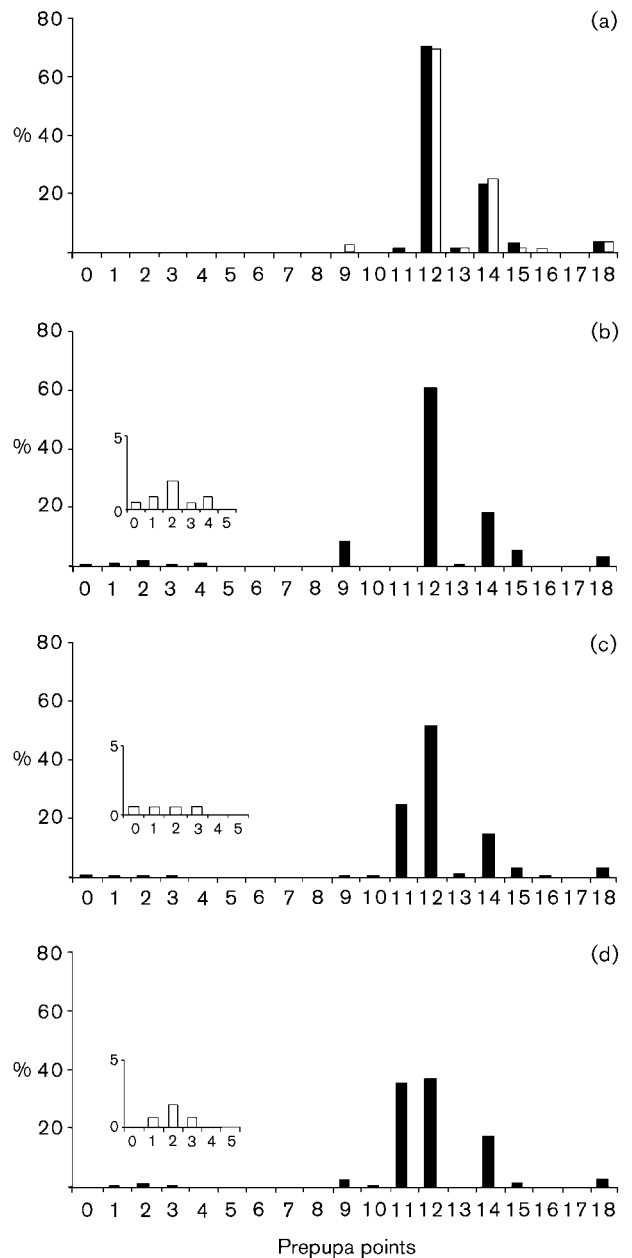
**Fig. 2.** (a) Southern blot. Undigested and *Hind*III-digested calyx DNA (1–3  $\mu$ g) was separated by FIGE. Radiolabelled 12g1, 12g2 and 16.8g1 cDNAs were used as probes. A linear size marker is shown on the left. Exposure time was 6 (12g1, 16.8g1) or 4 (12g2) days. (b) Northern blot. Total RNA (20  $\mu$ g) of sixth-instar X-ray-parasitized larvae at early cell-formation stage or of non-parasitized sixth-instar larvae (negative control) was hybridized with radiolabelled 12g2 cDNA. An RNA size marker is shown on the left. (c) As a loading control, the membrane was stripped and hybridized with a fragment of *S. littoralis* actin.



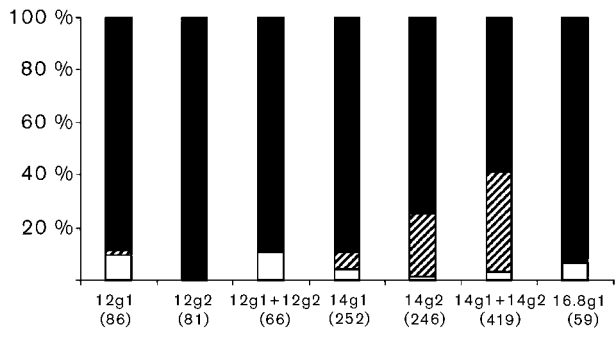
**Fig. 3.** (a) Pupa and typical representatives of developmentally arrested larval-pupal intermediates as seen after CiV/venom injection into *S. littoralis* eggs. Prepupa points (pp, underneath) were determined according to the scoring system described by Soller & Lanzrein (1996). X-ray-parasitized larvae scored mostly 12 and, to a lesser extent, 14 pp. (b) Examples of developmentally arrested larval-pupal intermediates observed after injection of dsRNA (14g1 and 14g2) into X-ray-parasitized *S. littoralis* eggs. Bar, 5 mm.

revealed that the effect of dsRNA injection was significant for both genes (for  $\chi^2$  and  $P$  values, see legend to Fig. 4). These data suggest that, to some extent, it is possible to silence polydnavirus genes *in vivo* and that 14g1 and 14g2 play a role in inducing developmental arrest in the prepupal stage.

CiV genes 12g1 and 12g2 alone and in combination, as well as 16.8g1, were analysed in the same manner in X-ray-parasitized larvae. 12g1 and 12g2 have sequence similarity to 14g1 and 14g2, respectively (Wyder *et al.*, 2002), but the expression pattern is somewhat different for 12g1, as it is expressed early and late, whereas 14g1 is expressed only late in both parasitized and X-ray-parasitized hosts; 16.8g1, on the other hand, is expressed throughout parasitization and X-ray parasitization (Bonvin *et al.*, 2004). The effect of dsRNA injection of these genes is shown for the three classes of effects in Fig. 5, along with the corresponding results of 14g1 and 14g2; the values obtained with the control injections were subtracted for each class. Thus, effects of dsRNA are directly comparable between the various genes. Fig. 5 illustrates only weak and statistically not significant effects for 12g1 ( $\chi^2=1.42$ ,  $P=0.231$ ), for 12g1 together with 12g2 ( $\chi^2=1.2$ ,  $P=0.273$ ) and 16.8g1 ( $\chi^2=0.183$ ,  $P=0.669$ ) and no effect for 12g2.



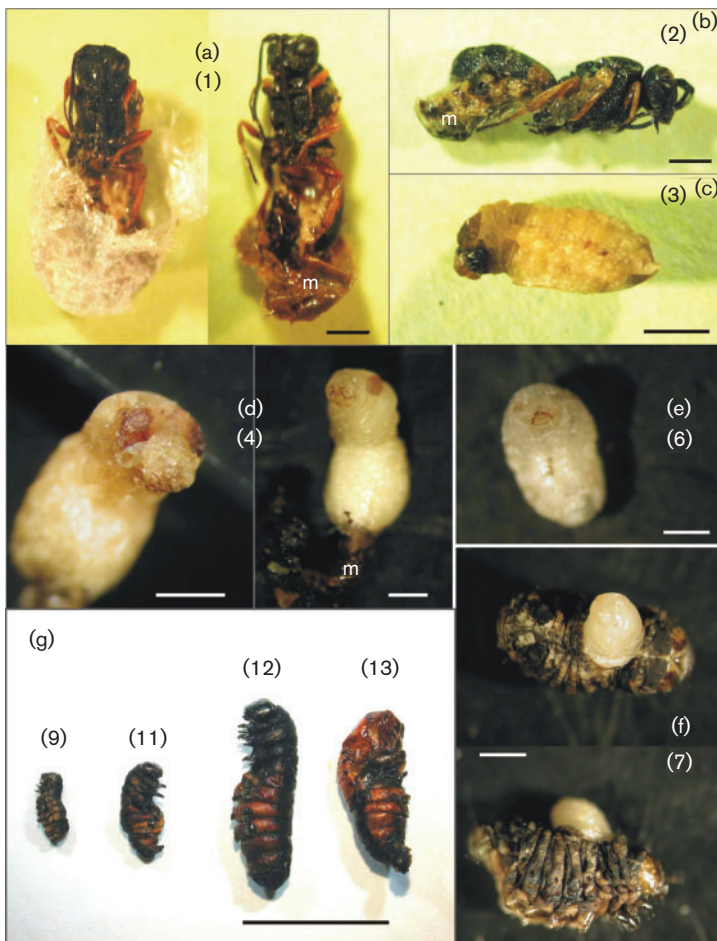
**Fig. 4.** Effects of injection of dsRNA on development of X-ray-parasitized larvae. (a) Control injections (ssRNA or TE; filled bars,  $n=271$ ) and no injection (empty bars,  $n=98$ ). (b, c, d) Injection of (b) 14g1 ( $n=261$ ), (c) 14g2 ( $n=254$ ) or (d) 14g1 together with 14g2 ( $n=430$ ) dsRNA into X-ray-parasitized eggs. Prepupa points (pp) were determined according to Soller & Lanzrein (1996). The inset shows the 0–5 pp region at higher magnification. For statistical evaluation, pp scores were grouped into three classes (12–17, 6–11 and 1–5 pp) and a  $\chi^2$  test was performed: 14g1,  $\chi^2=6.36$ ,  $P=0.012$ ; 14g2,  $\chi^2=20.18$ ,  $P<0.0001$ ; 14g1+14g2,  $\chi^2=40.65$ ,  $P<0.0001$ .



**Fig. 5.** Effect of injection of dsRNA of CiV genes 12g1 and 12g2 alone and in combination, of 14g1 and 14g2 alone and in combination and of 16.8g1 into X-ray-parasitized eggs. Scores were grouped into three classes (12–17 pp, filled bars; 6–11 pp, hatched bars; 1–5 pp, empty bars) and values obtained with the control injections were subtracted for each class. Numbers of evaluated insects are given in parentheses. A  $\chi^2$  test against the controls gave the following: 12g1,  $\chi^2=1.43$ ,  $P=0.231$ ; 12g1+12g2,  $\chi^2=0.183$ ,  $P=0.273$ ; 16.8g1,  $\chi^2=0.183$ ,  $P=0.669$ . Statistical data for 14g1 and 14g2 were given with Fig. 4.

### RNAi with parasitized *S. littoralis*

The same approach was then used with parasitized eggs that were injected with dsRNA of the same five genes alone or in combination (0.2  $\mu$ M). In parasitized larvae, effects of RNAi with viral genes can potentially become manifest with respect to the development of both the parasitoid and the host. Thus, a scoring system taking into account both parasitoid and host development had to be created. In analogy to the pp system used in the case of X-ray parasitization (Soller & Lanzrein, 1996), a dfp system was established. The various phenotypes that may arise as a consequence of developmental failures are described in Table 1 and Fig. 6. Non-affected adult wasps are in category I and score 0 dfp. In category II, the parasitoid died as a non-eclosed adult (1 dfp) or as a cocoon-enveloped pupa (2–3 dfp). In category III, the parasitoid died as a naked pupa or prepupa (4–5 dfp). In category IV, the parasitoid died as a third-instar larva (6 dfp) or as a second larval-instar larva trying to emerge from the host (7 dfp) or still inside the host (8–10 dfp). In category V, the parasitoid died as a first-instar larva or never hatched. In the case of host prepupae scoring 11 dfp, one can deduce from parasitoid removal and transplantation experiments



**Fig. 6.** Phenotypes obtained by RNAi of CiV genes in parasitized hosts with corresponding scores in dfp. In (a), (b) and (c), wasp pupae were enveloped in a cocoon. The wasp in (a) has no defect except that it did not egress from the cocoon and thus scores 1 dfp; m, meconium. In (b), wings are defective (2 dfp). In (c), pupal development has hardly occurred (3 dfp). The pupa in (d) has red-coloured eyes and ocelli, but did not spin a cocoon (4 dfp), whilst (e) is still larval (6 dfp). In (f), the anterior part of the parasitoid is emerging dorsally from the host, (dorsal view on top, lateral view at bottom) (7 dfp). Stationary host prepupae are shown in (g), from the small fifth-instar larva scoring 9 dfp, to large sixth-instar larvae, similar to X-ray-parasitized prepupae (12 dfp) or even closer to non-parasitized pupae (13 dfp). Bars, 1 mm [(g), 1 cm].

(Pfister-Wilhelm & Lanzrein, 1996) that the parasitoid larva died in the third or fourth instar of the host. The developmentally arrested L6 prepupae scoring 12 dfp are typical after X-ray parasitization, which means that the parasitoid did not hatch or died very early. Individuals scoring 13 dfp represent larval–pupal intermediates with 9 pp, as observed after injection of 14g1 dsRNA into X-ray-parasitized eggs (Fig. 4b).

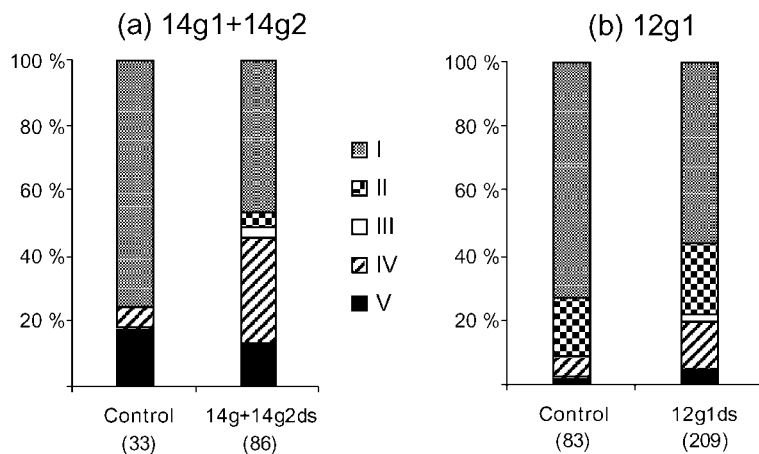
The mean hatching rate of injected eggs was 43 %, but the differences between egg clutches ranged from 10 to 65 %. Also, non-injected eggs can display great mortality and we know from earlier experiments that the manipulation of the eggs (brushing, attaching on glass slide etc.) already reduces the hatching rate of the host (Lanzrein *et al.*, 2001). Parasitoid mortality was also occasionally considerable and varied greatly between egg clutches; from studies on nutritional physiology, we know that the parasitoid larva and pupa are very sensitive to the nutritional status of the host larva (M. Kaeslin, personal communication). Thus, some parasitoids fail to develop fully, even without experimental disturbance. In the dsRNA experiments with parasitized eggs, we thus only evaluated egg clutches in which over 70 % successful parasitoid development was observed. After injection of 14g1 and 14g2 dsRNA together, fewer adult wasps emerged than in controls ( $\chi^2=8.593$ ,  $P=0.003$ ) and the majority were in category IV (Fig. 7); most scored 7–9 dfp, which indicates that the parasitoid did not manage to emerge from the host. Also, separate injection of 14g1 and 14g2 dsRNA increased the number of parasitoid larvae not emerging from the host, but the effect was not statistically significant (data not shown). Injection of 12g1 dsRNA significantly reduced the number of adult wasps ( $\chi^2=7.52$ ,  $P=0.006$ ) and increased the proportion of parasitoids that died before or at emergence from the host (Fig. 7b). With 12g2 dsRNA, no effect was seen and with 16.8g1 dsRNA, the number of adult wasps was only weakly reduced (data not shown). These data show that injection of dsRNA of viral genes into parasitized eggs can reduce the developmental success of the parasitoid; however, this approach is even more difficult than

injection into X-ray-parasitized eggs, as parasitoid larvae are very vulnerable under experimental conditions.

## DISCUSSION

Analysis of the newly cloned CiV genes 12g1, 12g2 and 16.8g1 revealed almost no similarity to already known genes, with the exception of 12g1 and 12g2 having high similarity to 14g1 and 14g2, respectively; this was not surprising, as the entire CiV12 and CiV14 were found to be closely related (Wyder *et al.*, 2002). A signal peptide was not detected for any of the predicted proteins. 14g1 and 12g1 have also some similarity to putative recombinases of *Chironomus pallidivittatus* (GenBank accession nos AJ311054–AJ311057; Rosén *et al.*, 2002), but it is conceivable that they are pseudogenes, as no translation could be demonstrated (J.-E. Edström, personal communication). For 16.8g1, a low-abundance splice variant was observed, in which the shifting of the frame results in a premature termination codon downstream. Such transcripts are apparent targets for nonsense-mediated mRNA decay (NMD) and it has been postulated that coupling of alternative splicing and NMD is a means of regulating protein expression (Lewis *et al.*, 2003). The CiV genes analysed up to now cannot be assigned to any of the described polydnavirus gene families (Kroemer & Webb, 2004), nor can they be grouped into new families. The three CiV genes analysed here localize only to their proper segment (Fig. 2) and we know that these segments are unique (Wyder *et al.*, 2002); in contrast, a large proportion of other polydnavirus genes appear to occur on more than one segment (Chen & Gundersen-Rindal, 2003; Chen *et al.*, 2003; Falabella *et al.*, 2003; Kroemer & Webb, 2004; Malva *et al.*, 2004; J. M. Drezen, personal communication).

Measurement of relative amounts of transcripts showed that 14g1, 14g2 and 12g2 are transcribed only towards the end of parasitization or X-ray parasitization, when the CiV-dependent developmental arrest in the prepupal stage is induced and 12g1 is expressed early and late (Johner & Lanzrein, 2002; Bonvin *et al.*, 2004). Injection of dsRNA of



**Fig. 7.** Effect of injection of dsRNA of 14g1 together with 14g2 (a) and of 12g1 (b) into parasitized eggs as compared to control injections. Categories were assigned according to Table 1 and Fig. 6. Number of insects evaluated is given in parentheses. For evaluation with a  $\chi^2$  test, values of classes II–IV were compared with values of class I between control injection and dsRNA injection. 14g1 + 14g2,  $\chi^2=8.593$ ,  $P=0.0034$ ; 12g1,  $\chi^2=7.52$ ,  $P=0.0061$ .



14g1 and 14g2 alone or in combination into X-ray-parasitized eggs could partially reverse the developmental arrest in a way such that several individuals became more pupa-like; interestingly, dsRNA of 14g1 affected mainly the anterior part and that of 14g2, mainly the posterior part (Figs 3 and 4). These observations indicate a role of these viral genes in preventing pupation of the host. This is the first *in vivo* analysis of polydnavirus gene function by RNAi, but only 10–15 % of the individuals showed a strong and up to 40 % a weak effect. This could be due to poor penetration of host cells, dilution in the course of host growth or degradation of the dsRNA. It has to be kept in mind that these dsRNA molecules must be taken up into cells of the embryo and maintained for over 10 days, possibly in the intermediary form of small interfering RNAs, before any target mRNA is synthesized. Attempts to measure the extent of gene silencing by real-time PCR with reverse-transcribed RNA of potentially affected last-instar larvae were inconclusive until now. With the early- and late-expressed gene 12g1 (Bonvin *et al.*, 2004), the rescue effect after injection of dsRNA into X-ray-parasitized eggs was only very weak, and no effect was seen with 12g2 or 16.8g1 (Fig. 5). RNAi has recently been applied successfully to a lepidopteran cell line to study the role of polydnavirus genes in immunosuppression (Beck & Strand, 2003). With respect to comparable *in vivo* applications of RNAi, an effectiveness of approximately 50 % was observed after injection of dsRNA of a developmental gene into early embryos of a spider (Schoppmeier & Damen, 2001). Injection of dsRNA into syncytial blastoderm-stage embryos was very efficient in *Drosophila*, *Tribolium*, *Bombyx* and *Gryllus* (Brown *et al.*, 1999; Quan *et al.*, 2002; Miyawaki *et al.*, 2004) but, in *Drosophila*, it was not effective in later embryonic stages (Brown *et al.*, 1999). It is thus conceivable that the low and variable effectiveness observed here has to do with the time point of injection and possibly also with the site of deposition of the dsRNA in the egg. Interestingly, injection of dsRNA was effective in adult *Drosophila* (Goto *et al.*, 2003), adult *Anopheles* (Blandin *et al.*, 2004) and planarians (Sánchez Alvarado & Newmark, 1999). Parental RNAi was applied successfully in *Hyalophora* (Bettencourt *et al.*, 2002). It is still unclear how the dsRNA penetrates the cells. Silencing by RNAi of baculovirus genes that are essential for virus propagation was seen to protect *Tenebrio* larvae from infection (Valdes *et al.*, 2003) and it was found that this effect is strongly dose-dependent (Flores-Jasso *et al.*, 2004). Comparison of the expression patterns of the eight CiV genes analysed up to now reveals four different patterns and indicates a stage-dependent regulation (Bonvin *et al.*, 2004). We assume the existence of at least 20–35 CiV genes and it is thus very likely that the CiV-induced developmental arrest results from the combined effect of several known and unknown viral genes. This would mean that dsRNA of all CiV genes involved in developmental arrest would have to be introduced successfully to fully reverse the developmental arrest.

With parasitized eggs, the situation is even more complex.

The experimental procedures cause some mortality and we know that this parasitoid is susceptible to various stress conditions: developmental failures as described in Table 1 can occasionally even occur in the rearing colony. Variability in parasitoid developmental success has also been observed in other systems (Boulétreau, 1986). The combination of limited efficiency of the dsRNA application with a variable background mortality hampered the RNAi approach in parasitized eggs. Nevertheless, the injection of 14g1 and 14g2 dsRNA together or 12g1 alone significantly reduced parasitoid survival; the majority of affected parasitoid larvae could not emerge from the host (Fig. 7). These three late-expressed CiV genes could thus play a role in keeping the host cuticle soft to allow egression of the parasitoid and feeding on the host. In non-parasitized larvae, the cuticle becomes tougher in the stage of pupal cell formation and the delay of this process by virus/venom can be considered the initial phase of the developmental arrest. In this stage, ecdysteroid titres are reduced in both parasitized (R. Pfister-Wilhelm & B. Lanzrein, unpublished data) or in X-ray-parasitized (Grossniklaus-Bürgin *et al.*, 1998) larvae and it is conceivable that these three viral genes act through inhibition of ecdysone production. Induction of host developmental arrest before pupation by polydnaviruses is very common (Lawrence & Lanzrein, 1993) but, in many cases, the parasitoid larva emerges from the host before it approaches pupation. Interestingly, *Campoletis sonorensis* parasitization or ichnovirus injection induces degeneration of the host's prothoracic gland only in the last instar (Dover *et al.*, 1988), but not if parasitization occurred in the penultimate instar; under natural conditions, *C. sonorensis* hardly attacks last-instar larvae (Dover & Vinson, 1990). It thus appears that the developmental arrest comes mainly into effect in the case of parasitoid larvae not having reached maturity when the host approaches pupation. It could thus be considered a means of extending successful parasitoid development and egression. Alternatively, these three viral genes might play a role in parasitoid survival by manipulating the nutritional milieu of the host to the parasitoid's benefit. Work in progress aims at increasing the effectiveness of RNAi *in vivo*, and we are also attempting to study the role of early- and constitutively expressed CiV genes with this method.

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