

# Ovary development and polydnavirus morphogenesis in the parasitic wasp *Chelonus inanitus*. I. Ovary morphogenesis, amplification of viral DNA and ecdysteroid titres

Dorothee Marti, Christa Grossniklaus-Bürgin, Stefan Wyder, Toni Wyler and Beatrice Lanzrein

Institute of Cell Biology, University of Berne, Baltzerstrasse 4, CH-3012 Bern, Switzerland

Correspondence  
Beatrice Lanzrein  
beatrice.lanzrein@izb.unibe.ch

Polydnaviruses are unique symbiotic viruses that are replicated in the calyx cells of the ovary of some parasitic wasps. They have a segmented genome of circular double-stranded DNA and are injected along with the wasp's egg into the host, where they are essential for successful parasitism.

Polydnaviruses replicate from integrated proviral DNA, and after excision of viral segments, flanking DNA is rejoined. Little is known about ovarian morphogenesis, the mode of amplification of the viral DNA and the involvement of ecdysteroids. Here we have analysed these parameters in the course of pupal–adult development in the braconid wasp *Chelonus inanitus*. Immediately after pupation, ovarian cells proliferated and calyx cells began to differentiate; at this stage ecdysteroids, in particular 20-hydroxyecdysone, were highest. Thereafter, calyx cells began to increase in size and DNA content and eventually became gigantic. Amplification of non-viral DNA (actin) and viral DNA in its integrated and excised form and of corresponding rejoined flanking regions was measured by quantitative real-time PCR. In the early phase of calyx cell differentiation, copy numbers of actin and integrated viral DNA increased to a similar extent. This, along with the increase in nuclear volume and DNA content in the absence of extensive cell proliferation, suggested polyploidization of the early stage calyx cells. In the following phase, integrated viral DNA was selectively and intensively amplified and eventually excised and circularized. As copy numbers of excised circular viral DNA and rejoined flanking DNA reached similarly high levels, excised viral DNA appeared not to replicate. After adult eclosion, amplification of viral DNA declined.

Received 16 September 2002

Accepted 20 January 2003

## INTRODUCTION

Many parasitic wasps of the families Braconidae and Ichneumonidae form symbiotic relationships with polydnaviruses (bracoviruses and ichnoviruses), which are injected along with the wasp eggs into the host (Webb *et al.*, 2000). In the host, these viruses prevent encapsulation of the parasitoid eggs and larvae (Lavine & Beckage, 1995; Schmidt *et al.*, 2001) and often also cause a developmental arrest of the host before pupation (Lawrence & Lanzrein, 1993; Soller & Lanzrein, 1996). The polydnaviruses have a unique genome structure consisting of multiple segments of double-stranded circular DNA; proviral DNA is integrated in the wasp's genome and is transmitted vertically through the germ line (Stoltz, 1993). Recent findings with two bracoviruses have indicated that viral segments are clustered in the wasp's genome (Belle *et al.*, 2002; Wyder *et al.*, 2002).

Polydnaviruses replicate from the integrated proviral DNA in particular cells of the wasp ovary, the calyx cells; no replication is seen in the parasitized host (Webb, 1998). The initiation of virus replication has been shown to be associated with a specific phase in pupal–adult development in the ichneumonid *Camponotus sonorensis* (Norton & Vinson, 1983) and the two braconids *Chelonus inanitus* and *Cotesia congregata* (Albrecht *et al.*, 1994; Gruber *et al.*, 1996; Savary *et al.*, 1999; Pasquier-Barre *et al.*, 2002). In *C. sonorensis* it has been proposed, on the basis of ligation and *in vitro* application of 20-hydroxyecdysone (20E), that this hormone may regulate polydnavirus replication (Webb & Summers, 1992). However, no data on ecdysteroid identity and titre changes in pupal–adult development of a polydnavirus-carrying wasp are yet available to support or question this hypothesis. The morphogenesis of the ovary, the various developmental stages of calyx cells and their DNA content, and the process of bracovirus release have not yet been analysed. With respect to replication of viral DNA, it is unclear to what extent it is amplified and whether it is

The EMBL accession numbers of the sequences in this paper are: Z58828, Z58831, Z58832, AJ278676, AJ278677, AJ319653 and AJ439880.

the proviral integrated viral DNA, or the excised viral DNA, or both, that is replicated.

To address these questions we have made an extensive study, presented here and in the accompanying paper (Wyler & Lanzrein, 2003), encompassing histological, ultrastructural, endocrinological and molecular analyses of the process of ovary and calyx cell differentiation and polydnavirus formation. We used the braconid wasp *C. inanitus* and its polydnavirus (CiV) as a model system. The excision sites of two viral segments (CiV12 and CiV14) have been analysed and it has been shown that excision occurs through a site-specific recombinational event and that, after excision, flanking regions are rejoined (Gruber *et al.*, 1996; Wyder *et al.*, 2002). Proviral CiV segments appear to be clustered in the genome of the wasp, as flanking regions of CiV12 and CiV14 have been shown to hybridize to other viral segments (Wyder *et al.*, 2002). The morphogenesis of the ovary in the course of pupal–adult development is strictly correlated with the pigmentation pattern of the pupae and has been subdivided into stages 1–6 (Albrecht *et al.*, 1994). Excision and circularization of viral DNA has been shown to commence at stage 3b (Gruber *et al.*, 1996; Wyder *et al.*, 2002).

In this paper, we have illustrated the morphological and histological changes of the ovary in the course of pupal–adult development and the differentiation of the calyx cells. In the same period of time, we measured the copy number of viral DNA in the integrated and excised form and rejoined flanking DNA by real-time quantitative PCR; as a measure for replication of non-viral DNA, copy numbers of an actin gene were measured. In addition, the fluctuation of various ecdysteroids was measured in the course of pupal–adult development and into adulthood. The data show that ecdysteroids were highest immediately after pupation when ovarian morphogenesis and calyx cell differentiation begin. Subsequently, the nuclei of calyx cells started to increase and the real-time PCR data indicated that calyx cells first become polyploid and that the proviral cluster is then selectively amplified followed by excision of viral segments. The excised circular segments appeared not to replicate. In the accompanying paper (Wyler & Lanzrein, 2003), we have described the DNA content and the ultrastructure of the various developmental stages of calyx cells on their way to becoming virus-producing cells and have related these changes to the molecular data presented here.

## METHODS

**Insects and staging of pupae.** *C. inanitus* (Braconidae, Hymenoptera) is a solitary egg–larval parasitoid, and was reared on its natural host *Spodoptera littoralis* (Noctuidae, Lepidoptera). Adult *S. littoralis* were kindly given to us by Syngenta AG, Stein, Switzerland. Details of the biology and rearing of parasitoid and host are given in Grossniklaus-Bürgin *et al.* (1994). Assignment of stages in pupal–adult development was as described by Albrecht *et al.* (1994).

**Laser confocal microscopy and DNA staining.** Ovaries were fixed in 2% formaldehyde in PBS and rinsed twice in PBS containing

0.5% Tween 80. RNA was digested by the addition of RNase A (3 mg ml<sup>-1</sup>). DNA was stained with propidium iodide (10 µg ml<sup>-1</sup>) in distilled water, rinsed in distilled water and mounted in Glycergel (Dako). Stained ovaries were analysed using a laser confocal microscope Zeiss 310; excitation lasers were set at 488 and 514 nm.

**Histology.** Ovaries were fixed in sodium cacodylate buffer (pH 7.4) containing 2% glutaraldehyde and 0.8% formaldehyde. They were rinsed in sodium cacodylate buffer, dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus-Kulzer). Using glass knives, 2 µm sections were cut and stained using the Feulgen reaction and 1% toluidine blue in distilled water.

**Cloning of *C. inanitus* actin cDNA.** Forward (5'-gtggaagctTCA-CCAAGTGGATGACATG-3') and reverse (5'-gtggaagcttACTCCT-GCTTCGAGATCCAC-3') primers containing *Hind*III sites were designed in highly conserved coding regions of known insect actins with their 3' ends positioned in a non-degenerated codon. One µg of a *C. inanitus* first instar cDNA library in λgt11 was used as a template in a 50 µl PCR reaction with 200 nM of each primer, 100 µM each dNTP and 2.5 units *Taq* polymerase (Qiagen). The reaction proceeded for 5 min at 95 °C, then for 35 cycles of 30 s at 95 °C, 30 s at 54 °C and 1 min at 72 °C, with 10 min termination at 72 °C. A PCR product of approximately 850 bp was gel-purified, digested with *Hind*III and ligated into a *Hind*III-cut pBluescript KS+ vector. Three positive clones were sequenced with the Thermo Sequenase Sequencing kit (Amersham) on a GeneReadIR 4200 apparatus (Licor). Sequence analysis was carried out using BLAST (Altschul *et al.*, 1997). Two clones were identical and were used to design primers for the real-time PCR. Their sequence showed significant similarity to known insect actins (>86% at the mRNA level and 98% at the protein level). The sequence is available under accession no. AJ439880. The third clone was similar to other actin sequences.

**Isolation of ovarian DNA and real-time quantitative PCR.** Female pupae of stages P2, P3a, P3b, P4, P5, P6 and 4-day-old adults were anaesthetized on ice and their abdomina were cut away and placed in a drop of PBS. The ovaries were dissected out and the lower part containing only the calyx region and the oviducts was removed and placed in an Eppendorf tube with 200 µl of buffer (100 mM NaCl, 10 mM Tris/HCl, 25 mM EDTA, 0.5% SDS, pH 8). Ovaries from three females per stage were combined. For each stage, three independent isolations were made. After addition of 10 µl proteinase K (10 mg ml<sup>-1</sup>), samples were incubated for 2 h at 37 °C. The DNA was purified by phenol and chloroform extractions and subsequent ethanol precipitation.

To determine the copy number of CiV12 and CiV14 in the integrated and excised form and the rejoined DNA, quantitative PCR was performed. For integrated CiV12, the primers used were 12RR/12RL, for excised CiV12, primers were 12LL/12RR and for the corresponding rejoined DNA, primers were 12LR/12RL, yielding amplicons of 232, 265 and 267 bp, respectively (see Gruber *et al.*, 1996, and accession nos Z58828, Z58831 and Z58832). For integrated CiV14, primers were 14RL/14RR, for excised CiV14, primers were 14LL/14RR and for the corresponding rejoined DNA, primers were 14LR/14RL, yielding amplicons of 318, 264 and 386 bp, respectively (see Wyder *et al.*, 2002, and accession nos AJ278676, AJ278677 and AJ319653). To analyse amplification of non-viral wasp DNA, an actin gene of *C. inanitus* was used with the actin forward primer 5'-CCTTCAACAGCCCAGCCAT-3' and reverse primer 5'-CACGGACAATTTCTCGCTCA-3', yielding an amplicon of 255 bp. The PCR product obtained with these primers did not span an intron, as the size of the PCR product obtained with genomic DNA was the same as with cDNA.

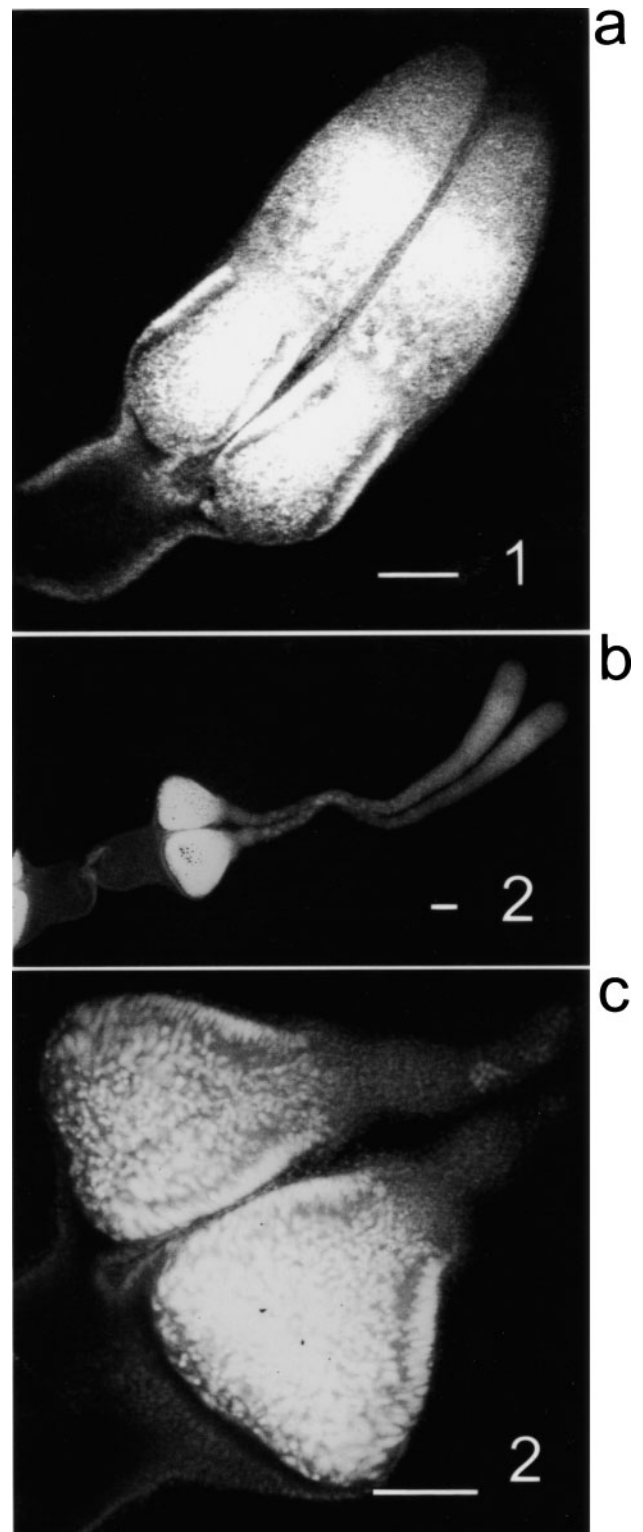
PCR was performed with the Sybr Green I Reaction System (Eurogentec) in 96-well Optical Reaction Plates (Applied Biosystems). On each plate, each reaction was run in duplicate. To

correct for variable primer efficiencies between different runs, a dilution series of P6 DNA was run on each plate as an internal standard. To allow determination of absolute copy numbers of integrated and excised CiV12 and CiV14 and their corresponding rejoined DNA, known amounts of clones were amplified on the same plate. In the case of CiV12, this was clone  $\lambda$ A21 for the integrated form, clone 1G10 for the excised form and clone pCR1 for the rejoined form (for description of clones, see Gruber *et al.*, 1996). In the case of CiV14, this was clone  $\lambda$ 1B231 for the integrated form, a 1300 bp *Hind*III subclone of 2A6 for the excised form and clone CiV14LR/RL for the rejoined form (for description of clones, see Wyder *et al.*, 2002). On each plate, non-template controls were carried out for each primer pair. For each reaction 2  $\mu$ l of an appropriate dilution of template DNA was amplified in a volume of 30  $\mu$ l containing 200  $\mu$ M each dNTP, 3.5 mM MgCl<sub>2</sub>, 0.75 units HotGoldStar DNA polymerase, 200 nM of each primer and 0.9  $\mu$ l of 1:2000-diluted Sybr Green I stock. Cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 1 min and 60 °C for 1 min. PCR analyses were performed with the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). The quality of primers was checked by analysis of melting curves and analysis of the PCR products on a 3.5% MetaPhor (BioWhittaker) agarose gel. PCR data were analysed with the GeneAmp 5700 SDS Software, version 3.1. Each reaction was carried out in duplicate or triplicate with each of the three independent DNA isolations per stage.

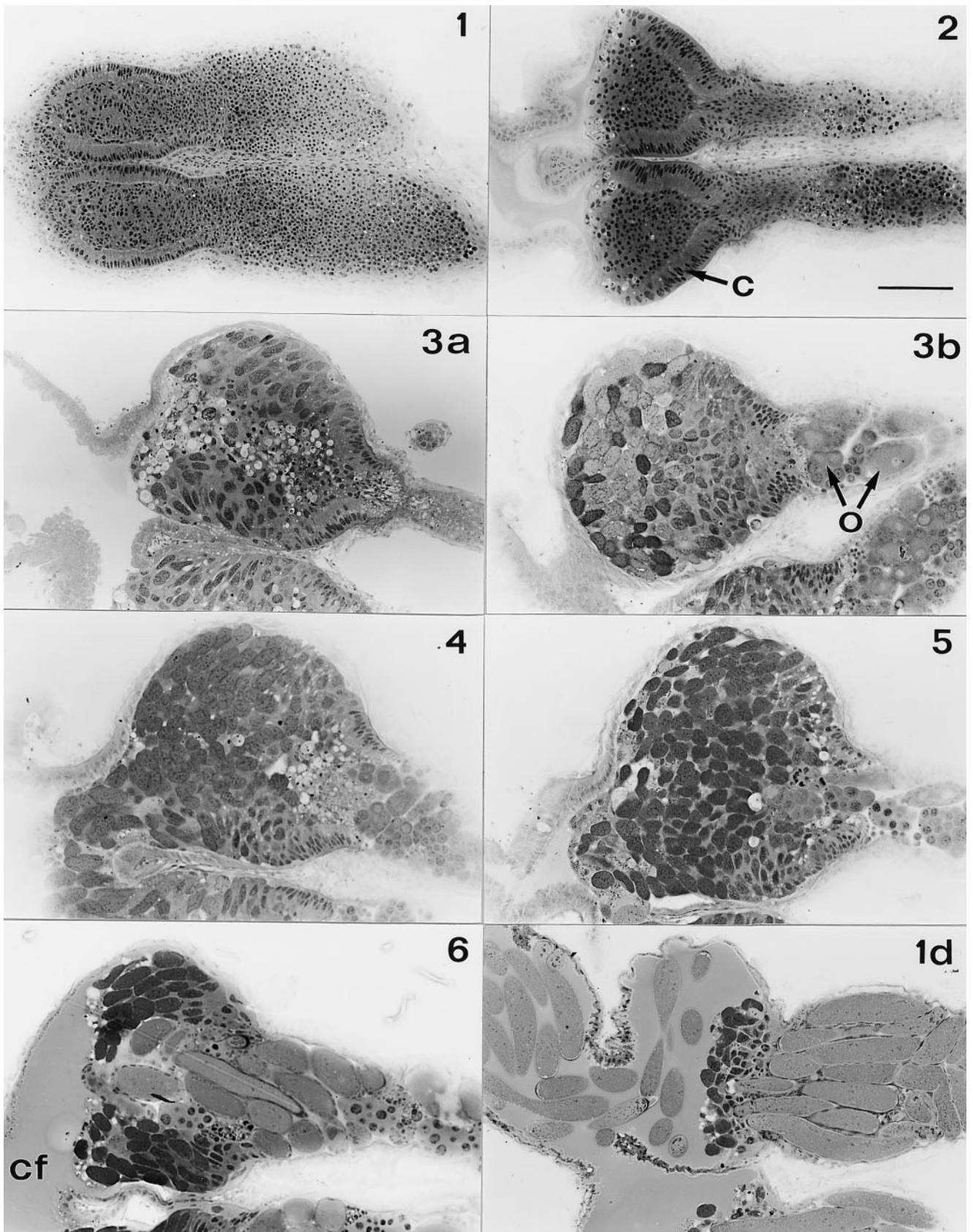
**Ecdysteroid analyses.** Solvents were of pro-analysis grade or high performance liquid chromatography (HPLC) grade and were purchased from Romil Chemicals or Merck. [23,24-<sup>3</sup>H(N)]-ecdysone (specific activity 58.8 Ci mmol<sup>-1</sup>) was purchased from New England Nuclear, and ecdysone (E) and 20-hydroxyecdysone (20E) were from Simes. Other ecdysteroids were kindly provided by R. Lafont (Paris). Standard abbreviations for ecdysteroids are used (Lafont *et al.*, 1993).

The ecdysteroid analyses were in principle carried out as described in Grossniklaus-Bürgin & Lanzrein (1990a). For measurements of Sep-Pak-purified ecdysteroids (see Fig. 6), three to five females were homogenized in 1 ml acetonitrile; for measurement of HPLC-purified ecdysteroids (see Fig. 7), six to ten females were homogenized in 2 ml acetonitrile. After centrifugation the extract was washed once with 1 ml acetonitrile and twice with 1 ml 50% acetonitrile in water. The supernatant was then partitioned against hexane to remove lipids and each phase was washed with an equal volume of the counterphase. The aqueous phase was evaporated in a Speed Vac concentrator and the residue taken up in 5 ml of water. The aqueous extract was then passed through a C18 Sep-Pak (Waters) cartridge and after washing with water, ecdysteroids were either eluted with 5 ml 100% methanol and then separated by HPLC (see below) or else first eluted with 5 ml 25% methanol and subsequently with 60% methanol. Polar ecdysteroids, such as ecdysone (E) and 20-hydroxyecdysone (20E), elute in the 25% methanol fraction, while mid-polar ecdysteroids, such as E, 20E and 20,26-dihydroxyecdysone (20,26E), elute in the 60% methanol fraction (Grossniklaus-Bürgin & Lanzrein, 1990a; Grossniklaus-Bürgin *et al.*, 1998). Aliquots of the 25% and 60% methanol fractions or the HPLC fractions were quantified by radioimmunoassay (RIA) as described previously (Grossniklaus-Bürgin & Lanzrein, 1990a). Two different antibodies were used, one specific for the side chain (H-2) and the other specific for the ecdysone nucleus (H-22); both were generous gifts from L. I. Gilbert. Data were expressed in 20E equivalents or in their corresponding equivalents (Warren & Gilbert, 1986).

Ecdysteroids were separated by ion suppression-reversed phase HPLC on a Nucleosil RP-18 column as described by Grossniklaus-Bürgin *et al.* (1998). For elution, a linear gradient of 8–40% acetonitrile in 20 mM Tris/HClO<sub>4</sub> buffer (pH 7.5) within 45 min at a flow rate of 1 ml min<sup>-1</sup> was used, followed by 100% acetonitrile for 10 min. Fractions were



**Fig. 1.** Propidium iodide-stained ovaries (whole mounts) from pupae at stage 1 (a) and 2 (b, c) as seen by laser confocal microscopy. In (c) the calyx region is shown at higher magnification. Designation of pupal stages is according to Albrecht *et al.* (1994). All pictures show one half of an ovary with the oviduct oriented to the left. Bar, 100  $\mu$ m.



collected every 0.5 min and assayed by RIA with two different antibodies (see above). The elution pattern of E, 20E, 20,26E and 20Eoic was established as described previously (Grossniklaus-Bürgin *et al.*, 1998).

## RESULTS

### Morphological changes in the ovary and calyx region in pupal–adult development

An overview of the morphological and histological changes occurring in the ovary and calyx region in the course of pupal–adult development is presented in Figs 1 and 2. Fig. 1 shows the appearance of stage 1 and 2 ovaries as illustrated by laser confocal microscopy after treatment with the DNA stain propidium iodide. At stage 1, the ovary is a small compact organ in which the ovarioles are still fused; the intensity of DNA staining is high in both the future calyx region and the future reproductive part (vitellarium and germarium). At stage 2, the separation into four ovarioles is manifest; each ovariole now consists of the calyx region (highly stained) and the vitellarium and germarium (Fig. 1b). The higher magnification of the calyx region (Fig. 1c) reveals separation of an epithelial layer and intense staining of the nuclei. The histological changes of the calyx region are shown in Fig. 2. At stage 1, the future reproductive part is a bud with undifferentiated cells, while in the calyx region an epithelial layer of future calyx cells is visible. In both parts, cells appear to be proliferating. At stage 2, the peripheral calyx cells are elongated and form a layer around a mass of central cells. At stage 3a, the calyx cells in the middle and lower part have developed into large cells with very large nuclei and many of the central cells appear to have degenerated. In the upper part of the calyx region, a zone of proliferation with smaller elongated calyx cells is visible. At stage 3b, the nuclei of the calyx cells in the lower part have become enormous, the proliferative region in the upper part still exists and in the vitellarium maturing oocytes with nurse cells appear. At stage 4, calyx cells with excessively large nuclei are predominant and this becomes even more obvious at stage 5. A small region of proliferating calyx cells, maturing oocytes and some central cells are visible in the upper part. At stage 6, mature oocytes are visible, some passing through the calyx region. The oviduct now contains calyx fluid and the proportion of proliferating calyx cells and degenerating central cells has decreased. In 1-day-old adult females, the oocytes predominate and are also seen in the oviduct, surrounded by calyx fluid. Mature calyx cells are still present but appear to diminish and proliferating calyx cells are no longer visible.

To get an estimate of the increase in size of the calyx region, length and width were measured on histological sections;

assuming a globular shape, the following volumes in  $\text{mm}^3$  were obtained (means  $\pm$  SD of five determinations per stage): stage 2,  $0.007 \pm 0.001$ ; stage 3a,  $0.011 \pm 0.003$ ; stage 3b,  $0.017 \pm 0.006$ ; stage 4,  $0.024 \pm 0.008$ ; stage 5,  $0.025 \pm 0.008$ ; stage 6,  $0.029 \pm 0.008$ . Thus, the major increase in the calyx region occurs from stage 2 to 4 when the volume grows by a factor of 3.4. The histological pictures in Fig. 2 show that this is due to enlargement of the calyx cells and not an increase in cell number.

### Replication of non-viral DNA and integrated and excised viral DNA in pupal–adult development

We measured replication of non-viral and viral DNA in the course of pupal–adult development. To analyse whether replication of viral DNA occurred in the integrated proviral or the excised form, or both, real-time quantitative PCR was carried out with primers specific for integrated and excised viral segment CiV12 and for the rejoined flanking DNA of CiV12. Replication of non-viral DNA was measured with primers specific for an actin gene of *C. inanitus*. DNA from calyx regions of ovaries from pupae at stages 2–6 and from 4-day-old adult females was used as templates and the data are shown in Table 1 and Fig. 3. As the calyx region is not yet separated from the reproductive part of the ovary at stage 1 (see Figs 1a and 2), analyses obtained with this stage were not included. The amplicon copy number of actin and integrated CiV12 increased from stage 2 to 3a to a similar extent, i.e. tenfold, to approximately  $4.6 \times 10^5$  and  $1.5 \times 10^5$ , respectively, while excised CiV12 and corresponding flanking DNA were absent. Since in this phase many calyx cell nuclei have greatly increased in size and DNA content (Fig. 2; see also Wyler & Lanzrein, 2003), this increase in both actin and integrated CiV12 suggested that initially calyx cells become polyploid. In stage 3b, actin had increased in copy number to  $10^6$  per ovary, while integrated CiV12 had increased even more to  $1.3 \times 10^7$ ; excised CiV12 and corresponding rejoined DNA appeared with very similar copy numbers of approximately  $1.1 \times 10^5$  and  $2.5 \times 10^5$ , respectively. From stage 3b up to stage 6, actin remained at a similar level while integrated CiV12 increased massively up to  $5 \times 10^8$ , suggesting selective amplification of the proviral cluster; excised CiV12 and rejoined flanking DNA both increased by three orders of magnitude to  $3 \times 10^8$  and  $2 \times 10^8$ , respectively. The fact that excised CiV12 and rejoined flanking DNA are present in similar quantities up to stage 6 suggests that CiV12 is only amplified before excision. In 4-day-old adult females, excised CiV12 was highest, integrated CiV12 somewhat lower and rejoined DNA dropped to  $2.6 \times 10^5$  and actin to  $2.9 \times 10^3$ . To investigate whether the situation is similar for CiV14, quantitative PCR measurements were carried out with CiV14 primers. Fig. 4 shows that at stage 6, copy numbers of

**Fig. 2.** Histological sections (2  $\mu\text{m}$ ) of ovaries from pupae at stages 1–6 and from a 1-day-old adult female (1d). Staining was carried out using the DNA stain of Feulgen and 1% toluidine blue. Designation of stages is according to Albrecht *et al.* (1994). In all pictures the oviduct is oriented to the left and magnification was the same; bar (given in 2), 100  $\mu\text{m}$ . o, Oocytes; c, calyx cells; cf, calyx fluid.

**Table 1.** Copy numbers per ovary of a *C. inanitus* actin gene and the viral segment CiV12 in its integrated and excised form and the corresponding rejoined flanking DNA during pupal–adult development (stages 2–6) and in 4-day-old adult females.

Designation of pupal stages is according to Albrecht *et al.* (1994). Values were determined by quantitative PCR from three independent DNA isolations per stage, each being amplified on two to three separate reaction plates. Data are means  $\pm$  SE ( $n=6-9$ ). The background copy number calculated using non-template controls was subtracted.

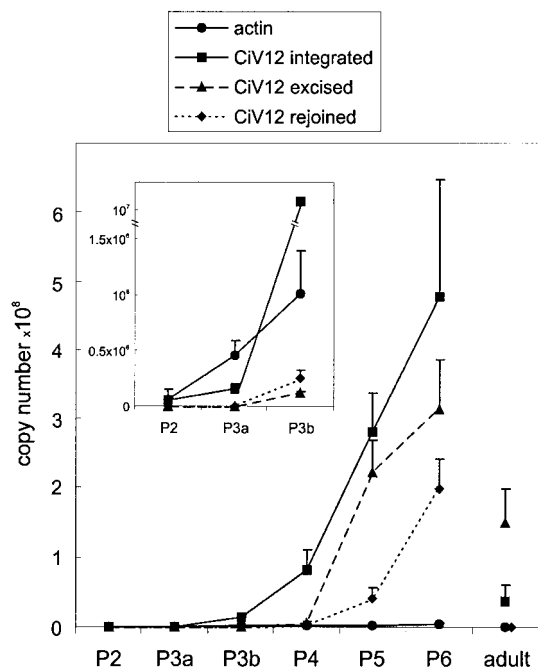
Stage	Actin	CiV12 integrated	CiV12 excised	Rejoined flanking DNA
2	$5.6 \pm 1.3 \times 10^4$	$2.5 \pm 1.6 \times 10^4$	0	0
3a	$4.6 \pm 1.3 \times 10^5$	$1.5 \pm 0.5 \times 10^5$	0	0
3b	$1.0 \pm 0.4 \times 10^6$	$1.3 \pm 0.4 \times 10^7$	$1.1 \pm 0.2 \times 10^5$	$2.5 \pm 0.8 \times 10^5$
4	$1.6 \pm 0.7 \times 10^6$	$8.2 \pm 2.8 \times 10^7$	$6.8 \pm 1.7 \times 10^6$	$2.2 \pm 0.7 \times 10^6$
5	$1.7 \pm 0.5 \times 10^6$	$2.8 \pm 0.6 \times 10^8$	$2.2 \pm 0.5 \times 10^8$	$4.0 \pm 1.6 \times 10^7$
6	$4.0 \pm 1.4 \times 10^6$	$4.8 \pm 1.7 \times 10^8$	$3.1 \pm 0.7 \times 10^8$	$2.0 \pm 0.4 \times 10^8$
Adult	$2.9 \pm 1.6 \times 10^3$	$3.6 \pm 2.3 \times 10^7$	$1.5 \pm 0.5 \times 10^8$	$2.6 \pm 1.2 \times 10^5$

integrated and excised CiV14 and corresponding flanking DNA were in a similar range to those of CiV12 although slightly lower. From all these data the following hypothetical model for CiV DNA replication is proposed (Fig. 5): (i) calyx cells first become polyploid; (ii) the proviral cluster is then selectively amplified; this could be due to local amplification of chromosomal DNA or a rolling circle mechanism; (iii) after amplification single segments are

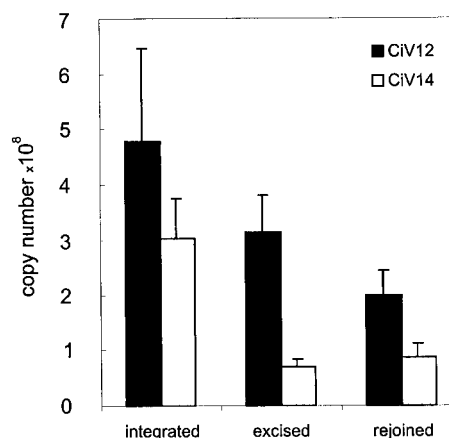
excised and circularized by a recombination process. Excised circular viral segments are not further amplified.

### Ecdysteroids in the course of pupal–adult development

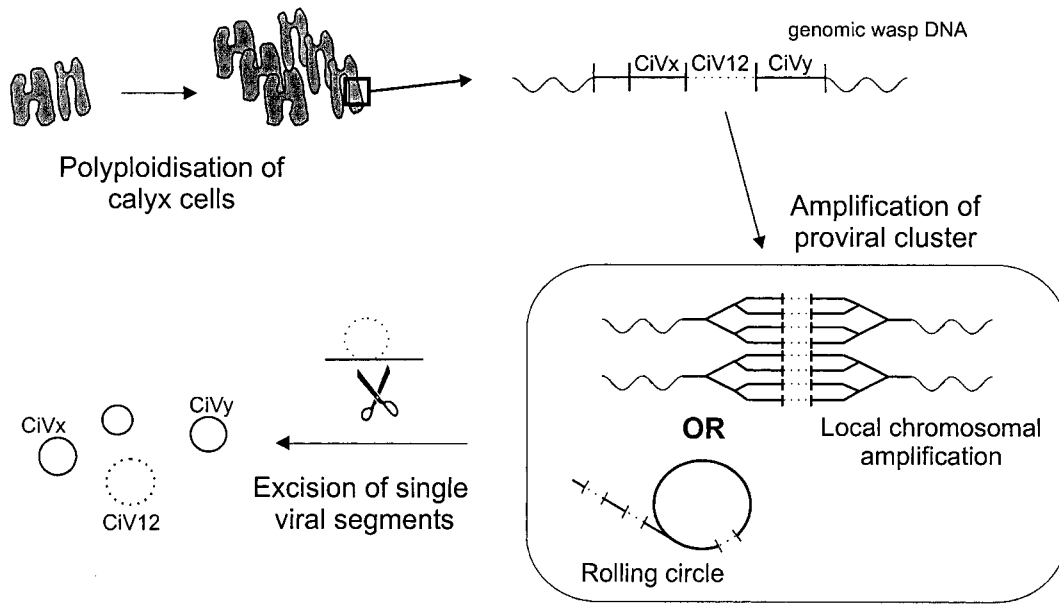
On the basis of *in vitro* application of 20E it has been proposed that this hormone regulates polydnavirus replication (Webb & Summers, 1992) but no ecdysteroid measurements have been made to support this hypothesis. To get an overview of the ecdysteroid changes in the course of pupal–adult development, ecdysteroids in 60% and 25% methanol fractions were analysed. Ecdysteroids in the 60% methanol fraction, which contains E, 20E, 20,26E, increased rapidly after the pupal moult, reached a peak between stages 1 and 2 and then dropped continuously (Fig. 6). Ecdysteroids in the 25% methanol fraction, which contains the metabolites Eoic and 20Eoic, increased towards stage 2, then remained at a similar level and slowly dropped towards adult eclosion. In order to elucidate the composition of



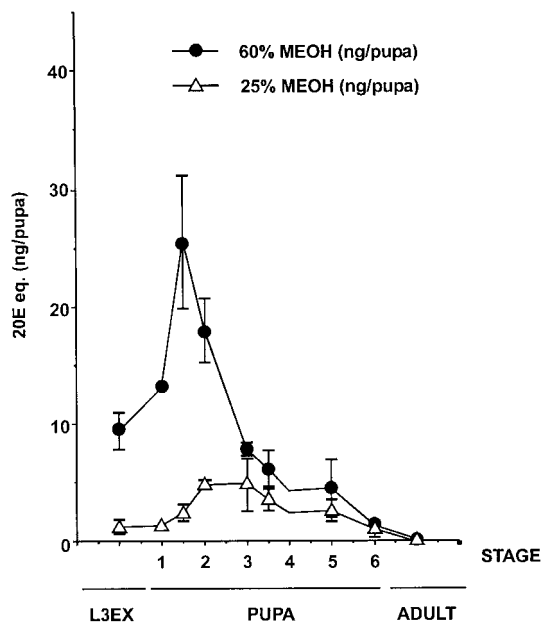
**Fig. 3.** Copy numbers per ovary (calyx region only) of *C. inanitus* actin, integrated and excised CiV12 and the corresponding rejoined flanking DNA in pupal stages 1–6 and 4-day-old adult females that were not allowed to oviposit. The inset shows stages 2–3b at a lower scale. Designation of stages is according to Albrecht *et al.* (1994). Data are means  $\pm$  SE of six to nine determinations.



**Fig. 4.** Copy numbers per ovary (calyx region only) of integrated and excised CiV12 and CiV14 and their respective rejoined flanking DNA in pupal stage 6. Data are means  $\pm$  SE of six to nine determinations.

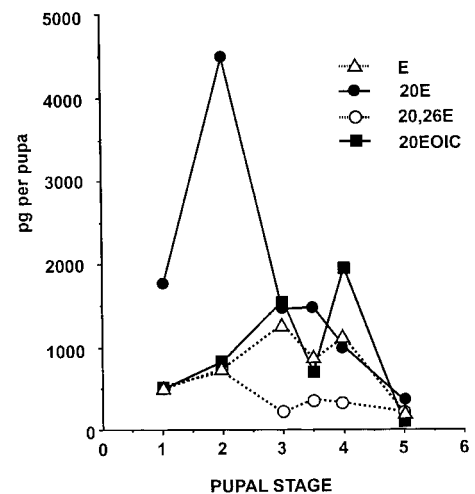


**Fig. 5.** Model illustrating a hypothetical mode of CiV replication in calyx cells. Proviral segments are integrated in tandem arrays in the wasp genome. After polyploidization, the region containing the proviral cluster is amplified either by local chromosomal amplification or by a rolling circle mechanism. Viral segments are then excised through a recombinational event and are not further amplified.



**Fig. 6.** Ecdysteroids in whole-body extracts of *C. inanitus* external third instar larvae (L3EX), female pupae from stage 1–6 and 1-day-old adult females. Designation of stages is according to Albrecht *et al.* (1994) with a refinement in very young pupae: stage 1 was subdivided into a and b, the former having unpigmented ocelli and the latter pigmented ocelli. Ecdysteroids in the 25% ( $\Delta$ ) and 60% ( $\bullet$ ) methanol fraction of C18 Sep-Pak are expressed in 20E equivalents per insect as determined with the H-22 antibody. Data are means  $\pm$  SE of three to five determinations.

ecdysteroids, we analysed HPLC-separated ecdysteroids by RIA and use of two different antibodies, one specific for the ecdysteroid side-chain (H-2) and the other for the ecdysteroid nucleus (H-22). In Fig. 7, values for E, 20E, 20,26E and 20Eoic are given in their corresponding equivalents which were calculated from cross-reactivity data for the



**Fig. 7.** Quantities of E, 20E, 20,26E and 20Eoic from stage 1–5. Each symbol represents the analysis of pooled extracts from four to ten pupae after HPLC separation and quantification of the fractions with two different antibodies; quantities are expressed in their corresponding equivalents as determined with the H-22 antibody (Warren & Gilbert, 1986).

two antibodies (Warren and Gilbert, 1986). 20E was by far the predominant ecdysteroid in stages 1–2 when ovarian morphogenesis and calyx cell differentiation take place (see Figs 1 and 2). E was present from stage 1 up to stage 4. In third larval instar *Chelonus* sp., the presence of E and 20E has been confirmed by gas chromatography–mass spectrometry analysis (Grossniklaus-Bürgin & Lanzrein, 1990b). Among the tentatively identified 20,26E and 20Eoic, the former was present at low quantities from stage 1 up to stage 5 and the latter increased after stage 2 when 20E levels fell.

## DISCUSSION

The real-time quantitative PCR data showed that integrated as well as excised DNA of viral segments CiV12 and CiV14 were massively amplified in the course of pupal–adult development, reaching copy numbers in the range of  $10^8$  per ovary (Table 1, Figs 3 and 4). A hypothetical model describing how CiV DNA might be amplified is given in Fig. 5. Up to stage 3a, the integrated proviral form of CiV12 is amplified to a similar extent as that of the non-viral actin DNA. In this phase, the calyx cells increase massively in size and DNA content, and the nuclei of the most advanced cells become highly lobulated (Figs 1 and 2; see also Wyler & Lanzrein, 2003). This indicates that calyx cells initially become polyploid. The copy number of actin in the calyx region at stage 1 is estimated to be around  $3 \times 10^4$  because the entire ovary contains  $7 \times 10^4$  copies and the calyx region comprises somewhat less than half of the ovary at that stage (Figs 1 and 2). With an increase up to  $10^6$  copies in stage 3b, one can thus calculate a degree of polyploidization of approximately 30. Polyploidization is widespread in arthropods and often seen in highly specialized large cells (Brodsky & Uryvaeva, 1977; Edgar & Orr-Weaver, 2001); other specialized cells of parasitic wasps, the teratocytes, also become polyploid (Strand & Wong, 1991; Hotta *et al.*, 2001).

From stage 3a onwards, integrated proviral CiV12 was approximately 100 times more amplified than actin, as it reached a copy number of around  $5 \times 10^8$  compared with  $4 \times 10^6$  for actin. This indicates selective amplification of proviral CiV12. As CiV segments appeared to be clustered in the wasp's genome (Wyder *et al.*, 2002) and since proviral CiV14 reached a similar copy number (Fig. 4), it seems likely that after stage 3a, the entire proviral cluster is specifically amplified. Similarly, *Drosophila* follicle cells first become polyploid and then specifically amplify regions containing the chorion genes (Osheim *et al.*, 1988; Calvi *et al.*, 1998). In baculoviruses, palindromic structures have been reported to act as origins of replication (Pearson *et al.*, 1992; Ahrens *et al.*, 1995; Kool *et al.*, 1995). In CiV, several inverted repeats have been found on all sequenced segments (Wyder *et al.*, 2002) and it is conceivable that some of the palindromic structures might serve as origins of replication. A large palindromic structure was also found in the EP1 circle of the *C. congregata* bracovirus (Savary *et al.*, 1997). Another way of replication of the viral cluster could be a

rolling circle mechanism in which DNA synthesis proceeds unidirectionally (Fig. 5). In this case, a large circular molecule containing the viral cluster and an origin of replication should exist. For the bracovirus of *C. congregata*, it was recently shown that a large molecule containing at least two viral segments and downstream sequences is amplified prior to excision of individual segments (Pasquier-Barre *et al.*, 2002).

Excised circular CiV12 DNA and corresponding rejoined flanking DNA was first detected at stage 3b and both then increased in a similar manner (Table 1, Fig. 3). At stage 6, copy numbers of excised circular and flanking rejoined DNA were similar for both CiV12 and CiV14 (Fig. 4). This suggests that excised circular segments do not replicate. Also recent observations with the *C. congregata* bracovirus support this assumption, as the quantity of excised EP1 never exceeded that of the large precursor molecule containing EP1 (Pasquier-Barre *et al.*, 2002). Coincident with the first appearance of excised circular viral DNA in stage 3b, calyx cells with a huge and swollen nucleus containing viral envelopes and virogenic stroma appear (Wyler & Lanzrein, 2003); these cells appear to be the ones in which amplification of the proviral cluster and excision of viral DNA take place.

The copy number of proviral and excised viral DNA increases massively from stage 3b up to stage 6 (Table 1, Fig. 3). A comparison with histological (Fig. 2) and ultrastructural observations (Wyler & Lanzrein, 2003) indicates that in this phase calyx cells of all developmental stages occur, the most advanced being located near the oviduct. The proportion of virion-containing cells increases steadily, and at stage 6 release of virions has commenced and calyx fluid, which contains virions, can be seen in the oviduct. In 4-day-old adult females, which were not allowed to oviposit, the copy number of integrated proviral CiV12 had dropped to  $3.6 \times 10^7$  suggesting that amplification of integrated proviral DNA decreases in the adult. The copy number of excised circular CiV12 was still at  $1.5 \times 10^8$ , while that of rejoined flanking DNA had dropped to  $2.6 \times 10^5$  and that of actin to  $2.9 \times 10^3$ . This shows that in the adult stage the excised circular form of viral DNA is predominant. This is the form that is encapsidated in viral particles and released into the oviduct as calyx fluid (Fig. 2). All these data show that virus production is most intense in the second half of pupal–adult development and diminishes in the adult, which agrees with ultrastructural observations (Wyler & Lanzrein, 2003). A comparison with the egg-laying behaviour of the wasp revealed the following. Under our rearing conditions, adult females live for 25–30 days and may lay eggs until they die. On average a female lays  $1757 \pm 945$  eggs ( $n = 12$ ) in its lifetime but some lay up to 2800 eggs (F. von Niederhäusern & B. Lanzrein, unpublished). With DNA dot blot analyses it was found that the calyx fluid collected from one female contained 150–250 ng viral DNA at adult eclosion (F. von Niederhäusern & S. Wyder, unpublished) and extrapolation from copy numbers of excised CiV12 gave values in the range of 600 ng per ovary. As we have shown



that approximately 0.1 ng viral DNA is injected along with each egg (Lanzrein *et al.*, 2001), the amount of virus present at adult eclosion would already be sufficient for all ovi-positions in the life-time of a female. In *C. congregata*, the intensity of polydnavirus replication remained high until 3 days after adult eclosion (Pasquier-Barre *et al.*, 2002).

Ecdysteroids increased immediately after the pupal moult and 20E was predominant; thereafter ecdysteroids decreased (Figs 6 and 7). Here we have used data obtained with whole body homogenates, as collection of haemolymph, especially from young pupae, is difficult. However, for stages 2, 3a, 3b and 5, some analyses with haemolymph have been made, which indicated that the majority of ecdysteroids in the 60% methanol fraction circulates in haemolymph (data not shown). These are the first data on ecdysteroid identification and quantification in pupal–adult development of a polydnavirus-carrying wasp. In the ichneumonid *C. sonorensis*, it was proposed, on the basis of *in vitro* application of 20E, that this hormone regulates polydnavirus replication (Webb & Summers, 1992). However, the titre data shown here do not support this hypothesis, since at stage 3b, when excised viral DNA first appears, 20E has already decreased and continues to drop, whereas polydnavirus replication is most intense up to stage 6. The level of 20E is highest when the reproductive tract is in a phase of intensive cell proliferation and differentiation, which suggests a role in early ovarian morphogenesis, as has been reported for *Drosophila* (Audit-Lamour & Busson, 1981; Hodin & Riddiford, 1998). Furthermore, ecdysteroid titres were also highest shortly after pupation in pupae of an ichneumonid ectoparasitoid, which does not harbour polydnaviruses (Gelman *et al.*, 2000). Thus, a specific role of 20E in regulating polydnavirus replication appears rather unlikely; it is conceivable that the *in vitro* 20E application made by Webb & Summers (1992) accelerated calyx cell differentiation and thus indirectly caused an increase in viral DNA.

## ACKNOWLEDGEMENTS

We should like to thank Syngenta (Stein, Switzerland), for providing us with adult *Spodoptera littoralis* and the diet for rearing the larvae. We also thank Dr V. Meyer, Institute of Chemistry and Biochemistry of the University of Berne, for allowing us to use the HPLC equipment, Dr L. Gilbert, Chapel Hill, for ecdysone antibodies and Dr R. Lafont, Paris, for ecdysteroid reference compounds. Financial support from the Swiss National Science Foundation (grants 3100-041828.94, 31-52399.97 and 3100-063444.00 to B.L.) is gratefully acknowledged.

## REFERENCES

Ahrens, C. H., Pearson, M. N. & Rohrmann, G. (1995). Identification and characterization of a second putative origin of DNA replication in a baculovirus of *Orgyia pseudotsugata*. *Virology* **207**, 572–576.

Albrecht, U., Wyler, T., Pfister-Wilhelm, R., Gruber, A., Stettler, P., Heiniger, P., Kurt, E., Schümperli, D. & Lanzrein, B. (1994). Polydnavirus of the parasitic wasp *Chelonus inanitus* (Braconidae): characterization, genome organization and time point of replication. *J Gen Virol* **75**, 3353–3363.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Audit-Lamour, C. & Busson, D. (1981). Oögenesis defects in the *ecd-1* mutant of *Drosophila melanogaster*, deficient in ecdysteroid at high temperature. *J Insect Physiol* **27**, 829–837.

Belle, E., Beckage, N. E., Rousset, J., Poirié, M., Lemeunier, F. & Drezen, J. M. (2002). Visualization of polydnavirus sequences in a parasitoid wasp chromosome. *J Virol* **76**, 5793–5796.

Brodsky, W. Y. & Uryvaeva, I. V. (1977). Cell polyploidy: its relation to tissue growth and function. *Int Rev Cytol* **50**, 275–332.

Calvi, B. R., Lilly, M. A. & Spradling, A. C. (1998). Cell cycle control of chorion gene amplification. *Genes Dev* **12**, 734–744.

Edgar, B. A. & Orr-Weaver, T. L. (2001). Endoreplication cell cycles: more for less. *Cell* **105**, 297–306.

Gelman, D., Carpenter, J. E. & Greany, P. (2000). Ecdysteroid levels/profiles of the parasitoid wasp, *Diapetimorpha introita*, reared on its host, *Spodoptera frugiperda* and on an artificial diet. *J Insect Physiol* **46**, 457–465.

Grossniklaus-Bürgin, C. & Lanzrein, B. (1990a). Qualitative and quantitative analysis of juvenile hormone and ecdysteroids from the egg to the pupal molt in *Trichoplusia ni*. *Arch Insect Biochem Physiol* **14**, 13–30.

Grossniklaus-Bürgin, C. & Lanzrein, B. (1990b). Endocrine interrelationship between the parasitoid *Chelonus* sp. and its host *Trichoplusia ni*. *Arch Insect Biochem Physiol* **14**, 201–216.

Grossniklaus-Bürgin, C., Wyler, T., Pfister-Wilhelm, R. & Lanzrein, B. (1994). Biology and morphology of the parasitoid *Chelonus inanitus* (Braconidae, Hymenoptera) and effects on the development of its host *Spodoptera littoralis* (Noctuidae, Lepidoptera). *Invertebr Reprod Dev* **25**, 143–158.

Grossniklaus-Bürgin, C., Pfister-Wilhelm, R., Meyer, V., Treiblmayr, K. & Lanzrein, B. (1998). Physiological and endocrine changes associated with polydnavirus/venom in the parasitoid–host system *Chelonus inanitus*–*Spodoptera littoralis*. *J Insect Physiol* **44**, 305–321.

Gruber, A., Stettler, P., Heiniger P., Schümperli, D. & Lanzrein, B. (1996). Polydnavirus DNA of the braconid wasp *Chelonus inanitus* is integrated in the wasp's genome and excised only in later pupal and adult stages of the female. *J Gen Virol* **77**, 2873–2879.

Hodin, J. & Riddiford, L. M. (1998). The ecdysone receptor and ultraspiracle regulate the timing and progression of ovarian morphogenesis during *Drosophila* metamorphosis. *Development Genes Evol* **208**, 304–317.

Hotta, M., Okuda, T. & Tanaka, T. (2001). *Cotesia kariyai* teratocytes: growth and development. *J Insect Physiol* **47**, 31–41.

Kool, M., Ahrens, C. H., Vlak, J. M. & Rohrmann, G. (1995). Replication of baculovirus DNA. *J Gen Virol* **76**, 2103–2118.

Lafont, R., Koolman, J. & Rees, H. (1993). Standardized abbreviations for common ecdysteroids. *Insect Biochem Mol Biol* **23**, 207–209.

Lanzrein, B., Pfister-Wilhelm, R. & von Niederhäusern, F. (2001). Effects of an egg–larval parasitoid and its polydnavirus on development and the endocrine system of the host. In *Endocrine Interactions of Insect Parasites and Pathogens*, pp. 95–109. Edited by J. P. Edwards & R. Weaver. Oxford: BIOS Scientific Publishers.

Lavine, M. D. & Beckage, N. E. (1995). Polydnaviruses: potent mediators of host insect immune dysfunction. *Parasitol Today* **11**, 368–378.

Lawrence, P. O. & Lanzrein, B. (1993). Hormonal interactions between insect endoparasites and their host insects. In *Parasites and*

- Pathogens of Insects*, pp. 59–86. Edited by N. E. Beckage, S. N. Thompson & B. A. Federici. San Diego: Academic Press.
- Norton, W. N. & Vinson, S. B. (1983).** Correlating the initiation of virus replication with a specific pupal developmental phase of an ichneumonid parasitoid. *Cell Tissue Res* **231**, 387–398.
- Osheim, Y. N., Miller, O. L. & Beyer, A. L. (1988).** Visualization of *Drosophila melanogaster* chorion genes undergoing amplification. *Mol Cell Biol* **8**, 2811–2821.
- Pasquier-Barre, F., Dupuy, C., Huguet, E., Monteiro, F., Moreau, A., Poirié, M. & Drezen, J.-M. (2002).** Polydnavirus replication: the EP1 segment of the parasitoid wasp *Cotesia congregata* is amplified within a larger precursor molecule. *J Gen Virol* **83**, 2035–2045.
- Pearson, M., Bjornson, R., Pearson, G. & Rohrmann, G. (1992).** The *Autographa californica* baculovirus genome: evidence for multiple replication origins. *Science* **257**, 1382–1384.
- Savary, S., Beckage, N., Tan, F., Periquet, G. & Drezen, J. M. (1997).** Excision of the polydnavirus chromosomal integrated EP1 sequence of the parasitoid wasp *Cotesia congregata* (Braconidae, Microgastrinae) at potential recombinase binding sites. *J Gen Virol* **78**, 3125–3134.
- Savary, S., Drezen, J. M., Tan, F., Beckage, N. E. & Periquet, G. (1999).** The excision of polydnavirus sequences from the genome of the wasp *Cotesia congregata* (Braconidae, Microgastrinae) is developmentally regulated but not strictly restricted to the ovaries in the adult. *Insect Mol Biol* **8**, 319–327.
- Schmidt, O., Theopold, M. & Strand, M. R. (2001).** Innate immunity and its evasion and suppression by hymenopteran endoparasitoids. *BioEssays* **23**, 344–351.
- Soller, M. & Lanzrein, B. (1996).** Polydnavirus and venom of the egg–larval parasitoid *Chelonus inanitus* (Braconidae) induce developmental arrest in the prepupa of its host *Spodoptera littoralis* (Noctuidae). *J Insect Physiol* **42**, 471–481.
- Stoltz, D. B. (1993).** The polydnavirus life cycle. In *Parasites and Pathogens of Insects*, pp. 167–187. Edited by N. E. Beckage, S. N. Thompson & B. A. Federici. San Diego: Academic Press.
- Strand, M. R. & Wong, E. A. (1991).** The growth and role of *Microplitis demolitor* teratocytes in parasitism of *Pseudoplusia includens*. *J Insect Physiol* **37**, 503–516.
- Warren, J. T. & Gilbert, L. I. (1986).** Ecdysone metabolism and distribution during the pupal–adult development of *Manduca sexta*. *Insect Biochem* **16**, 65–82.
- Webb, B. A. (1998).** Polydnavirus biology, genome structure, and evolution. In *The Insect Viruses*, pp. 105–139. Edited by L. K. Miller & L. A. Ball. New York & London: Plenum.
- Webb, B. A. & Summers, M. D. (1992).** Stimulation of polydnavirus replication by 20-hydroxyecdysone. *Experientia* **48**, 1018–1022.
- Webb, B. A., Beckage, N. E., Hayakawa, Y., Krell, P. J., Lanzrein, B., Stoltz, D. B., Strand, M. R. & Summers, M. D. (2000).** Polydnaviridae. In *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*, pp. 253–260. Edited by M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle & R. B. Wickner. San Diego: Academic Press.
- Wyder, S., Tschannen, A., Hochuli, A., Gruber, A., Saladin, V., Zumbach, S. & Lanzrein, B. (2002).** Characterization of *Chelonus inanitus* polydnavirus segments: sequences and analysis, excision site and demonstration of clustering. *J Gen Virol* **83**, 247–256.
- Wyler, T. & Lanzrein, B. (2003).** Ovary development and polydnavirus morphogenesis in the parasitic wasp *Chelonus inanitus*. II. Ultrastructural analysis of calyx cell development, virion formation and release. *J Gen Virol* **84**, 1151–1163.