

Ovary development and polydnavirus morphogenesis in the parasitic wasp *Chelonus inanitus*. II. Ultrastructural analysis of calyx cell development, virion formation and release

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Polydnaviruses are unique symbiotic viruses that are formed only in calyx cells in the ovary of parasitic wasps in the families Braconidae and Ichneumonidae; accordingly, two genera, *Bracovirus* and *Ichnovirus* are recognized. We have presented a detailed ultrastructural analysis of ovary and calyx cell differentiation and virion morphogenesis, together with the first data on virion release in a bracovirus. Differentiation of the ovary into germarium/vitellarium and the calyx region begins immediately after pupation. In the periphery and central part of the calyx, some cells and their nuclei begin to enlarge and the DNA content increases. The calyx cell nuclei then further increase and become highly lobulated, nuclear pores become very abundant and the cytoplasm is rich in ribosomes. This suggests synthesis and import of viral envelope proteins as viral envelopes appear in the nuclei shortly later. The appearance of viral envelopes is accompanied by a swelling of the nucleus and a change in electron density. Thereafter, the calyx cells reach the final stage with a highly swollen nucleus containing virogenic stroma and mature virions with nucleocapsids. Up to this stage, the DNA content of nuclei increases 120-fold and the volume 45-fold. The mature calyx cells are positioned in the vicinity of the oviduct lumen; for release of virions first the nuclear and then the plasma membrane disintegrate. On the border of the oviduct lumen, cells of an epithelial layer become phagocytic and remove debris, leading to a calyx fluid that contains only densely packed virions.

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INTRODUCTION

Polydnaviruses are remarkable viruses as they are obligatorily associated with parasitic wasps and are formed only in a particular cell type of the wasp's ovary, the calyx cells. In the family *Polydnaviridae* two genera are recognized, the genus *Ichnovirus*, harboured by ichneumonid wasps, and the genus *Bracovirus*, harboured by braconid wasps (Webb *et al.*, 2000). Molecular and morphological data suggest a single origin of the bracoviruses (Whitfield, 1997), which is estimated to have occurred 73.7 ± 10 million years ago (Whitfield, 2002). Much less is known about the phylogeny of the ichnoviruses, but the association of ichneumonids with viruses seems to have occurred independently from the association of braconids with viruses (Whitfield, 1997). The members of the genera *Ichnovirus* and *Bracovirus* are unrelated by serological and hybridization analyses (Webb *et al.*, 2000).

The characteristics of ichnoviruses and bracoviruses are described in Webb *et al.* (2000) and illustrate the differences in their morphology and the process of virion formation. Ichnovirus virions consist of nucleocapsids of uniform size

having the form of a prolate ellipsoid, surrounded by two unit-membrane envelopes. The inner envelope appears to be assembled *de novo* within the nucleus of calyx cells, while the outer envelope is acquired by budding of virions through the plasma membrane into the oviduct lumen. Bracovirus virions consist of cylindrical nucleocapsids of uniform diameter but of variable length, surrounded by a single envelope, which appears to be assembled *de novo* within the nucleus. Depending on the species, a virion contains one or several nucleocapsids. The mode of release of bracoviruses is not clarified but probably involves lysis of virus-carrying cells. The genome of both ichnoviruses and bracoviruses is segmented and consists of various circles of double-stranded DNA. The viral genome is transmitted in proviral linear form integrated in the wasp's chromosomes (reviewed in Webb, 1998; Belle *et al.*, 2002) and for two bracoviruses it was recently shown that proviral segments are clustered (Belle *et al.*, 2002; Wyder *et al.*, 2002).

Excision and circularization of viral DNA has been shown to begin at a particular stage in pupal–adult development for the ichnovirus of *Campoletis sonorensis* (Webb & Summers, 1992) and the bracoviruses of *Chelonus inanitus* (Albrecht

et al., 1994; Gruber *et al.*, 1996; Wyder *et al.*, 2002) and *Cotesia congregata* (Savary *et al.*, 1999; Pasquier-Barre *et al.*, 2002). A comparison with ultrastructural observations (Norton & Vinson, 1983; Pasquier-Barre *et al.*, 2002) suggests that excision of viral DNA is concomitant with the appearance of virions in calyx cells. Very little is known about the early development of calyx cells and the sequence of events leading to polydnavirus formation. In the accompanying paper (Marti *et al.*, 2003) on ovary development and polydnavirus morphogenesis in *C. inanitus*, we showed that calyx cells begin to differentiate and increase in size shortly after pupation, and that non-viral DNA (actin) and integrated proviral DNA are amplified to the same extent, which suggests polyploidization of calyx cells at this early stage of development. Calyx cells then massively increase in size, their nuclei become very large and proviral DNA is specifically amplified followed by excision of viral segments. Here we present a detailed ultrastructural investigation of the development of calyx cells and the processes associated with virion formation and release. We have also analysed the DNA content of calyx cell nuclei by measurement of fluorescence after Hoechst staining. The data indicate that calyx cells undergo dramatic developmental changes, which involve a massive increase in volume and DNA content of the nucleus, the appearance of viral envelopes and nucleocapsids in the nucleus and finally lysis of cells leading to release of virions into the lumen of the oviduct.

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).

Preparation of ovaries. Ovaries were fixed in 2% glutaraldehyde and 0.8% paraformaldehyde in 0.08 M sodium cacodylate buffer, pH 7.2, at 4°C. After several washes in 0.1 M sodium cacodylate buffer, samples for LR-White embedding and for epoxy resin embedding were treated differently. LR-White was used as it has the advantage of allowing analysis of consecutive sections by fluorescence microscopy after Hoechst staining and electron microscopy. However, in combination with Hoechst staining, osmium tetroxide fixation cannot be used and thus membranes are not visualized. The LR-White samples (see Figs 2 and 3) were dehydrated through a graded series of ethanol and embedded in LR-White (Sigma). Polymerization was for 14 h at 60°C. The epoxy resin samples (see Figs 1 and 4–7) were post-fixed in 1% osmium tetroxide in sodium cacodylate buffer and stained in 0.5% (w/v) uranyl acetate in 0.05 M maleate buffer, pH 5.0. They were washed with 0.05 M maleate buffer, dehydrated in acetone and embedded in low viscosity epoxy resin (Spurr, 1969). Sections (1 µm for fluorescence microscopy and 60–100 nm for electron microscopy) were cut on a Sorvall MT2-B ultramicrotome using diamond knives (Diatome). Sections for electron microscopy were transferred on to carbon-coated Parlodion films on copper grids or on to single slot grids coated with Formvar films. LR-White-embedded sections were stained for 10 min in 2% phosphotungstic acid, pH 6.8, and for 5 min in

1% uranyl acetate in water. Epoxy resin-embedded sections were stained with lead citrate (Reynolds, 1963). The sections were analysed on a Phillips EM300 electron microscope operated at 60 kV.

Hoechst staining and quantification of fluorescence. LR-White-embedded 1 µm sections were stained for 1 min with 2 µg of the DNA stain bisbenzimidazole (Hoechst 33258) ml⁻¹, rinsed in PBS and mounted in fluorescence mounting medium (Dako). Sections were analysed on a Nikon Eclipse E600 fluorescence microscope and fluorescence intensity per area was quantified using Advanced Image Data Analyser software (AIDA) from Raytest Isotopengeräte. Due to fading, values were always determined relative to nuclei of non-virus-producing cells.

For measurement of nuclear volumes, nuclei cut in the centre were selected. For the reference nuclei (non-virus-producing cells in the ovarian sheath or oviduct), the area was quantified using the AIDA software and the volume was calculated by assuming a spherical shape. For mature calyx cell nuclei, the length and width was measured and the volume was calculated assuming an ellipsoid shape.

RESULTS

In the course of pupal–adult development of *C. inanitus*, the pigmentation of the pupa increases in a characteristic manner and development of the ovary is strictly correlated with the pigmentation pattern; various developmental stages (1–6) of pupae and their ovaries have been defined (Albrecht *et al.*, 1994). The morphological and histological changes in the ovary are shown in the accompanying paper (Marti *et al.*, 2003) and reveal that from stage 1 to stage 2, the ovary develops from a compact stump with proliferating cells into a complex structure consisting of a long germarium/vitellarium, the calyx region with a peripheral layer of elongated cells and the oviduct. In Fig. 1 we have shown an overview of the lower part of a stage 2 ovary (a) and details of the calyx region (b–d) as seen by electron microscopy. The oviduct and the calyx are covered by an ovarian sheath and the oviduct is lined with an epithelial layer. The calyx of the two ovarioles is separated by a septum and along the septum and in the periphery elongated cells are seen. The calyx is separated from the oviduct lumen by a very thin epithelial layer (Fig. 1a, c). In Fig. 1(b), a group of elongated peripheral cells and the covering ovarian sheath are shown at higher magnification; the nuclei of the elongated cells contain many patches of dense heterochromatin. Fig. 1(c) and (d) show regions of the more central part of the calyx in the vicinity of the oviduct lumen; two types of cells can be seen, one showing typical signs of degeneration and the other resembling the peripheral cells in that the nuclei contain many patches of dense heterochromatin; the latter cells and the elongated peripheral cells will develop into virus-producing calyx cells.

In the accompanying paper (Marti *et al.*, 2003), the Feulgen-stained ovaries showed that nuclei of mature calyx cells have a very high DNA content. We thus investigated the DNA content of calyx cell nuclei at various stages of their differentiation into virus-producing cells by treating ovaries of stages 3a–6 with the Hoechst DNA stain. Fig. 2 shows that at stage 3a, the nuclei of the peripheral elongated and

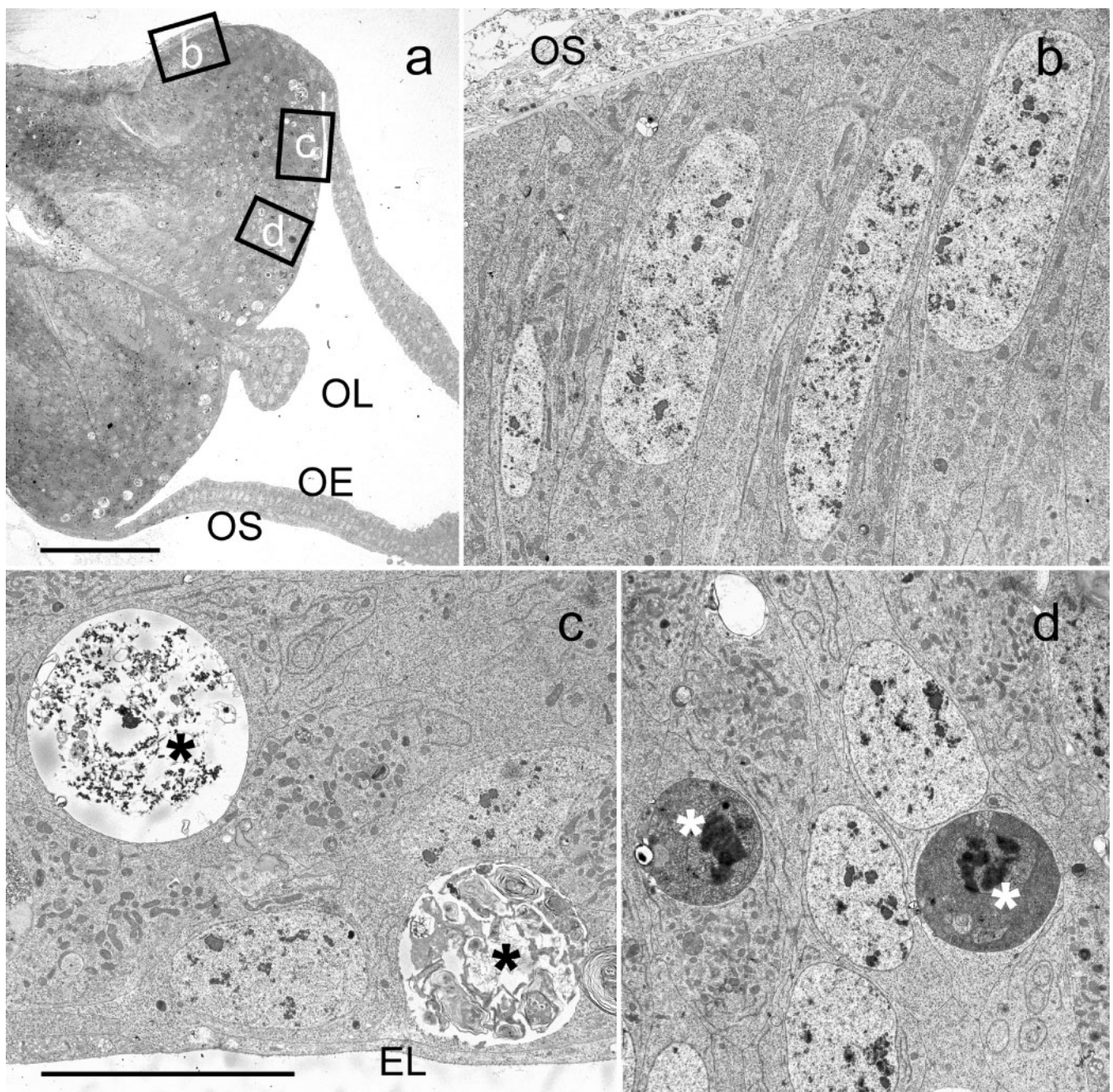


Fig. 1. Ultrastructure of a stage 2 ovary. (a) Overview of the calyx region of two ovarioles and the oviduct. The positions of (b)–(d) are indicated. Bar, 100 μm . (b) Peripheral calyx region at higher magnification: elongated calyx cells and nuclei surrounded by the ovarian sheath. (c) Region in the lower calyx showing a thin layer of epithelial cells forming the border of the oviduct lumen. Calyx cells in this region are roundish and seen together with degenerating cells (asterisks). (d) Another region in the lower calyx with calyx cells next to degenerating cells (asterisks). OS, Ovarial sheath; OE, oviduct epithelium; OL, oviduct lumen; EL, epithelial layer. Magnification of (b)–(d) is the same; bar, 10 μm .

roundish central cells are much larger and brighter than the nuclei of the septum, oviduct or ovarial sheath, indicating an increase in DNA content. At stage 3b, several nuclei in the lower part of the calyx have become excessively large and bright, indicating a further increase in DNA content. At stage 4, the proportion of enlarged and very bright nuclei

has increased and at stage 6 these large and bright nuclei predominate. At this stage, oocytes appear in the calyx and the oviduct is bright, indicating the presence of calyx fluid. To characterize the various stages of calyx cells seen with the Hoechst stain, we analysed consecutive sections by either Hoechst staining or electron microscopy. For this purpose,

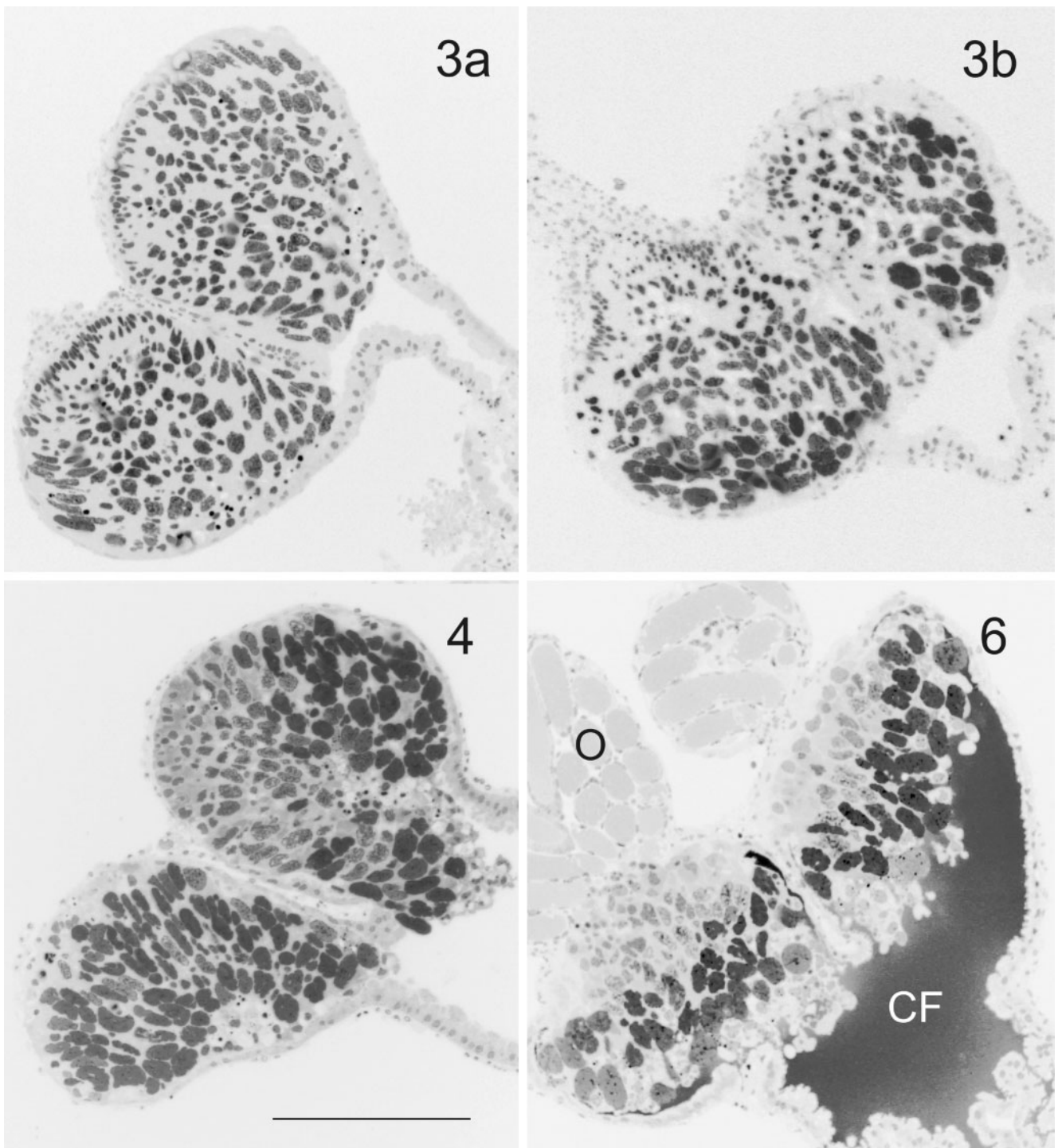


Fig. 2. Calyx region of ovaries at stages 3a, 3b, 4 and 6 by fluorescence microscopy following Hoechst staining. The signal is inverted so that the intensity of brightness in fluorescence is proportional to the darkness shown here. The oviduct is at the bottom right in all pictures. At stage 3a, brighter and larger nuclei are seen in the periphery and in the central part than in the oviduct. At stage 3b, nuclei in the lower part of the calyx become very large and bright, while the ones in the upper part are of intermediate size and brightness. At stage 4, the number of very large and bright nuclei is very high, but the oviduct lumen still empty. At stage 6, oocytes appear, the number of nuclei with intermediate size has decreased and calyx fluid is seen in the lumen of the oviduct. O, Oocytes; CF, calyx fluid. Bar, 100 μ m.

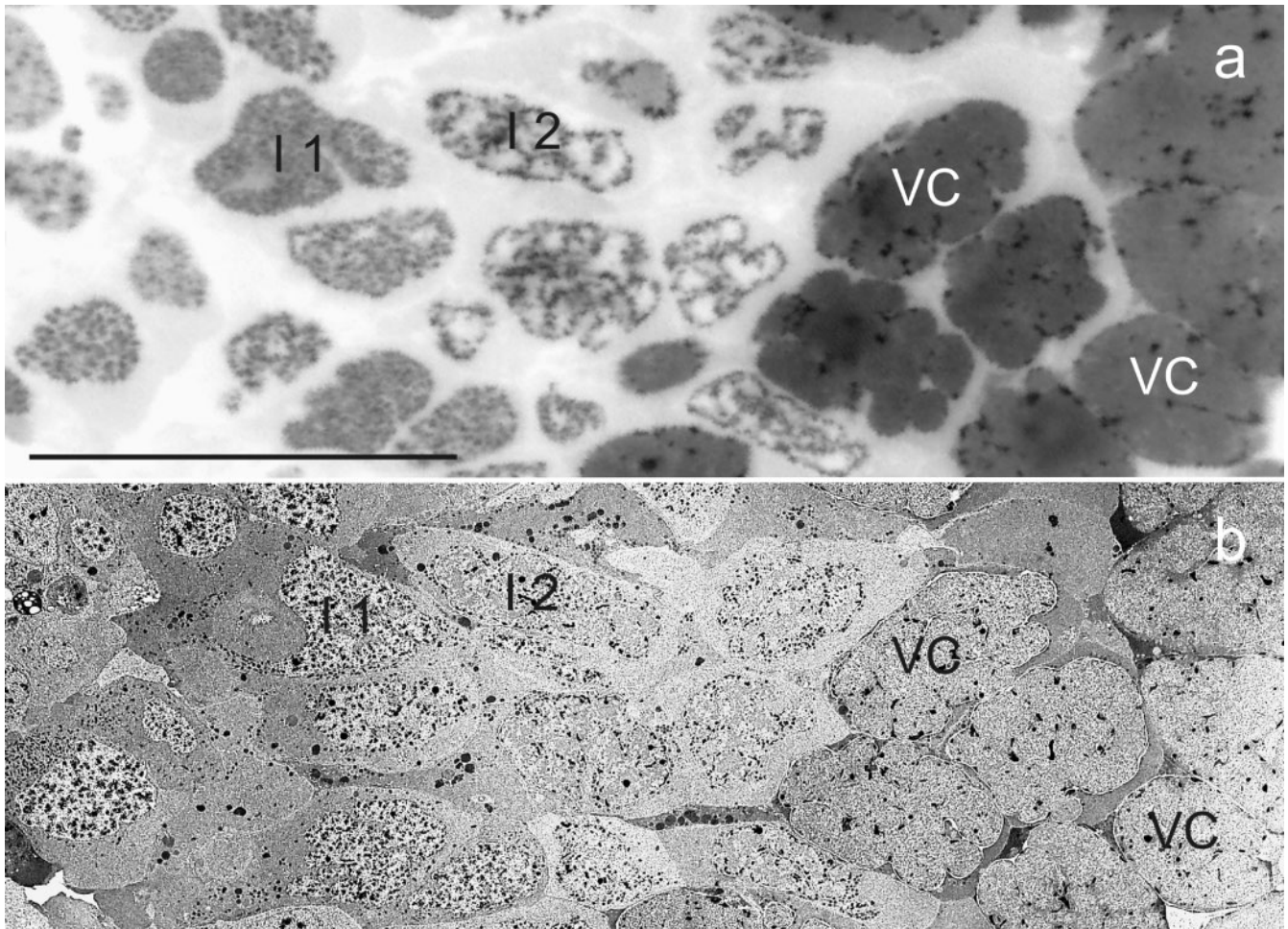


Fig. 3. Comparison of a Hoechst-stained section (a) with a consecutive section observed by electron microscopy (b) using an LR-White-embedded ovary. The same cells are seen in both sections of a region in a stage 6 ovary. The same early intermediate stage (I 1) and late intermediate stage (I 2) and mature virion-containing calyx cells are labelled in both pictures. The I 1 cells have a highly lobulated nucleus containing patches of dense heterochromatin, the I 2 cells have a roundish nucleus with a similar electron density to that of the cytoplasm and the mature calyx cells are swollen and lobulated and contain virions. I 1, Early intermediate-stage calyx cell; I 2, late intermediate-stage calyx cell; VC, mature virion-containing calyx cells. Magnification in (a) and (b) is the same; bar, 50 μm .

LR-White-embedded ovaries had to be used (see Methods). A direct comparison of Hoechst-stained cells with the ultrastructural analysis is shown in Fig. 3, illustrating a region of the stage 6 ovary presented in Fig. 2, which contains several developmental stages of calyx cells (from left to right). The partially enlarged nuclei, which appear roundish and dotted in the Hoechst stain, are also roundish in the electron microscope and the dots represent patches of dense heterochromatin. The irregularly shaped nuclei of intermediate size and brightness in the Hoechst stain are either nuclei that are highly lobulated and rich in heterochromatin (early intermediate stage, I 1) or swollen and lobulated and with a similar electron-density in the nucleus and cytoplasm (late intermediate stage, I 2). The very large and bright nuclei in the Hoechst stain appear as highly swollen and lobulated in the electron microscope and

contain virogenic stroma and virions (VC). Details are shown in Fig. 4. To get an idea of the increase of DNA in calyx cells in the course of their development, densities of Hoechst-stained sections of ovaries at stages 3b, 4 and 6 were quantified (Table 1). The densities per area are presented relative to that of non-virus cells, which were given the value 1. The average density in nuclei of intermediate-stage calyx cells (I 1 and I 2) was about 1.5 times higher than that in nuclei of non-virus cells. In virus-containing nuclei, the density was between 2.15 (stage 3b) and 2.65 (stage 6) times higher than in non-virus cell nuclei. In the calyx fluid, the density was lower than in virus-containing nuclei and was similar to that of intermediate-stage calyx cells. We also measured the increase in volume of calyx cell nuclei. For non-virus cells, the mean volume of 40 measured nuclei was $109.5 \pm 32.5 \mu\text{m}^3$, while for

Table 1. Relative DNA content per area of nuclei of non-virus cells (ovarial sheath, septum or oviduct), intermediate-stage calyx cells, virus-containing calyx cells and calyx fluid.

Densities were measured on Hoechst-stained sections of ovaries at stages 3b, 4 and 6 as described in Methods and are given as values relative to non-virus cells. The number of measured nuclei or areas (calyx fluid) is given in parentheses.

Stage	Relative DNA content per area			
	Non-virus cell	Intermediate-stage calyx cell*	Virus-containing calyx cell	Calyx fluid
3b	Mean	1 (83)	1.53 (113)	2.15 (64)
	Range		1.14–2.14	1.77–2.92
4	Mean	1 (67)	1.47 (47)	2.42 (79)
	Range		1.20–2.04	1.39–3.97
6	Mean	1 (52)	1.64 (74)	2.65 (75)
	range		1.37–1.91	2.32–3.28
				1.44 (54)
				0.96–1.89

*Early and late intermediate-stage calyx cells (I 1 and I 2) were not separately measured because they cannot easily be distinguished by Hoechst staining alone.

virus-containing calyx cells the mean volume of 20 measured nuclei was $4807.5 \pm 2305.3 \mu\text{m}^3$. Thus, the nuclear volume increased approximately 45-fold. Along with the increase in density of approximately 2.65 (Table 1), this means that the DNA content in calyx cell nuclei increased approximately 120-fold.

The development of calyx cells through their characteristic stages and the release of virions was analysed in detail at the ultrastructural level using conventional epoxy resin-embedded and osmium tetroxide post-fixed ovaries at various stages (Figs 4–6). A typical calyx cell at an early intermediate stage of development (I 1) is shown in Fig. 4(a) and (b). This type of calyx cell is first seen in ovaries at stage 3a and has a nucleus of intermediate size and DNA content. The nucleus is highly lobulated, occupies a large portion of the cell and contains many patches of dense heterochromatin. In the cytoplasm, ribosomes are very abundant. Fig. 4(b) illustrates patches with high abundance of nuclear pores, which is typical for calyx cells at this stage of development. Fig. 4(c) and (d) shows a slightly later stage of development, a late intermediate-stage calyx cell (I 2). This stage is characterized by a roundish and swollen appearance of the nucleus and a very similar electron density of nucleus and cytoplasm. Furthermore, the dense patches of heterochromatin are less abundant and in the nucleus patches of virogenic stroma and envelopes (Fig. 4d, inset) appear. The mitochondria accumulate in a row along the nuclear membrane (Fig. 4d) and are no longer distributed in the cytoplasm. Fig. 4(e)–(g) shows the final stages of calyx cell development. These cells first appear at stage 3b and have a very high DNA content. Their nucleus is extremely large and contains patches of virogenic stroma, viral envelopes and mature virions with nucleocapsids. In the younger cells of this stage, viral envelopes are more abundant than nucleocapsid-containing virions and in the oldest stages almost only mature virions are visible.

The position of the various stages of calyx cells together

with the epithelial layer and the calyx fluid in a 1-day-old adult female are shown in Fig. 5. The overview of the lower outer part of the calyx (Fig. 5a) shows that the oviduct lumen is filled with virions (in the calyx fluid) and that various stages of calyx cells are present, the latest stage being situated nearest to the oviduct lumen and the younger stages in the upper part. The epithelial cells forming the border of the oviduct lumen have differentiated into cells with apparently phagocytic activity. The higher magnifications (Fig. 5b, c) show that these cells have protrusions into the oviduct lumen and contain lysosome-like vesicles and various types of vacuoles, some also containing virions. The release of the virions from the calyx cell nuclei into the oviduct is illustrated in Fig. 6. A calyx cell in which the nuclear membrane has disintegrated is shown in Fig. 6(a) and (b). At this stage virions and cytoplasmic organelles are mixed. The next step is illustrated in Fig. 6(c) and (d) showing a calyx cell at the border of the oviduct lumen with a disintegrated nuclear membrane and a partially disintegrated plasma membrane. Toward the oviduct lumen, this disintegrating cell is lined by one of the phagocytic epithelial cells. We assume that these cells remove cellular debris from lysed calyx cells, which results in the very homogeneous calyx fluid consisting only of virions. This is the first illustration of the process of release for a bracovirus. Another section of the calyx region of a 1-day-old adult female, where eggs passing through the calyx region are visible, is shown in Fig. 7(a). This reveals that mature eggs with a chorion pass between the calyx cells and the layer of phagocytic cells on the border of the oviduct lumen. The higher magnification of two adjacent eggs (Fig. 7b) clearly shows that virions are not attached to the surface of the egg chorion. A comparison of the density of virions in a mature calyx cell, where virogenesis is complete, and in calyx fluid shows that the density is higher in calyx fluid (Fig. 7c). Fig. 7(d) shows a higher magnification of *C. inanitus* bracovirus particles in calyx fluid. The virions have a roundish to pear-like shape and contain a single cylindrical nucleocapsid of varying length.

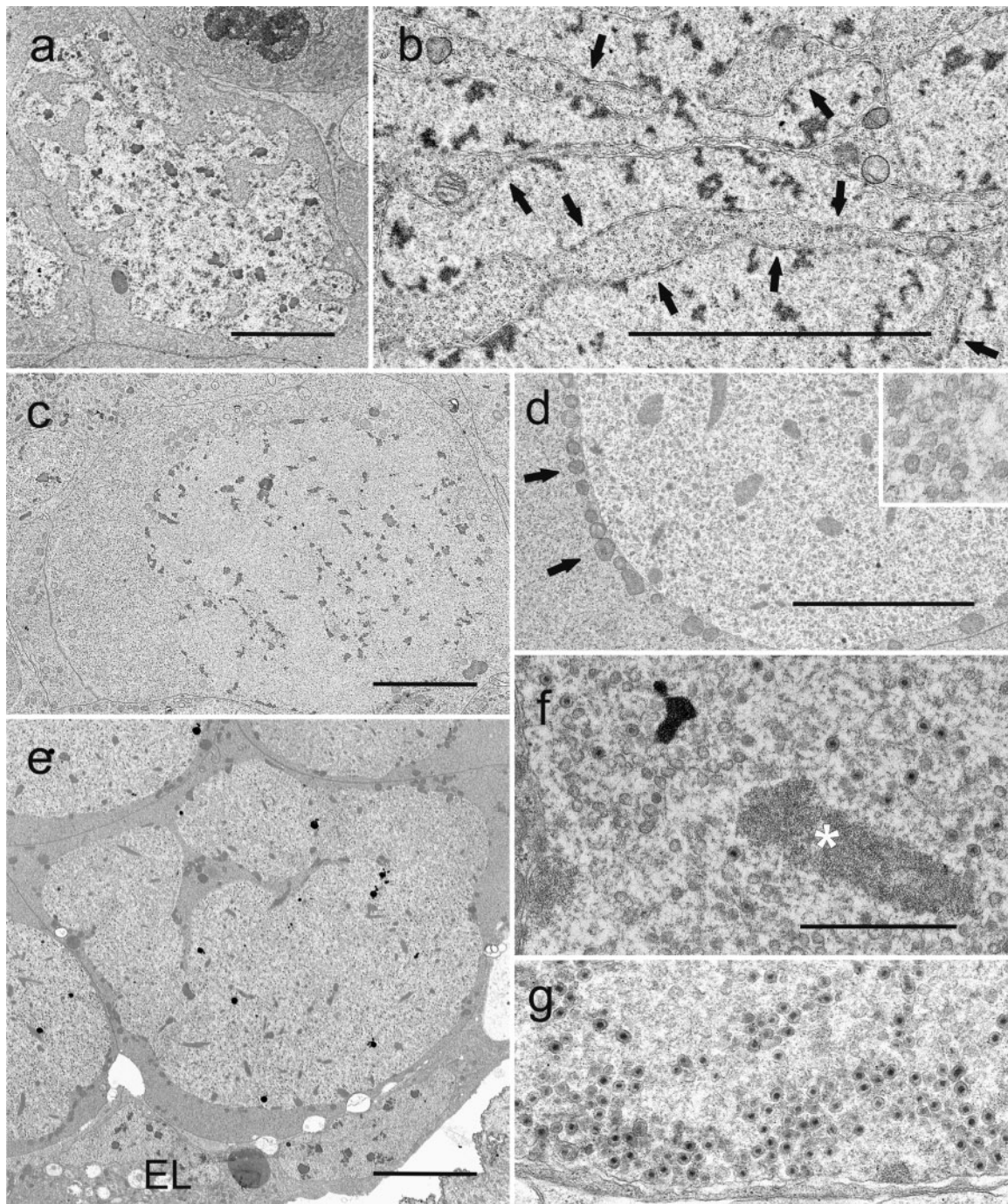


Fig. 4. Development of calyx cells as seen by electron microscopy (epoxy-embedded ovaries). Parts (a), (c) and (e) are at the same magnification and show the increase in size and the change in shape of the calyx cell nucleus during the course of development from the early intermediate stage I 1 (a) to the late intermediate stage I 2 (c) and the mature virion-containing stage (e). Parts (a) and (b) show an I 1 stage calyx cell with cytoplasm rich in ribosomes and a highly lobulated nucleus containing patches of dense heterochromatin. Part (b) shows part of a highly lobulated nucleus illustrating patches with abundant nuclear pores (arrows). Parts (c) and (d) show an I 2 stage calyx cell with a swollen nucleus having a similar electron density to that of the cytoplasm. The mitochondria (arrows) are arranged along the nuclear membrane. The inset in (d) shows a higher magnification of the nucleus and reveals the presence of viral envelopes. Parts (e)–(g) show a mature calyx cell with a large and swollen nucleus and very little cytoplasm. In (e) a cell of the epithelial layer (EL) on the border of the oviduct can be seen; (f) and (g) show higher magnifications of the nucleus with virogenic stroma (asterisk) and mature virions along with envelopes. (a)–(e) Bar, 5 μm ; magnification in (f) and (g) is the same, bar, 1 μm .

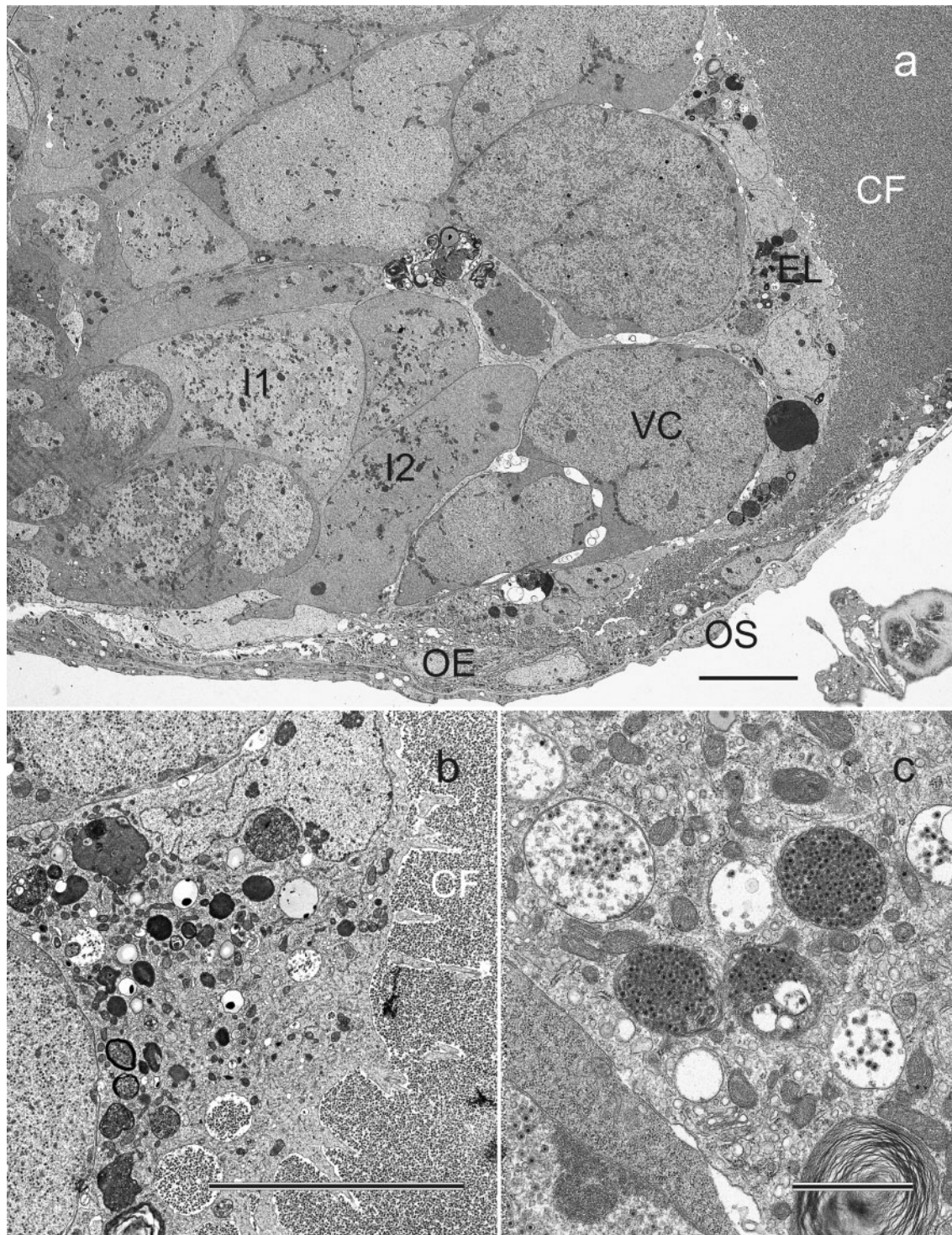


Fig. 5. Overview of part of the calyx region of a 1-day-old adult female (a) and details of the cells of the epithelial layer (b, c). (a) Outer lower part of calyx region and upper part of oviduct filled with calyx fluid. Various stages of calyx cells are seen, the younger stages in the upper part and the mature stages in the lower part; on the border of the oviduct lumen, the epithelial layer is seen and in the periphery the oviduct epithelium and the ovarian sheath, which surround the calyx region. (b) Epithelial cell on the border of the oviduct with protrusions into the calyx fluid and several types of inclusion bodies. (c) Epithelial cell on the border of the oviduct with vacuoles containing virions. EL, Epithelial layer on border of oviduct lumen; I 1, early intermediate-stage calyx cell; I 2, late intermediate-stage calyx cell; VC, virion-containing mature calyx cell; CF, calyx fluid; OE, oviduct epithelium; OS, ovarian sheath. (a, b) Bar, 10 μ m; (c) bar, 1 μ m.

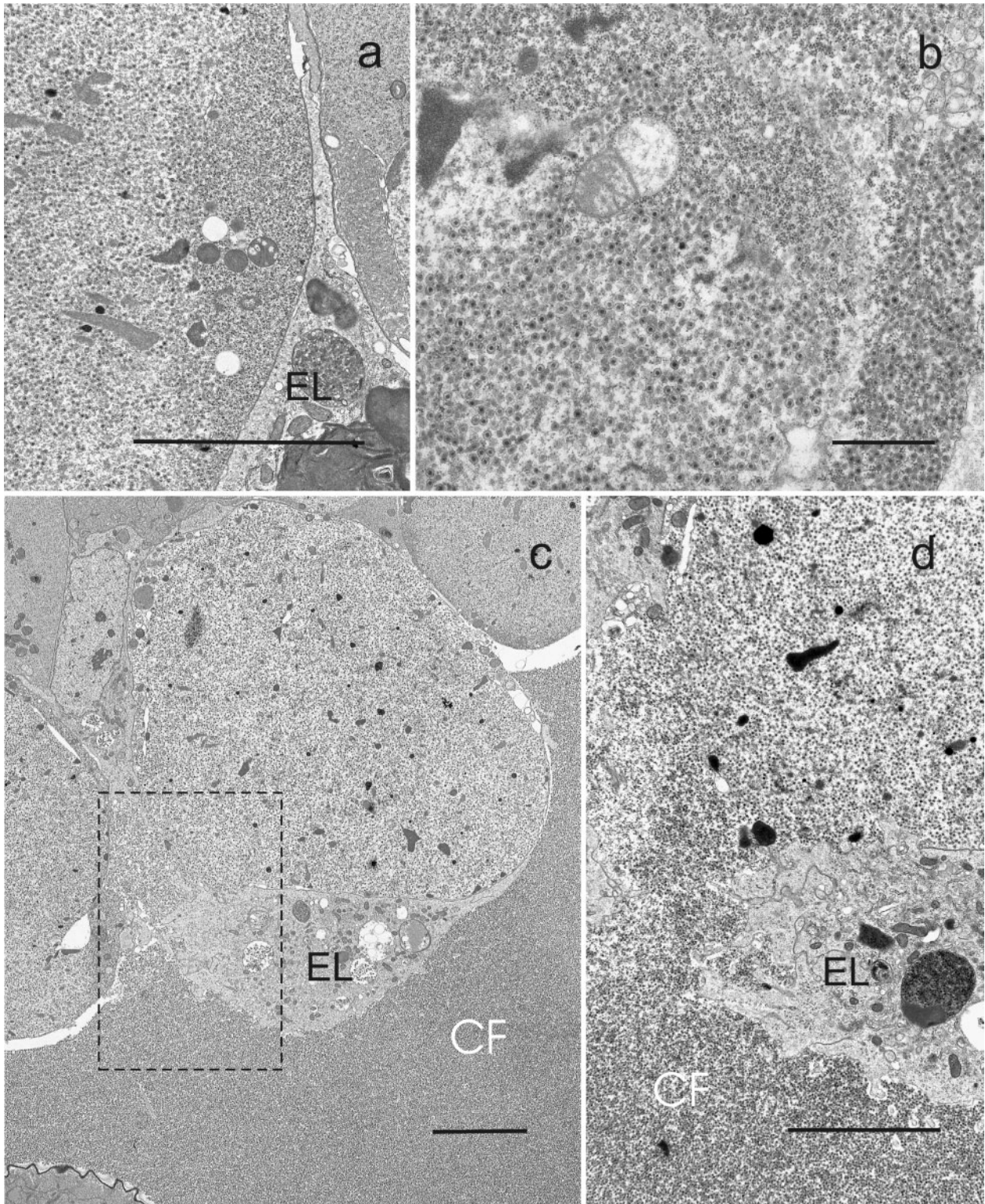


Fig. 6. Release of virions from calyx cells. (a) Calyx cell with disintegrated nuclear membrane but with the plasma membrane still intact, flanked by a cell of the epithelial layer on the border of the oviduct lumen. (b) Calyx cell with disintegrated nuclear membrane at higher magnification. Cytoplasmic organelles and virions are mixed. (c) Overview of a mature calyx cell with disintegrated nuclear membrane and partially disintegrated plasma membrane flanked by a phagocytic cell of the epithelial layer. The frame denotes the area shown at higher magnification in (d), which illustrates the flow of virions towards the calyx fluid. EL, Epithelial layer on border of oviduct lumen; CF, calyx fluid. (a, c–d) Bar, 5 μm ; (b) bar, 1 μm .

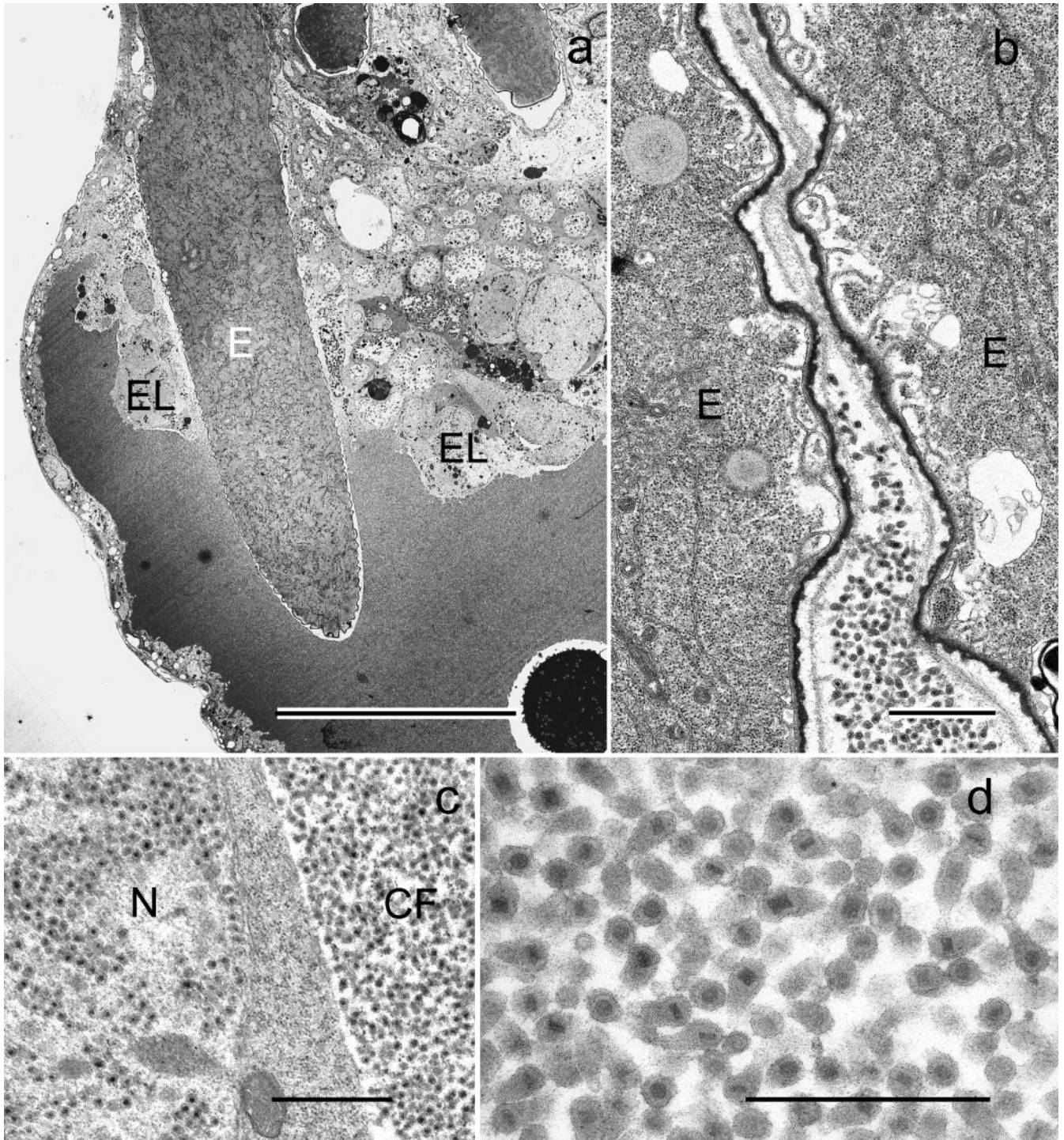


Fig. 7. Eggs, calyx cells and calyx fluid in a 1-day-old adult female. (a) Passage of egg between calyx cells and cells of the epithelial layer. (b) Higher magnification of two adjacent eggs shows that virions are freely floating and not attached to the egg surface. (c) Mature calyx cell that has not yet lysed and calyx fluid showing that virion density is higher in calyx fluid. (d) Densely packed virions in calyx fluid at higher magnification revealing pear-shaped virions with cylindrical capsids of variable length. E, Egg; EL, epithelial layer on border of oviduct lumen; CF, calyx fluid; N, nucleus of calyx cell. (a) Bar, 50 μm ; (b, c) bar, 1 μm ; (d) bar, 0.5 μm .

DISCUSSION

We have presented the first complete description of calyx cell differentiation and development, virion morphogenesis and virion release in a bracovirus. Along with the endocrinological data, the histological observations and the data on replication of non-viral DNA and proviral integrated DNA and excision of viral DNA (accompanying paper, Marti *et al.*, 2003), it is now possible to develop a composite picture of ovary and calyx cell development and the processes associated with bracovirus morphogenesis. Immediately after pupation, ecdysteroids (mainly 20-hydroxyecdysone) are high and probably induce development of the ovary from a tiny stump into a complex structure consisting of germarium/vitellarium and the calyx region (Marti *et al.*, 2003). At stage 2, ecdysteroids begin to decrease and the calyx cells are already discernible at the periphery and in the central part of the calyx of each ovariole; some calyx cell nuclei have started to increase in size and DNA content, while cell proliferation is limited and restricted to a very small region. This, along with data on replication of non-viral DNA and integrated proviral DNA (Marti *et al.*, 2003), indicates that in this early stage of development calyx cells become polyploid. In the central part, degenerating cells are seen along with calyx cells and a thin epithelial layer separates the calyx from the oviduct lumen (Fig. 1). In the next phase, stages 2–3a, nuclear volume and DNA content of the most advanced calyx cells increase further (Figs 2 and 3, Table 1) and non-viral and proviral DNA are rapidly amplified (Marti *et al.*, 2003) indicating a further increase in ploidy. The nuclei of these calyx cells (I 1) become highly lobulated and contain many patches of heterochromatin; patches with abundant nuclear pores are visible and the cytoplasm is rich in ribosomes (Fig. 4a, b). We suggest that at this stage, proteins of viral envelopes are synthesized in the cytoplasm and then imported into the nucleus. This assumption is based on the observation that immediately after this stage envelopes appear in the nucleus (Fig. 4d). In this stage (I 2), nuclei are roundish and swollen, virogenic stroma appears and the density of nuclei and cytoplasm is very similar (Fig. 4c), while the mitochondria accumulate along the nuclear membrane (Fig. 4c, d). As selective amplification of viral DNA commences only from stage 3a onwards (Marti *et al.*, 2003), these observations suggest that viral envelope proteins are encoded by wasp DNA (which is amplified through polyploidization) and not by viral DNA, which is encapsidated.

At stage 3b, the first calyx cells with virions appear; the DNA content in the nuclei of these cells is very high (Figs 2 and 3, Table 1), ploidy has reached the final level of approximately 30 and viral DNA is now selectively and intensely amplified and excised (Marti *et al.*, 2003). The volume of these nuclei is approximately 45 times greater than that of cells in the ovarial sheath or oviduct and together with the DNA density measurements (Table 1), a total increase in DNA content of approximately 120 can be calculated. Up to stage 6, amplification of proviral

DNA and excision of viral DNA continue (Marti *et al.*, 2003) and more and more calyx cells reach the final stage of development, which is characterized by the presence of mature virions in the nucleus. The increase in average relative DNA content per area of virus-containing cells from 2·15 in stage 3b to 2·65 in stage 6 (Table 1), along with the fact that excised viral DNA does not replicate (Marti *et al.*, 2003), suggests that amplification of proviral DNA and excision of viral DNA and encapsidation can occur in parallel. This is in accordance with ultrastructural data, which show that young virus-containing calyx cells contain mainly envelopes and few virions, while the oldest virus-containing cells (often located nearest to the oviduct) are filled with virions.

At the end of stage 5, some mature calyx cells in the proximity of the oviduct begin to disintegrate whereby first the nuclear membrane and then the plasma membrane fall apart leading to the release of virions into the oviduct (Figs 5 and 6), which has been demonstrated here for the first time. The calyx fluid in the oviduct consists of very densely packed virions, the density being even higher than in the nucleus (Fig. 7c). It appears that the layer of epithelial cells that form the border of the oviduct lumen play an important role and remove debris (Figs 5 and 6).

A comparison with other bracoviruses revealed the following. The situation in *C. inanitus* is similar to that in *Chelonus texanus* (Stoltz *et al.*, 1976, Stoltz & Vinson, 1977), as only one type of virion is formed and as each virion contains only one nucleocapsid. In *C. inanitus* we could show that each nucleocapsid contained only one segment of viral DNA (Albrecht *et al.*, 1994). In *C. congregata*, *Cotesia marginiventris* and *Cotesia kariyai* virions contain several nucleocapsids (Stoltz & Vinson, 1977; Hamm *et al.*, 1990; De Buron & Beckage, 1992; Tanaka *et al.*, 2002) and it is not known whether single nucleocapsids consist of single segments of viral DNA. In *C. congregata*, middle and late pupal stages and adults have been analysed and ovary development was correlated with a characteristic increase in pigmentation (Pasquier-Barre *et al.*, 2002) as is the case with *C. inanitus* (Albrecht *et al.*, 1994). Similarly, a stage of calyx cell development with a highly lobulated nucleus and patches of heterochromatin was also seen, followed by a stage with the appearance of virogenic stroma (Pasquier-Barre *et al.*, 2002). In *C. marginiventris* only adult females were analysed (Hamm *et al.*, 1990), but the different stages of calyx cells described and the changes in shape and size of the nucleus were similar to those seen in *C. inanitus*. These authors also proposed that mature calyx cells disintegrate and mentioned epithelial phagocytic cells; phagocytic cells were also seen in *C. congregata* (De Buron & Beckage, 1992) but their function remained unclear. In *C. kariyai*, a 13-fold increase in the rate of DNA synthesis in the calyx region was observed in the course of pupal–adult development (Tanaka *et al.*, 2002).

A comparison with other ichnoviruses revealed the following. In two ichneumonids, ovary development and

ichnovirus morphogenesis have been analysed, namely *Campoletis sonorensis* (Norton *et al.*, 1975, Norton & Vinson, 1983) and *Hyposoter didymator* (Volkoff *et al.*, 1995). As in *C. inanitus*, the development of the ovary and the appearance of polydnviruses and excision of viral DNA are correlated with the pigmentation pattern of the pupae (Norton & Vinson, 1983; Webb & Summers, 1992; Volkoff *et al.*, 1995) and the calyx cell differentiation begins shortly after pupation (Volkoff *et al.*, 1995). Calyx cell nuclei of *H. didymator* first increase in size, then pass through a lobulated stage with patches of heterochromatin followed by a stage with virogenic stroma and less heterochromatin (Volkoff *et al.*, 1995), similar to the situation in *C. inanitus*. However, the final stages of virogenesis are different, as release of ichnoviruses involves budding through the nuclear and plasma membranes (Norton *et al.*, 1975; Volkoff *et al.*, 1995). A major difference in the situation with *C. inanitus* is the finding that some calyx cells also appear to be secretory in addition to producing polydnviruses and that the calyx fluid contains flocculent material in addition to polydnviruses (Volkoff *et al.*, 1995).

In *C. inanitus*, polydnviruses are not attached to the surface of the mature eggs (Fig. 7a, b). Similar observations were made with the *C. marginiventris* bracovirus (Hamm *et al.*, 1990), the *C. congregata* bracovirus (De Buron & Beckage, 1992) and the ichnoviruses of *Diadegma terebrans* (Krell, 1987) and *C. sonorensis* (Norton *et al.*, 1975). In contrast, the ichnovirus of *Tranosema rostrale* was found to form a particulate coat around the egg (Cusson *et al.*, 1998). In conclusion, it appears that ovarian morphogenesis and the early steps in calyx cell development are similar in polydnvirus-producing braconids and ichneumonids. Thus, despite the fact that the association of braconids and ichneumonids with viruses seems to have occurred independently (Whitfield, 1997), the way of propagating these viruses is similar and involves the development of a particular cell type, the calyx cells, which are directed to produce these unique viruses.

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