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Replication of a herpes-like virus in larvae of the flat oyster *Tiostrea chilensis* at ambient temperatures

P. M. Hine*, B. Wesney, P. Besant

National Institute of Water and Atmospheric Research, PO Box 14-901, Kilbirnie, Wellington, New Zealand

ABSTRACT: Veligers removed from brooding *Tiostrea chilensis* (Philippi, 1845) experienced ~95% mortalities over 3 to 4 d at 16 to 18°C that appeared to be associated with a herpes-like virus. Ultrastructural observations of post-removal veligers showed the presence of early viral replication or putative latent stages at 4 h, all stages of replication at low levels at 24 h, which increased to high levels at 48 h, followed by mortalities at 72 h onwards. Initially, infected interstitial or epithelial cells had an enlarged nucleus with a wavy outline in which heterochromatin was marginated. With continued increase in size, nuclei became smooth in outline with reduction or loss of heterochromatin. Capsids with lucent cores (LCC) and empty capsids appeared in the nucleus, often in association with tubular structures ~65 nm in diameter that were composed of subunits in a helical configuration that contained a tubular core ~35 nm in diameter. Empty capsids and LCC sometimes occurred in paracrystalline arrays. Partial nucleolar disaggregation and encapsidation of dense fibrillar material preceded envelopment entering and de-envelopment leaving the perinuclear cisterna, tegumentation in cytoplasmic vesicles, and egress. Groups of dense cytoplasmic filaments 30 to 35 nm in diameter occurred in some infected cells. Apparently normal cells with a few intranuclear empty capsids and/or LCC at 4 h post-removal may represent latent infections. Replication was not observed in larvae held at 24 to 27°C, but a few cells had enlarged hypochromatic karyolytic nuclei, and 1 to 2 capsids were observed in them, at 48 h. Viral replication was similar to that of ranid herpesvirus 1 (Lucké tumour) infections. This is the fifth ostreid species from which herpesviruses have been reported.

KEY WORDS: Herpesvirus · Mortality · Oysters · *Tiostrea chilensis* · Replication · Ultrastructure · Cytopathology · Latency

INTRODUCTION

Replication of herpes-like viruses has been reported from *Crassostrea gigas* larvae (Hine et al. 1992, Nicolas et al. 1992, Le Deuff et al. 1994, 1995, 1996, Renault et al. 1994b, 1995) and spat (Renault et al. 1994a), *Crassostrea virginica* adults (Farley et al. 1972), *Ostrea edulis* spat (Comps & Cochennec 1993) and *Ostrea angasi* adults (Hine & Thorne 1997). Mortalities are usually associated with elevated temperatures (Farley et al. 1972, Hine et al. 1992, Nicolas et al. 1992, Renault et al. 1994a, b, 1995, Le Deuff et al. 1996), with only low levels of virogenesis at lower temperatures (Farley et al. 1972, Le Deuff et al. 1996). Details of replication vary between species, but it is not known whether 1 or more herpes-like viruses are involved.

To investigate whether herpes-like virus transmission can occur between larval *Crassostrea gigas* and larval flat oysters *Tiostrea chilensis*, we cohabited larvae at 24 to 27°C, the permissive temperature for *C. gigas* herpesvirus replication (Hine et al. 1992, Le Deuff et al. 1996). Larvae were also cohabited at the *C. gigas* virus non-permissive temperature of 16 to 18°C, and larvae of both species were cultured separately at both temperature ranges, as controls. Two successive cohabitation experiments had to be terminated in <4 d because of high mortalities among flat oysters, including the controls. A further attempt at culturing flat oyster larvae alone at both temperatures also had to be terminated in <4 d because of high (80 to 90%) mortalities. Examination of moribund flat oyster

*E-mail: m.hine@niwa.cri.nz

larvae held at 16 to 18°C showed the presence of herpes-like viruses and, late in the experiment, numerous bacteria. A fourth attempt at holding flat oyster broods at 16 to 18°C did not result in mortalities.

This paper describes the replication and course of infection of a herpes-like virus in *Tiostrea chilensis* larvae at 16 to 18°C, and the associated cytopathology, and considers whether the virus occurs as a latent infection in oysters.

MATERIALS AND METHODS

The data presented are based on 2 cohabitation experiments and the third experiment with only *Tiostrea chilensis* larvae. Flat oysters *T. chilensis* were collected from Wellington harbour by divers, opened, and late stage veligers from 3 to 5 broods were pooled. Larvae of *Crassostrea gigas* were obtained from natural spawning at the hatchery at Mahurangi, the site of earlier herpesvirosis (Hine et al. 1992). Larvae were placed into 8 tanks, 4 of which were stocked at 1 larva ml^{-1} at 16 to 18°C, with 4 other tanks stocked at 5 larvae ml^{-1} at 24 to 27°C. These densities span the 3 larvae ml^{-1} used by Le Deuff et al. (1994). *C. gigas* larvae were always stocked at the same densities as *T. chilensis* larvae. In the third and fourth experiments in which *T. chilensis* larvae were cultured alone, larvae were stocked at 5 larvae ml^{-1}. *T. chilensis* and *C. gigas* larvae were separated by a 25 µm mesh gauze membrane. UV-filtered seawater was used and larvae held without feeding to reduce bacterial contamination. Flat oyster larvae have rich lipid reserves and can survive without feeding for several days. On initial removal, and at 4, 8, 16, 24, 48, 72 and 80 h, a small sample of larvae of each species swimming in the lower water column was fixed in 2.5% glutaraldehyde in 0.22 µm filtered seawater, and processed for transmission electron microscopy (TEM) as previously described (Hine et al. 1992). At 24, 48, 72 and 96 h, the living and dead larvae in each tank were gently mixed, and the proportion of live larvae to dead larvae among 50 larvae/time determined by trypan blue exclusion. Subsequently a fourth experiment was carried out in which larvae from 3 oysters were maintained separately at 16 to 18°C for 6 d.

RESULTS

Mortality

The mortality rates over 1 to 3 d among *Tiostrea chilensis* larvae in cohabitation experiments were similar in each tank at both temperatures (Fig. 1), and

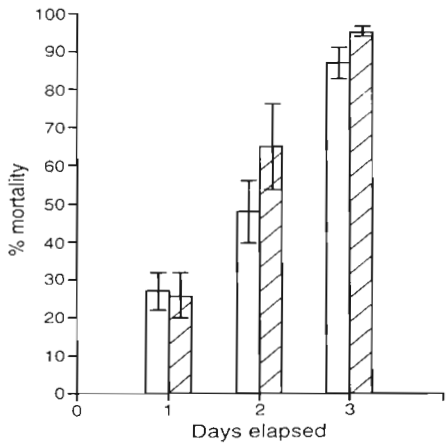


Fig. 1. *Tiostrea chilensis*. Daily mortality at 16 to 18°C (open histogram) and 24 to 26°C (hatched histogram) based on 6 tanks at each time and temperature

from 2 d onwards bacterial contamination was apparent in both groups. Similar mortality rates, but less bacterial contamination, occurred in the third experiment in which *T. chilensis* larvae were cultured separately. In contrast, in the fourth experiment no mortality resulted over 6 d and TEM failed to show any signs of viral infection. No mortalities occurred among cohabited and control *Crassostrea gigas* larvae, and no viruses were observed by TEM. The description below is therefore based on *T. chilensis* infections.

Pathogenesis and virogenesis at 16 to 18°C

Virus replication was common in mantle epithelial and interstitial cells, but in digestive tract epithelial cells intranuclear developmental stages were less common. Normal mantle epithelial and interstitial cells had a central elongated ovoid nucleus with a few large islets of heterochromatin, blocks of marginated heterochromatin, and a prominent round nucleolus comprising a dense nucleolonema enclosing a lighter pars amorpha (Fig. 2a). The cytoplasm contained a few long strands of rough endoplasmic reticulum (rER), many ribosomes, Golgi cisternae, and a few mitochondria.

Replication occurred in the following stages; the temporal occurrence of stages is shown in Table 1. Sizes given are based on 50 measurements. In stage I, the nuclei of a few apparently normal interstitial cells contained 1 to 4 empty capsids per section, pentagonal or hexagonal in cross-section [Fig. 2a (inset)], or capsid-like particles 97 ± 6 nm in diameter enclosing electron-lucent ring-shaped cores 55 ± 4 nm in diameter (lucent core capsids, LCC). Stage II cells had normal nuclei, but either virions apparently entering the cell (Fig. 3), or nucleocapsids (Fig. 4) and empty capsids in the cyto-

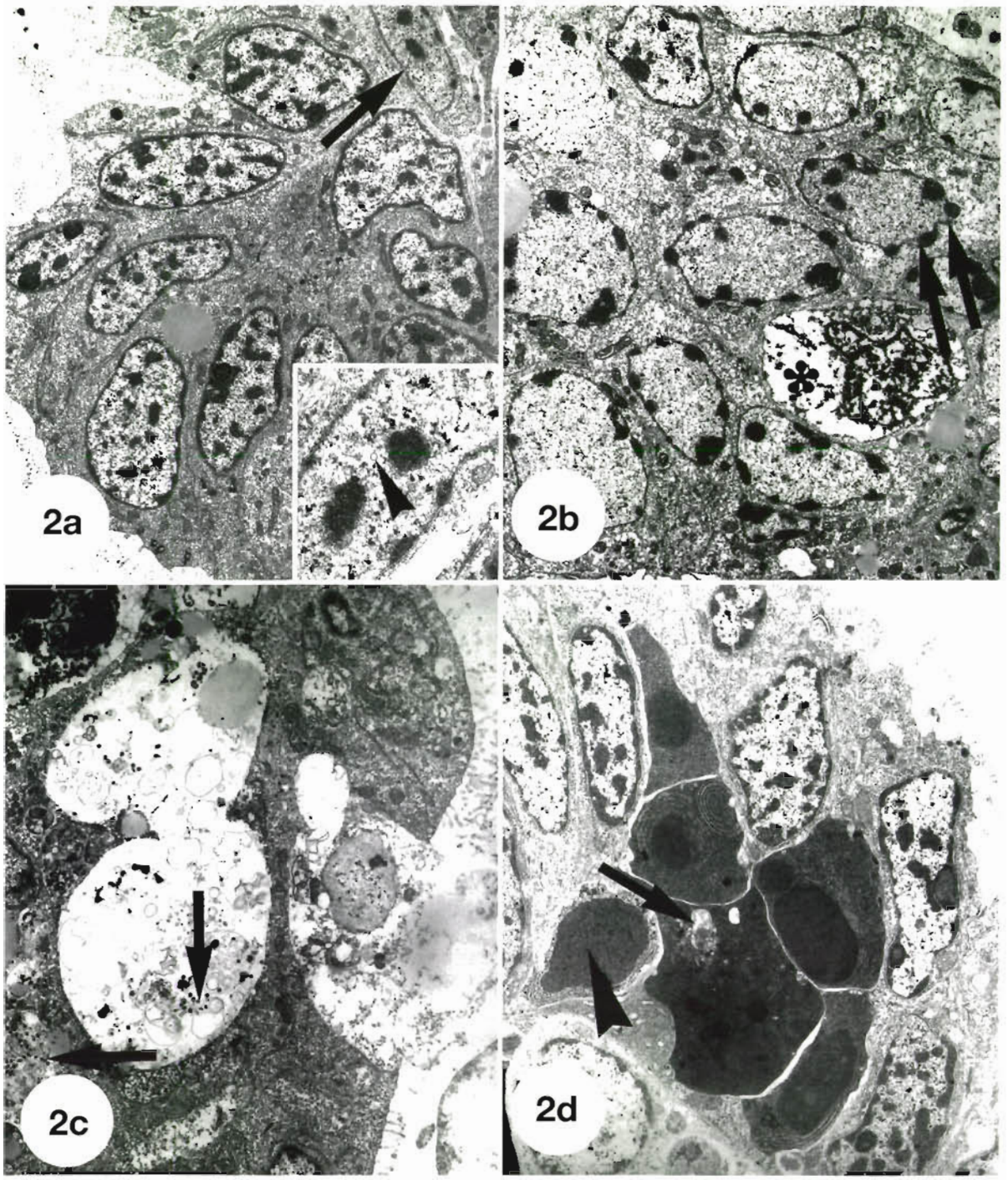


Fig. 2. *Tiostrea chilensis*. Appearance of mantle epithelial and interstitial cells; 16 to 18°C (a) Apparently normal epithelial and interstitial cells with nuclei containing a nucleolus, islets of heterochromatin (4 h), ×4430 Inset: Nucleus of a cell (arrow in Fig 2a) containing an empty capsid (arrowhead in inset), ×10 500 (b) Margination of heterochromatin in interstitial cell nuclei, sometimes into small lobes (arrows). Most cells at stage III, and 1 lysed (stage V) cell (✱) (2 d), ×4340 (c) Spaces left by lysed (stage V) interstitial cells, containing cellular debris and virions (arrows) (2 d); ×5030. (d) Cluster of hyperdense (stage VI) interstitial cells, one with only a dense nucleus (arrowhead) and one containing empty capsids (arrow) (3 d); ×6450

plasm. Nucleocapsids also occurred in secondary lysosomes near the apical surface of digestive tract epithelial cells (Fig. 5), suggesting they had entered the cell from the gut lumen. Stage III included all nuclear stages: (a) nuclear membrane proliferation and folding; (b) heterochromatin margination, often into lobes, with sub-

Table 1. Herpes-like virus in *Tiostrea chilensis*. The numbers of cells at infection stages I to VI in relation to temperature and sampling time. Based on 2 sections through 5 larvae/time/temperature, each examined for 15 min. Descriptions of the stages are given in 'Results'

Time fixed	Stages I	II	III	IV	V	VI
Ambient 16–18°C						
0 h	2					
4 h	4	2	2		1	1
8 h		1				
16 h					3	1
24 h	3		6	2	8	6
36 h			8	8	2	1
48 h		1	42	30	14	1
72 h			2	4	1	2
80 h						
Elevated 24–27°C						
0 h	1					
4 h		6			3	
8 h	1					
16 h						
24 h						
36 h						
48 h			3			
80 h						

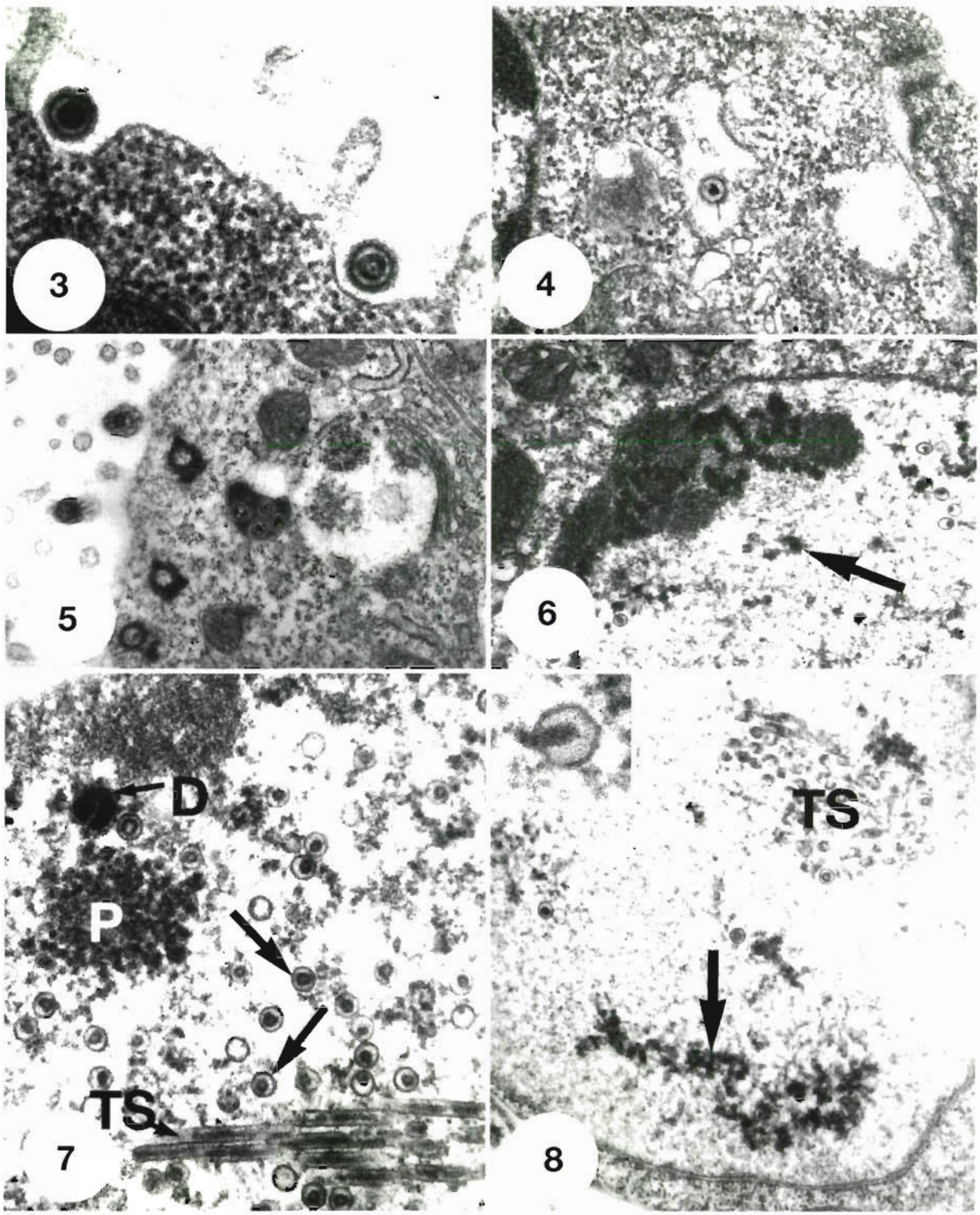
sequent reduction or loss of chromatin (Fig. 2b); (c) partial nucleolar disaggregation into an unravelled elongated strands of nucleolonema in close apposition to heterochromatin (Fig. 6), with eventual fragmentation of the nucleolonema; (d) appearance of groups of perichromatin granules and a dense body with dark granular content (Fig. 7); (e) presence of LCC (Figs. 7 & 9) and empty capsids 97 ± 5 nm in greatest diameter; (f) appearance of dense flocculent material (Fig. 8) which became encapsidated (Fig. 8 inset); (g) formation of a rod around which a dense core formed; (h) occasional occurrence of a large ovoid body of fine granular material (Fig. 10); (i) occurrence of tubular structures (TS), comprising an outer tubule 66 ± 12 nm and inner tubule 30 ± 5 nm in diameter (Figs. 7, 9 & 10), with a helical substructure in the outer tubule. These sometimes extended from the nuclear membrane across the nucleus to the nuclear membrane opposite. Transverse sections of TS showed a tube within a tube arrangement similar in appearance to LCC, which were usually abundant near TS (Fig. 8). Tubule components occasionally rearranged to form tubules 95 to 100 nm in width (Fig. 11). In ~5% of 100 infected cells, groups of TS surrounded large paracrystalline arrays comprising mainly LCC, but also some empty capsids, and a few capsids with partially formed cores and nucleocapsids 107 ± 6 nm with nucleoids 62 ± 4 nm in diameter (Fig. 9).

At stage IV, development in the cytoplasm was apparent. Nucleocapsids were enveloped in folds of the inner nuclear membrane, which sometimes formed intranuclear sacs (Fig. 12). The nuclear membrane was often dilated due to a dense matrix and contained bilaminar vesicles 70 nm in diameter (Fig. 9), which also occurred in rER, with which the perinuclear cisterna appeared to be continuous. Ribosomes were arrayed along the cytoplasmic face of the outer nuclear membrane. Mitochondria appeared normal. Large concentric arrays of rER were rarely seen. Bundles of dense cytoplasmic filaments 30 to 35 nm in width occurred in ~5% of 100 infected cells (Fig. 13), but were never observed in apparently uninfected cells.

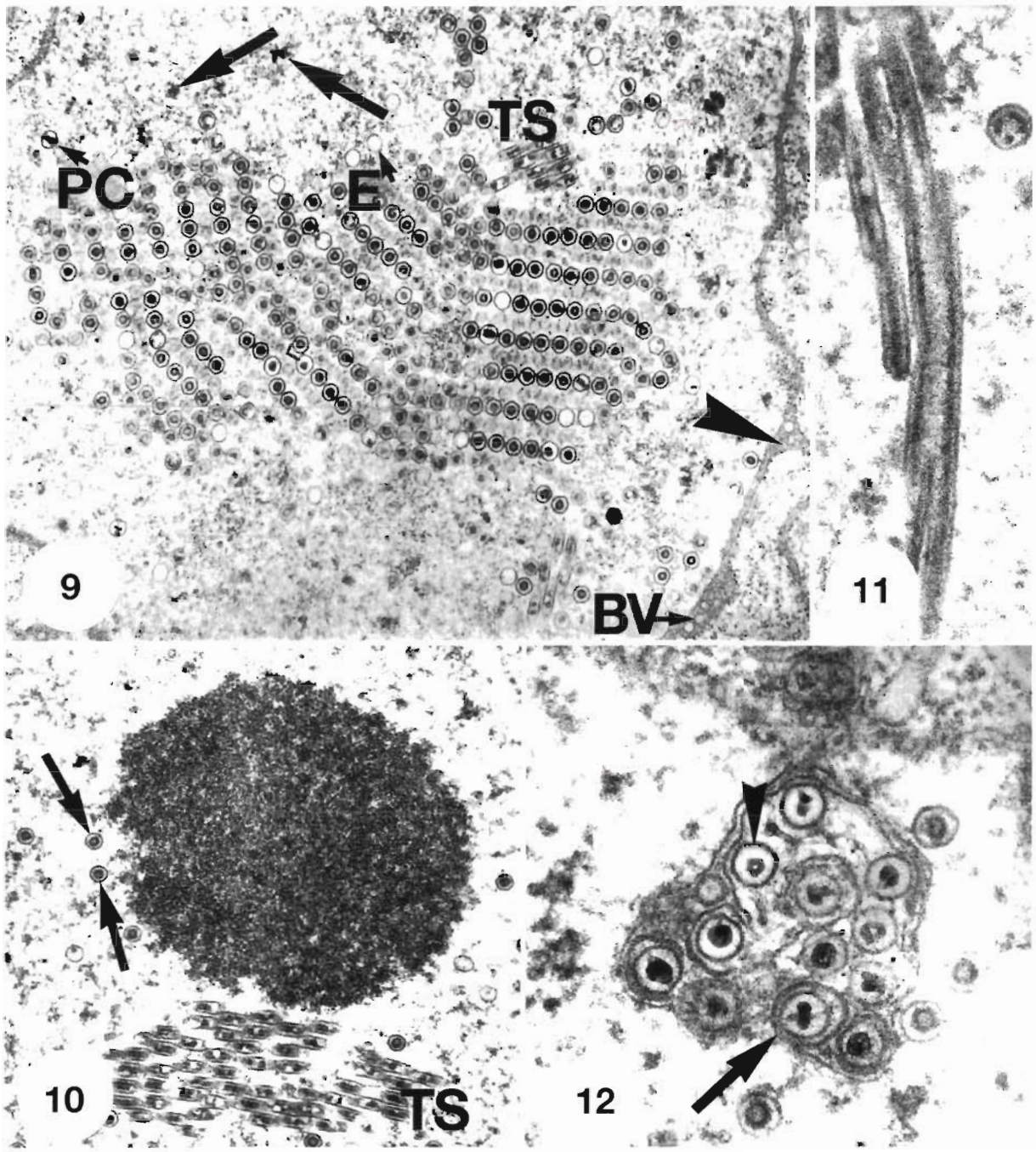
Nucleocapsids were present in the cytoplasm, but empty capsids and LCC rarely occurred in the cytoplasm, except following karyolysis. Nucleocapsids were in contact with vermiform smooth vesicles containing moderately osmiophilic material, or enveloped in ovoid vesicles containing a similar substance (Fig. 14). At this stage a tegument became apparent between the capsid and envelope. The vesicular material appeared to condense, leaving tegumented virions in lucent vacuoles. Empty capsids and LCC did not enter vesicles or become enveloped. Apparent budding from the plasma membrane was infrequently observed (Fig. 15), but extracellular virions containing capsids 101 ± 9 nm, enclosing nucleoids 63 ± 5 nm, were abundant (Fig. 16).

At stage V, necrotic cells, often with a very convoluted lysed nucleus, harboured a few intranuclear and cytoplasmic empty capsids, or capsids with abnormal cores. Spaces left by lysed cells contained necrotic debris, nucleocapsids and virions (Fig. 2b, c). A different process of cell death was observed at stage VI. Infected nuclei were homogeneous in content and dense, and the cytoplasm comprised dense background substance, particularly ribosomes, and organelle remnants including concentric arrays of endoplasmic reticulum (ER). A few viral particles at different stages of development were apparent in many of these hyperdense cells (Fig. 17), which were frequently grouped in foci (Fig. 2d).

These stages were applicable to most infected cells, the main exception being cells in which cytoplasmic stages occurred, but few nuclear stages were apparent. It appeared that these cells produced few virions, but that these viruses were possibly produced continuously over a long period. In some larvae, many adjacent cells showed synchronous replication (Fig. 2b). In others development was asynchronous in adjacent cells. This biased the numbers in Table 1, which should only be taken as a general indication of the stages present. Even so, it appeared that early stages occurred at 4 h, nuclear and cytoplasmic stages were present at 24 h and were abundant by 48 h, after which most larvae died. However, infected lysing and hyperdense cells were also present during the first 24 h.



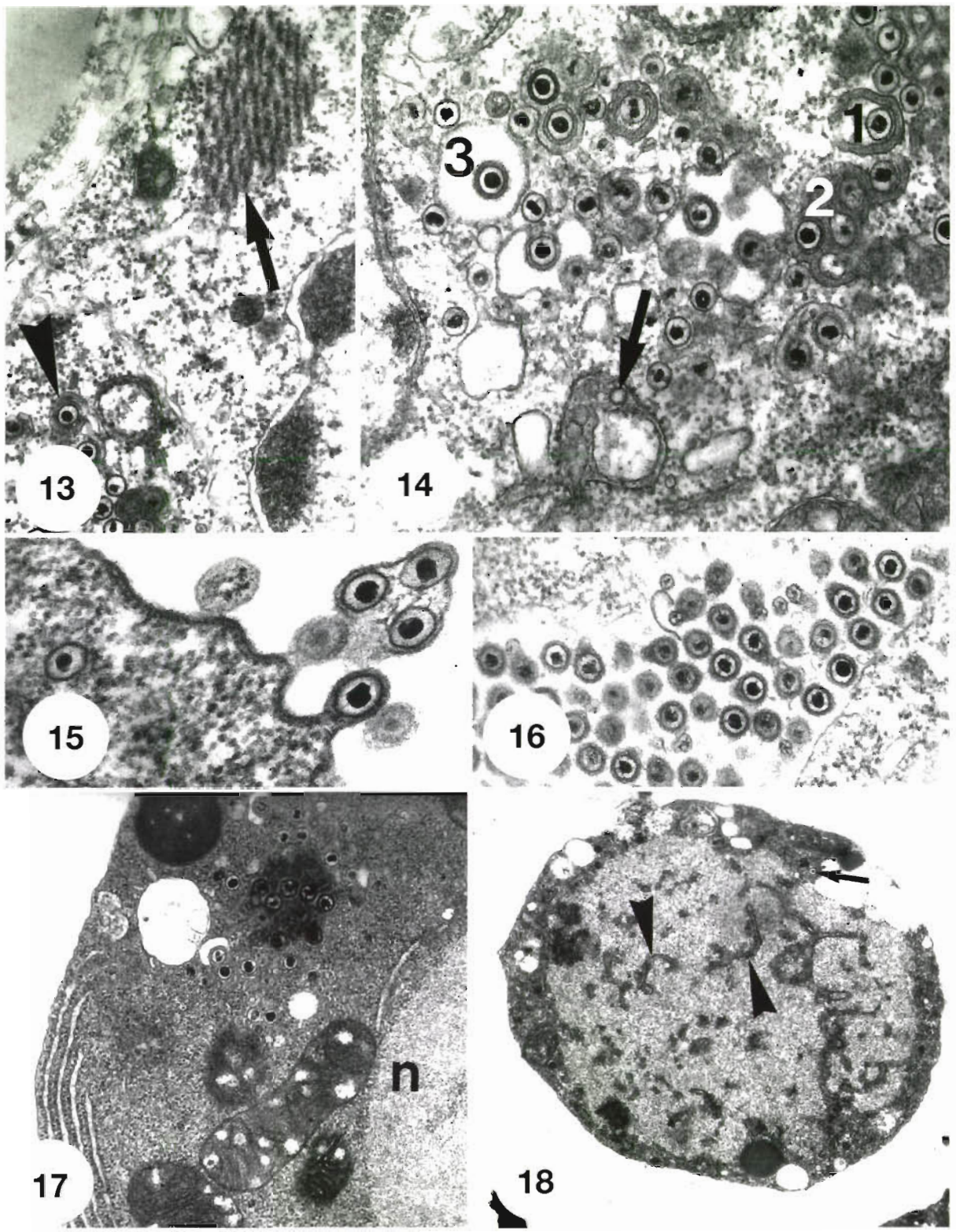
Figs. 3 to 8. Herpes-like virus in *Tiostrea chilensis*. Early stages of replication at 16 to 18°C. Fig. 3. Virions in indentations of the cell surface between mantle epithelial cell microvilli; apparently prior to entry (2 d); ×77 140. Fig. 4. Coated nucleocapsid in the cytoplasm of a mantle epithelial cell with a normal nucleus (stage II) (4 h); ×45 000 Fig. 5. Apical surface of a ciliated digestive tract epithelial cell showing a lysosome-like body containing 4 nucleocapsids (stage II) (2 d); ×22 200 Fig. 6 Disaggregation of the nucleolus of an infected interstitial cell into fibrillar and granular components Some capsids with partial cores lie next to putative viral DNA (arrow) (stage III) (2 d); ×23 130 Fig. 7. Edge of nucleus of an interstitial cell showing penchromatin granules (P), a dense body (D), tubular structures (TS), and lucent core capsids (LCC) (arrows) (stage III) (2 d); ×45 710 Fig. 8. TS, LCC, and flocculent material thought to be viral DNA (arrow) (2 d); ×25 560. Inset. encapsidation of dense material (stage III) (2 d). ×106 120



Figs 9 to 12 Herpes-like virus in *Tiostrea chilensis* Intranuclear features at stage III (Figs. 9 to 11) and stage IV (Fig. 12) (16 to 18°C) Fig. 9 Paracrystalline array of mainly lucent cores (LCC). Also present are. empty capsids (E), capsids with partial cores (PC), tubular structures (TS), putative viral DNA (arrows), dark matrix in the nuclear membrane (arrowhead) containing bilaminar vesicles (BV) (3 d), ×27 380. Fig 10. Ovoid fine granular mass, TS, and LCC (arrows) (stage III) (2 d); ×26 920. Fig. 11. TS 100 to 65 nm in diameter with a 35 nm inner tubule (1 d); ×68 570 Fig. 12 Intranuclear sac surrounded by LCC, containing nucleocapsids with (arrow) and without (arrowhead) envelopes (2 d), ×71 140

Pathogenesis and virogenesis at 24 to 27°C

Except for initial entry stages and lysed cells at 4 h, and a cell at stage I at 8 h, neither viral developmental stages or cytopathology suggesting the presence of virus were observed until 48 h (Table 1). At that time, of a few dense cells seen with enlarged hypochromatic nuclei, one was observed with perinuclear capsids and abnormal membranous inclusions in the nucleoplasm (Fig 18).



Figs. 13 to 18. Herpes-like virus in *Tiostrea chilensis* Stages IV to VI at 16 to 18°C, except Fig 18 (24 to 27°C) Fig. 13. Cytoplasmic dense filaments (arrow), and nucleocapsids acquiring a tegument (arrowhead) (stage IV) (2 d), ×39 000 Fig. 14. 1: Nucleocapsids by vermiform or circular vesicles, 2. enveloped nucleocapsids in vesicles with a dense matrix; or 3: lucent vesicles. Note the dense matrix in rough endoplasmic reticulum (rER) containing bilaminar vesicles (arrow) (stage IV) (2 d); ×58 150. Fig. 15. Virion apparently budding from the plasma membrane of a mantle epithelial cell (stage IV) (2 d); ×67 060. Fig. 16 Extracellular virions (2 d); ×40 010. Fig. 17. Hyperdense (stage VI) cell with an homogeneous nucleus (n), and cytoplasmic nucleocapsids, some apparently arrested while budding into vesicles (16 h), ×23 370. Fig. 18. Dense cell showing karyolysis and intranuclear membranes (arrowheads) probably due to convolutions of the nuclear membrane. An empty capsid and a nucleocapsid lie at the edge of the nucleoplasm (arrow) (2 d). ×13 940

DISCUSSION

The virions observed here have the features of herpesviruses of higher vertebrates, and as previously reported from oysters (Table 2) (Hine et al. 1992, Hine & Thorne 1997). Some of the features previously reported in replication of oyster herpes-like viruses were seen in *Tiostrea chilensis* (Table 3). The initial wavy folding of the nuclear membrane, and paracrystalline arrays of empty capsids have been reported from *Crassostrea gigas* (Renault et al. 1994b), but the large paracrystalline arrays of LCC have not. Ovoid bodies of fine granular material occur in *C. gigas* and *Ostrea angasi* infections (Hine et al. 1992, Hine & Thorne 1997). Although LCC in oysters have not been distinguished from nucleocapsids with mature toroidal cores, they have been illustrated in *C. gigas* infections (Renault et al. 1994a, b). The dense body with granular content resembles the groups of dark flecks reported from *O. angasi* (Hine & Thorne 1997). Envelopment in perinuclear cisternae, intranuclear sacs, and in ovoid cytoplasmic vesicles have also been reported (Hine et al. 1992, Comps & Cochennec 1993, Hine & Thorne 1997) and are common features of herpesviruses.

Virogenesis in *Tiostrea chilensis* differs from that reported in other oysters in: (1) replication at ambient temperatures, (2) partial disaggregation of the nucleolus, (3) the presence of TS, (4) LCC in paracrystalline arrays, (5) the dark matrix and vesicles in the nuclear membrane, and the continuity of the nuclear membrane with rER, (6) bundles of cytoplasmic filaments, and (7) budding from the cell surface. Some of these features are compared between oyster species, and with well-characterized herpesviruses in Table 3. As noted by Farley et al. (1972), oyster viruses resemble ranid herpesvirus 1 (Lucké tumour virus). In this study, in particular, replication at lower temperatures but inhibition of replication at higher temperatures, epitheliotropism, the partial disaggregation of the nucleolus, TS, LCC, paracrystalline arrays, and cytoplasmic filaments of 30 to 35 nm diameter resemble replication of the Lucké tumour virus (Lunger et al. 1965, Mizell et al. 1968, Stackpole & Mizell 1968, Stackpole 1969, Skinner & Mizell 1972, Wong & Tweedell 1975, McKinnell & Cunningham 1982). The TS of *T. chilensis* herpes-like virus were composed of an outer tubule with helical substructure that was occasionally 100 nm, but usually 65 nm, in diameter, containing a tubular core 35 nm in diameter. These resemble the 95, 65, and 35 nm diameters respectively reported for TS in ranid herpesvirus 1 infections (Stackpole & Mizell 1968, Stackpole 1969, Skinner & Mizell 1972). The subunits with a helical configuration have been identified as capsomeres, and it appears that LCC form from these capsomere tubules (Stackpole & Mizell 1968, Tumilowicz & Powell 1990). In turn, LCC appeared to develop into empty capsids. This interpretation is supported by the observations of Weiland et al. (1986) that electron-lucent capsids in murine cytomegalovirus (CMV) infection develop to empty

Table 2. Comparison of reported bivalve herpes-like viruses. *Tiostrea chilensis* data based on 50 measurements. MNC: mature nucleocapsid

Species/cell type	Size		Virions tailed	Study
	MNC	Virions		
Crassostrea gigas larvae and spat				
Paraspherical cells	70 ± 2	90 ± 5		Nicolas et al. (1992)
Unidentified/myocytes	97 ± 4	131 ± 9	+	Hine et al. (1992)
Connective cells of the velum	72–75			Le Deuff et al. (1994)
Interstitial cells/myocytes	80			Renault et al. (1994a)
Connective cells of velum mantle, gills. In myocytes, haemocytes	70–75	120		Renault et al. (1994b, 1995)
Velar interstitial and epithelial cells	80			Le Deuff et al. (1996)
Crassostrea virginica adults				
Cells around haemolymph sinuses	70–90			Farley et al. (1972)
Ostrea edulis spat				
Interstitial cells, haemocytes, fibroblasts	80	140–150	+	Comp & Cochennec (1993)
Ostrea angasi adults				
Haemocytes, fibroblasts	98 ± 4	135–140	+	Hine & Thorne (1997)
Tiostrea chilensis larvae and spat				
Interstitial cells, mantle and digestive tract epithelial cells	101 ± 9	130 ± 11		This study

Table 3. Comparison between α-herpesviruses, β-herpesviruses, Lucké herpesvirus and oyster herpesviruses

	α	β	Lucké herpesvirus	*Tiostrea chilensis*	*Ostrea angasi*	*Crassostrea gigas*
Nuclear membrane folding	+[5,9,11]	+[20]	+[8,16]	+[25]	–	±[22]
Reduplicated nuclear membrane	+[5,9,11]	–	–	–	–	–
Nucleolar disaggregation	+[7,9,13]	–	±[4]	±[25]	–	–
Aligned fibrils (rods)	±[9,13]	±[14]	–	–	+[24]	–
Skein-like viral matrix	–[11]	+[2,11,14,17]	–	–	+[24]	–
Dark flecks or mass	+[11,13]	±[16]	–	+[25]	+[24]	–
Intranuclear tubules	+[3,5]	+[14,19,21]	+[6,8,10]	+[25]	–	–[a]
Lattice tubules	+[3,5,7]	–	–	–	–	–
Paracrystalline arrays	+[3,5,7,9,18]	±[2]	+[1,8,10]	+[25]	–	±[22]
Latency in central nervous system	+[12,23]	–	–	?	?	?
Latency in leukocytes	–	+[23]	–	?	+?[24]	?
Latency in epithelium	–	–	+[15]	?	?	?
Cytoplasmic filaments	–	–	+[1,8,10,15]	+[25]	–	–

[1]Lunger et al. (1965), [2]McGavran & Smith (1965), [3]Murphy et al. (1967), [4]Jacob (1968), [5]Nii et al. (1968), [6]Stackpole & Mizell (1968), [7]Schwartz & Roizman (1969a), [8]Stackpole (1969), [9]Cook & Stevens (1970), [10]Skinner & Mizell (1972), [11]Smith & De Harven (1973), [12]Baringer & Swoveland (1974), [13]Luetzeler & Heine (1978), [14]Fong (1982), [15]McKinnell & Cunningham (1982), [16]Papadimitriou et al. (1984), [17]Weiland et al. (1986), [18]Wigdahl et al. (1986), [19]Biberfeld et al. (1987), [20]van Bruggen et al. (1989), [21]Tumilowicz & Powell (1990), [22]Renault et al. (1994b), [23]Murphy et al. (1995), [24]Hine & Thorne (1997), [25]This study.

[a]Present in *Crassostrea virginica* (Farley et al. 1972)

capsids. Similarly, empty capsids appear after LCC in herpes simplex virus (HSV) infection (Nii et al. 1968), and the lucent cores of LCC are not composed of DNA (Friedmann et al. 1975).

The data in Table 1 suggest that at 16 to 18°C virogenesis may be initiated following removal of veligers from the brood chamber with replication at 24 h, abundant virion production at 48 h, and larval death and reduced viral production at 72 h. This course of disease is similar to the 2 to 4 d in *Crassostrea gigas* mentioned by Le Deuff et al. (1994, 1996), but more rapid than the 8 to 10 d usually observed in *C. gigas* at ~25°C (Hine et al. 1992, Renault et al. 1994b, Le Deuff et al. 1996). It is intermediate in time between the 8 to 20 h needed for HSV to reach high virion production (Friedmann et al. 1975), and the 4 to 9 d usually required for CMV virion production (Smith & De Harven 1973). However, CMV development in macrophages can be as rapid as 24 h for envelopment in the perinucear cisterna, and cytoplasmic stages at 48 h (van Bruggen et al. 1989). It also resembles the 72 h replication time of ranid herpesvirus 1 (Stackpole 1969). The occurrence of paracrystalline arrays in some cells after 2 to 3 d, and in hyperdense cells, is similar to their occurrence late in the infection cycle of HSV (Nii et al. 1968, Schwartz & Roizman 1969a) and ranid herpesvirus 1 (Stackpole 1969).

Virus replication stages II to V resemble those of herpesviruses. The nucleocapsids in secondary lysosomes of digestive epithelial cells may represent endocytosis and destruction of virions in the gut. Similar association between nucleocapsids and lysosomes has been reported in varicella-zoster virus (VZV; Gershon et al. 1973), CMV (Lussier et al. 1974), and ranid herpesvirus 1 (Stackpole 1969) infection. However in VZV, nucleocapsids replicating in the host cell were enclosed in Golgi vesicles and lysosomes, and were inactivated prior to egress. Similarities in structure and appearance between the nuclear membrane and rER in this study, and ducts leading from the perinuclear cisterna in HSV infection (Schwartz & Roizman 1969b), suggest that the oyster herpes-like virus may effect egress by this route. However, it appeared necessary for the nucleocapsids to pass into vesicles containing dense material, thus acquiring a tegument, before envelopment and shedding. It may be that the dense matrix and bilaminar vesicles in dilated perinuclear and rER cisternae permit tegument acquisition and envelopment in the dilated ducts, prior to egress from them.

Some hyperdense (stage VI) cells were infected, but similar condensation occurred in apparently uninfected cells at death. Viral levels in the nuclear and cytoplasmic compartments of hyperdense cells were similar to those seen in some stage IV cells in which all developmental stages were present at low levels. It may be that infection can follow a chronic/low virogenesis course leading to hyperdense cells, or an acute/high virogenesis course leading to cell lysis. Quantitative differences may occur if different cell types are infected, as in fibroblasts and neurons infected with VZV (Wigdahl et al. 1986). If so, different cell types may explain asynchronous replication among some groups of cells, and synchronous replication may occur among groups of the same cell type.

Veligers dying at 16 to 18°C had extensive lesions associated with high levels of virogenesis, but in the fourth experiment at 16 to 18°C, where there was no mortality, there were no indications of viral infection. Mortality at 24 to 27°C was similar to that at 16 to 18°C, but the cause of mortality was not apparent. The elevated temperatures (24 to 27°C) may be at the upper limit of tolerance for a cold temperate oyster, such as New Zealand *Tiostrea chilensis*, which in the wild survives at 6 to 25°C (Buroker et al. 1983). If so, lack of replication at 24 to 27°C may be due to temperature making host cells abnormal and non-permissive. Interestingly, although mortality rates among herpesvirus-infected French *Crassostrea gigas* larvae held at 25 to 26°C were higher than at 22 to 23°C, at which temperature viral replication was not observed, the latter group also experienced relatively high progressive mortalities (Le Deuff et al. 1996). Dense intranuclear bodies were the only alteration seen in *C. gigas* larvae at 22 to 23°C. They were interpreted as being either an expression of viral protein in a latent infection or as representing an aborted viral cycle (Le Deuff et al. 1996). The cell with an enlarged hypochromatic nucleus and capsids seen at 48 h at 26 to 27°C in this study may also represent latent infection, as α-herpesviruses may establish latent infections at supraoptimal temperatures (Russell & Preston 1986). In HSV-infected rabbits, 1 or 2 hyperdense cells per ganglion, with a few intranuclear empty capsids, putative cores, and nucleocapsids have been reported from explanted trigeminal ganglia, the site of latent infection (Baringer & Swoveland 1974).

Stage I cells may provide further evidence of latency. Empty capsids and LCC occurred in normal host cell nuclei, whereas nuclear enlargement and heterochromatin margination normally preceded capsid formation, as in other herpesviruses (Murphy et al. 1995). The LCC and empty capsids are unlikely to derive from recent entry as unenveloped nucleocapsids move from the point of entry to a nuclear pore where the capsid remains and only viral DNA enters the nucleus (Lycke et al. 1988, Murphy et al. 1995). Stage II is consistent with this process. Normal cells with intranuclear LCC and empty capsids were patently infected, but infection appeared to be static. In latency, the viral genome may be integrated into the host cell genome or in free forms inside the nucleus without viral expression, or low levels of virus might be produced and shed in excretions (Murphy et al. 1995). The few capsids in apparently normal cells are similar to the few empty capsids, nucleocapsids and pleomorphic capsids observed in neurons infected with VZV (Wigdahl et al. 1986). Reactivation from latency and subsequent virogenesis may occur under conditions of stress, such as removal from the brood chamber in this study, or elevated temperatures (Le Deuff et al. 1996). Similarly, stress reactivates HSV and VZV infections in humans.

The virus in *Tiostrea chilensis* occurred in the absence of *Crassostrea gigas* herpesvirus (data not shown), and the *T. chilensis* virus shows several qualitative differences in replication to herpesviruses in *C. gigas* and *Ostrea angasi* (Table 3). Differences in replication in relation to host cell type or virus strain are often quantitative (Schwartz & Roizman 1969a), but differences between virus species are usually qualitative. Differences in replication in *T. chilensis* are unlikely to be due to lower temperature, as herpesvirus replication is usually consistent at an optimal temperature with mutant strains showing incomplete replication outside that range (Atkinson et al. 1978). However, it would be premature to conclude that similarities in the ultrastructure of replication between the virus reported here and ranid herpesvirus 1 necessarily mean they are more closely related to each other than are the herpesviruses of oysters. Many factors influence the details of replication, such as host species, temperature, and the cell types involved. A more experimental approach and molecular techniques are needed to clarify the inter-relationships of oyster herpesviruses, and their affinities with herpesviruses of vertebrates.

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