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Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*

R. M. Le Deuff[1], T. Renault[1,*], A. Gérard[2]

[1]Unité de Recherche en Pathologie et Immunologie Générales, [2]Unité de Recherche en Génétique et Ecloserie; Laboratoire de Génétique, Aquaculture et Pathologie, IFREMER, BP 133, F-17390 La Tremblade, France

ABSTRACT: This paper describes effects of temperature on herpes-like virus detection in Pacific oyster *Crassostrea gigas* larvae held at different temperatures. Intranuclear, intracytoplasmic and extracellular viral particles were observed in velum and mantle connective tissues of oyster larvae reared at 25–26°C. In larvae held at 22–23°C, although nuclear lesions were observed, the presence of viral particles was not detected. Results were obtained for oyster larvae with 4 different parental origins. Herpesvirus infection was found in 3 of the 4 groups of oyster larvae held in the same conditions at the higher temperature.

KEY WORDS: Herpesvirus · Pacific oyster · *Crassostrea gigas* · Larval culture · Mortality · Temperature

INTRODUCTION

The Pacific oyster *Crassostrea gigas* is the most important species of mollusc reared in the world (FAO 1989) and is of particular importance for French mariculture. However, cultivation may be endangered by the occurrence of epizootics, especially viral diseases, which are considered one of the major risks to production. Indeed, oyster mortalities were described and associated with the presence of several families of morphologically described viruses among different species of ostreids. The first was reported in adult American oysters *Crassostrea virginica* and described as a member of the family Herpesviridae (Farley 1972). More remarkably, mass mortalities of adult Portuguese oyster *Crassostrea angulata* among French livestock were caused by an iridovirus between 1967 and 1970 (Comps & Duthoit 1976, Comps et al. 1976, Comps & Bonami 1977). Other important viruses described from ostreids are members of the families Iridoviridae, Papovaviridae, Togaviridae, Retroviridae and Reoviridae (Elston 1979, Elston & Wilkinson 1985, Farley 1976, 1978, Meyers 1979).

More recently, in 1991, other viruses belonging to the Herpesviridae were associated with high mortalities of hatchery-reared larvae of *Crassostrea gigas* in France (Nicolas et al. 1992) and in New Zealand (Hine et al. 1992). Subsequently, sporadic high mortalities of larval *C. gigas* were observed in 1992, 1993 and 1994 associated with a herpes-like virus in some French hatcheries (Renault et al. 1994b). The pathogenicity of this virus was demonstrated earlier by experimental transmission of the disease to axenic *C. gigas* larvae (Le Deuff et al. 1994). Additionally, mortalities of oyster spat of *Ostrea edulis* and *C. gigas* were also associated with herpes-like viruses (Comps & Cochennec 1993, Renault et al. 1994a). These herpes-associated mortalities among ostreids all occurred during hotter summer periods. Thus, it appeared necessary to investigate the effects of temperature on the induction of these viral infections. For this purpose, we cultured *C. gigas* larvae at high (25–26°C) and low (22–23°C) temperatures and we examined the susceptibility to the disease of larvae from broodstocks of different geographical origins.

*E-mail: trenault@ifremer.fr

MATERIAL AND METHODS

Specimens. Pacific oyster *Crassostrea gigas* broodstocks originated from 4 different French locations (Marennes, La Tremblade, Brest and Arcachon) indicated in Fig. 1. Oysters from La Tremblade originated from Arcachon, but were transferred and kept in the hatchery at La Tremblade for 12 mo before the experiments. Oysters from Marennes (Charente-Maritime), La Tremblade (Charente-Maritime) and Brest (Brittany) were conditioned at the IFREMER laboratory at 20 to 22°C for 3 wk in order to improve gamete quality before spawning. Cultured oysters from Arcachon (Gironde) were used for spawns directly after being caught, without the step of conditioning in the hatchery.

Spawns and larval cultures. Gametes were recovered by strip dissection of gonads. Fertilization was performed with males and females of the same geographic origin. These respective spawns were divided in 2 groups held either at 22–23 or at 25–26°C, for 15 d. Survival rates were estimated by counting oyster larvae in a Sedgewick-Rafter counting cell S50. Dead larvae were differentiated from healthy and moribund larvae on the basis of swimming activity. For each culture, 30 larvae were measured with a micrometer and the average size calculated. Samples of larvae were fixed for transmission electron microcopy as described below.

Transmission electron microscopy. Samples of larvae were fixed (1 h, 4°C) in 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2), post-fixed (1 h, 4°C) with 1% osmium tetroxide in the same buffer, dehydrated through an ascending ethanol series (70°, 95° and 100°), partially rehydrated by two 10 min baths of cacodylate buffer and decalcified overnight with 2% EDTA in cacodylate buffer. After a second dehydration, samples were cleared with propylene oxide and embedded in Epon resin. Thick sections (1 µm) for light microscopy were stained in 0.5% toluidine blue in 1% aqueous sodium borate solution. Ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate. These sections were then examined with a JEOL JEM 1200EX transmission electron microscope at 60 kV.

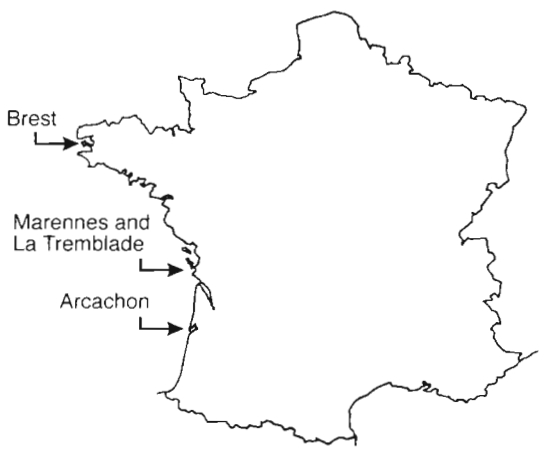


Fig. 1. Origin of cultured Pacific oyster broodstocks in France

RESULTS

Larval mortalities and growth rates

Dead larvae were differentiated from healthy (swimming actively) and moribund (swimming slowly in circles) larvae. Moreover, rounded cells and portions of velum could be seen detaching from dead larvae. Survival rates of the different larval cultures are shown in Fig. 2. Different results were obtained for groups

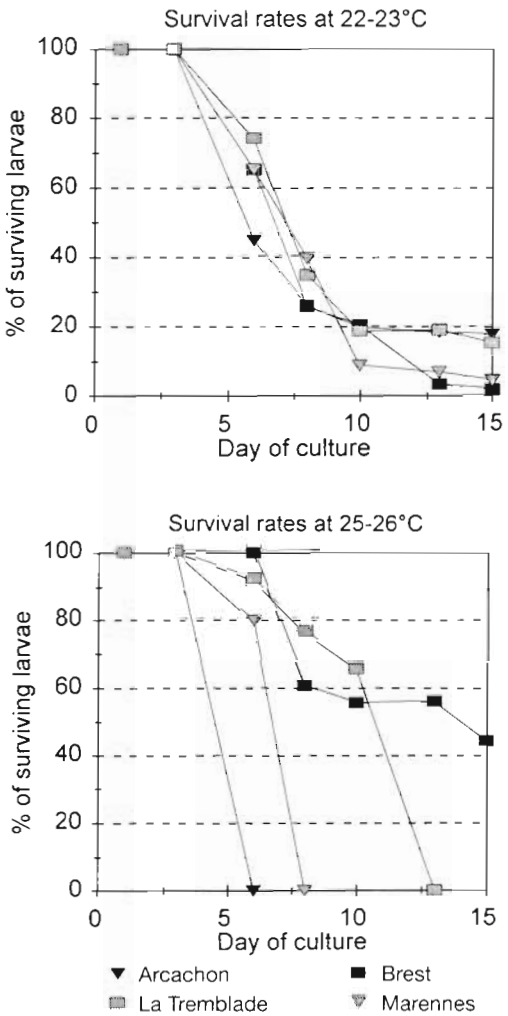


Fig. 2. *Crassostrea gigas*. Survival rates of Pacific oyster larvae held at either 22–23°C or 25–26°C. Spawns were performed with broodstocks from 4 different French sites: Arcachon, La Tremblade, Brest and Marennes

of larvae reared under exactly the same conditions. At 25–26°C, mortalities of oyster larvae from Arcachon, Marennes and La Tremblade started on Day 4 post fertilization. Survival rates at 25–26°C decreased suddenly and reached 0% on Day 6 for oyster larvae from Arcachon, on Day 8 for larvae from Marennes and on Day 13 for larvae from La Tremblade. In contrast, good survival rates on Day 15 of culture (44.4%) were observed for larvae from Brest.

Mortality rates in groups of oyster larvae cultured at 22–23°C were more progressive and, for larvae from Arcachon, Marennes and La Tremblade, lower than in the corresponding 25–26°C groups. On Day 15 post fertilization, survival rates were 1.6% for oyster larvae from Arcachon, 14.8% for larvae from Marennes, 4.2% for larvae from La Tremblade and 17.4% for larvae from Brest.

Growth rates did not show significant differences between groups of larvae held at the same temperatures (Fig. 3). High mortalities occurred on some groups of larvae held at 25–26°C between Days 4 and 11, when these larvae had an average size of 95 to 162 µm. On Days 1 to 11 of culture, growth rates for groups of larvae reared at 25–26°C were higher than for those reared at 22–23°C. However, average larval length reached similar values on Day 15 of culture. Indeed, larvae from Brest held at 25–26°C measured 230 µm, while larvae from the different groups held at 22–23°C measured 220 to 255 µm.

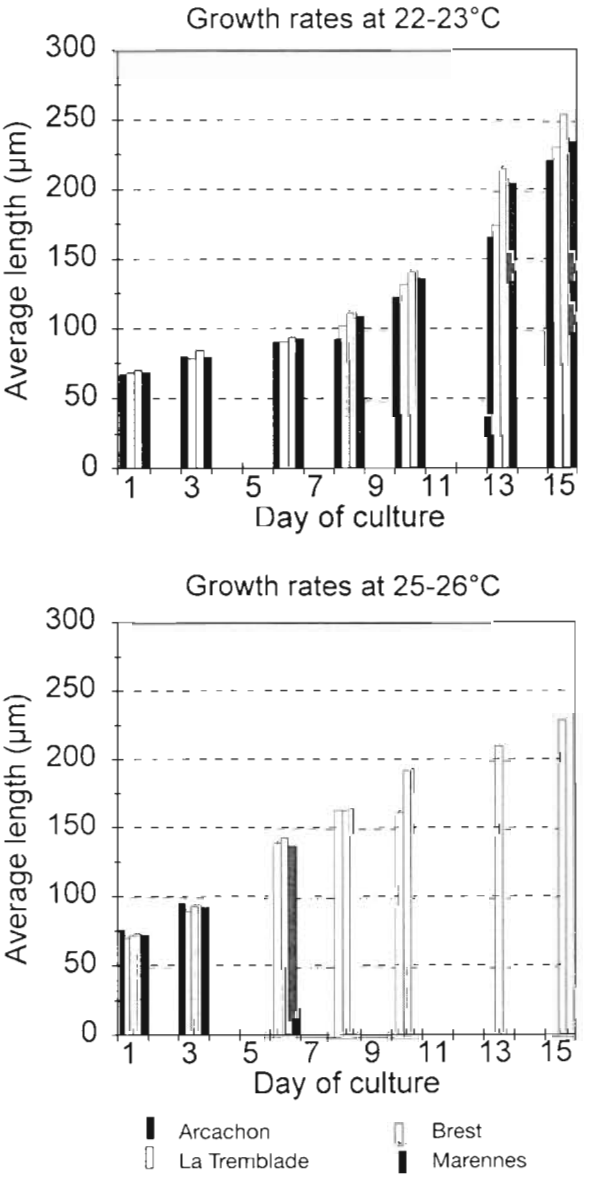


Fig. 3. *Crassostrea gigas*. Growth rates of Pacific oyster larvae held at either 22–23°C or 25–26°C. Larvae were obtained from broodstocks originating in Arcachon, La Tremblade, Brest and Marennes

Histological and transmission electron microscope analysis

Analysis of semithin sections under light microscopy (Table 1) revealed nuclear alterations in oyster larvae from Arcachon, Marennes and La Tremblade cultivated at 22–23°C and 25–26°C. No lesions were observed in either group of oyster larvae from Brest. These alterations included condensed nuclei and nuclei presenting marginated chromatin (Fig. 4).

Analysis by transmission electron microscopy (Table 1) revealed the presence of herpes-like viral particles (Figs. 5 to 9) only in samples of oyster larvae from Arcachon, Marennes and La Tremblade held at higher temperatures (25–26°C). Under transmission electron microscopy, interstitial and epithelial cells of the velum exhibited intranuclear and intracytoplasmic virus-like particles. In infected cells, the nucleus contained spherical or polygonal particles 80 nm in diameter. Some particles appeared empty and consisted of structures assumed to be capsids, others contained an electron-dense core and were interpreted as being nucleocapsids (Figs. 5 & 6). Enveloped single virions were observed in the cytoplasm (Fig. 7) and in extracellular spaces (Fig. 8). These particles consisted of a capsid with an electron-dense core that was in turn surrounded by an envelope with a typical membrane-like trilaminar appearance. In oyster larvae cultivated at 25–26°C, viral particles were first detected on Day 6 post fertilization for oysters from Marennes and Arcachon, and on Day 8 for oysters from La Tremblade. Good survival rates were obtained in larvae of parents originating from Brest, and herpes-like virus was not detected in these larvae reared at either 22–23°C or 25–26°C.

Moreover, herpes-like viral particles were not detected in any larvae reared at 22–23°C. However, some

Table 1 *Crassostrea gigas*. Transmission electron microscope and light microscope examination of fixed samples of Pacific oyster larvae held at different temperatures, 22–23 (22) or 25–26°C (25). Observation of nuclear alterations (NA) and/or the presence of herpes-like viral particles (VP) are indicated as a percentage calculated from the indicated number of oyster larvae examined in each case (N). nd: not determined; –: 100% mortality occurred, no analysis was performed later

	Light microscopy				Transmission electron microscopy					
	NA		N		NA		VP		N	
	22	25	22	25	22	25	22	25	22	25
Arcachon										
Day 6	0%	100%	100	50	0%	100%	0%	100%	6	4
Day 8	0%	–	70	–	0%	–	0%	–	10	–
Day 10	10%	–	40	–	8%	–	0%	–	12	–
Day 13	25%	–	32	–	0%	–	0%	–	6	–
Day 15		–		–		–		–		–
La Tremblade										
Day 6	0%	0%	90	60	0%	0%	0%	0%	6	4
Day 8	0%	49%	95	45	0%	100%	0%	100%	9	4
Day 10	nd		nd		8%		0%		6	
Day 13	3%	100%[a]	35	45[a]	0%	[b]	0%	[b]	2	4
Day 15	10.5%	–	19	–	0%	–	0%	–	2	–
Brest										
Day 6	0%		90		0%		0%		5	
Day 8		0%		70		0%		0%		3
Day 10	0%		35		0%		0%		5	
Day 13										
Day 15	0%	0%	22	31	0%	0%	0%	0%	5	4
Marennes										
Day 6	6%	29%	117	80	60%	100%	0%	100%	5	5
Day 8		–		–		–		–		–
Day 10	nd	–	nd	–	40%	–	0%	–	5	–
Day 13		–		–		–		–		–
Day 15	0%	–	14	–	40%	–	0%	–	5	–

[a] Among 45 larvae observed, 41 were empty shells, but 100% nuclear alterations were found in the 4 shells of larvae containing tissues

[b] Only empty shells of larvae were found, observation of viral particles was therefore not possible

oysters in these 22–23°C sets exhibited nuclear alterations (Fig. 9) similar to the nuclear lesions observed in the larvae reared at 25–26°C (Fig. 6). These nuclear alterations were characterized by condensed nuclei appearing very electron dense, but with some round, electron-lucent areas (Fig. 9). However, in no set of larvae held at 22–23°C were these abnormalities associated with the detection of herpes-like viral particles. Moreover, these nuclear alterations were not found in any larvae from Brest.

Comparing these results to survival rates (Fig. 2), herpes-like viral particles were found in those 25–26°C sets in which sudden high mortalities occurred, while nuclear alterations in 22–23°C sets were correlated with more progressive mortalities. Moreover, in the La Tremblade set reared at 25–26°C, larvae exhibiting nuclear lesions observed by light microscopy were 0% on Day 6, 49% on Day 8 and 100% on Day 13 (Table 1). 100% mortality in this group also occurred on Day 13, while the presence of viral particles detected by transmission electron microscopy was found in 0% of the larvae on Day 6 and 100% on Day 8. For larvae from Marennes at 25–26°C, 29% exhibited nuclear lesions on Day 6, while viral particles were found in 100% by transmission electron microscopy on the same day. In the group of larvae from Marennes, 100% mortality occurred on Day 8, but no analysis by light or transmission electron microscopy could be performed since all larval shells were empty. For larvae from Arcachon at 25–26°C on Day 6, 100% exhibited nuclear lesions under light microscopy, 100% contained viral particles and 100% mortality occurred.

DISCUSSION

In the present study, effects of temperature were investigated by comparing survival and growth rates of *Crassostrea gigas* spawns reared at either 22–23°C or 25–26°C. These results were related to the histological and ultrastructural examination of oyster larvae. Although samples of all sets of oyster larvae were fixed each day, we performed transmission electron microscopy only for samples corresponding to the

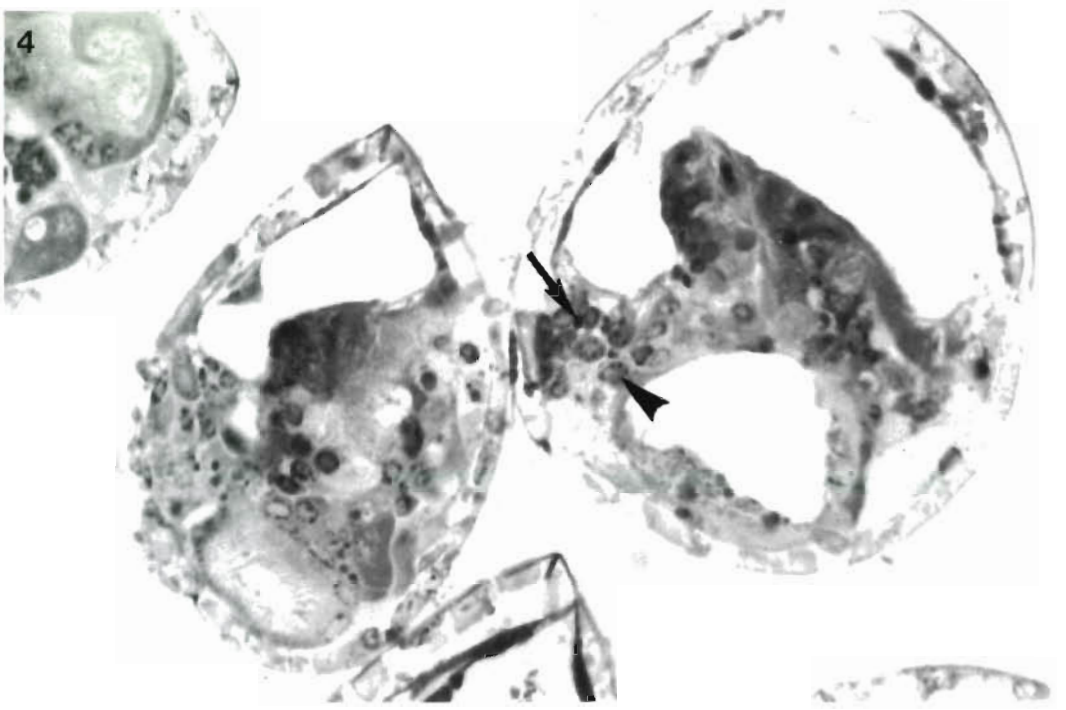


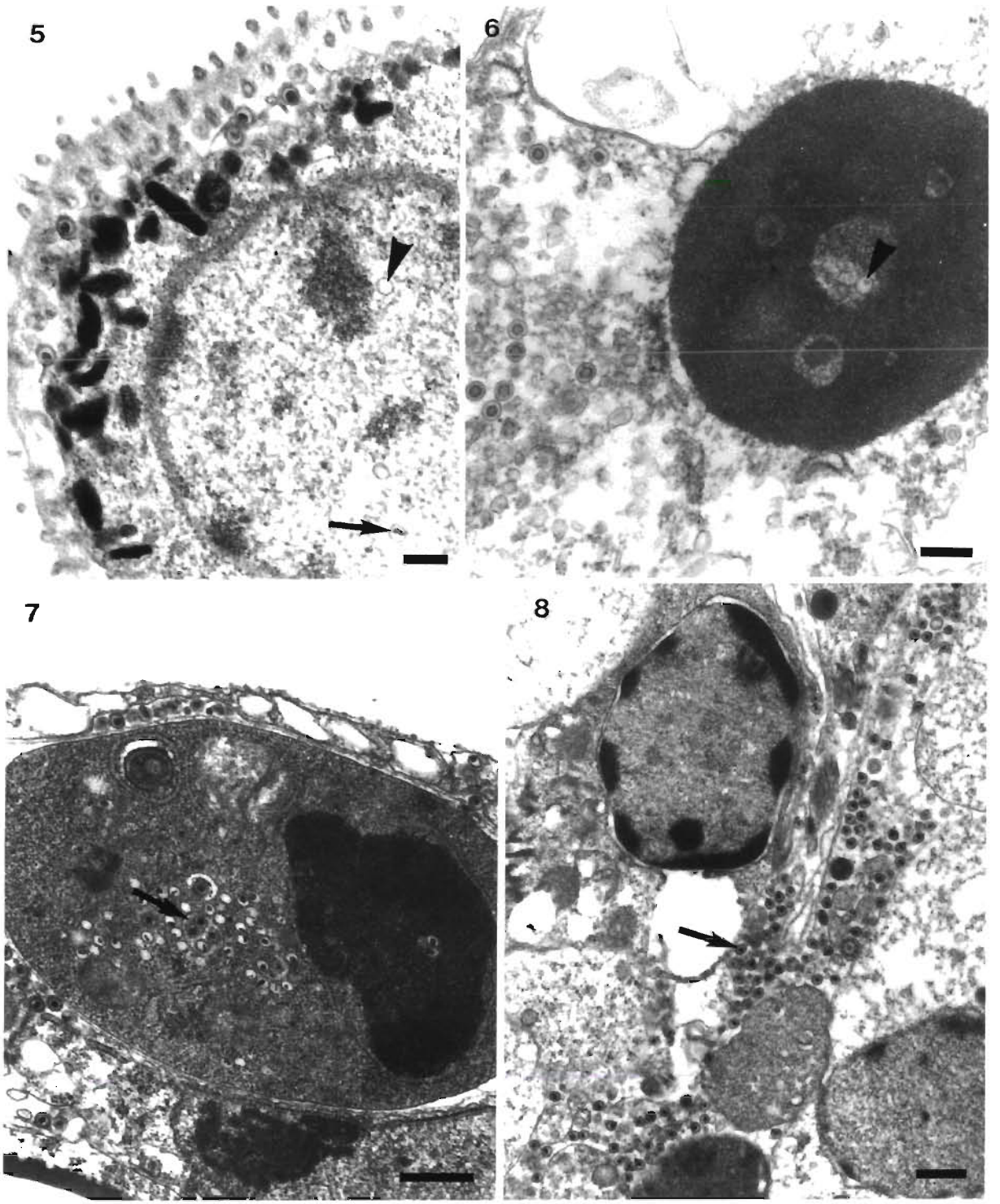
Fig. 4. *Crassostrea gigas*. Toluidine-blue-stained semithin sections of Pacific oyster larvae. Larvae from parents originating from Arcachon, La Tremblade and Marennes held at 22–23°C or 25–26°C exhibited nuclear lesions. Arrow: condensed, hyperbasophilic nuclei. Arrowhead: enlarged nuclei presenting marginalisation of chromatin

development of mortalities. Although it was difficult to handle numerous samples using this method, this procedure enabled us to compare survival rates and observations of nuclear lesions and viral particles. Moreover, observation of later viral infection could not be performed as infected cells detach from the larvae, and the shells of the oyster larvae were empty a few days after the beginning of the infection.

Viruses related to the family Herpesviridae were previously described in Pacific oyster *Crassostrea gigas* larvae (Hine et al. 1992, Nicolas et al. 1992) and associated with high and sudden mortalities occurring early in oyster larvae development, usually between Days 6 and 8 post fertilization. But sometimes 100% larval mortalities associated with herpesvirus infection were observed as soon as Day 4 post fertilization (T. Renault pers. comm.). These results point out the importance of checking survival rates of oyster larvae daily. Moreover, we tried to relate these survival rates with the presence of lesions associated with herpesvirus infection. Indeed, Nicolas et al. (1992) and Renault et al. (1994b) previously described the main histological changes in herpesvirus-infected larvae as abnormal basophily of connective cells, presence of enlarged nuclei that show abnormal shape and chromatin pattern and condensed hyperbasophilic nuclei. These authors also reported herpesvirus particles in association with these cellular alterations. Moreover, viral particles were often observed in enlarged nuclei, but only occasionaly in condensed nuclei (Renault et al. 1994b). For diagnostic purposes, the nuclear alterations, particularly marginated chromatin in hypertrophied nuclei, can be detected by either light or transmisson electron microscopy. Although transmission electron microscopy is actually the most reliable method for diagnosis of marine mollusc viruses, light microscopy allows one to look at a greater number of specimens in each sample (Table 1) and thus increases the likelihood of detecting those specimens potentially carrying the infection.

The demonstration of herpesvirus pathogenicity was performed by inoculating axenic oyster larvae with viral suspensions (Le Deuff et al. 1994). Sudden and high mortalities occurred within 2 to 4 d post inoculation. Viral particles were observed by transmission electron microscopy in moribund axenic oyster larvae, and histological and ultrastructural changes in these larvae were identical to the lesions described in naturally infected oysters.

In the present study, mortalities in sets of oyster larvae from Marennes, La Tremblade and Arcachon held at 25–26°C occurred suddenly compared to mortalities in sets held at 22–23°C. The mortalities of oyster larvae held at 25–26°C reached 100% on Days 6 to 13 of culture, which was in accordance with previous reports of herpesvirus infections (Hine et al. 1992,



Figs. 5 to 8. *Crassostrea gigas*. Transmission electron micrographs of herpes-like virus infected cells of Pacific oyster larvae held at 25–26°C. Figs. 5 & 6. Intranuclear spherical or polygonal viral particles. Some particles appeared empty (arrowheads) and others contained an electron dense core (arrow). Scale bar = 200 nm. Fig. 7. Enveloped viruses (arrow) within cytoplasmic vesicles in an infected cell of a Pacific oyster larva. Scale bar = 500 nm. Fig. 8. Enveloped herpes-like viral particles (arrow) found in extracellular spaces. Scale bar = 500 nm

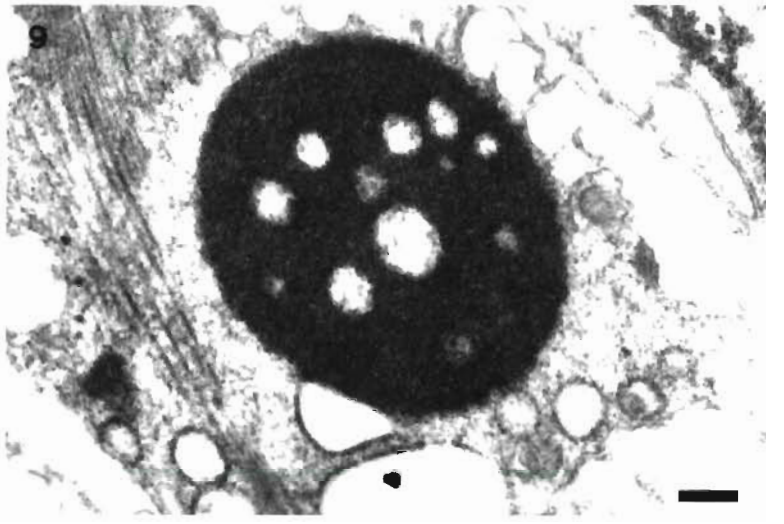


Fig. 9. *Crassostrea gigas*. Transmission electron micrograph of nuclear alterations observed in Pacific oyster larvae held at 22–23°C. The nucleus appeared very electron dense, but with some electron-lucent areas. Scale bar = 200 nm

Nicolas et al. 1992) and with results concerning experimentally infected larvae (Le Deuff et al. 1994) which suggest that this virus may have a short multiplication cycle and may spread quickly.

Best survival rates were obtained for groups of larvae from Brest. Survival levels on Day 15 of culture were higher for larvae held at 25–26°C (44.4%) than for larvae held at 22–23°C (17.4%). These results could be expected since high temperatures (25–26°C), in the absence of pathological problems, are optimal for the development and survival of *Crassostrea gigas* larvae. In optimal conditions (25–26°C), a survival rate of 40 to 50% is expected on Day 15 of culture, while it decreases to 10–20% at 22–23°C for spawns obtained by gonad strip dissection method (A. Gérard pers. comm.). Moreover, growth rates could not be related to the development of herpesvirus infection, since no noticeable difference was observed between groups of oyster larvae held at the same temperature. But, it can be expected that at low temperatures (22–23°C) larval growth is slower than at high temperatures (25–26°C) and, considering the respective breeding temperatures, larval growth rates obtained here were standard (A. Gérard pers. comm.).

When comparing survival rates, we noted that herpes-like viral particles were found in groups of oyster larvae in which high and sudden mortalities occurred. A further comparison of results from light and transmission electron microscope analyses in conjunction with the time course of survival at 25–26°C may also shed light on the rate of viral development in larvae. This is well illustrated with oyster larvae from La Tremblade reared at 25–26°C. In this group, viral particles could be observed in 100% of the larvae as early as Day 8, while light microscopy revealed nuclear lesions in only 49%. This difference is thought to reflect that weak viral infection could not be detected easily by light microscopy. On Day 13, at a further stage of viral infection, 100% of the larvae examined by light microscopy were found to exhibit nuclear lesions; these larvae were indeed heavily infected and larval mortality reached 100%. Observations made on groups of larvae from Marennes and Arcachon reared at 25–26°C are in agreement with this hypothesis.

In this study, viral particles were observed only in larvae held at 25–26°C. A similar feature was reported by Farley (1972) when adult *Crassostrea virginica* suffered a high mortality (52%) associated with herpes-like viral infection when held at 28 to 30°C, versus control oysters reared at 12 to 18°C (18% mortality) in which no viral infection was found. However, nuclear alterations were histologically observed in this study in oysters reared at 22–23°C, although not associated with the presence of viral particles at the ultrastructural level. Moreover, no other pathogen which could provoke such lesions was observed. These nuclear abnormalities could result either from a low productive viral cycle in which viral particles are very rare, or from a nonproductive state, in which viral particles are not produced. In this last case, 2 hypotheses may be developed. First, these nuclear alterations could result from a viral protein expression associated with a true latent viral phase (Meyers 1979, Garcia-Blanco & Cullen 1991). Second, nuclear alterations could result from an abortive viral cycle, in which only some early phases of the viral cycle are achieved, with synthesis of structural or functional proteins and eventually viral DNA replication, but without any production of virions (Girard & Hirth 1989). Consequently, structural aspects of the infected cell nuclei would change. It is important to note that, for viruses belonging to the Herpesviridae,

a low productive or an abortive viral cycle is generally associated with a further switch of the virus to a latent state (Girard & Hirth 1989).

For these reasons, larval rearing at 22–23°C is potentially hazardous because, although acute infection is avoided, limiting larval mortalities, latent, asymptomatic carrier larvae could be produced and the virus disseminated. In other words, larvae held at low temperatures (22–23°C) which exhibit no evidence of viral particles under transmission electron microscopy could nonetheless be infected by the herpes-like virus; these carriers may represent reservoirs of the virus and could transmit this agent. Oysters produced by such larval breedings could also be susceptible to a switch from a latent to a productive infection at a later time.

In conclusion, it seems that both temperature and origin of parents, when breeding temperatures are the same, apparently influence expression of herpes-like virus in *Crassostrea gigas* larvae. Higher temperatures promote early production of viral particles in association with high mortalities. The result is a fast spread of the disease to the whole brood and high sudden mortalities. Larvae originating from some oyster broodstocks, particularly from Brest, grew well at both rearing temperatures. Since neither herpes-like virus nor nuclear alterations were detected in these larvae, parental origin might be important in relation to appearance of the disease among larvae. As all the larval breedings were performed at the same time and in the same conditions at both temperatures, this result suggests that one mode of transmission of this virus could be vertical, from parent to larvae. Moreover, with the gonad strip dissection procedure, this possible vertical transmission could occur either via the gamete cells or via the many extraneous, non-gamete cells.

Although no mortalities occurred among the different broodstocks of *Crassostrea gigas* used in this study, a further hypothesis is that the virus is present in a latent phase in some of the parents and is activated by stress in these hosts (Garcia-Blanco & Cullen 1991), such as a modification of rearing temperature. Adults are usually held at 22°C, generally warmer than in the natural environment, to accelerate the gonadal maturation process. Other stressors include manipulation or transport of animals, crowding or a modification of feeding. The virus induced by this stress would be transmitted to larvae. Consequently, in larvae reared at high temperatures (25–26°C), the viral productive cycle would be faster and the virus would spread quickly to the whole brood, leading to high and sudden mortalities of larvae. In contrast, in larvae reared at lower temperatures (22–23°C) and presenting nuclear alterations, the viral replication may remain in an abortive viral cycle (Girard & Hirth 1989).

Our plans for testing this last hypothesis will probably indicate the mechanisms of transmission of the disease and its relations to temperature or other stressors. We expect to establish a breeding procedure that could avoid the production and dissemination of larvae carrying the virus and which might be susceptible to developing the disease in a later spat stage or transmitting the herpes-like virus to their progeny. Our future plans also include the purification of herpes-like virus of *Crassostrea gigas* and further development of diagnostic methods based on the use of nucleic acid probes or antibodies specific for this virus in order to study the possibility of associating the detection of nuclear abnormalities with the presence of viral DNA and/or viral proteins. Establishment of such diagnostic methods would enable not only epidemiological study and survey of the disease, but also verification of the hypotheses developed in this study.

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