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Viral gametocytic hypertrophy of *Crassostrea gigas* in France: from occasional records to disease emergence?

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ABSTRACT: Viral gametocytic hypertrophy was reported for the first time in 2001 in Pacific oyster *Crassostrea gigas* in France. Since this date, the number of reported cases and the distribution area have increased every year; however, the cases are not associated with macroscopic signs or increased mortality rates. Both male and female gametes were hypertrophied and basophilic inclusions were observed in gamete nuclei. Transmission electron microscopy revealed the presence of viral particles in these intranuclear basophilic inclusions. These particles had characteristics similar to those of the *Papillomaviridae* and *Polyomaviridae* families: they were small, non-enveloped, icosahedral, and 44 to 56 nm in diameter. The viral particles were found in male, female and hermaphrodite oysters and no significant difference in viral infection was observed between those groups. The frequency of detection and the intensity of infection were low and no host defence reaction was recognised, suggesting that the viral particles had a weak impact on *C. gigas*. The viral particles described in the present study seem to be similar to these described in *C. virginica* in the USA and Canada and in *C. gigas* in Korea, but further studies are required to confirm their identity. The issue of a possible emergence of this infection is discussed.

KEY WORDS: *Crassostrea gigas* · Pacific oyster · Viral gametocytic hypertrophy · Gonad · *Papillomaviridae* · *Polyomaviridae*

INTRODUCTION

Since the 1970s, viruses belonging to various families have been reported in bivalve molluscs: *Herpesviridae*, *Iridoviridae*, *Picornaviridae*, *Reoviridae*, *Birnaviridae*, *Retroviridae* and *Papovaviridae* (Elston 1997). This last family was originally composed of the 2 genera, *Papillomavirus* and *Polyomavirus*, which are now recognized as 2 separate families, *Papillomaviridae* and *Polyomaviridae* (Van Regenmortel et al. 2000). These 2 families share morphological characteristics: they are viruses which are non-enveloped, icosahedral, and 40 to 55 nm in diameter. Replication and assembly occur in the nucleus of the host cell and virions are released by cell destruction. Members of the families *Papillomaviridae* and *Polyomaviridae* are generally host-specific and are transmitted by contact or by airborne particles. Many have been reported as oncogenic (Van Regenmortel et al. 2000). They infect a wide range of animals including marine vertebrates and invertebrates (Farley 1976, Edwards et al. 1977).

A papilloma-like virus responsible for viral gametocytic hypertrophy (VGH) was first reported in 1973 in adults of the eastern oyster *Crassostrea virginica* collected in the USA from Maine (Farley 1976). Similar viral particles were also observed in *C. virginica* on the east coast of North America (Sparks 1985), from the Gulf of Mexico (Winstead & Courtney 2003) and from Atlantic Canada (McGladdery & Stephenson 1994). Papilloma-like or polyoma-like viruses were detected in other bi-

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valve species such as *Mya arenaria* in Massachusetts (USA), *Pinctada maxima* in Australia, *Ruditapes philippinarum* in Spain (Elston 1997, Montes et al. 2001) and more recently in *C. gigas* in Korea (Choi et al. 2004).

Bivalve papilloma-like and polyoma-like viruses apparently induce hypertrophy of the host cell nucleus associated with dense nucleic inclusions. Viral particles have been observed in haemocytes, connective tissues and gill epithelium of *Mya arenaria*; in labial palps epithelial cells of *Pinctada maxima*; in gill epithelium, striated muscular fibres, neurons, granulocytes, endothelium and connective tissue of *Ruditapes philippinarum* and in gametocytes of *Crassostrea virginica* and *C. gigas* (Elston 1997, Montes et al. 2001, Choi et al. 2004). Similar gametocyte lesions have been reported in several ostreid species such as *Saccostrea glomerata*, *C. rhizophorae*, *Ostrea edulis* and *O. conchaphila* (= *O. lurida*), but the presence of viral particles was not confirmed by transmission electron microscopy (TEM) (Farley 1978, Bower et al. 1994). From these reports, it is difficult to assess the actual impact of papilloma-like and polyoma-like viruses on their molluscan host. Another pending question is whether or not reported infections of molluscs are caused by a unique virus. We report here the presence of VGH in French populations of Pacific oyster *Crassostrea gigas*.

MATERIALS AND METHODS

From 2001 to 2004, 156 samples of *Crassostrea gigas*, making a total of 3980 individuals, were collected along French coasts as part of the national zoosanitary surveillance programme (Fig. 1). Out of these 156 samples, 76 were spat and 80 were adults.

Oyster individuals were cut into 2 parts; one was fixed in Davidson's fixative for histological examination and the other was fixed in Carson's fixative for further TEM. Tissues in Davidson's fixative were dehydrated and embedded in paraffin. Two sections per individual (2 to 3 µm thick) were cut; the first was stained with hematoxylin-eosin for routine examination and the second with Feulgen's stain for DNA when lesions were present.

The Carson-fixed tissues were transferred into cold 2.5% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.2 for 1 h and rinsed twice in 0.2 M cacodylate buffer at 4°C for 48 h. Tissues were then postfixed twice in 1% osmium tetroxide in the same buffer at 4°C for 10 min. They were dehydrated, cleared twice in propylene oxide for 15 min and infiltrated for 1 h in 50:50 propylene oxide:Epon resin. After 1 h infiltration in pure Epon resin, they were embedded in resin and cured for 48 h at 60°C. Ultrathin (20 nm thick) sections were stained in 5% uranyl acetate in 50% ethanol for 20 min and in 5% lead citrate in fresh boiled distilled water for 3 min. They were examined with a JEOL JEM 1200 EX transmission electron microscope at 80 kV.

The infection frequency is defined as the proportion of infected individuals in a given sample (Ancelle 2002). Frequencies of the infection in male and female oysters were compared using a χ^2 test (Scherrer 1984). Only the years 2002 to 2004 were considered because samples were comparable and representative.

RESULTS

From 2001 to 2004, 36 individuals displayed gonadal lesions that were characterised by an abnormally increased size of gametes. Ovocyte size could reach 100 µm in diameter (normal size of a ripe ovocyte: 36 ± 4.4 µm; Lango-Reynoso et al. 2000), and spermatozoa could reach 52.1 µm in head diameter (normal size of a mature spermatozoa without flagella: 2.5 µm in diame-

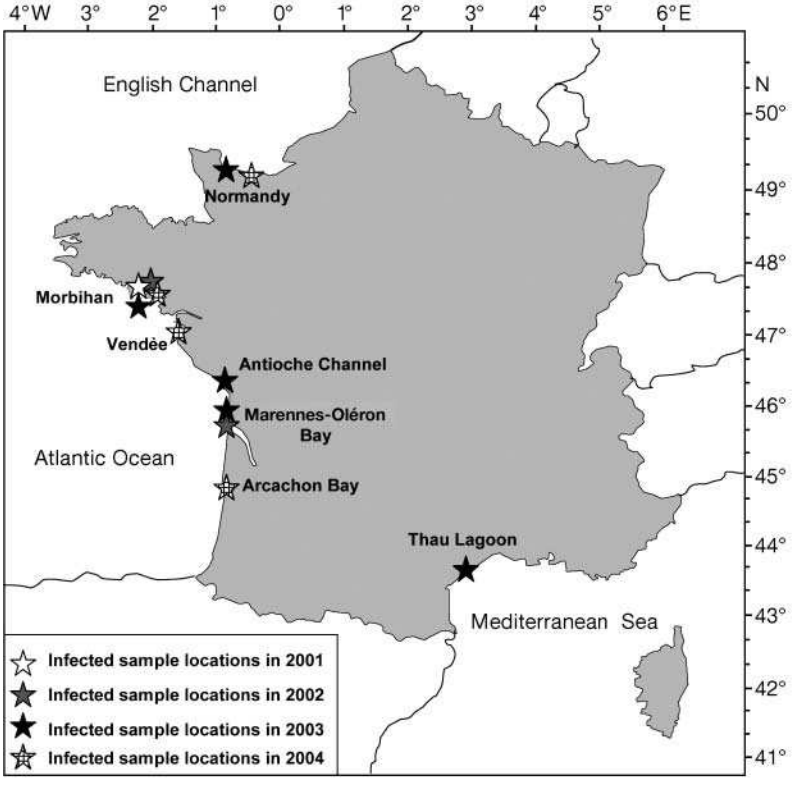
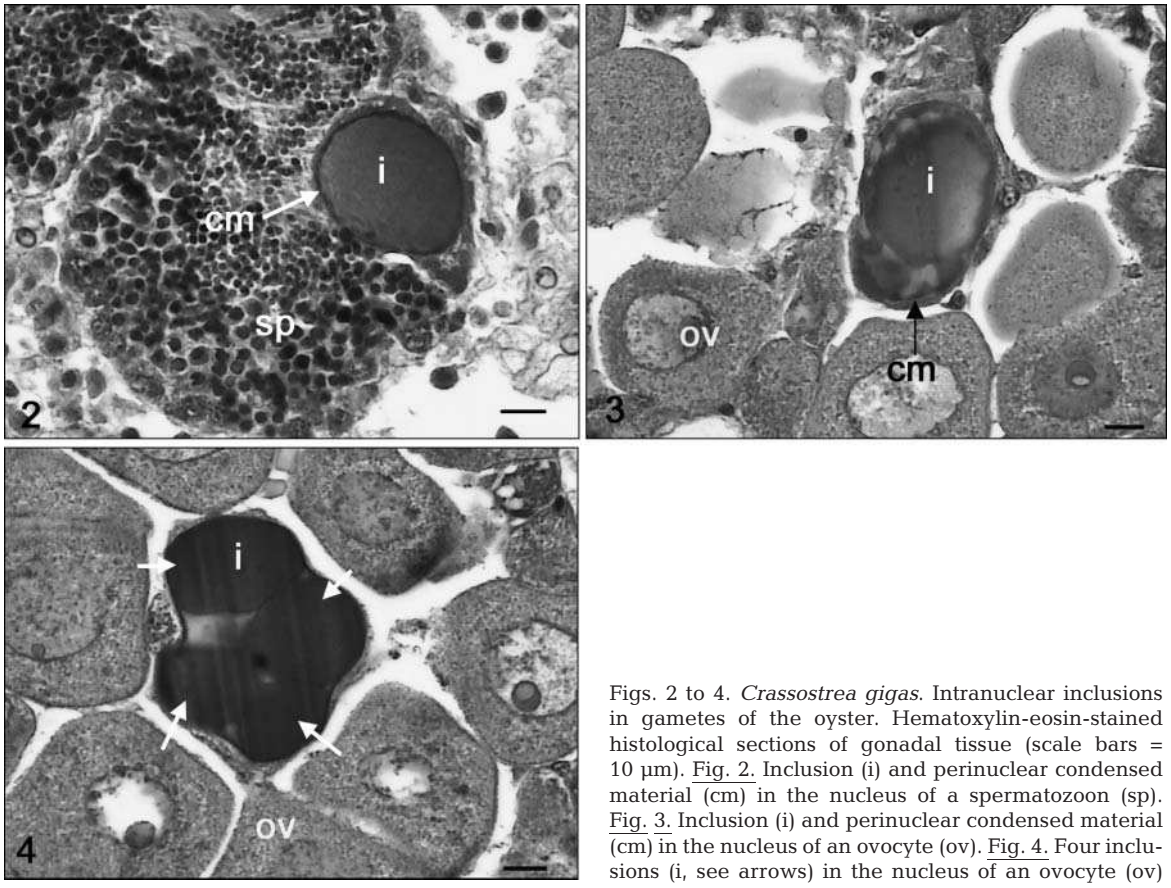


Fig. 1. Location of the infected *Crassostrea gigas* samples in France collected between 2001 and 2004



Figs. 2 to 4. *Crassostrea gigas*. Intranuclear inclusions in gametes of the oyster. Hematoxylin-eosin-stained histological sections of gonadal tissue (scale bars = 10 µm). Fig. 2. Inclusion (i) and perinuclear condensed material (cm) in the nucleus of a spermatozoon (sp). Fig. 3. Inclusion (i) and perinuclear condensed material (cm) in the nucleus of an ovocyte (ov). Fig. 4. Four inclusions (i, see arrows) in the nucleus of an ovocyte (ov)

ter; Bozzo et al. 1993). In hematoxylin-eosin-stained sections, infected male and female gametes exhibited basophilic hypertrophied nuclei with perinuclear condensed material (Figs. 2 & 3). An amorphous matrix, corresponding to a basophilic finely granulous inclusion, was observed in the central area of abnormal nuclei (Fig. 2). Cytoplasm was frequently reduced due to nucleus hypertrophy (Fig. 3). One female oyster showed 4 inclusions in a single ovocyte nucleus (Fig. 4). Inclusions in both ovocytes and spermatozoa were moderately Feulgen-positive, indicating the presence of DNA (Fig. 5) whereas the perinuclear condensed material was strongly Feulgen-positive. In all cases, no major haemocyte infiltration or other lesions were found associated with the infection.

At the ultrastructural level, nuclear membranes of infected gametes appeared normal and an interrupted ring of marginated chromatin was observed; the central area of infected nuclei contained numerous viral particles (Fig. 6). Viral particles were non-enveloped and small; their size varied between 44 to 56 nm in diameter (average 48 ± 1 nm). They showed 5 or 6 sides in section, suggesting an icosahedral symmetry (Fig. 7). Both empty and full capsids could be observed (Fig. 8). Viral particles were also detected in extracellular spaces of the gonad follicles (Fig. 9). No cytoplasmic abnormality could be observed in ovocytes or spermatozoa.

The infection intensity was generally low with an average of 16 infected gametes per slide (1 to 136 infected cells per section). The frequency of

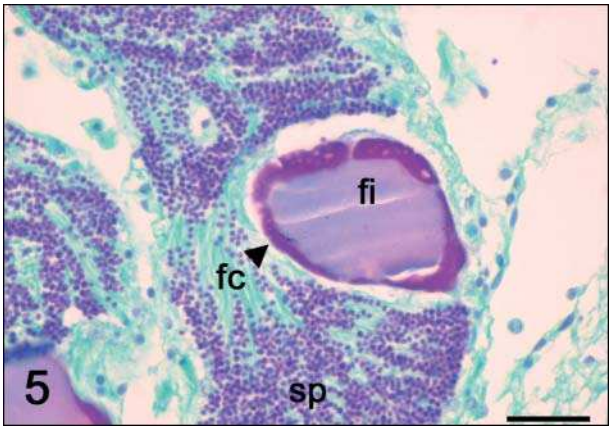
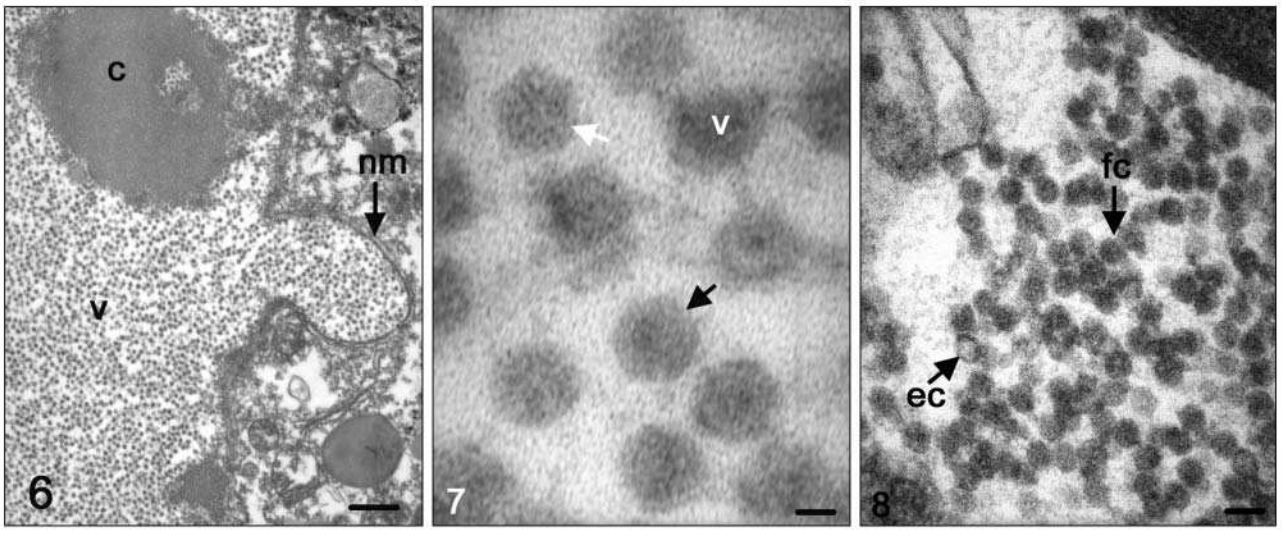


Fig. 5. *Crassostrea gigas*. Intranuclear inclusions in a spermatozoon of the oyster. Feulgen-stained histological section of gonadal tissue (scale bar = 20 µm). Moderate Feulgen-positive inclusions (fi) in the nucleus centre and strong Feulgen-positive condensed material (fc, see arrowhead) at the nucleus periphery of a spermatozoon (sp)



Figs. 6 to 8. *Crassostrea gigas.* Intranuclear viral particles in oyster gamete. Ultrathin sections of the gonadal tissue. Fig. 6. Intranuclear viral particles (v) in an ovocyte with a normal nuclear membrane (nm) and chromatin masses (c); scale bar = 500 nm. Fig. 7. Intranuclear 5-sided (white arrow) and 6-sided (black arrow) viral particles (v) in a spermatozoa; scale bar = 20 nm. Fig. 8. Intranuclear viral particles in an ovocyte. Presence of empty (ec) and full capsids (fc); scale bar = 50 nm

the viral infection varied between 3.3 and 13.3% (Table 1). Gonad lesions were observed in *Crassostrea gigas* in several production areas (Fig. 1) mainly during summer when gonad maturation is maximum; however, viral particles were also found in 2 occurrences in winter in the early stages of gametogenesis, ovogonia or spermatogonia from non-mature individuals. VGH infected spat and adults in males, females, hermaphrodites and individuals in the early stages of gametogenesis (Fig. 10, Table 1). In one of the 2 infected hermaphrodite individuals, which were predominantly male, only the ovocytes were infected whereas in the other hermaphrodite individual, both ovocytes and spermatozoa were infected. No significant difference at the 5% significance level was noted in the infection frequency between males and females during the years 2002 to 2004 ($\chi^2 = 1.22$; $p = 0.27$).

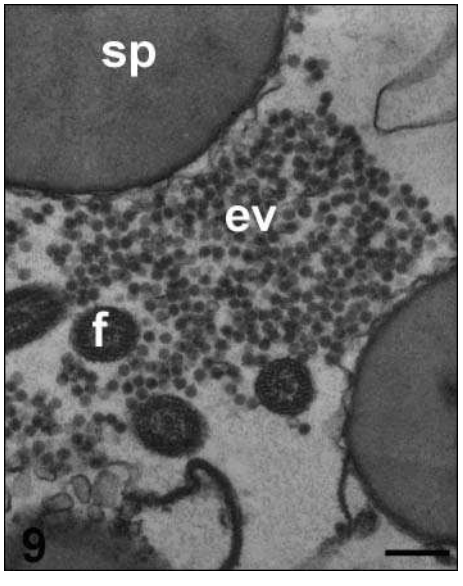


Fig. 9. *Crassostrea gigas*. Extracellular viral particles in gonad follicles of the oyster. Ultrathin section of the gonadal tissue. Extracellular viral particles (ev) between sperm flagella (f) and spermatozoa (sp) section; scale bar = 200 nm

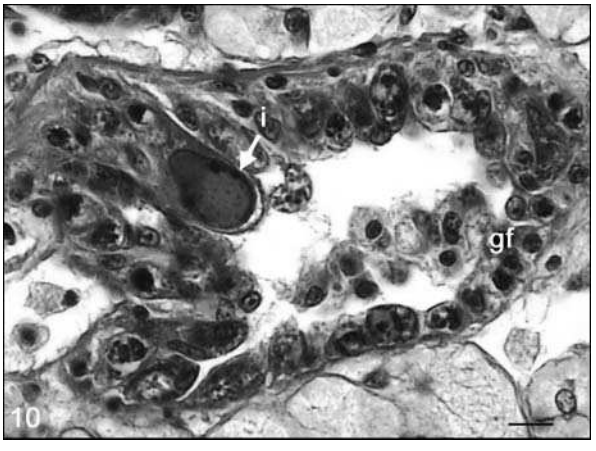


Fig. 10. *Crassostrea gigas*. Intranuclear inclusion in a gonad follicle of the oyster. Histological section in the gonadal tissue stained with hematoxylin-eosin (scale bar = 10 µm). Intranuclear inclusion (i) in a gonad follicle (gf) at the early stages of gametogenesis

Table 1. *Crassostrea gigas*. Viral gametocytic hypertrophy in the Pacific oyster. Frequency of the disease detection in oysters from different French locations

Year	Month	Sampling location	Oyster stage	No. of ind.	No. of infected ind.	Frequency (%)	No. of infected males/ No. of males	No. of infected females/ No. of females	No. of infected hermaphrodites/ No. of hermaphrodites	No. of infected ind. in early stages of gametogenesis/No. of ind. in early stages of gametogenesis
2001	Jun	Morbihan	Spat	30	2	6.7	2/14	0/3	0/1	0/12
2002	Jul	Marennes-Oléron Bay	Spat	30	1	3.3	1/18	0/4	0/0	0/8
2002	Jul	Morbihan	Spat	216	13	6.0	11/140	2/50	0/1	0/25
2003	Feb	Marennes-Oléron Bay	Spat	8	1	12.5	1/4	0/4	0/0	0/0
2003	May	Morbihan	Adult	60	1	3.3	0/18	0/27	1/4	0/11
2003	Jun	Marennes-Oléron Bay	Spat	59	4	6.8	3/33	1/18	0/1	0/7
2003	Jul	Marennes-Oléron Bay	Spat	29	1	3.5	1/11	0/8	0/0	0/10
2003	Aug	Thau lagoon	Adult	30	1	3.3	1/12	0/8	0/3	0/7
2003	Aug	Normandy	Adult	30	1	3.3	0/8	0/15	1/3	0/4
2003	Dec	Antioche channel	Adult	15	2	13.3	0/3	1/1	0/0	1/11
2004	Mar	Normandy	Adult	30	1	3.3	0/1	0/1	0/0	1/28
2004	Jun	Morbihan	Spat	30	1	3.3	0/18	1/12	0/0	0/0
2004	Jun	Vendée	Spat	30	1	3.3	1/19	0/6	0/0	0/5
2004	Jun	Morbihan	Spat	30	1	3.3	0/15	1/11	0/1	0/3
2004	Jun	Arcachon Bay	Adult	30	1	3.3	1/10	0/19	0/1	0/0
2004	Jul	Morbihan	Spat	15	1	6.7	1/8	0/6	0/1	0/0
2004	Jul	Arcachon Bay	Adult	30	3	10	0/4	3/21	0/5	0/0

DISCUSSION

Farley (1976) was the first author to report gametocyte hypertrophy associated with papilloma-like viruses in marine bivalves. Since then, similar conditions have been detected and reported from different oysters in North America and Asia (McGladdery & Stephenson 1994, Elston 1997, Choi et al. 2004). This report is the first record of gametocytic hypertrophy in *Crassostrea gigas* in Europe.

Ultrastructural observations have shown that, as previously reported, lesions detected in histology are associated with the presence of viral particles. The morphological characteristics displayed by these viral particles, including size and capsid symmetry, strongly suggest their close relationship with the families *Papillomaviridae* and *Polyomaviridae* (Van Regenmortel et al. 2000). Unlike viral particles previously described in *Crassostrea virginica* (McGladdery & Stephenson 1994, Farley 1985) and in *C. gigas* (Choi et al. 2004), the viral particles described in our study cannot be formally assigned to one of these families.

Papilloma-like and polyoma-like viruses have already been described from several bivalve species in different organs and tissues (Elston 1997). All cases were reported as inducing massive hypertrophy of infected cell nuclei associated with nuclear inclusions. Neither histopathology nor ultrastructure studies allow discrimination of these viral particles that were reported from different host species in different part of the world. The question of whether one or more types of virus are responsible for these infections remains open.

The frequency of VGH in *Crassostrea gigas* reported in this study (3.3 to 13.3%) is similar to those previously observed but slightly below the frequency for *C. virginica* in the USA (1 to 30%; Farley 1985, Winstead & Courtney 2003) and slightly above that for *C. gigas* in Korea (3.3 to 7.1%; Choi et al. 2004). These differences may not be significant. The infection intensity of the disease in French populations of *C. gigas* was usually low (up to 136 infected cells per section) compared to the maximum infection reported in *C. virginica* (up to 350 infected cells; Farley 1985). However, the overall infection intensity (16 infected cells per section on average) was higher in the present study compared to that reported in *C. virginica* (4 infected cells per section on average; Farley 1985). The 2 species of cupped oysters are well known for displaying clear differential lesions in response to parasitic infections. VGH equally affected *Crassostrea gigas* males and females, unlike *C. virginica*, in which females were more often reported infected (Farley 1985).

The low recorded frequency and intensity of this condition suggest that VGH probably has no lethal impact on oysters. This assertion is also supported by

the absence of a haemocytic reaction, as was similarly observed in *Crassostrea gigas* in Korea (Choi et al. 2004). However, although VGH has no or limited impact at population levels, the virus may affect the viability of oyster gametes and consequently oyster fecundity, as has been proposed for other pathogens affecting gametes such as *Steinhausia* spp. in *Mytilus galloprovincialis* (Figueras et al. 1991) or *Marteilioides chungmuensis* in *C. gigas* (Park et al. 1998). The viral particles in our study were also observed in an extracellular location within the gonad follicles of *C. gigas*, as was also the case in *C. virginica* (Farley 1985). This extracellular presence in the gonad follicle strongly suggests a potential for vertical transmission of the virus as well as horizontal transmission to progeny during the spawning process. Vertical transmission is proposed for oyster herpesvirus (Arzul et al. 2002) and also for human *Papillomavirus* and *Polyomavirus* (Pietropaolo et al. 1998, Tenti et al. 1999).

Although the impact of VGH on *Crassostrea gigas* populations and stocks seems to be limited, caution should be taken with regards to the oncogenic capacity of many other members of the *Papillomaviridae* and *Polyomaviridae* families (Van Regenmortel et al. 2000). For example, in *Mya arenaria*, such viruses were also suspected in gonadal neoplasia of the soft-shell clam (Harshbarger et al. 1979). Beyond their potential pathogenic impact, these viruses may open new avenues in the development of molluscan cell lines. The study of an oncogenic virus could bring new impetus to the development of cell lines, as some authors proposed with human *Papillomaviruses* for the immortalization of cell lines (Oda et al. 1996).

VGH is readily detected during the later stages of gametogenesis, suggesting an underestimation of the infection rates in the early stages of gametogenesis or after gamete release. In France, the number of infected individuals, as well as the distribution area, has apparently increased between 2001 and 2004, but the frequency at a given site remained low. Despite an intensive survey of Pacific oyster populations in France (between 1990 and 2004, a total of 43 418 oysters were analysed by the National Network for Surveillance and Monitoring of Mollusc Health, REPAMO), VGH had never been reported before 2001, suggesting a possible recent emergence of the disease. However, both low frequency and low intensity of the infection associated with the difficulty of detection from non-mature stages could also explain why it has only recently been detected. It is usually accepted that once a condition is described, it will then be increasingly recognised and recorded by diagnosticians; such a process may explain an apparent increase in the prevalence of VGH in French stocks. Since drafting of this manuscript, 7 cases were recorded by the REPAMO network in 2004, which shows a relatively stable number of cases detected annually. However, with 2 cases reported from Arcachon Bay and Vendée in 2004, sites where VGH had not previously been detected, the distribution area of this virus appears to be increasing. This increase could possibly be explained by transfers between the different production areas; the lower frequency of transfers in Arcachon Bay and Vendée than in other production areas, such as Marennes-Oléron Bay, Brittany and Normandy, could explain the late appearance of VGH in these areas. Therefore, VGH is currently flagged by the REPAMO network as a potential concern and a series of 3 measures are being undertaken: (1) communication with European counterparts within the existing network of the National Reference Laboratories for Mollusc Diseases; (2) implementation of a targeted survey to assess the potential spread and impact of VGH; and (3) retrospective analysis of archived material to assess the most probable status of oyster populations prior to initial recognition in 2001.

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