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Ostreid herpes virus 1 infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: Differences in viral DNA detection and quantification using real-time PCR

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Abstract:

Ostreid herpes virus 1 (OsHV-1) infections, notably reported in Europe and the USA, are closely associated with significant mortalities of the Pacific oyster, Crassostrea gigas, especially during its early stages of life. In summer 2006, we monitored mortality by strict daily verification of three full-sib families of oysters reared under common conditions. We quantified OsHV-1 using real-time PCR in dead and living individuals during and after a mortality event. Mortality events were severe and brief, but significantly different between tested families (cumulative mortality ranging from 1.2 to 49%). Real-time PCR assays revealed different viral DNA loads in dead individuals from different families ($P < 0.001$). Moreover, the mean level of infection among families was correlated with mortality ($P < 0.05$). Living oysters showed a significantly lower amount of viral DNA compared with dead ones. This is the first experiment showing the daily changes of individual OsHV-1 DNA load during a mortality outbreak. Our results also support the previously reported high genetic basis underlying the variance of resistance of Pacific oyster to summer mortality, suggesting that there might be a possibility to improve resistance to OsHV-1 by selective breeding.

Keywords: *Crassostrea gigas*; Mortality; OsHV-1; Real-time PCR; Viral DNA quantification

1. Introduction

Pacific oyster (*Crassostrea gigas*) production on the French coasts has experienced periodic mass mortalities during the summer months for at least 20 years (Renault *et al.*, 1994; Goulletquer *et al.*, 1998; Soletchnik *et al.*, 1999, 2007). This syndrome is known as 'summer mortality' and as been reported in most Pacific oyster producing countries, such as Japan, USA and Australia. The first description of this phenomenon was made in the 1940s in Japan, where *C. gigas* is endemic. According to Koganezawa (1974), the mortality outbreaks began occurring in 1945 with the large-scale use of hanging culture methods. In the late 1950s, *C. gigas* mortality episodes were also reported on the West coast of the USA (Glude, 1974 in Cheney *et al.*, 2000), where *C. gigas* had been imported for aquaculture production. These mortality outbreaks had many of the same features as those observed in Japan (Koganezawa, 1974). A summer mortality episode in Alaska in 1987 involved unusually high temperatures (~20°C) for this area (Meyers *et al.*, 1990).

In France, factors such as food limitation, oxygen depletion, salinity and temperature variations do not appear to act alone as single direct causative factors of the summer mortality syndrome (Soletchnik *et al.*, 1998, Ropert *et al.*, 2008). It has been suggested that mortality outbreaks occurring in *C. gigas* are the result of multiple factors or stressors, including elevated temperatures, physiological stress associated with maturation, aquaculture practices, pathogens or pollutants (Goulletquer *et al.*, 1998; Samain and McCombie, 2008). Although efficient defence mechanisms normally help to limit the proliferation of pathogens in oysters (Harris-Young *et al.*, 1995; Cheng, 1996), because they are benthic filter-feeders they are permanently exposed to pathogens and are therefore vulnerable to such mortality risks.

A multidisciplinary project known as 'Morest' was recently conducted in France, including studies on genetics, physiology, immunology and pathology, to improve our understanding of the causes of summer mortality in oysters (Samain and McCombie, 2008). This project notably studied the complex interactions between environmental factors, oysters and pathogens, including a herpes virus (Renault *et al.*, 1994) and different species of bacteria belonging to the genus *Vibrio* (Le Roux *et al*., 2002; Le Roux *et al.*, 2004; Labreuche *et al.*, 2005, Garnier *et al.*, 2007). A high heritability of resistance to summer mortality was demonstrated (Dégremont *et al.*, 2005, 2007), and divergent selection for survival was performed over several generations (Boudry *et al.* 2008). Interestingly, high ("R": Resistant) and low ("S": Susceptible) selected lines showed a differential pattern for reproduction, with S groups investing more in reproduction and/or delaying spawning for a longer time than R groups (Samain *et al.,* 2007). These results agree with those obtained in the USA (Beattie *et al.*, 1980), supporting the hypothesis that gonad maturation and loss of carbohydrate reserves are involved in summer mortality (Perdue *et al.*, 1981).

Herpes-like viruses have been detected during summer mortality outbreaks in France (Renault *et al.*, 1994; Nicolas *et al.*, 2008) and in the USA (Friedman *et al.*, 2005; Burge *et al.*, 2007). Because of the economic importance of oyster aquaculture, molecular diagnostic tools were developed to detect these viruses (Renault and Lipart, 1998; Renault *et al.*, 2000; Arzul *et al.*, 2002; Lipart and Renault, 2002) based on the complete genome sequence of the virus infecting *C. gigas* in France (ostreid herpes virus 1, OsHV-1) obtained by Davison *et al.* (2005). Recently, the development of a real-time PCR assay provided a method of quantifying OsHV-1 DNA (Pépin *et al.*, 2008). This new diagnostic tool offers new opportunities for the study of viral replication mechanisms, management of the disease or selective breeding.

In summer 2006, an experiment was performed to investigate the daily kinetics of summer mortality in full-sib families of oysters. The experiment was designed as part of a study of the genetic basis resistance. We quantified OsHV-1 DNA using real-time PCR in live and dead juveniles from three oyster families sampled during the survey and in individuals surviving to the end of the experiment. Viral DNA detection was first carried out in 3 different tissues (mantle, digestive gland and adductor muscle) from a number of individuals in order to assess the best organ for viral detection. Using real-time PCR, we characterized and quantified viral load as the number of viral DNA copies per mg of fresh tissue. This allowed us to investigate the daily kinetics of the viral infection before, during and after the mortality outbreak.

2. Materials and methods

2.1 Oysters

The mating design relied on three successive generations (F0, F1 and F2). The F0 individuals were sampled within the third generation of the divergent selective breeding lines for survival (G3R and G3S), showing contrasted survival to summer mortality both in the field and in laboratory experiments (Boudry *et al.*, 2008). For each generation, reproduction of oysters was performed as follows. To induce gametogenesis, oysters were placed in a favourable environment for reproductive maturation, with an enriched diet of algae and gradual increase of water temperature to reach 22-24°C during the first four weeks of a total conditioning period of 12 weeks. At the end of this period, the maturation of gametes was assessed by photonic microscopy. When fully mature, gametes were obtained by scarification of the gonads and were filtered and counted. These gametes were mixed in a volume of 500mL filtered seawater at a ratio of 100 spermatozoids per oocyte (Boudry *et al.*, 2002) to fertilize 3 millions eggs for each cross. Then, 3 hours post-fertilisation, fertilized eggs were transferred to a rearing room and put in 30L tanks with water temperature maintained around 24°C. Oysters were reared during their larval stage without any selective culling (i.e. no sieving to discard smallest individuals), following the protocol described in Taris *et al.* (2006). On the 28 July 2004, bi-parental crosses, were performed in the Ifremer experimental research station of the Invertebrate Physiology Laboratory in Argenton (Finistère, France) between eight F0 individuals from the low ("S") and high ("R") selected lines, to produce F1 hybrid families. Following settlement and metamorphosis, when oysters reached a minimal size of 2 mm they were transferred to the Ifremer nursery in Bouin (Vendée, France). Families were placed for 12 weeks in high-growth environmental conditions with an enriched diet of the alga *Skeletonema costatum* (Baud and Bacher, 1990). Following the same protocol, F1 individuals were crossed with another one to produce three F2 families (R*O, Z*AC and B*X). These F2 crosses were performed at the Ifremer experimental hatchery of the Ifremer Genetics and Pathology Laboratory in La Tremblade (Charente Maritime, France) on the 22 February 2006 (day 0). Juveniles were sampled when their size ranged from 16 to 60 mm. This range of size is usually sufficient to obtain enough fresh tissue from dead individuals to extract nucleic acids. The mortality monitoring experiment started on July 8 (i.e. 138 days after fertilization) and was ended 86 days later (October 15, day 224), once no more mortality had been observed for several weeks.

2.2 Experimental design to investigate the kinetic of summer mortality

On 29 June 2006 (day 128), three groups of 1500 juveniles, randomly sampled within each family, were placed in a single $30m^3$ raceway, in the experimental facilities of the Ifremer Genetics and Pathology Laboratory. Seawater temperature was automatically recorded every 10 minutes during the course of the experiment using a multi-parametric *in situ* monitoring system (YSI 6920S). The water inflow rate corresponded to two total renewals of the raceway water per day. Individuals were put in standard oyster-growing bags (6mm mesh) as follows: (*i*) one thousand oysters were put in a first bag where dead oysters were removed daily for study (*ii*) five hundred individuals were placed in a second bag from which 30 different living juveniles (as our protocol is destructive) were sampled daily during the first four weeks of the monitoring and then twice a week until the end of the experiment. The bags of oysters were randomly arranged on iron racks at 40 cm height above the bottom, as is the commonly practice used in oyster aquaculture in the field, and were turned over every week to avoid algal development and subsequent feed depletion.
Prior to the beginning of the experiment (day 128), a set of 96 individuals was first assessed by real-time PCR to evaluate the viral prevalence of OsHV-1. During the experiment, bags were checked daily to remove dead oysters, which were stored immediately at -20°C to conserve DNA for later extraction if a sufficient amount of tissue remained. Dead oysters could be identified by their open valves, and mortality was quantified daily by counting the number of individuals that had died.
Individual viral quantifications were performed by real-time PCR for (*i*) the first 150 individuals that died in each of the two families where significant mortality occurred (i.e. families R*O and Z*AC), (*ii*) 12 of the 30 living individuals sampled daily from each of the three families during the mortality outbreak, (*iii*) 150 individuals per family that had survived through the mortality outbreak to day

224. We were careful to sample the same type of tissue on dead, living and surviving oysters to allow the direct comparison of the viral load and reduce variance in its measurement as much as possible.

2.3 OsHV-1 detection and quantification using real-time PCR

Total DNA was extracted using the Qiagen Qiamp® tissue mini kit according to the manufacturer's protocol and as previously reported by Robledo *et al.*, (2000). The DNA quality was firstly checked by spectrophotometry (Eppendorf®) and run on a 1.5% agarose gel. Low quality ($DO_{260/280}$>1.8) and quantity (<50 $\mu g.\mu L^{-1}$) DNA samples were removed and replaced by another one for the following steps.
The real-time PCR protocol applied for OsHV-1 DNA detection and quantification has been published recently (Pépin *et al.*, 2008). Briefly, DNA samples were diluted to a concentration of $2ng.\mu L^{-1}$. The reaction mix was composed of 5µL total DNA sample, 12.5µL Full-Velocity® SYBR Green Master Mix reagent (Stratagene), 2.5µL [2µM] forward (C9) and reverse primers (C10), and 2.5µL distilled water, in a final reaction volume of 25µL. The primer pair generates a 197 bp amplicon and targets the C region of the viral genome (Barbosa-Solomieu *et al.*, 2004). Amplifications were performed in 96-well plates on a Mx3000P real-time PCR thermocycler sequence detector (Stratagene), according to the following conditions: initial denaturation 1min at 95°C, followed by 40 cycles of 15s at 95°C and 30s at 60°C. The specificity of the PCR products was systematically checked with the melting temperature (Tm) value from the dissociation curve (Bustin, 2000). The quantification of the samples was based on a standard five-fold dilution curve derived from a stock solution of OsHV-1 genomic DNA (5.10^6 copies.μL^{-1}) extracted from purified virus particles (Le Deuff and Renault, 1999). Efficiency (E) and linearity (R^2) were calculated from the standard curve with MxPro v3.0 software (Stratagene), and tested for each run. All samples were analysed in triplicate.
Individual viral DNA quantification was first performed on a set of seven individuals that died during a previous mortality event associated with OsHV-1 infection, using mantle, digestive gland and adductor muscle to study the variance in real-time PCR viral DNA quantification among these different tissues. As a second step, the viral DNA was individually quantified in mantle from three types of individuals (i.e. dead, living and surviving) for each family. The results were expressed as a $\log_{10}$ of the viral DNA copy number per mg of tissue.

2.4 Statistical analysis

Descriptive statistics on individual viral DNA quantification data were carried out using SPSS v11.5 statistical software. The normality of the data was tested using a Shapiro-Wilk test. A non-parametric Kendall W test was used to investigate the differences in viral DNA quantification between the different types of tissue, and a non-parametric Kruskal-Wallis test was performed to analyse the differences in variance of viral DNA quantities between (1) tissues, (2) families and (3) the type of individuals (dead, living or surviving). A Spearman rho test was used to analyse the correlation between viral DNA quantification and mortality data. P values lower than 0.05 were used to identify significant effects or differences.

3. Results

3.1 Oyster mortality and seawater temperature

No significant mortality was recorded prior to day 138. High mortalities were then observed on day 139 (Fig. 1). Cumulative mortality over the twelve following weeks ranged from 1.2 (family B*X) to 49% (family Z*AC) and was significantly different between families ($p<0.001$). Mortality was sudden, as more than 60% of dead individuals were observed during the first week of the survey and very little mortality was observed after day 159 (Fig. 1). During the mortality outbreak (day 139

to 159), the mean seawater temperature (± SD) was 23.8 ± 1.02°C, ranging from 21.5 and 26.8°C (Fig. 1).
During the experimental monitoring period, mean water temperature was not significantly different from temperatures observed in other years. However, it should be noted that the outbreak was preceded by an increase in the seawater temperature of 2.3°C over two days.

3.2 Detection of OsHV-1 and individual viral quantification

Quantification of viral DNA was optimised by first comparing viral detection obtained after DNA extraction from different tissues sampled from oysters that died during a previous mortality event associated with OsHV-1 infection. A comparison was made between adductor muscle (AM), mantle (M) and digestive gland (G). Pieces of the different tissues ranging from 20 to 25mg were precisely weighed to allow the viral DNA load of individuals to be estimated as the number of viral genomic DNA copies per milligram of tissue. A sample of 20-25mg represents 6‰ to 1.5% of the total fresh weight, depending the size of the individual (2-4g). The comparison among three different tissues did not show any significant differences (W=8.97; P>0.05). The viral load was estimated as $1.03\ 10^8 \pm 7.67\ 10^7$, $1.15\ 10^8 \pm 8.50\ 10^7$ and $9.79\ 10^7 \pm 7.26\ 10^7$ viral DNA copies.mg^{-1} in the adductor muscle, mantle and digestive gland tissues, respectively. We therefore decided to extract nucleic acids from a single tissue only during the mortality experiment, and chose mantle because the amount of this tissue remaining on dead individuals is usually sufficient to achieve reliable DNA extraction.
The raw data obtained from the analysis of samples were carefully examined to verify that Ct values (Cycle threshold) obtained had the correct Tm value corresponding to the amplicon. This method allows incorrect PCR amplification to be detected visually. The detection of OsHV-1 in the set of 96 randomly chosen individuals prior to the experiment (day 128) did not reveal any significant viral infection. Only three of these animals displayed positive detection results, with viral DNA copies.mg^{-1} inferior to 1.10^3.
In our experiment, the virus was detected in both living and dead individuals. In dead individuals, the prevalence was estimated as 94.7% (142/150) and 95.3% (143/150) in the R*O and Z*AC families, respectively. In living individuals, the prevalence ranged from 9.4% (9/96 – B*X family) to 83.2% (89/107 – Z*AC family). Qualitative data for OsHV-1 detection on the three oyster families and associated prevalence are presented in Table 1.
The individual viral load was significantly different between dead, living and surviving oysters (P<0.001). In dead individuals, the viral load showed a high variance both between families and within each family (P<0.001 – Table 2). The highest level of infection was detected in dead animals of the Z*AC family (Z=27.25; P<0.001) with a mean viral load estimated at $1.14\ 10^7$ DNA copies mg^{-1} (Fig. 2). In the 20 individuals of the B*X family that died during the experiment, quantitative data could not be estimated because the amount of tissue remaining in the dead oysters was not sufficient to extract viral DNA. In living individuals, the average viral load showed no significant difference between families (P=0.07) but was highly variable, especially in the Z*AC family where it ranged from 0 to $1.10\ 10^8$ DNA copies mg^{-1} tissue. The lowest mean level of infection was observed in the B*X family with $2.5\ 10^2$ DNA copies mg^{-1} of tissues. In individuals that survived to the end of the experiment, the viral load was significantly lower from that observed in dead and living individuals sampled during the experiment (P<0.001), but there was no significant difference between the three families (P=0.069). The highest and lowest levels of quantification were detected in the R*O and B*X families with $3.34\ 10^2$ and $1.02\ 10^2$ copies of viral DNA mg^{-1} of tissue, respectively (Table 2). The correlation between the level of mortality that occurred in families and the mean level of OsHV-1 DNA detection was highly significant (Spearman's ρ=0.338; P<0.001).

4. Discussion

4.1 Factors involved in oyster mortality

The present experiment was designed to monitor oyster mortality and sample live and dead individuals on a daily basis. Furthermore, oysters were reared under common environmental conditions throughout their lives, including when mortality occurred. This allowed individual viral DNA loads to be recorded daily, thus providing a measure of the viral infection level that could be compared between dead, living and surviving oysters, and also within and between families. The fact that the mortality episode occurred very suddenly suggests that an infectious agent, such as OsHV-1, may be the causal factor. Similarly, Burge *et al.* (2006) observed a comparable temporal pattern in mortality of Pacific oysters reared in the field along the West coast of the USA. In this study, mortalities ranged from 5.7 to 64% during a 12 days period. Although our experiment might not fully reflect environmental conditions in the field, our results are likely to be relevant to summer mortality observed in field conditions (Burge *et al.*, 2006).
Additionally, a positive effect of high temperature on OsHV-1 infection have also been reported, notably in hatchery-reared larvae of Pacific oyster (Le Deuff *et al.*, 1996). Our monitoring of the seawater temperature supports these results, as do those reported by Burge et al. (2006). A higher temperature influences the expression of herpes virus by promoting an earlier production of viral particles, resulting in a rapid spread of the disease that could be capable of causing sudden and massive mortalities. OsHV-1 may thus usually act in synergy with environmental parameters, particularly temperature, to affect oysters (Moal *et al.*, 2008). In our experiment, the mortality outbreak occurred after a 2°C increase in seawater temperature. The two-day delay before the mortality outbreak may reflect the time required for the virus to initiate an intense replication phase. In the following days, when massive mortality was occurring, seawater temperature reached 23.8°C±1.02°C. This clearly illustrates that temperature is one of the major factors acting on the mechanisms of OsHV-1 infection. Interestingly, temperature is also one of the main factors involved in gametogenesis in the Pacific oyster (Fabioux *et al.*, 2005). In autumn (i.e. after the reproductive period), decreasing water temperature may be related to the observed decrease in viral load in oysters and absence of mortality. Temperature is clearly a significant factor related to mortality outbreaks, but it is not the only one (as notably documented by Samain *et al.*, 2008). Significant correlations are found between water temperature gametogenesis, physiological and immunological weakening, prevalence of pathogens, and oyster mortality. A similar relationship was also demonstrated in the infection of the eastern oyster by the parasite *Perkinsus marinus* (Ragone Calvo *et al.*, 2003).

4.2 Quantification of OsHV-1 DNA

A number of different techniques (PCR, ISH and immunochemistry) were employed to detect OsHV-1 in 30 asymptomatic individuals of the Pacific oyster, *Crassostrea gigas* in a previous study by Arzul *et al.* (2002). This study used different types of tissue from adult oysters for OsHV-1 detection. When mantle and gill tissues were used, >76% of individuals tested positive whereas, when gonadic tissue was used, OsHV-1 was detected in a far less significant number of individuals (33%, Arzul *et al.*, 2002). To further examine the hypothesis of differential viral load between tissues, we compared the OsHV-1 in three types of tissue (mantle, digestive gland and adductor muscle), this time taken from infected animals. The gonadic tissue was not analysed due to the young age of the oysters (<1 year). Although gametogenesis can be significant in one-year old oysters, it is usually more limited and variable than in older individuals. As a result, this tissue is also harder to sample from one-year-old oysters than the tissues we tested. Furthermore, the oysters in our study did not present mature gonads during the sampling period. Very few quantitative data are yet available regarding OsHV-1 infection in oysters (Renault *et al.*, 2004), which may partly be due to the lack of cell lines in bivalve molluscs on which to cultivate this infectious agent. Usually, conventional PCR is used to characterize OsHV-1 prevalence (Renault *et al.*, 2000, 2004 ; Burge *et al.*, 2006 ; Webb *et al.*, 2007). Our study is the first showing quantitative data over time for the characterization of viral prevalence during a mortality event. This was made possible by the use of real-time PCR quantification recently developed (Pépin *et al.*, 2008). Our study describes the evolution and spread of the viral infection at the individual and the family level. Prior to our study, Pépin *et al.* (2008) showed that an average number of 1.10^3 copies of viral DNA mg^{-1} of tissue do not represent an infective status associated with mortality. Similarly low viral loads have been reported for the koi herpes virus (KHV) in asymptomatic fish and it was suggested that they could be associated with latency rather than productive infection (Gilad *et al.*,

2004 ; Goodwin *et al.*, 2006). In our study, the viral load, in living or dead oysters reached up to 10^7-10^8 viral DNA copies mg^{-1} tissue but was highly variable. High variations in viral DNA quantification between individuals may indicate different stages of viral infection. The detection of a great amount of viral DNA in some living oysters (10^7 - 10^8 copies of viral DNA mg^{-1} tissue) may represent the final stage of infection before death, in which case detecting such an amount of OsHV-1 DNA may be interpreted as a negative prognostic factor. However, further knowledge of the kinetics of viral infection is still needed. The study of the temporal evolution of the OsHV-1 DNA load in a single adult individual during viral infection would be of great interest. This would require repetitive sampling of a few mg of mantle tissue from the same individuals, which is a common practice in adult oysters where it does not induce a higher mortality rate (>1%; Suquet *et al.*, 2009). Attempts to quantify *Perkinsus marinus*, which affects the eastern oyster *C. virginica*, have previously been made in the water column using a molecular approach (Audemard *et al.*, 2006). Real-time PCR assay appears to be a powerful tool that would be appropriate for investigating the abundance of OsHV-1 DNA in water samples to monitor virus presence in the environment over seasonal changes.

4.3 Genetically based resistance to OsHV-1 and its transmission

Our results suggest there is a genetic basis for resistance to OsHV-1 infection in the Pacific oyster. First, our data showed a high variance in the viral prevalence between the 3 full-sib oyster families. This suggests that each group of oysters may be composed of individuals with different degrees of tolerance to the viral infection. Their parental origin may lead to a differential segregation of alleles linked to sensitivity or resistance to mortality and/or OsHV-1 infection. In the B*X family, we could argue that both F1 parents carried alleles linked to the resistance to mortality and/or infection by OsHV-1 whereas, for the Z*AC family, the same alleles were not carried by the parents and were therefore not transmitted to the progeny.
Previous studies suggest that a genetic basis may underlie resistance to OsHV-1 infection in the Pacific oyster. Le Deuff *et al.* (1996) showed that the geographic origins of parents, in relation to the appearance of the disease, might have influenced the cumulative percentage of mortality in their progeny.
The persistence of OsHV-1 in asymptomatic adults was demonstrated by the detection of viral DNA and proteins, suggesting that OsHV-1 has a high prevalence among adult oysters and may persist in its host after a primary infection (Arzul *et al.*, 2002). The vertical transmission of OsHV-1 through gametes released during experimental crosses, was investigated in another study made over three successive generations of Pacific oysters (G0 and G1 parental oysters, G1 and G2 larvae - Barbosa-Solomieu *et al.*, 2005). Results strengthened the hypothesis of a vertical transmission, although it was not possible to predict the issue of a particular type of cross. The detection of viral DNA in parental oysters did not systematically correspond to a productive infection or result in any successful transmission to the progeny. Moreover, Batista *et al.* (2007) argue that negative PCR results do not necessarily indicate that oysters are not infected, because when levels of viral DNA in the tissues are low, they may be below the threshold of the detection method.
Other means of viral transmission should be considered. Latent infection in oysters may be reactivated by the water temperature stimulus or other environmental factors allowing the transmission of the virus to the nearest neighbours. Like other herpes viruses, OsHV-1 seems to be able to persist in its hosts (Arzul *et al.*, 2002). Infected oysters may play the role of carriers and reservoirs of the virus, promoting virus transmission from infected to non-infected ones, as previously hypothesized (Le Deuff *et al.*, 1996). Considering this possibility, we decided to examine the seawater in which the oysters were growing in an attempt to see whether OsHV-1 could be detected using real-time PCR in samples collected during the mortality outbreak (day 139 to day 159). The results demonstrated the presence of viral DNA in the seawater around the oysters (1.10^3 to 2.10^3 viral DNA copies.L^{-1}). Although this method still requires some technical improvement, real-time PCR assay appears to be a valuable tool for investigating the abundance of OsHV-1 DNA in water samples and could therefore be used to monitor virus presence in the environment over seasonal changes. The detection of viral DNA in sea water samples and the observed variance of viral load among dead and living individuals suggest that (*i*) sea water may act as a vector in the horizontal transmission of OsHV-1 and (*ii*) some individuals play a reservoir

role in the spread of the disease as previously proposed (Arzul *et al.*, 2002). Heavily infected oysters may have been sites of intense viral replication until their death, and virus release from moribund and dead oysters allowed horizontal transmission to living ones. Moribund and dead infected oysters may thus act as a major source of the virus. In our experiment, variation in survival within and between families suggests that such horizontal transmission did not lead to the mortality of newly infected oysters during the study period.

Improved knowledge about transmission of OsHV-1 may lead to practical epidemiological recommendations that would limit the impact of viral infection on the oyster industry. The screening for OsHV-1 in stocks of oysters during their transportation between different geographical zones of culture could help to prevent the spread of the virus. Similarly, larvae and seed produced by hatcheries and nurseries could be screened prior to their transfer to the field. In Maryland and Virginia (USA), the introduction of a non-native species, the cupped oyster *Crassostrea ariakensis* originating from China, was discussed to overcome the drastic decline of eastern oyster *Crassostrea virginica* populations due to over-fishing, diseases and habitat destruction in Cheasapeake Bay (Moss *et al.*, 2007). Prior to the introduction, the pathogen survey of *C. ariakensis* and other sympatric oyster species was conducted on samples collected in the PR China, Japan and Korea using molecular diagnostics and histopathology. This study revealed the presence of two *Perkinsus* species not currently found in USA waters and three particular strains of molluscan herpes viruses. As a result, there is a strong interest in increasing the use of this kind of study prior to any restoration or introduction attempts with oysters or marine bivalves in general. The knowledge gained with the Pacific oyster model can be extended to other species affected by herpes-like viral infections, such as the abalone whose production in Taiwan and in Australia has been affected by massive mortalities over the last few years (Chang *et al.*, 2005; Hooper *et al.*, 2007; Tan *et al.*, 2008).

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Tables

Table 1. Qualitative data for OsHV-1 detection and associated prevalence obtained using real-time PCR in dead and living oysters of the three studied oyster families.
N = number of samples analysed by real-time PCR.

Status	Family	N	Positive	Negative	Prevalence (% of infected individuals)
Dead individuals	B*X	0*			
	R*O	150	142	8	94.7
	Z*AC	150	143	7	95.3
Living individuals	B*X	96	9	87	9.4
	R*O	107	89	18	83.2
	Z*AC	102	81	21	79.4

*** No mortality recorded in the B*X family**

Table 2. Viral load quantification in oyster samples using real-time PCR, expressed as viral DNA copy number mg^{-1} of fresh tissue, in dead, living and surviving oysters.

Status	Family	Cumulative percentage of mortality (n= 1500)	Number of individuals analysed	Minimal individual viral load	Maximal individual viral load	Average viral load	Standard error
Dead individuals	B*X	1.2	0	ND	ND	ND	ND
	R*O	38	150	5	$2.95\ 10^{07}$	$2.04\ 10^{06}$	$3.86\ 10^{06}$
	Z*AC	49	150	0	$4.84\ 10^{08}$	$1.14\ 10^{07}$	$4.66\ 10^{07}$
Living individuals	B*X	-	96	0	$3.40\ 10^{03}$	$2.50\ 10^{02}$	$1.34\ 10^{03}$
	R*O	-	107	0	$6.57\ 10^{07}$	$1.08\ 10^{05}$	$6.70\ 10^{06}$
	Z*AC	-	150	0	$1.10\ 10^{08}$	$1.71\ 10^{05}$	$1.11\ 10^{06}$
Surviving individuals	B*X	-	150	0	$2.35\ 10^{03}$	$1.02\ 10^{02}$	$2.48\ 10^{02}$
	R*O	-	150	0	$1.71\ 10^{03}$	$3.34\ 10^{02}$	$1.47\ 10^{02}$
	Z*AC	-	150	0	$2.46\ 10^{02}$	$1.86\ 10^{02}$	$2.54\ 10^{02}$

ND: no individuals were analysed

Figures

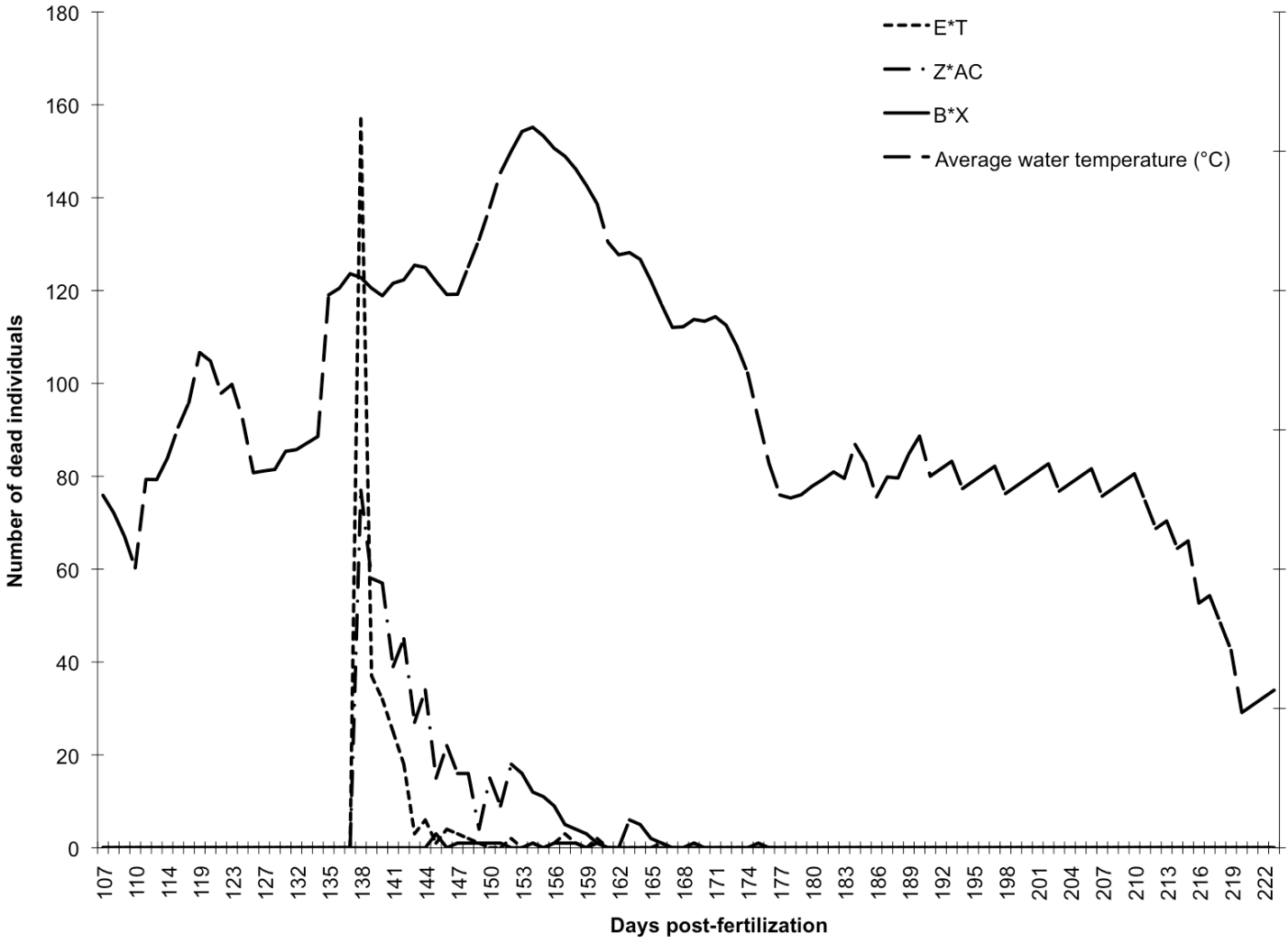

Figure 1. Kinetics of mortality during the outbreak in summer 2006, shown with concomitant seawater temperature (°C)

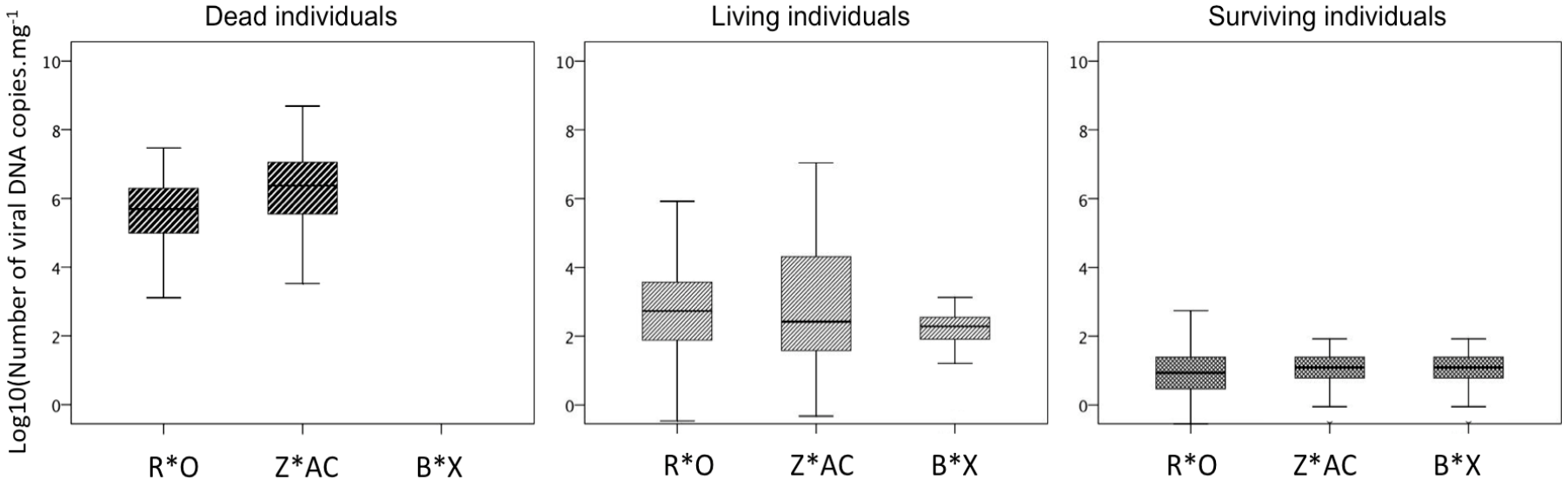

Figure 2. Box-plots representing the variance in mean viral quantification between families and sample types