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Insights on the association between somatic aneuploidy and ostreid herpesvirus 1 detection in the oysters *Crassostrea gigas*, C. angulata and their F1 hybrids

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Abstract :

Cytogenetic abnormalities associated with viral infections, including from viruses of the Herpesvirales order, have been reported in vertebrate species. Ostreid herpesvirus 1 (OsHV-1) has been detected worldwide during mortality outbreaks of the Pacific oyster *Crassostrea gigas*. On the other hand, a high proportion of aneuploid cells in somatic tissues have been observed in *C. gigas*. In this study, we analysed the putative association between aneuploidy levels and the detection of OsHV-1 in gills of *C. gigas*, the Portuguese oyster *C. angulata* and their F1 hybrids cultured in Ria Formosa (Portugal). OsHV-1 was detected by PCR in 5.4% of the total of oysters analysed (n = 111) namely in 11.1%, 8.0% and 1.7% of *C. gigas*, *C. angulata* and F1 hybrid respectively. Sequencing analysis of a viral fragment amplified with the C2/C6 primer pair revealed a high similarity with the OsHV-1 reference type. Moreover, *in situ* hybridization confirmed the presence of OsHV-1 in gill tissue. Oysters where OsHV-1 was detected had a significantly higher mean percentage of aneuploid cells (25%) than the ones where the virus was not detected (18%). However, the overall low percentage of positive samples contrasted with the high mean percentage of aneuploidy observed, with 50% of the oysters analysed showing a percentage of aneuploid cells between 20% and 30%. We hypothesize that somatic aneuploidy may adversely affect oysters making them more prone to OsHV-1 infection, but the virus is unlikely to be the cause of somatic aneuploidy.

Keywords : Aneuploidy, Ostreid herpesvirus 1, Oyster, Crassostrea

Introduction

Chromosomal instability and damage have been reported in association with viral infections (Fortunato & Spector 2003). Viruses can interfere with cellular DNA-damage response, which encompasses a complex series of pathways responsible for the maintenance of genome integrity (Weitzman, Lilley & Chaurushiya 2010). Most of our knowledge about virus-host interactions and their associations with chromosomal abnormalities comes from studies in humans and other vertebrates (Yasunaga & Jeang 2009). This is notably the case in herpesviruses such as Epstein-Barr virus, human cytomegalovirus, herpes simplex virus and human herpesvirus 8. During latent infections herpesviruses can interfere with cell cycle checkpoints and cell death pathways, which can result in chromosomal abnormalities (Yasunaga & Jeang 2009). Among the different gross chromosomal abnormalities associated with herpesvirus infections are chromosome deletions, translocations, inversions and numerical alterations (Pan, Zhou & Gao 2004)).

Detection of herpes-like virus associated with mortality of the Pacific oyster *Crassostrea gigas* larvae were first described by Hine, Wesney & Hay (1992) and Nicolas, Comps & Cochennec (1992). The genome characterization of a herpes-like virus infecting *C. gigas* allowed its classification as a member of the *Malacoherpesviridae* family under the name Ostreid herpesvirus 1 (OsHV-1) (Le Deuff & Renault 1999; Minson, Davison, Eberle, Desrosiers, Fleckstein, McGeoch, Pellet, Roizman & Studdert 2000; Davison, Trus, Cheng, Steven, Watson, Cunningham, Le Deuff & Renault 2005; Davison, Eberle, Ehlers, Hayward, McGeoch, Minson, Pellett, Roizman,·Studdert &·Thiry 2009). OsHV-1 is presumed to be one of the main pathogens involved in mass mortality outbreaks of *C. gigas* reported worldwide, namely in several European countries, USA, New Zealand and Australia (Renault 2012,

Segarra, Pépin, Arzul, Morga, Faury & Renault 2010; Burge, Griffin & Friedman 2006; Jenkins, Hick, Gabor, Spiers, Fell, Read, Go, Dove, O'connor, Kirkland & Frances 2013), which has adversely affected aquaculture production of this species.Mortality outbreaks have been observed in association with OsHV-1 detection in larvae and juvenile of *C. gigas* (Arzul, Renault, Lipart & Davison 2001; Renault & Arzul 2001). OsHV-1 is presumed to establish latent/persistent infections in *C. gigas* adults (Arzul, Renault, Thébault & Gérard 2002), but little is known about its interaction with the host.

Aneuploidy can be defined as a condition in which the number of chromosomes of a cell is not a multiple of the haploid number of the species due to a gain and/or loss of chromosomes. Cytogenetic abnormalities in mitosis can result in a mosaic of normal and aneuploid cells in somatic tissues, and the degree of somatic aneuploidy can vary substantially among individuals. Some of the factors responsible for aneuploidy have already been identified in studies carried out mainly on vertebrates, especially in mice and human (e.g. Cimini & Degrassi 2005). Aneuploidy has also been identified in different mollusk bivalve species (Thiriot-Quievreux 1986, Le Grand, Kraffe, Montaudouin, Villalba, Marty & Soudant 2010; Teixeira de Sousa, Matias, Joaquim, Ben-Hamadou & Leitão 2011). In *C. gigas* and in the Portuguese oyster *Crassostrea angulata*, somatic aneuploidy has been reported and targeted to specific chromosome pairs (Leitão, Boudry & Thiriot-Quiévreux 2001; Teixeira de Sousa, Joaquim, Matias, Ben-Hamadou & Leitão 2012). Moreover, it has been shown that some pollutants can significantly increase aneuploidy levels in *C. gigas* (Bouilly, Leitão, McCombie & Lapègue 2003). However, the etiology of somatic aneuploidy in oysters remains mostly unknown.

In the context of the GENEPHYS (FAIR 95-421) European research project high aneuploidy levels were observed in *C. gigas* progenies in which herpes-like virus was

103 detected by transmission electron microscopy, which was the only available detection
104 tool at the moment (unpublished data). Based on this previous observation the present
105 study was carried out to investigate the putative association between OsHV-1 detection
106 and the percentage of aneuploid cells in gill tissue of oysters.

Material and methods

Biological material and aneuploidy scoring

A total of 111 oysters were analyzed, namely 25 *C. angulata*, 27 *C. gigas* and 59 *C. angulata* X *C. gigas* F1 hydrids that were collected in November 2004 from an oyster farm in Ria Formosa (Portugal). The oysters were produced and reared as described in Batista, Leitão, Fonseca, Ben-Hamadou, Ruano, Henriques, Guedes-Pinto & Boudry (2007). Water temperature and salinity at the time of oyster sampling were approximately 15ºC and 35 gL-1, respectively. The *C. angulata* individuals analyzed had a mean (±standard deviation) live weight of 19.3 (±8.3) g and a mean height of 46.6 (±8.6) mm. The *C. gigas* individuals analyzed had a mean live weight of 26.9 (±9.3) g and a mean height of 57.0 (±8.3) mm. The F1 hybrids analyzed had a mean live weight of 26.1 (±7.9) g and a mean height of 56.4.0 (±8.1) mm. The 111 oysters analyzed were 472 days old and were at the post-spawning stage (histological examination, data not shown). In the 30 days prior to sampling no mortality was observed. Aneuploidy was scored in gill tissue as described in Batista *et al*. (2007a). Briefly, the level of aneuploidy was estimated by counting the number of aneuploid metaphases in 30 metaphases per individual and hence a total of approximately 3330 metaphases were analysed.

DNA extraction, PCR and sequencing

A piece of gill tissue was removed from each oyster and stored in 70% ethanol. DNA was extracted from gill tissue using a modification of a phenol/chloroform method described by Sambrook, Fritsch & Maniatis (1989). Briefly, DNA was extracted by a combination of proteinase K/SDS digestion followed by phenol/chloroform extraction and ethanol precipitation. In order to assess the quality of the DNA extracted and the

132 presence of inhibitors, a fragment of the cytochrome c oxidade subunit I gene with 584
133 bp of *C. angulata* and *C. gigas* was PCR amplified using the primer pairs COI3 (5'-
134 GTA TTT GGA TTT TGA GCT GT-3') and COI4 (5'-GAG GTA TTA AAA TGA CGA
135 TC-3'). Reactions were performed in 25 µL volume composed of 2.5 µL of 10x PCR
136 reaction buffer, 2 µL of dNTP (2.5 mM), 0.5 µL of each primer (10 µM), 0.2 µL of Taq
137 polymerase (5 u/ µl) and 1.0 µL of template DNA (ca. 100 ng). PCR reactions were
138 performed for one cycle at 94°C for 2 min, 30 cycles consisting of denaturation at 94°C
139 for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, followed by a final
140 extension for 7 min at 72°C. Detection of OsHV-1 DNA was performed by PCR using
141 the primer pair C2 (5'- CTC TTT ACC ATG AAG ATA CCC ACC -3')/C6 (5'- GTG
142 CAC GGC TTA CCA TTT TT -3') to amplify a 709 bp fragment as described by Arzul
143 *et al*. (2001) but with an annealing temperature of 60ºC as described by Batista, Arzul,
144 Pepin, Ruano, Friedman, Boudry & Renault (2007). Reference OsHV-1 DNA (supplied
145 by Ifremer, La Tremblade, France) was used as positive control and distilled water as
146 negative controls. The C2/C6 primer was chosen for detection of OsHV-1 DNA based
147 on its high specificity and sensitivity that allows the systematic detection of 10 fg of
148 purified viral DNA in the presence of up to 500 ng of oyster DNA (Batista *et al*. 2007b).
149 The PCR products obtained using the C2/C6 primer pair were digested with the
150 restriction endonuclease *HaeIII* to confirm their specificity. Digestion reactions were
151 performed in 20 µl volumes composed of 2 µl of digestion buffer, 10 µl of double-
152 distilled water, 0.5 µl of *HaeIII* (10 U/ µl) and 10 µl of amplicons. Amplicons were
153 separated by electrophoresis in 1.5 % agarose gels with ethidium bromide staining. The
154 PCR products from two positive samples (one *C. angulata* and one *C. gigas*) were
155 purified using 0.2 U of Exonuclease I (*ExoI*), 1 U of Shrimp Alkaline Phosphatase
156 (SAP) and 8.8 µL of water, and incubated for 37°C for 60 min, followed by 80°C for 20

157 min. The purified PCR products were sequenced using both primers (C2 and C6) with
158 the BigDye kit (Applied Biosystems) and an automatic sequencer (ABI-PRISM, model
159 377, Applied Biosystems). The nucleotide sequences obtained were edited using Bioedit
160 version 7.0.9.0. (Hall 1999) and aligned with Clustal W (Thompson, Higgins & Gibson
161 1994).
162
163 ***In situ* hybridization (ISH)**
164 *In situ* hybridization was performed as described in López-Sanmartín, Webb & Navas
165 (2013) in samples that yield positive results using the C2/C6 primer pair. Briefly, DNA
166 probes were produced using the C2/C6 primer pair and according to instructions of the
167 Roche "PCR DIG Probe Synthesis Kit". Approximately 5 µm sections were cut from
168 paraffin blocks of oyster tissue fixed with Davison solution and placed in silanised
169 slides. Tissues were dewaxed (65ºC during 10 min), rehydrated and treated with
170 proteinase K (50 µg/ml in PBS) at 37 °C during 15 min. Prehybridation was carried out
171 using 100 µl of pre-hybridization solution (50% formamide, 4× SSC, 5× Denhardt's
172 solution, 0.50 mg yeast tRNA/ml, 0.25 mg Salmon testes DNA/ml) and covered with
173 acetate and placed at 42 °C during 60 min in a humid chamber. Afterwards, 60 µl of
174 hybridization solution (30% formamide, 2.4× SSC, 3× Denhardt's solution, 0.30 mg
175 yeast tRNA/ml, 0.15 mg Salmon testes DNA/ml and 0.3 ng/µl of C2/C6 probe-DIG)
176 was added and the acetate replaced. Slides were maintained at 95 °C during 15 min
177 followed by 1 min at 4°C (cold plate). Slides were then left overnight in a humid
178 chamber at 42 °C. Sections were washed twice with 2x SSC and 1x SSC solutions for 5
179 min and again twice with 0.5 SSC solution at 37 °C for 10 min. A final wash was
180 performed with buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) at 20°C (2 x 5 min)
181 and with buffer 2 (blocking solution) for 60 min. Probe detection was performed

according to the instructions of the kit Anti-digoxigenin-AP Fag Fragments Kit (Roche). Slides were then counterstained using Bismarck brown Y (0.5 % in water) during 1 min and mounted in Shandon aqueous mounting media and left to dry for 24 h.

Statistical analysis

Due to the low number of positive samples the data from the 3 groups (*C. angulata*, *C. gigas* and their F1 hybrids) was pooled. Student's t-test was used to determine if there were significant differences in mean aneuploidy levels between OsHV-1 positive and negative samples. Levene's test was used to test for homogeneity of variance. In addition, a permutation test with a total of 10,000 permutations was performed to estimate the probability that the difference between the mean aneuploidy of OsHV-1 positive and negative samples was equal to or greater than the mean difference observed.

Results and discussion

Detection and characterization of OsHV-1

OsHV-1 was detected by PCR using the C2/C6 primer pair in 6 out of the 111 oysters analysed, namely in 3 out of the 27 *C. gigas*, in 2 out of the 25 *C. angulata* and in 1 out of 59 F1 hybrids. The amplicons obtained with the C2/C6 primer pair had the expected sizes (Fig. 1). Digestion of amplicons obtained with *HaeIII* yielded the expected pattern (ca. 255 and 454 bp fragments) matching the size obtained using amplicons for the positive control. Additionally, oyster DNA was amplified in all samples (n=111) with the primer pair COI3/COI4, which indicates that there were no inhibitory factors that could result in false-negatives (Fig. 1). The comparison of the 2 sequences obtained in the present study (GenBank accession number XXXXXX) with OsHV-1 reference type

(accession no. AY509253) for the region comprised between position 4180 bp and 4784 bp revealed the insertion of a CTA motif in the microsatellite zone. Moreover, 100% similarity was observed between the sequences obtained in the present study and OsHV-1 sequences (JN800075, JN800077, JN800078 and JN800080) described in France in 2003 by Renault, Moreau, Faury, Pepin, Segarra & Webb (2012) in juvenile *C. gigas*. The results of the present study identify for first time the presence of OsHV-1 in Portugal in adults of *C. gigas* and *C. angulata*. *In situ* hybridization revealed positive labeled gill cells, confirming the results obtained by PCR, but also the presence of OsHV-1 in other tissues namely in the mantle (Fig. 2) and in labial palps. The percentages of positive samples observed in adults of *C. gigas* (10.3 %), *C. angulata* (8.0%) and F1 hybrids (1.7%) was considerably lower than the 63.3% reported by Arzul *et al.* (2002) in *C. gigas* adults (France) using the same primer pair and DNA also extracted from gills. In the present study oysters were collected in late November, whereas in Arzul *et al.* (2002) sampling was performed in August, which may explain the different prevalence levels observed. Indeed, monthly variation in OsHV-1 detection in *C. gigas* spat has been reported (Garcia, Thébault, Dégremont, Arzul, Miossec, Robert, Chollet, François, Joly, Ferrand, Kerdudou & Renault 2011), with the virus being detected mainly during the spring/summer period when water temperature increases. However, other factors cannot be ruled out, such as specific environmental conditions and/or a low viral presence in the culture site in Ria Formosa (Portugal).

Association between aneuploidy and OsHV-1

The percentage of aneuploid cells in *C. gigas*, *C. angulata* and F1 hybrids analyzed ranged between 7 and 30 % with a mean (±standard deviation) of 18 (±6) % (Fig. 3). Previous studies have showed that levels of somatic aneuploidy in *C. gigas* individuals

232 can vary from 0 up to 43 % with mean values for populations between 9 and 34%
233 (Thiriot-Quiévreux, Pogson & Zouros 1992; Leitão, Boudry & Thiriot-Quiévreux
234 2001). The mean somatic aneuploidy of 18% observed in the present study was
235 relatively high (with a maximum individual aneuploidy of 30%), which contrasts with
236 the low number of OsHV-1 positive individuals observed (5.4 %, total n=111). The
237 virus was not detected in 89% of the oysters with the highest levels of aneuploidy levels
238 observed, namely between 20 and 30%. However, a significantly higher mean
239 aneuploidy level (t-test; t=2.911; p=0.0044) was observed in oysters in which OsHV-1
240 was detected (mean of 25%) in comparison with oysters in which the virus was not
241 detected (mean of 18%). Significant differences were also observed using the
242 permutation test (two-tailed $p<0.01$, confidence interval of 0.0034 and 0.0062) for mean
243 aneuploidy difference between OsHV-1 positive and OsHV-1 negative oysters. A
244 deleterious effect of aneuploidy on a large number of cells, making the host eventually
245 more susceptible to pathogens such as OsHV-1, may explain the detection of OsHV-1 in
246 some of the oysters showing high aneuploidy levels (between 20 and 27%). Indeed,
247 several authors have observed a negative correlation between aneuploidy and growth
248 rate in both oyster taxa (Leitão A., Boudry P. & Thiriot-Quiévreux C. 2001; Batista,
249 Leitão, Fonseca, Ben-Hamadou, Ruano, Henriques, Guedes-Pinto & Boudry 2007) but
250 the nature of this relationship remains unknown. The results obtained in the present
251 study suggest that there is a weak association between aneuploidy levels and the
252 detection of OsHV-1 in gill tissue of oysters. We hypothesize that somatic aneuploidy
253 may adversely affect oysters making them more prone to OsHV-1 infection. Our results
254 do not support the hypotheses that the virus is the cause of somatic aneuploidy. Hence,
255 if our hypothesis is correct, an increase in somatic aneuploidy due to pollutants (Bouilly
256 *et al*. 2003) and/or other factors can result in an increase in OsHV-1 infection. One

limitation of the present study was the low number of OsHV-1 positive oysters detected (6 out of 111 individuals), despite the high number of oysters showing high aneuploidy levels. Future studies focusing on scoring aneuploidy in experimentally infected oysters with OsHV-1 in comparison with non infected oysters should shed more light on the putative association between somatic aneuploidy and OsHV-1 infection.

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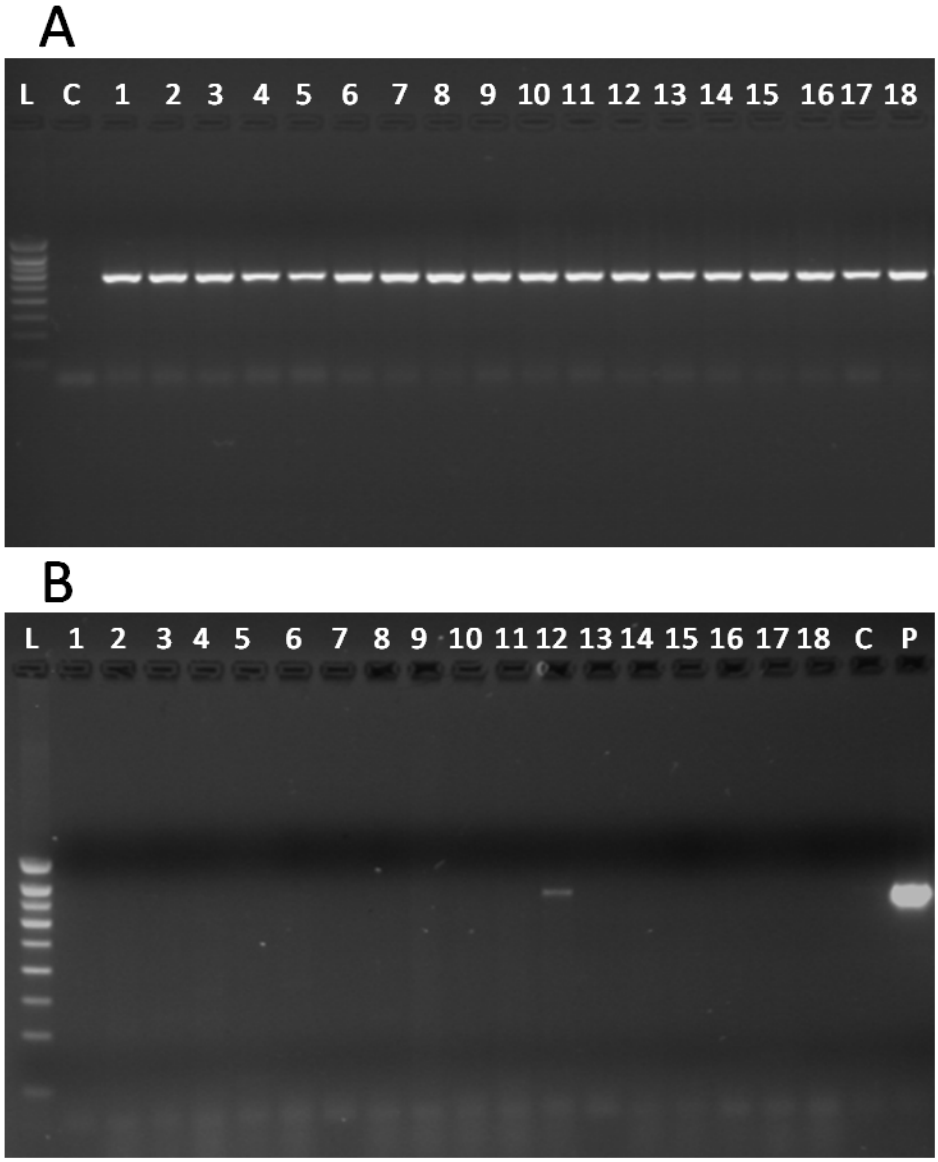

Figure 1. (A) Amplification of a COI fragment by PCR using the primer pair COI3/COI4 (PCR inhibition control). (B) OsHV-1 DNA detection by PCR using the C2/C6 primer pair. Lanes 1-18, *C. gigas* samples; Lanes C, negative control (water instead of DNA); Lane P, positive control (OsHV-1 DNA); Lane L, 100 bp DNA ladder (Promega).

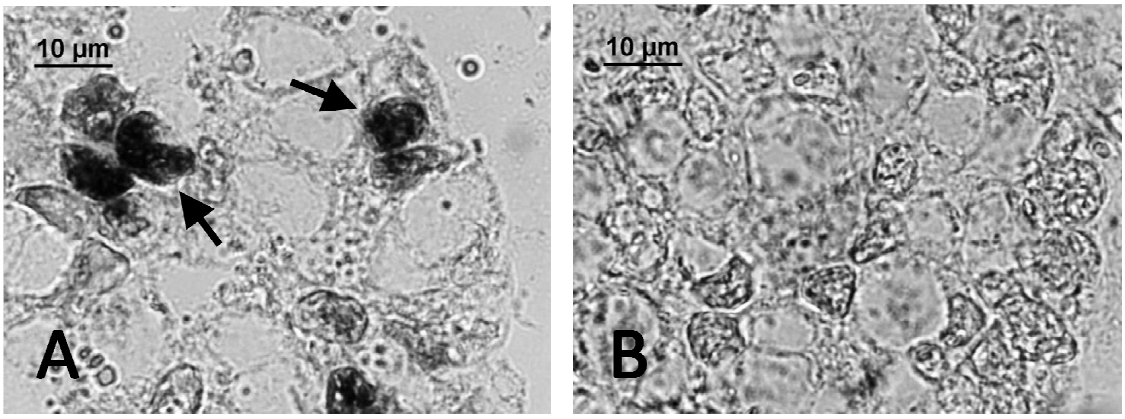

Figure 2. Mantle tissue of *C. angulata*. (A) *In situ* hybridization using the C2/C6 DIG-labelled probe and Bismarck brown Y counterstain and (B) without probe and Bismarck brown Y counterstain. Arrows indicate positive reactions.

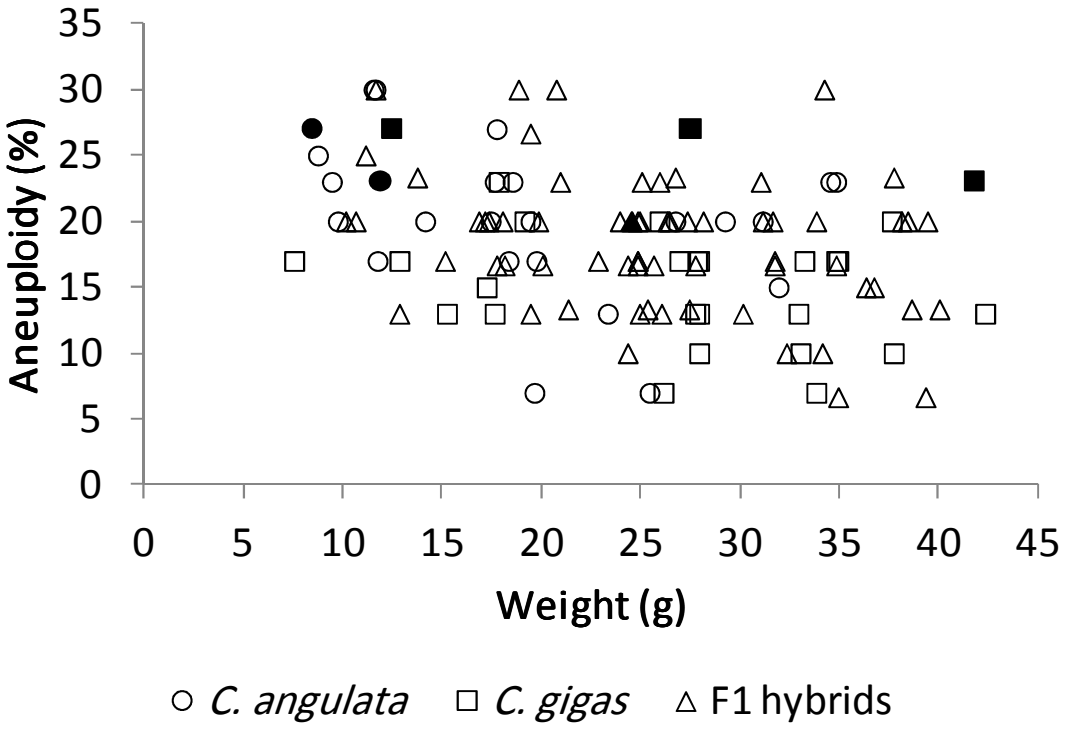

Figure 3. Aneuploidy percentage, live weight and detection of OsHV-1 in *Crassostrea angulata*, oyster *C. gigas* and F1 hybrids. Fill markers represent oysters in which OsHV-1 was detected.