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First detection of OsHV-1 in the cephalopod *Octopus vulgaris*. Is the octopus a dead-end for OsHV-1?

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ABSTRACT

The ostreid herpes virus (OsHV-1), associated with massive mortalities in the bivalve *Crassostrea gigas*, was detected for the first time in the cephalopod *Octopus vulgaris*. Wild adult animals from a natural breeding area in Spain showed an overall prevalence of detection of 87.5% between 2010 and 2015 suggesting an environmental source of viral material uptake. Overall positive PCR detections were significantly higher in adult animals ($p = 0.031$) compared to newly hatched paralarvae (62%). Prevalence in embryos reached 65%. Sequencing of positive amplicons revealed a match with the variant OsHV-1 μVar showing the genomic features that distinguish this variant in the ORF4. Gill tissues from adult animals were also processed for *in situ* hybridization and revealed positive labelling. Experimental exposure trials in octopus paralarvae were carried out by cohabitation with virus injected oysters and by immersion in viral suspension observing a significant decrease in paralarval survival in both experiments. An increase in the number of OsHV-1 positive animals was detected in dead paralarvae after cohabitation with virus injected oysters. No signs of viral replication were observed based on lack of viral gene expression or visualization of viral structures by transmission electron microscopy. The octopus response against OsHV-1 was evaluated by gene expression of previously reported transcripts involved in immune response in *C. gigas* suggesting that immune defences in octopus are also activated after exposure to OsHV-1.

1. Introduction

The common octopus *Octopus vulgaris* is widely distributed around the world and it is highly appreciated for human consumption (Roper et al., 1984). In 2016, total landings of *O. vulgaris* in Spain reached 4782 t (ICES, 2018). Since natural resources have been generally over-exploited, there is nowadays a high interest in new culture alternatives to satisfy the global demand. Due to fast growth and a short life cycle, *O. vulgaris* has been postulated as a good candidate for aquaculture purposes. However, disease and mortalities could seriously endanger the viability of these operations.

The main pathogen affecting the common octopus is the coccidian parasite *Aggregata octopiana* causing nuclear displacement, inflammation and ulceration (Gestal et al., 2002b). This pathogen, very common in wild octopus, does not seem to cause direct mortality, however it might make animals more susceptible to other pathogens (Gestal et al., 2002a). Several bacterial strains and a betanovirus have been recently described in skin lesions and the bacterium *Vibrio lentus* has been associated with diseased animals inducing significant mortalities under experimental conditions (Farto et al., 2003; Farto et al., 2019; Fichi et al., 2015). However, the disease progression associated with these pathogens is still unknown (Prado-Alvarez and García-Fernández, 2019). Lower octopus captures are typically attributed to environmental conditions; however unidentified factors, such as emerging diseases, might also be affecting the health and abundance of natural stocks.

The ostreid herpes virus (OsHV-1) is one of the most important pathogens in mollusc aquaculture, impacting the Pacific oyster *Crassostrea gigas* production globally. OsHV-1 was firstly described in moribund hatchery-reared larvae of *C. gigas* and also in other species displaying mortalities (Hine et al., 1992; Nicolas et al., 1992; Renault et al., 2000b; Renault and Arzul, 2001). OsHV-1 associated mortalities have been reported in numerous larval and juvenile bivalve species such

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Table 1
Detection of OsHV-1 in Wild Octopus Samples. Adult octopuses (A), embryos (E) and paralarvae (P). Specifications regarding life stage, age and mother origin are indicated in brackets for embryos and paralarvae samples. Asterisks show significantly lower overall prevalence of infection compared to adult animals (z-test, $p < 0.05$).

Life Stage	Number of samples	Location	Year of collection	Sample	OsHV-1 prevalence	Overall OsHV-1 prevalence
Adult	3	Vigo	2010	Gill	100% (3/3)	87.5%
Adult	11	Vigo	2011	Gill	72.7% (8/11)	
Adult	2	Vigo	2014	Gill	100% (2/2)	
Adult	8	Vigo	2015	Gill	100% (8/8)	
Embryo (stage XXIII, positive female 1)	5	Vigo	2014	Whole tissue (pool)	80% (4/5)	65%
Embryo (stage VIII, positive female 2)	5	Vigo	2014	Whole tissue (pool)	60% (3/5)	
Embryo (stage XV, positive female 2)	5	Vigo	2015	Whole tissue (pool)	60% (3/5)	
Embryo (stage XIII, positive female 3)	5	Vigo	2015	Whole tissue (pool)	60% (3/5)	
Paralarvae (0 dph, positive female 1)	6	Vigo	2015	Whole tissue	66% (4/6)	62% *
Paralarvae (2 dph, positive female 2)	10	Vigo	2015	Whole tissue	60% (6/10)	
Paralarvae (0 dph, positive female 3)	5	Vigo	2015	Whole tissue	60% (3/5)	

as the oysters *Crassostrea angulata*, *Crassostrea ariakensis, Tiostrea chilensis* and *Ostrea edulis*, clams such as *Ruditapes philippinarum* and *Ruditapes decussatus,* and the scallop *Pecten maximus,* larval and juvenile stages being most susceptible (Arzul et al., 2001a; Hine et al., 1998; 1997; Renault and Arzul, 2001).

Severe mass mortalities were observed in *C. gigas* juveniles in 2008, mainly associated with a new viral microvariant (OsHV-1 µVar) and elevated seawater temperature over 16 °C (EFSA, 2010; Segarra et al., 2010). The emergence of the disease was rapid, and microvariant and other genotypes affecting other species were also reported in a number of countries in both hemispheres (Friedman et al., 2005; Keeling et al., 2014; Lynch et al., 2012; Mineur et al., 2014; Morrissey et al., 2015; Xia et al., 2015). In Spain, OsHV-1 µVar was detected at the Mediterranean coast in a retrospective study including *C. gigas* samples from 2005 (Roque et al., 2012) and in the north in oysters collected in 2010 (Aranguren et al., 2012). In addition, OsHV-1 is a pathogen broadly distributed in different ecosystem compartments including sea water and sediments (Evans et al., 2017, 2014). Intra- and interspecific transmission between bivalves has been corroborated (Arzul et al., 2001c; Petton et al., 2013; Schikorski et al., 2011) and it was also hypothesised that the virus could be transmitted through suspended particulate material (Paul-Pont et al., 2013). For this reason the role of carriers and reservoirs is acquiring more importance to understand the expansion of the disease and try to mitigate the effect of the virus in culturing areas where multiple species coexist (Burge et al., 2011; O'Reilly et al., 2018; Pernet et al., 2016; Rodgers et al., 2018).

In the present work, we have detected OsHV-1 in different life stages of the common octopus *O. vulgaris* by molecular diagnosis and also by *in situ* hybridization. In order to ascertain viral transmission between species and adverse effects in early stages of octopus hosts, a battery of cohabitation experiments including octopus paralarvae and virus injected oysters were carried out.

2. Materials and methods

2.1. Archival *O. vulgaris* samples

Based on the principle of the Three Ŕs, to Replace, Reduce and Refine the use of animals for scientific purposes (European Animal Directive 2010/63/EU), we utilized a collection of tissues or DNA samples from adult *O. vulgaris* gills and whole embryo and paralarvae in this study. Table 1 shows the details concerning the date and location of collection of adult octopuses and the hatching of females. Before dissection, animals were anaesthetized using magnesium chloride and euthanized in anaesthetic overdose (Fiorito et al., 2015). A collection of different tissues from adults, including gills, were appropriately kept for histological

Table 2
Nucleotide sequences of primers used in this study, organism on which were tested and their function. Efficiency values of qPCR primers. Efficiencies of primers for viral transcription were previously determined by Morga et al., 2017.

Primer	Organism	Function	Sequence (5́- 3́)	Amplification Efficiency (%)
HVDP-F	OsHV-1	viral detection	ATTGATGATGTGGATAATCTGTG	99.2
HVDP-R	OsHV-1	viral detection	GGTAAATACCATTGGTCTTGTTCC	
C2	OsHV-1	viral detection	CTCTTTACCATGAAGATACCCACC	–
C6	OsHV-1	viral detection	GTGCACGGCTTACCATTTTT	
OHVC	OsHV-1	viral detection	AGGCGCGATTTGTCAGTTTAGAATCAT	–
OHVD	OsHV-1	viral detection	AGGTTCAGGTCTTTGCGTTCCGT	
ORF 42F	OsHV-1	viral transcription	GCAGGCATAACAGGTGAGCA	99.9 (Morga et al., 2017)
ORF 42 R	OsHV-1	viral transcription	TGAGAGGCGTGACAGGGAAT	
ORF 75F	OsHV-1	viral transcription	ATGATCTGCGCCACTCTGGT	100 (Morga et al., 2017)
ORF 75 R	OsHV-1	viral transcription	TGTGCCTGAAGGATGTGCAA	
ORF 80F	OsHV-1	viral transcription	AAGAGGATTTGGGTGCACAG	98.5 (Morga et al., 2017)
ORF 80 R	OsHV-1	viral transcription	TTGCATCCCAGGATTATCAG	
ORF 117F	OsHV-1	viral transcription	AATTTCCCGCCTCTGTGCTT	98 (Morga et al., 2017)
ORF 117 R	OsHV-1	viral transcription	TGATGACGGAAGTGGCAACA	
MyD88 F	*O. vulgaris*	gene expression	GGTTTGGCAGAATTGATTGG	93.0
MyD88 R	*O. vulgaris*	gene expression	TGGCAGTTGTATGGAAAGTAG	
LRR F	*O. vulgaris*	gene expression	CGTATTGAAATGCCACAGAATG	96.8
LRR R	*O. vulgaris*	gene expression	GGAAGGGTTTGAGGCTAAAG	
ADAR F	*O. vulgaris*	gene expression	CCGAACGTCTGGTGTTATC	98.1
ADAR R	*O. vulgaris*	gene expression	AGGACATTCCATCTTGCTATC	
DCL F	*O. vulgaris*	gene expression	AGTCCACCATTCAGACAAAG	93.4
DCL R	*O. vulgaris*	gene expression	GCAGTTAGTCCTAGTAATTGGG	
Ubiquitin OV F	*O. vulgaris*	gene expression	AGAAGGTTAAGTTGGCGGTTTTG	99.2
Ubiquitin OV R	*O. vulgaris*	gene expression	CCAGCTCCACATTCCTCGTT	

Table 3
Description of experimental trials carried out in *O. vulgaris* paralarvae including the biological material processed and the parameters evaluated.

	OsHV-1 experimental trials		
	Cohabitation trial	Immersion trials	
		0.22 μm undiluted viral suspension	Diluted viral suspension
Biological material	180*O. vulgaris* paralarvae (2 dph) 60*C. gigas* oysters	36*O. vulgaris* paralarvae (8 dph) –	72*O. vulgaris* paralarvae (26 dph) –
Dose	Viral suspension I 2.2×10^7 DNA viral copies injected in oysters	Viral suspension II 3.8×10^4 DNA viral copies/μl ASW	Viral suspension III 52 and 5.2×10^3 DNA viral copies/μl ASW
Sampling times	24, 48, 72 and 96 h	3 and 15 h	3, 6, 18 and 24 h
Replicates/condition	3 replicate tanks with 10 oysters + 30 paralarvae	12 paralarvae/well (1 control, 2 virus)	2 replicate wells (12 paralarvae/each)
Parameters	Mortality OsHV-1 detection	Mortality OsHV-1 detection Viral ORF transcription TEM	Mortality OsHV-1 detection Octopus gene expression TEM

procedures and DNA extraction. Embryo stages were assessed by stereoscopic microscopy (Naef, 1921) and the paralarvae age was calculated in days post-hatching (dph).

2.2. Cultured *O. vulgaris* paralarvae

Adult *O. vulgaris* (wet weight 1–1.5 kg) were collected by fishermen using artisanal traps in the Ria de Vigo, Spain (24°14.09′N, 8°47.18′W) and maintained in tanks (500 L) with circulating filtered seawater. Natural hatchings occurring under captivity in 2016 and 2018 were kept in tanks (100 L) and fed twice per day with the crustacean *Artemia salina* enriched with the microalgae *Isochrysis galbana* and the dietary supplements Red Pepper (Bernaqua) and Spirulin (Mc Algae, Kf Iber Frost). Paralarvae of 2, 8 and 26 dph age were used for experimental trials. Procedures for transportation, maintenance, anaesthesia and dissection were carried out in accordance with the principles published in the European Animal directive (2010/63/EU) for the protection of experimental animals and approved by the Spanish National Competent Authority ethics committee (Research Project ES360570202001/17/EDUC FORM 07/CGM01).

2.3. Viral suspensions and quantification of viral load

Dead *C. gigas* spat (fresh mortalities), 3.9 ± 0.2 cm in length, were frozen and held at −80 °C until detection of OsHV-1 by standard PCR. Mantle and gill tissues from OsHV-1 positive oysters were thawed, pooled and homogenized in artificial sea water (ASW), diluted and filtered through 10- and 5-μm nylon mesh (Biotek) and syringe nitrocellulose filters at 1.2 and 0.22 μm (MerkMillipore) (Paul-Pont et al., 2015; Schikorski et al., 2011). A total of three viral suspensions (VSI, VSII and VSIII) were prepared and maintained in sterile conditions at 4 °C for a maximum of three weeks. Viral suspensions were re-filtered through 0.22 μm before use. An aliquot of 200 μl was digested overnight at 56 °C in extraction buffer (100 mM NaCl, 25 mM EDTA, 0.5% SDS, and pH 8) containing proteinase K (1 mg/ml). DNA was extracted following the phenol–chloroform method, precipitated with ethanol and suspended in 20 μl of distilled water.

Absolute quantification of viral DNA copies was carried out by qPCR using HVDP-F and HVDP-R primers (Pepin et al., 2008; Webb et al., 2007) (Table 2). Reactions were performed in a QuantStudio3 real-time PCR System (Applied Biosystems) in triplicate in 20 μl final volume containing 12.5 μl of Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific), 2.5 μl of each primer (0.5 μM final concentration), 2.5 μl of distilled water and 5 μl of DNA at 5 ng/μl. The number of viral copies was extrapolated from a qPCR standard curve. Virus standards were prepared from an amplicon cloned into p-*GEM* plasmid (Promega) and transformed in JM109 Competent Cells (NZYtech). Positive clones were grown in liquid media and the plasmid was purified following the ZR Plasmid Miniprep™ kit (Zymo Research). DNA concentration and the number of molecules were estimated and eight serial dilutions (1:10) of this stock (2.37×10^9 viral copies/μl) were included as standards in the analysis (5 μl). The efficiency of the reaction was 99.2 ± 0.04, and the slope was −3.3 with an R^2 value of 0.996. The standard curve fulfilled the requirements of the Standard Operating Procedures published for OsHV-1 detection and quantification by real-time PCR (Pepin et al., 2008; Webb et al., 2007). The limit of detection was previously estimated in 10 viral copies/μl (Pepin, 2013). Negative controls using distilled water as a template were also included obtaining no amplification.

2.4. OsHV-1 experimental trials with viral suspension

Table 3 compiles the information about the biological material used for each experimental challenge in *O. vulgaris* paralarvae and *C. gigas* oysters and the parameters evaluated.

2.4.1. Cohabitation of *O. vulgaris* paralarvae with OsHV-1 injected *C. gigas*

Due to the impossibility to obtain naïve oysters from a hatchery, a shellfish farm provided the animals for the experiment. OsHV-1 was previously detected at the site where the farm was located (personal communication from the farmer). However, background prevalence at the moment of oyster collection was 10% and oysters were not experiencing mortalities. A total of 60 oysters (6 months old, 3.37 ± 0.17 cm) were anaesthetized with magnesium chloride (Suquet et al., 2009) before being injected in the adductor muscle. 30 oysters were injected with 100 μl of VSI (2.2×10^5 DNA viral copies/μl). A control group included the same number of animals injected with ASW. Three groups of 10 oysters per condition were placed into 3-L tanks with aeration containing 30 octopus paralarvae each (2 dph) and maintained in a constant temperature room at 22 °C. Paralarvae were feed daily with *A. salina* enriched with the microalgae *I. galbana* and the dietary supplements Red Pepper (Bernaqua) and Spirulin (Mc Algae, Kf Iber Frost). At 24, 48, 72 and 96 h post-exposure all dead animals and 5 sampled paralarvae were collected for DNA extraction.

2.4.2. Immersion of *O. vulgaris* paralarvae in viral suspension

Two immersion trials were carried out on 6-well culture plates in a 5-ml final volume at 22 °C (Table 3). Viral exposures were carried out in duplicate using 12 paralarvae per replicate. In trial 1, paralarvae (8 dph) were exposed to 0.22-μm undiluted VSII (3.8×10^4 DNA viral copies/μl) and dead animals were collected at 3 and 15 h post-exposure. One control replicate (ASW exposure) with the same number of animals was included in the experiment. In trial 2, paralarvae (26 dph) were exposed to two doses of VSIII (2.6×10^5 DNA viral copies/μl) by adding 100 μl of diluted VSIII (1/100) in low dose wells or 100 μl of VSIII in high dose wells. At 3, 6, 18 and 24 h post-exposure, mortality was monitored and 3 sampled animals were collected and fixed in RNAlater (ThemoFisher Scientific). Two control replicates (ASW exposure) were included in the experiment. In parallel, 100 μl of VSII and VSIII were injected into spat oysters which were then assessed for mortality.

2.5. DNA extraction

DNA from gills, paralarvae and embryo from *O. vulgaris* archival samples were extracted using the NucleoSpin Tissue extraction kit (Macherey-Nagel) following the manufacturer's instructions.

Gill tissues from *C. gigas* and paralarvae from experimental trials

were digested overnight at 56 °C in extraction buffer (100 mM NaCl, 25 mM EDTA, 0.5%SDS, pH 8) containing proteinase K (1 mg/ml). DNA was extracted following the phenol–chloroform method, precipitated with ethanol and suspended in 20 µl of distilled water. For comparative purposes, a selection of paralarval tissues were digested using CTAB buffer containing 100 mM Tris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA and 2% CTAB (Cetyltrimethyl Ammonium Bromide, SigmaAldrich).

All samples were individually processed with the exception of *O. vulgaris* embryos that were pooled (4 individuals per pool) and paralarvae of 8 and 26 dph that were longitudinally dissected and pooled in groups of 3–4 individuals. Samples were cleaned in 0.22 µm ASW before any processing.

2.6. Molecular detection of OsHV-1 by PCR and sequencing analysis

OsHV-1 screening in oyster DNA was carried out by PCR in 12.5 µl PCR Master Mix (ThermoFisher Scientific), 1 µl of forward and reverse primers (*C*2 and C6, 0.4 µM final concentration), 9.5 µl of distilled water and 1 µl of DNA template (100 ng/µl). Thermal cycling conditions started with an initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 20 *sec*, primer annealing at 58 °C for 60 *sec* and extension at 72 °C for 90 *sec*. A final extension at 72 °C was carried out for 10 min (Martenot et al., 2011; Renault and Arzul, 2001).

DNA from archival *O. vulgaris* samples and cultured paralarvae was amplified in a 25 µl final volume containing 2.5 µl of PCR buffer (1X), 1 µl of nucleotide mix (4 µM each, final concentration), 1 µl of forward and reverse primers (*C*2 and C6, 0.4 µM final concentration), 1 µl of $MgCl_2$ (1 mM final concentration), 0.625 U of DreamTaq polymerase (ThermoFisher Scientific), 16.875 µl of distilled water and 1 µl of DNA template (100 ng/µl) using the same thermal cycling as described above for oyster DNA.

A nested PCR was also carried out on DNA samples from cultured paralarvae using 1 µl of template (1:10 dilution) of the first PCR product using *C*2/C6 primers. The reaction contained 2.5 µl of PCR buffer (1X), 1 µl of nucleotide mix (4 µM each, final concentration), 1 µl of forward and reverse primers (OHVC and OHVD, 0.4 µM final concentration) (Lynch et al., 2013), 1 µl of BSA (0.004% final concentration), 16.875 µl of distilled water and 0.625 U of DreamTaq polymerase (ThermoFisher Scientific). All PCR reactions included known positive (DNA from an infected oyster) and negative controls (distilled water).

Fresh PCR products from archival *O. vulgaris* samples were cleaned using the ExoSAP-IT PCR product Cleanup reagent (ThermoFisher Scientific). PCR bands were also excised and purified following the QIA quick Gel extraction kit (Qiagen). Cleaned and purified products were unidirectionally sequenced using the *C*2 primer following the Sanger method (StabVida). Raw chromatograms were analysed with Chromas 231 software (Technelysium). Sequence assembly, translation, multiple alignment and searches of homology were performed using ExPaSy tools (http://us.expasy.org/tools), BioEdit v.7.2.5 (Hall, 1999) and GenBank databases using Blast algorithm (blastn) (http://ncbi.nlm.nih.gov/blast/). Sequences were submitted to the GeneBank database with the accession numbers: MT702617, MT702618 and MT702619.

2.7. Viral gene expression

Transcription of viral ORFs was evaluated in 8 dph paralarvae after immersion on VSII. Four lengthwise excised paralarvae were pooled for total RNA extraction. Procedures for RNA extraction, first-strand cDNA synthesis and relative expression of viral ORFs (ORF 42, ORF 75, ORF 80 and ORF 117) were carried out following procedures described in Morga et al. (2017). RNA was pooled to 1000 ng. cDNA from a heavily infected oyster (positive control) and NTCs (no template controls) were included in the analysis.

2.8. Octopus vulgaris gene expression

Three lengthwise excised paralarvae (26 dph) exposed to VSIII at high concentration or ASW for 24 h (Table 3) were pooled for total RNA extraction. Three pooled samples were homogenized in Trizol following manufactureŕs instructions (ThermoFisher Scientific). Genomic DNA digestion and RNA purification were made following the Direct-zol RNA MiniPrep (Zymo Research). RNA concentration was estimated in a NanoDrop 2000 (ThermoFisher Scientific) and 1000 ng of RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis kit (ThermoFisher Scientific).

Immune genes expressed in response to OsHV-1 infection in *C. gigas* were selected based on previously published studies (He et al., 2015; Renault et al., 2011; Rosani et al., 2014). The corresponding genome sequences were manually searched in a Blast database of *O. vulgaris* transcriptome (García-Fernández et al., 2019) against *C. gigas, Octopus bimaculoides* and *Homo sapiens* proteome from Uniprot (Bateman et al., 2017). The corresponding octopus nucleotide sequences were further analysed to corroborate similarity, protein translation and domain structure using Blast (http://www.ncbi.nlm.nih.gov/blast/) and ExPaSy tools (http://us.expasy.org/tools). Specific primers for qPCR were designed using Primer3 (http://primer3.ut.ee/) (Table 2) and tested in serial dilutions of a template consisting of a mix of cDNA from *O. vulgaris* paralarvae. Reactions were performed in a QuantStudio3 real-time PCR System (Applied Biosystems) in duplicates in 25-µl final volume containing 12.5 µl of Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific), 2.5 µl of each primer (3 µM), 6.5 µl of distilled water and 1 µl of cDNA (1:10 dilution). Thermal cycling was performed following the manufacturer's instructions including a final melting curve analysis. Data was analysed for relative expression following the Livak method (Livak and Schmittgen, 2001) using ubiquitin as housekeeping gene (García-Fernández et al., 2016) and control samples as calibrators. Negative controls (NTCs) were included in all reactions obtaining no amplification.

2.9. In situ hybridization and transmission electron microscopy

Gills tissues from three OsHV-1 positive octopuses and two paralarvae preserved in Davidsoń solution (Shaw and Battle, 1957) were processed for *in situ* hybridization. After dehydration, tissues were embedded in paraffin wax and blocks were cut in 7 µm sections (Prado-Alvarez et al., 2016). OsHV-1 was detected using specific DNA digoxigenin-labelled probes using *C*2/C6 primers and the DIG Nucleid Acid Detection Kit (Roche). Solutions and buffers were freshly prepared (Sambrook and Russell, 2001). Procedures for tissue digestion, hybridization and detection were carried out following Lynch et al., 2010 and Prado-Alvarez et al., 2016, with minor modifications. Briefly, Proteinase K incubation was reduced to 5 min at 37 °C and the probe (5 ng/µl) was incubated at 4% in hybridization buffer. For detection, 0.1% of Tween was added to nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution and incubation was extended for 90 min. Mounted slides were visualized under light microscopy (Leica DM 2500). Tissues from an infected oyster were included as positive control.

A total of six paralarvae from immersion trials were processed for transmission electron microscopy. Primary fixation for TEM was carried out in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.4 at 4 °C overnight, then samples were washed with 0.2 M sodium cacodylate buffer pH 7.4 at 4 °C and secondary fixation was done in 2% osmium tetroxide in 0.2 M sodium cacodylate buffer pH 7.4 (Castellanos-Martínez et al., 2014b). Paralarvae were dehydrated in a graded ethanol series and included in Epon resin (Sigma-Aldrich). Resin blocks were cut with a diamond knife to obtain semi-thin and ultra-thin sections. Semi-thin sections were stained with methylene blue. In the case of ultra-thin sections, the solutions used were uranyl acetate and lead citrate. A JEOL 100CXII TEM operated at 60 kV was used for the visualization of the different sections.

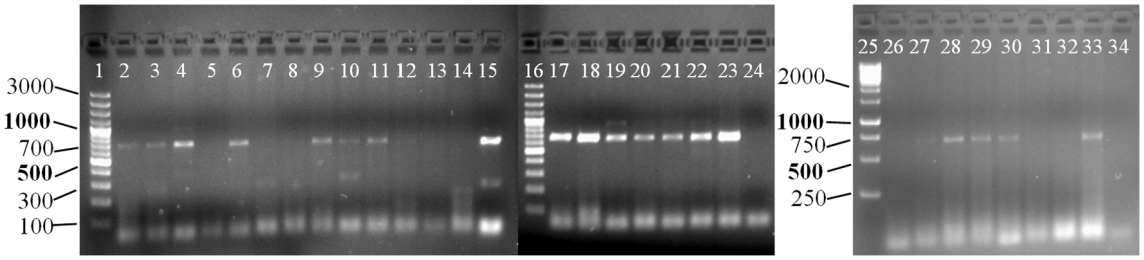

Fig. 1. Agarose gel image showing the positive bands corresponding to OsHV-1 amplified with C2/C6 primers. Molecular marker 100 pb (1 and 16), molecular marker 1 Kb (25), paralarvae samples (2–14), gill from adult octopuses (15, 17–22), embryo samples (26–32), positive controls *C. gigas* DNA (23 and 33) and negative controls (24 and 34).

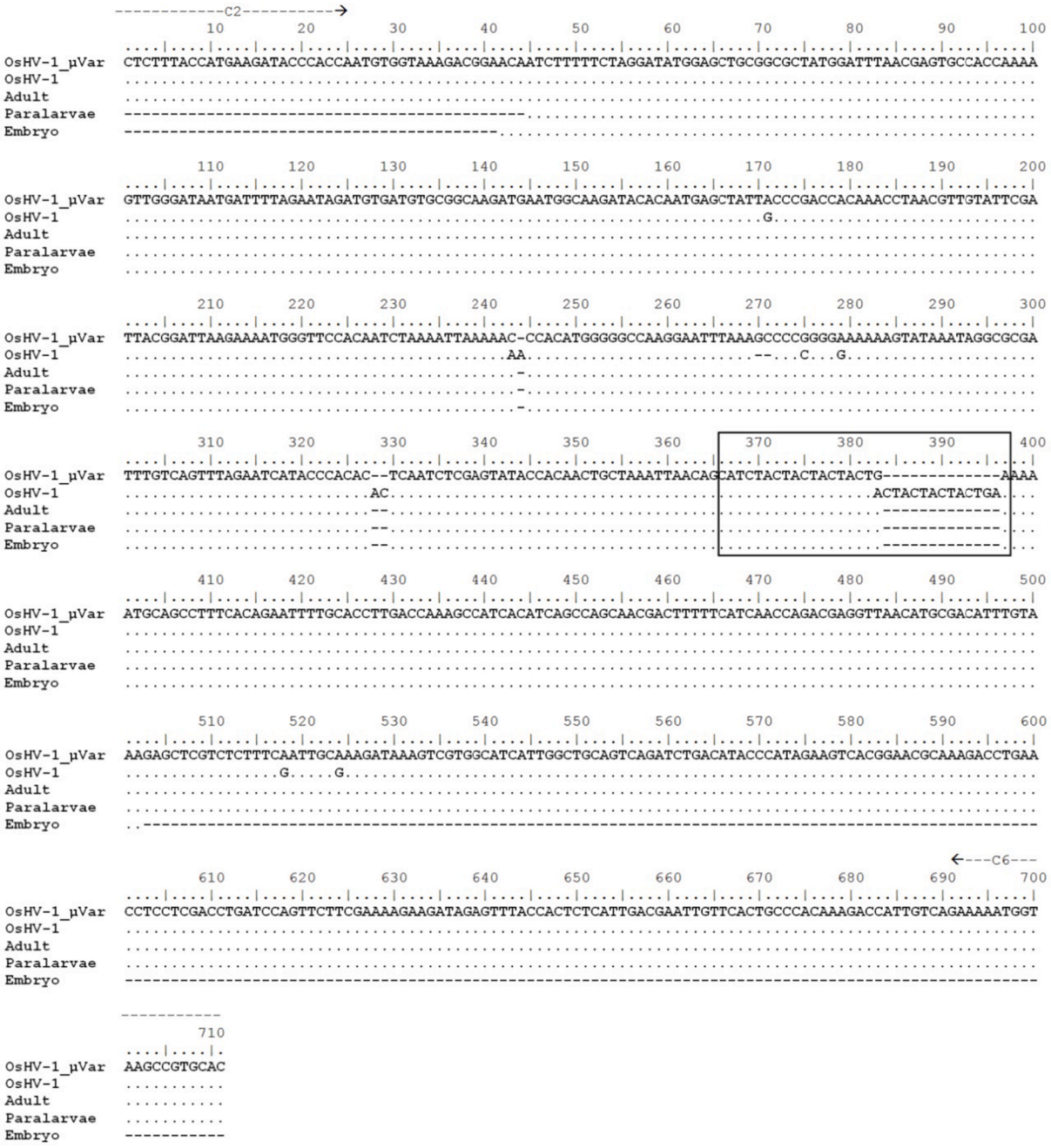

Fig. 2. Multiple alignment of contig sequences obtained in paralarvae, embryos and adult octopuses including the previously described ostreid herpes virus genotypes. (OsHV-1 reference genome, acc num AY509253; OsHV-1 µVar, acc num HQ842610.1). Primers used to amplify this fragment are indicated with arrows. Framed area indicates the polymorphism region that characterizes different virus genotypes.

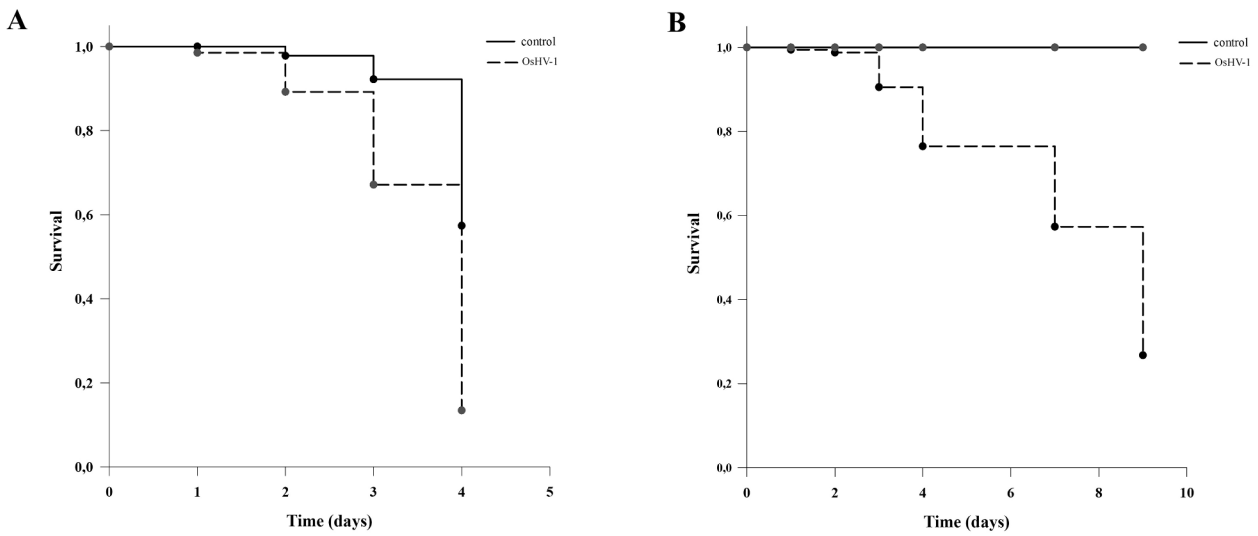

Fig. 3. Kaplan-Meier survival curve of paralarvae (A) and oysters (B) during 4 days-cohabitation experiment. Survival decrease in virus exposed animals was significantly different from controls (Log-Rank test, $p < 0.001$, $n = 3$).

2.10. Statistical analysis

Data are presented as mean ± standard error. Statistical analyses were carried out using SigmaPlot version 11.0. Differences in overall OsHV-1 prevalence were evaluated using the z-test for proportion analysis. Kolmogorov-Smirnov analysis was used to test data for normality before the comparison of groups. Attending to data distribution and equal variance test, the Students *t*-test or Mann-Whitney test were used in parametric or non-parametric analyses. Survival data were represented in Kaplan-Meier curves using the Log-Rank test for significance analysis and Holm-Sidak test for pairwise comparisons. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Detection of OsHV-1 in O. vulgaris in archival samples

Table 1 shows the details of the archival samples analysed in this study and the corresponding percentage of OsHV-1 detection. A total of 65 octopus samples were analysed including 21 paralarvae at 0 and 2 dph from 2015, 20 pooled embryo samples from 2014 and 2015 and 24 gill samples from adult animals collected from 2010 to 2015. PCR amplification using the C2/C6 primer pair yielded an amplicon product of the expected size in embryo, paralarvae and adult *O. vulgaris* samples compared to the positive control (genomic DNA from infected *C. gigas*) (Fig. 1). The overall detection of OsHV-1 in adult wild octopuses was 87.5% (Table 1). Overall viral prevalence in paralarvae (62%) was significantly lower compared to adult samples (z-test, $p = 0.031$). The offspring of three positive females was analysed in an embryonic stage and after hatching. Prevalence of OsHV-1 ranged between 60% and 80% (overall prevalence was 65%) in embryonic samples and between 60% and 66% in paralarvae (overall prevalence was 62%).

3.2. Identification of the OsHV-1 genotype in O. vulgaris

A total of 11 positive amplicons from different stages were unidirectionally sequenced using the specific primer C2 in 7 nucleotide sequences from adult animals, 3 from paralarvae samples and 1 from embryo. Raw chromatograms were manually examined to detect any erroneous reading. All sequences were identical. Using the blastn algorithm of the NCBI database, the OsHV-1 detected showed 100% identity to OsHV-1 µVar (Segarra et al., 2010). For easier comparative purposes, sequences from adult and paralarvae were grouped into two contigs. The multiple alignment including *O. vulgaris* sequences, reference OsHV-1 genome (accession number: AY509253) and OsHV-1 µVar (accession number: HQ842610.1) revealed no differences between *O. vulgaris* sequences and the µVar variant (Fig. 2). The characteristic features of µVar genotype corresponding to this genomic region appeared conserved in OsHV-1 detected in *O. vulgaris* including the identical number of consecutive repetitions in the microsatellite area (Fig. 2, framed area).

3.3. Paralarval survival and OsHV-1 detection after cohabitation with virus injected oysters

Survival of *O. vulgaris* and *C. gigas* after 96 h of cohabitation is represented in Fig. 3. Octopus paralarval survival was significantly lower in animals that cohabited with virus-injected oysters compared to survival in control tanks (Fig. 3A) (Log-Rank test, $p < 0.001$). Percentage of survival of virus exposed paralarvae decreased to 20% at the end of the trial. Control paralarvae in cohabitation with ASW injected oysters experienced mortality after 48 h of cohabitation reaching at the end of the trial 62.2% of survival.

Oyster survival in virus injected oysters was also significantly lower compared to controls (Fig. 3B) (Log-Rank test, $p < 0.001$). Mortality started at 72 h in virus injected animals whereas no dead animals were observed after ASW injection. A total of 16 dead oysters (53.3%) were collected at the end of the trial and all were OsHV-1 positive after PCR screening. Fig. 4A and 4B represent the mean of OsHV-1 positive detections (dark bars) compared to total animals screened (bright bars) in sampled and dead paralarvae, respectively. OsHV-1 was detected in sampled *O. vulgaris* from control and virus tanks at all sampling times. No significant differences were observed between conditions. On average, the number of dead paralarvae was significantly higher in virus than in controls tanks at 24 and 48 h post-exposure (Fig. 4B). Compared to controls, the number of OsHV-1 detections in dead paralarvae was significantly higher at 48 and 72 h post-exposure. No significant differences were observed after 96 h.

3.4. Paralarvae survival after OsHV-1 immersion challenge

The two viral suspensions utilized for the immersion trials caused significant mortality in octopus paralarvae and oysters. Undiluted VSII (3.8×10^4 DNA viral copies/µl) was used to expose paralarvae for a 15-h trial. Paralarval survival decreased to 50% after 3 h of immersion and all animals were dead at 15 h post-immersion (Fig. 5A) (Log-Rank test, $p < 0.001$). Octopus paralarvae were also exposed to dilutions of VSIII (2.6

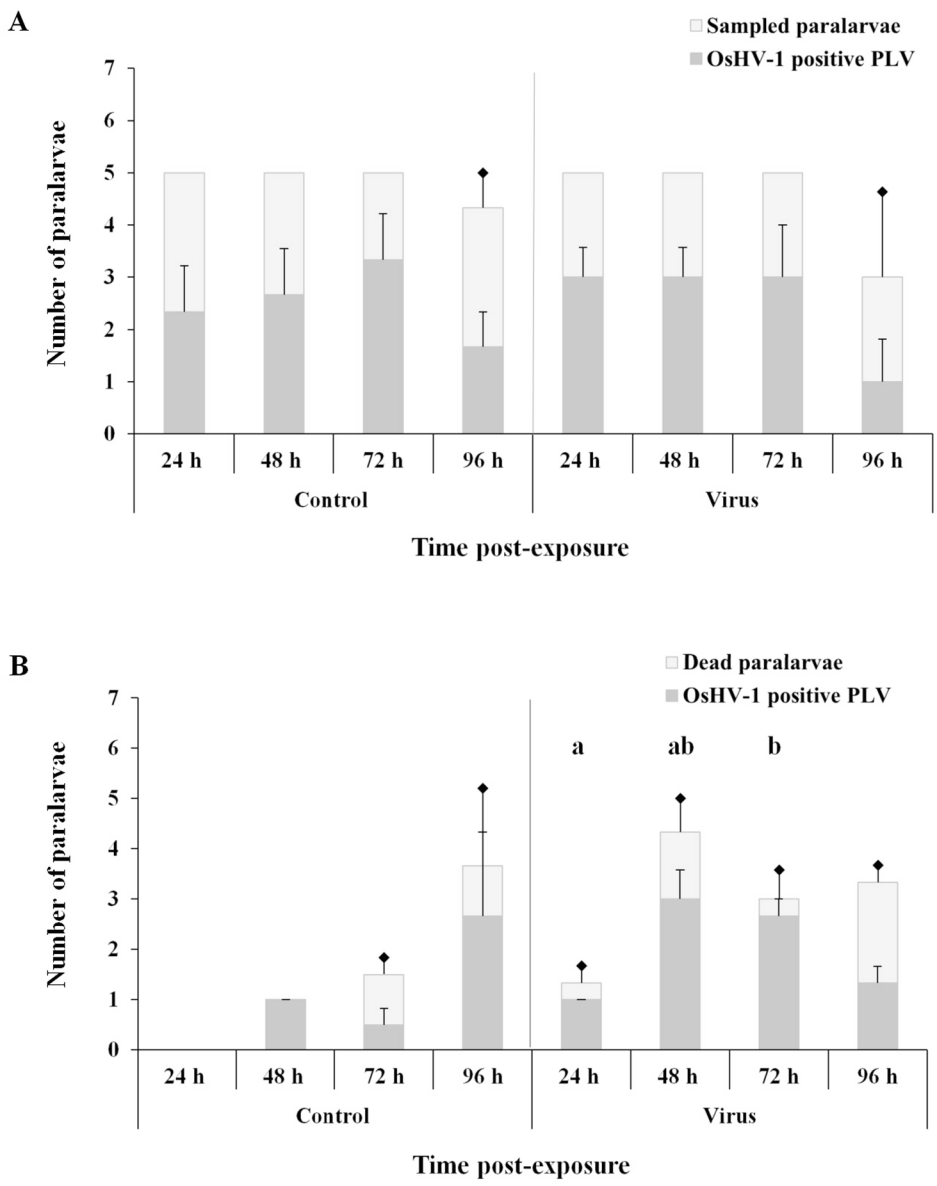

Fig. 4. Cumulative bar diagram of positive viral detections (dark bars) in sampled paralarvae (bright bars) after cohabitation with virus injected oysters and control oysters (A). Cumulative bar diagram of positive detections (dark bars) in dead paralarvae (bright bars) collected during cohabitation trial (B). Significant differences between control and virus exposed conditions are indicated with an "a" for total number of dead animals and with a "b" for positive detections (*t* test, $p < 0.05$, $n = 3$).

$\times 10^5$ DNA viral copies/µl) (Table 3). Over the 24 h trial, no dead animals were observed in the lowest concentration (Fig. 5B). However, compared to controls survival significantly decreased after exposure to the highest concentration (Holm-Sidak test, $p = 0.025$). Significant differences in survival were also detected after pairwise comparison of doses (Holm-Sidak test, $p = 0.017$). Oyster survival decreased to 68% after 6 days of intramuscular injection of VSII (Fig. 5C) (Log-Rank test, $p = 0.025$) whereas the percentage of oyster survival decreased to 16% at day 6 post-injection with VSIII (Fig. 5C) (Log-Rank test, $p < 0.001$).

3.5. Viral and O. vulgaris gene expression

Viral gene expression was assayed by qPCR on pooled 8 dph paralarvae (where OsHV-1 was previously detected) exposed to 0.22 µm undiluted viral suspension and controls for 3 and 15 h. No expression of viral ORFs was detected. However, expected amplification values were obtained in positive oysters used as controls (Data not shown).

Fig. 6 shows the relative expression assayed by qPCR of MyD88, LRR, ADAR and DCL genes in paralarvae exposed for 24 h to the high dose of VSIII. Data is presented as fold change of gene expression in virus exposed samples standardized against the corresponding control. Compared to expression in controls, a significant increase in the expression of MyD88 (*t*-test, $p = 0.003$), ADAR (*t*-test, $p = 0.033$) and DCL (*t*-test, $p = 0.009$) was observed in virus exposed samples. The relative expression of the gene coding of LRR containing protein was significantly lower to controls (*t*-test, $p = 0.039$). Samples assayed for gene expression were also screened for OsHV-1 detection by standard PCR obtaining negative results in controls and positive results in virus exposed samples.

3.6. Detection of OsHV-1 by in situ hybridization

Histological material from gill tissues from three OsHV-1 positive adult octopuses was tested for *in situ* hybridization. Light microscopy observation showed positive labelled deposits in one adult octopus after hybridization with C2/C6 probes (Fig. 7A–E). Blue labelled cells corresponded mainly to gill epithelium and compared to adjacent and unlabelled cells, the virus positive cells were larger. Compared to the positive oyster control (Fig. 7F) fewer cells were stained in *O. vulgaris* tissues and labelling was lighter. In positive control *C. gigas*, infected cells were observed in the connective tissue (based on labelling of cells), but was not observed in octopus connective tissue.

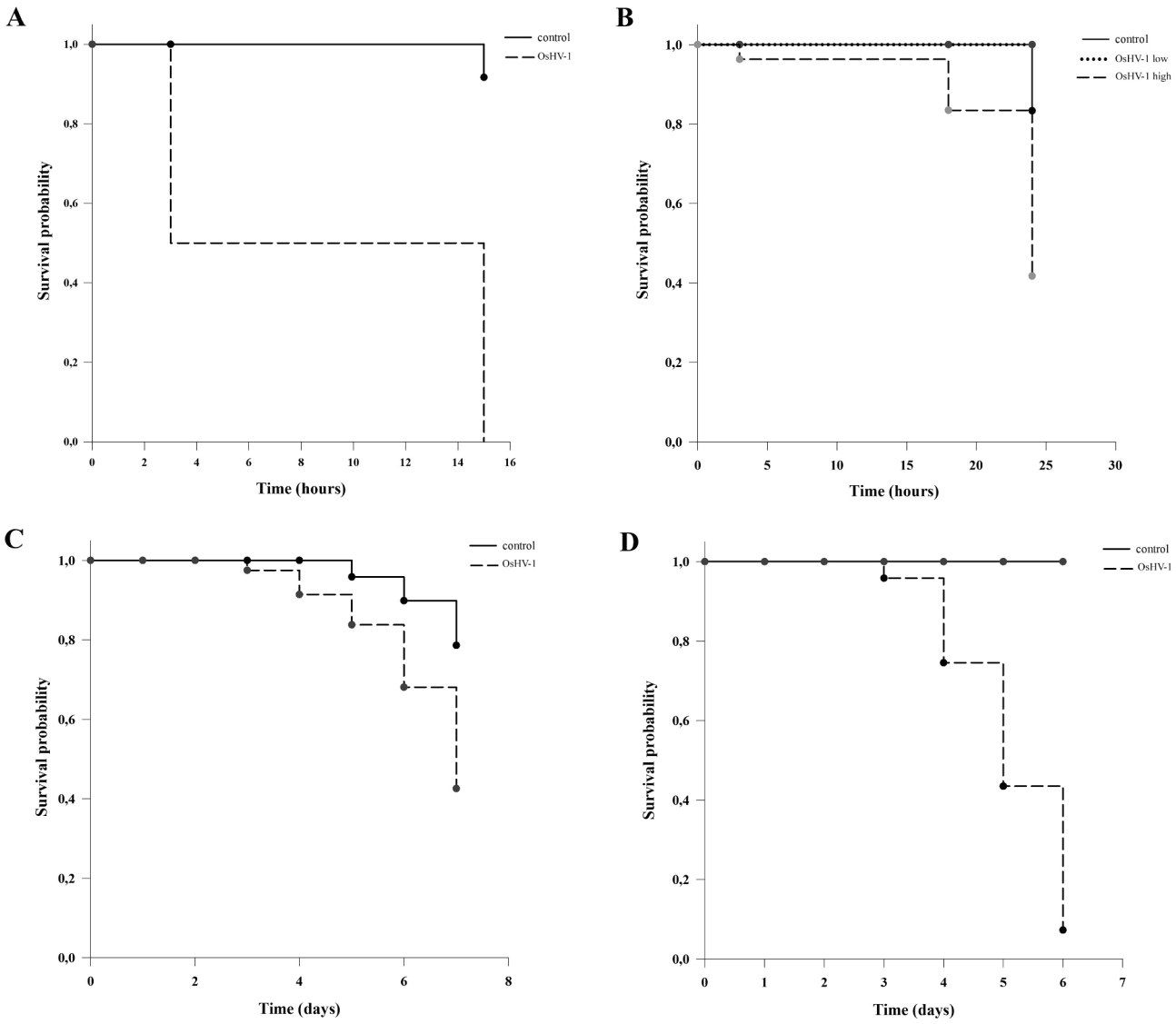

Fig. 5. Kaplan-Meier survival curve in paralarvae exposed by immersion to 0.22 μm undiluted VSII (A) and two dilutions of VSIII (B). Kaplan-Meier survival curve in intramuscularly injected oysters with VSII (C) and VSIII (D). Survival decrease in virus exposed animals were significantly different from controls (Log-Rank test, $p < 0.05$, $n = 2$).

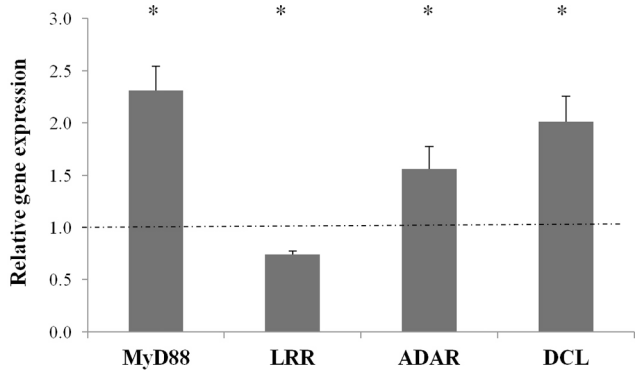

Fig. 6. Relative gene expression of MyD88, LRR containing protein coding gene, ADAR and DCL genes in *O. vulgaris* exposed for 24 h to viral suspension standardized against controls exposed to ASW. Significant differences compared to controls are indicated with and asterisk (*t* test, $p < 0.05$, $n = 3$).

3.7. Transmission electron microscopy analysis

After examination of 20 grids, viral capsids were not observed in any of the paralarvae exposed to viral suspension. Tissues appear well preserved and no obvious tissue lesions were observed in a total of 50 pictures examined. Fig. 8 shows a representative picture of connective tissue from paralarvae exposed by immersion to viral suspension.

4. Discussion

In this study, we have investigated the occurrence of OsHV-1 in the cephalopod *O. vulgaris* in field archival samples collected from 2010 to 2015. Conventional PCR using the C2/C6 primer pairs was used for molecular detection of OsHV-1 (Renault and Arzul, 2001). As previously reported by several authors, a number of adjustments were usually required for the obtaining of an unique and clear amplicon band (Batista et al., 2007; Friedman et al., 2005; Lynch et al., 2013; Martenot et al., 2011; Renault et al., 2000a). The PCR reaction in octopus samples was not completely efficient without the addition of the cationic detergent (CTAB) to the extraction buffer to avoid the interference related to mucopolyssacharides and related metabolites (Knebelsberger and

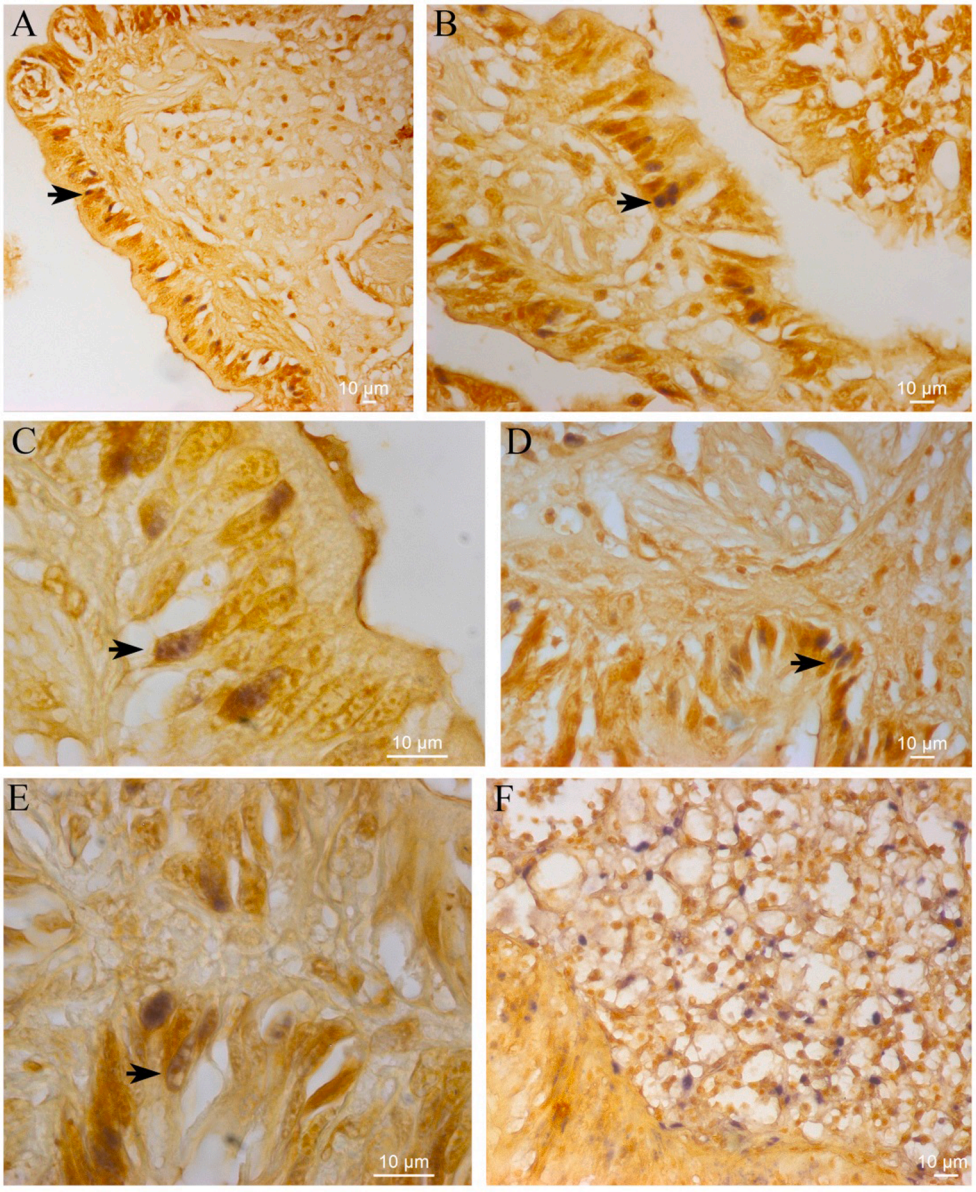

Fig. 7. *In situ* hybridization photomicrographs: (A-E) *O. vulgaris* gills, arrows show positive labelled cells, mainly in the epithelia of the gill. (F) Tissue from an infected oyster used as a positive control. Scale bar: 10 µm.

Stöger, 2012; Winnepenninckx et al., 1993). DNA detection and prevalence of OsHV-1 was similar in embryo and paralarvae octopus whereas a higher proportion of OsHV-1 positives was observed in adult animals. OsHV-1 detections were confirmed by direct sequencing of amplicons, with the OsHV-1 µVar genotype the unique variant found in the three life stages of *O. vulgaris*. The characteristic features of this region, including the microsatellite deletion, were conserved in *O. vulgaris* (Segarra et al., 2010). OsHV-1 infection in *O. vulgaris* determined by PCR was confirmed in 1 of 5 samples using *in situ* hybridization. However, labelling was not broadly distributed in the tissue, especially compared to the infected oyster used as positive control. Additional indicators of any associated pathology such as external injuries or losses in population densities did not offer more information. Although total landings per year in the area of collection revealed a drastic decrease throughout the years of our study (ICES, 2018; Pescadegalicia, 2020), such periodic declines are regularly observed in this species and can be attributed to multiple factors such as environmental conditions and climatological events (Caddy, 1983; Pierce et al., 2008; Vargas-Yáñez et al., 2009). This fact together with the reduced human control over cultivation hampers the detection of emerging diseases if there are not associated symptoms in animals with legal capture size.

The detection of viral DNA in gill cells together with the confirmation by DNA sequencing could indicate that a natural uptake of OsHV-1 µVar from the environment was occurring. OsHV-1 µVar was detected in *C. gigas* samples from 2005 and 2010 from the north and east coasts of Spain (Aranguren et al., 2012; Roque et al., 2012). However, information regarding OsHV-1 prevalence in the location where octopuses were collected was not available as oysters are not cultivated in this area. OsHV-1 containing waters from a nearby infected site, Ria de Arousa (Villalba et al., 2017), could be the source of viral DNA observed in our octopus samples. Indeed, water connections between embayments were confirmed by upwelling-downwelling episodes and coastal jet currents (Gilcoto et al., 2007; Souto et al., 2003). It has been noted that seawater movements through currents and tides can spread OsHV-1 infective particles and free viral DNA over kilometres, freely or attached to carriers such as phytoplankton (Evans et al., 2016, 2014; Pernet et al., 2012; Whittington et al., 2018). However the distance between oyster farming areas and the octopus population is 30 km, over which dilution

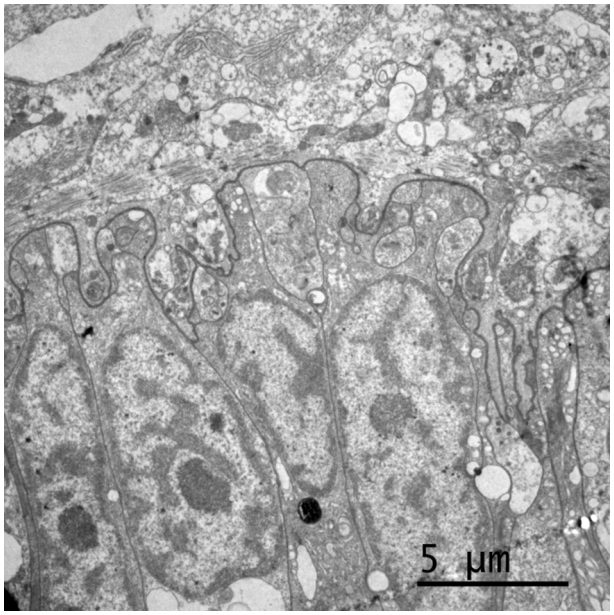

Fig. 8. Photomicrograph of *O. vulgaris* paralarvae exposed to virus suspension by transmission electron microscopy. No lesions or intact viral capsids were observed. Scale bar: 5 µm.

would be expected to reduce viral transmission (Evans et al., 2014). Unfortunately, no other studies were carried out in this confined area to corroborate virus presence in other species.

The origin of viral DNA in embryos cannot be resolved as gonad tissues from positive females were not available in the archival set of samples. Eggs are directly exposed to sea water and constantly ventilated by females (Guerra, 1992; Villanueva and D. Norman, 2008). Although the morphology of *O. vulgaris* eggs was not entirely described, other octopus species contain chorionic pores which favour interchange of fluids with the external media and could hypothetically serve as an entry route for viral material (Monsalvo-Spencer et al., 2013).

In order to decipher whether any harmful effect or disease progression associated with OsHV-1 was occurring in *O. vulgaris*, a number of experimental trials were carried out under controlled conditions. We used the paralarval stage for challenges presuming, as occurs in *C. gigas*, that early life stages could be more susceptible (Arzul et al., 2002; Burge et al., 2006; Prado-Alvarez et al., 2016; Renault et al., 1994; Renault et al., 2000b; Schikorski et al., 2011). Viral transmission between *C. gigas* and *O. vulgaris* was studied by cohabitation using virus injected oysters as the source of OsHV-1. Normal disease expression was observed in virus injected oysters corroborating the infectivity of the viral purification (Prado-Alvarez et al., 2016). Although significant paralarval mortality was observed in virus exposed animals, dead paralarvae were also observed under control conditions probably related to a deteriorated health status as a result of the inherent conditions of the cohabitation with oysters. Oyster excretion products and accidental physical contact with oyster shells could be harmful for octopus paralarvae in case of skin injury. Indeed, pathologies associated with skin lesions are very common in this species (Fichi et al., 2015; Gestal et al., 2019; Sykes et al., 2019). On the contrary, control paralarvae settled alone in cell culture plates for direct immersion trials remained in very good conditions and paralarval mortality, in this case, occurred almost exclusively in virus exposed animals.

Prevalence of OsHV-1 in living paralarvae was similar between experimental animals and archival paralarvae samples. Regarding prevalence of OsHV-1 in dead animals, the peak of dead and OsHV-1 positive animals was detected earlier in virus exposed animals than in controls, which leads us to suspect that virus exposed animals might uptake viral material released by moribund and dead oysters. Residual viral material could also be available under control conditions as control oysters were not naïve and could develop later infectivity processes. Although horizontal transmission of OsHV-1 has been previously described (Arzul et al., 2001c, 2001b; Petton et al., 2013; Schikorski et al., 2011), and we observed a high mortality and a greater number of positive detections in paralarvae exposed to OsHV-1, there was no evidence supporting infection of octopus paralarvae after cohabitation of injected oysters. However, viral capsids were not observed by TEM and vial ORFs were not expressed in dead paralarvae exposed to a high viral dose, leading us to conclude that no sign of viral replication was observed.

Considering previous information in gene expression after OsHV-1 infection (de Lorgeril et al., 2018; He et al., 2015; Renault et al., 2011), a search to find homologous transcripts was carried out in *O. vulgaris* transcriptomic data (García-Fernández et al., 2019). Among the components of the Toll-like receptor signalling pathway, we found a transcript with homology to myeloid differentiation primary response protein (MyD88) a cytosolic adaptor protein that mediated receptors signalling (Anderson, 2000). The classical MyD88 structure is composed by a death domain associated with a TIR (Toll/interleukin-1 receptor) domain (Anderson, 2000). Among the eight MyD88 adaptors identified in *C. gigas*, two of them are truncated and lack the death domain (Du et al., 2013; Wang et al., 2018). Previous studies on *C. gigas* showed that typical and truncated MyD88 are up-regulated after OsHV-1 challenges and poly I:C injection (Du et al., 2013; Green et al., 2014; Green and Montagnani, 2013; He et al., 2015; Renault et al., 2011). The homologous *O. vulgaris* transcript was also up-regulated after OsHV-1 exposure reaching more than two fold expression compared to controls at 24 h post-exposure. This result might indicate an activation of the TLR signalling pathway after OsHV-1 exposure. Other components of this route were previously studied in *O. vulgaris* and were up-regulated after parasite infection, which might indicate the importance of this route activation for octopus defence (Castellanos-Martínez et al., 2014a).

The leucine-rich repeat (LRR) containing protein has homology to Ran-GTPase-activating protein involved in mRNA processing and transport (Lui and Huang, 2009). The domain structure of the octopus transcript aligned to LRR-ribonuclease inhibitor superfamily (Kobe and Kajava, 2001). Ribonuclease inhibitors appeared down-regulated after OsHV-1 infection in *C. gigas* conforming part of an intricate mechanism for viral RNA elimination (He et al., 2015). In concordance to these conclusions, our results also showed a down-regulation of this transcript in *O. vulgaris*. This result might indicate that an activation of the machinery involved in RNA transformation after OsHV-1 exposure might be occurring in octopus. In relation to RNA edition and similarly to previous observations in OsHV-1 infected *C. gigas*, the double-stranded RNA specific editase transcript with homology to *Adar* gene was also higher regulated in *O. vulgaris* paralarvae exposed to viral suspension (He et al., 2015; Rosani et al., 2014). Mutations of the viral genome might disrupt viral open reading frames which constitutes an important defence against virus (Keegan et al., 2004). It would be interesting for a further corroboration of the antiviral defences in *O. vulgaris* to carry out a time course study under different conditions.

Regarding RNA interference machinery, we found a transcript with homology to *Dicer-like* gene. Dicer is an endoribonuclease involved in microRNAs and small interfering RNAs production that after complementary binding down-regulate viral expression (Aliyari and Ding, 2009; Müller and Imler, 2007). A *Dicer-like* transcript lacking some of the characteristic domains was up-regulated after OsHV-1 infection in *C. gigas* (He et al., 2015). Comparable results were observed in octopus paralarvae. Although key components for antiviral RNA interference were identified in the *C. gigas* genome, the complete system in molluscs is not fully understood (Green and Speck, 2018) but was proposed as an important tool for disease control in molluscs which might be mediated by conserved recognition domains with similar functions to the classical RNA silence pathway (Owens et al., 2015). The octopus response at transcriptional level might indicate that a reaction is occurring after

direct exposure and open the possibility to further investigate if octopuses are able to inhibit viral replication and what defences are these animals utilizing to evade OsHV-1.

5. Conclusions

To our knowledge, this is the first study reporting the detection of OsHV-1 in a cephalopod. Wild *O. vulgaris* might uptake viral material through the surrounding environment as direct contact to culturing oyster did not occurred in the area of study. It remains to be determined whether OsHV-1 is pathogenic to *O. vulgaris* or whether octopuses are susceptible or resistant to infection. On one hand, viral DNA was detected in field samples and an increase in viral detection was observed after experimental challenges. Sequencing analyses corroborate the genotype OsHV-1 µVar in *O. vulgaris* tissues. However, lesions at tissue and cellular level or signs of viral replication were not observed. Octopus paralarvae exposed to OsHV-1 expressed classical genes related to TLR signalling pathway and RNA edition, elimination and interference. Altogether, these results might suggest that the octopus *O. vulgaris* would not act as a reservoir for OsHV-1, being a putative dead-end for OsHV-1 virus. Further studies should be carried out to determine if *O. vulgaris* could be utilized as an animal model for antiviral response investigations.

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