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► **To cite this version:**

Timothy Green, Jean-Luc Rolland, Agnès Vergnes, David Raftos, Caroline Montagnani. OsHV-1 countermeasures to the Pacific oyster's anti-viral response. *Fish and Shellfish Immunology*, 2015, 47 (1), pp.435-443. 10.1016/j.fsi.2015.09.025 . hal-01260118

**HAL Id: hal-01260118**

**<https://hal.science/hal-01260118>**

Submitted on 12 Jan 2021

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## Full length article

## OsHV-1 countermeasures to the Pacific oyster's anti-viral response



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## ARTICLE INFO

## Article history:

Received 10 June 2015

Received in revised form

6 September 2015

Accepted 14 September 2015

Available online 15 September 2015

## Keywords:

*Crassostrea*

Anti-viral response

Apoptosis

Interferon-like

Poly I:C

## ABSTRACT

The host–pathogen interactions between the Pacific oyster (*Crassostrea gigas*) and Ostreid herpesvirus type 1 (OsHV-1) are poorly characterised. Herpesviruses are a group of large, DNA viruses that are known to encode gene products that subvert their host's antiviral response. It is likely that OsHV-1 has also evolved similar strategies as its genome encodes genes with high homology to *C. gigas* inhibitors of apoptosis (IAPs) and an interferon-stimulated gene (termed CH25H). The first objective of this study was to simultaneously investigate the expression of *C. gigas* and OsHV-1 genes that share high sequence homology during an acute infection. Comparison of apoptosis-related genes revealed that components of the extrinsic apoptosis pathway (TNF) were induced in response to OsHV-1 infection, but we failed to observe evidence of apoptosis using a combination of biochemical and molecular assays. IAPs encoded by OsHV-1 were highly expressed during the acute stage of infection and may explain why we didn't observe evidence of apoptosis. However, *C. gigas* must have an alternative mechanism to apoptosis for clearing OsHV-1 from infected gill cells as we observed a reduction in viral DNA between 27 and 54 h post-infection. The reduction of viral DNA in *C. gigas* gill cells occurred after the up-regulation of interferon-stimulated genes (viperin, PKR, ADAR). In a second objective, we manipulated the host's antiviral response by injecting *C. gigas* with a small dose of poly I:C at the time of OsHV-1 infection. This small dose of poly I:C was unable to induce transcription of known antiviral effectors (ISGs), but these oysters were still capable of inhibiting OsHV-1 replication. This result suggests dsRNA induces an antiviral response that is additional to the IFN-like pathway.

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## 1. Introduction

Anti-viral immunity in molluscs is poorly understood [1,2]. Most of our understanding of invertebrate anti-viral immunity stems from model systems, such as *Drosophila melanogaster*, mosquitoes (*Anopheles gambiae* and *Aedes aegypti*) and *Caenorhabditis elegans* [3–6]. RNA interference (RNAi) is the central part of the innate immune response of these model invertebrate species [4,6,7]. Additional anti-viral responses utilised by *Drosophila* and mosquitoes include programmed cell death (*i.e.* apoptosis and autophagy) [5,8] and a transcriptional response involving a set of genes that inhibit virus replication [9,10]. These anti-viral responses of model invertebrates are quite different to the vertebrate innate immune

response to viral infection. Vertebrate cells rarely utilise the RNAi-mediated anti-viral response [11]. Instead, the vertebrate innate immune system relies on recognising virus-derived nucleic acids through pathogen recognition receptors (*i.e.* Toll-like receptors, RIG-like receptors, cGAS) [12]. Engagement of these receptors activates the interferon (IFN)-pathway, which is an extremely powerful anti-viral response [12]. IFNs exert their anti-viral response by binding their cognate receptors on all nucleated cells, signal through the JAK-STAT pathway, and transcriptionally induce hundreds of IFN-stimulated genes (ISGs) that are collectively capable of controlling most, if not all, virus infections [12,13]. It was believed invertebrates lacked anti-viral systems homologous to the vertebrate IFN-response because model invertebrates (*Drosophila*, mosquitoes and *Caenorhabditis*) genomes do not encode IFN-cytokines or ISGs [14].

New evidence suggests the IFN-pathway evolved in the common metazoan ancestor with interferon-related genes present in

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the genomes of animals belonging to the phyla of Mollusca [2,15], Cnidaria [16] and Porifera [17,18]. Analysis of model invertebrate genomes reveal they have undergone substantial gene loss [19,20], which appears to also include genes related to the IFN-pathway. The genome of Pacific oyster (*Crassostrea gigas*) encodes many ISGs that are up-regulated in response to Ostreid herpesvirus type 1 (OsHV-1) [15,21–23]. The expression of these ISGs can be induced by injecting *C. gigas* with virus-associated nucleic acids (poly I:C) [24,25] and this response is protective against OsHV-1 infection [22]. Programmed cell death involving autophagy is also involved in the *C. gigas* response to experimental infection with OsHV-1 [26]. Autophagy is induced in the mantle tissue of *C. gigas* in response to OsHV-1 infection and survival assays incorporating known inhibitors of autophagy have demonstrated that this antiviral response has a protective role against OsHV-1 [26]. However, it is unknown if other evolutionarily conserved immune responses present in vertebrates and arthropods, such as RNAi and apoptosis also contribute to molluscan anti-viral immunity.

The incomplete understanding of anti-viral immunity in molluscs is hampering effective management of emerging viral diseases affecting aquaculture production of marine bivalves [27,28] and gastropods [29,30], mainly caused by viruses belonging to the Herpesvirales order [28,31–33]. Herpesviruses constitute a large family of enveloped, double-stranded DNA viruses that are responsible for many human and animal diseases. The genomes of mammalian herpesviruses encode many gene products that subvert the host's anti-viral response and exploit their host's cellular machinery for their own benefit [reviewed by Refs. [34–36]]. For example, the genome of Human herpesvirus-8 (HHV-8) contains 86 genes, of which at least 22 encode proteins that are immunomodulatory [34]. Since the IFN-response is the major innate antiviral response of humans, HHV-8 encodes at least seven proteins that suppress the IFN-response [reviewed by [34]]. It is likely that molluscan herpesviruses have also developed similar strategies to evade their host's innate immune response and persist. Identifying these strategies could provide insight into the major anti-viral pathways of molluscs.

The genome of Ostreid herpesvirus type 1 (OsHV-1) contains 124 open reading frames (ORFs) and eight of these ORFs encode proteins with high homology (E-value <  $1 \times 10^{-5}$ , Score > 200) to proteins encoded by the genome of the Pacific oyster, *C. gigas* [21]. These OsHV-1 ORFs include four inhibitors of apoptosis (ORF42, 87, 99, 106), two super-family type 2 helicases (ORF67, 115), ribonuclease reductase (ORF51), and transmembrane protein with high homology to *C. gigas* cholesterol-25-hydroxylase (ORF57) [33]. Vertebrate cholesterol-25-hydroxylase (CH25H) is an IFN-stimulated enzyme that converts cholesterol to a soluble antiviral factor, 25-hydroxycholesterol, that suppresses viral growth by blocking membrane fusion between virus and cell [37]. These observations suggest that OsHV-1 has the potential to modulate the anti-viral response of *C. gigas* by inhibiting apoptosis and interfering with ISGs. OsHV-1 also appears to be able to modulate host DNA synthesis by encoding enzymes involved in nucleotide biosynthesis (ribonuclease reductase) and DNA/RNA metabolism (SF2 Helicases) [38].

In this study, the first objective was to investigate OsHV-1 countermeasures to the oyster's anti-viral response by simultaneously comparing mRNA expression levels of *C. gigas* and OsHV-1 genes during an experimental infection. Genes investigated include host and virus genes that share high homology (IAPs, CH25H, SF2 Helicases & ribonucleotide reductase) and an additional set of host genes involved in the extrinsic apoptotic pathway (TNF, TNF receptors, FADD and caspases). Apoptosis was also quantified in gill tissue of *C. gigas* at discrete time-points using biochemical assays to measure caspase-3/7 activity and localise apoptotic cells in

histological sections. The second objective of this study was to identify if oysters have anti-viral responses induced by dsRNA that are additional to the already described IFN-like response [22,25]. To achieve this second objective, we simultaneously injected *C. gigas* with OsHV-1 and a low concentration of poly I:C ( $1 \text{ mg ml}^{-1}$ ). This concentration of poly I:C was chosen because it is insufficient to induce ISG expression in *C. gigas*.

## 2. Materials and methods

### 2.1. Animals and OsHV-1 inoculum

Pacific oysters (*C. gigas*) were produced at the IFREMER Oyster Hatchery in Argenton, France. Oysters were grown-out in a bio-secure nursery facility at the IFREMER facility of Bouin (France) before being transferred to IFREMER's Aquaculture Research Facility in Palavas-les-Flots (Laboratoire Aquaculture en Languedoc Roussillon, LALR), France. At the time of experimentation, these oysters were classified to be juveniles (age = 6 months, shell length =  $38.8 \pm 4.0 \text{ mm}$ ) and they were entering the early stages of spermatogenesis as assessed by histology (results not presented). These oysters were used for all experiments outlined in this manuscript.

An initial OsHV-1 homogenate was prepared according to Schikorski et al. [39], from ten dead oyster spat collected during an OsHV-1 mortality event (summer 2014) in Thau Lagoon, France. Dead spat were homogenised in 10-volumes of autoclaved seawater. The oyster homogenate was clarified by centrifugation ( $1000 \times g$  for 10 min,  $4 \text{ }^\circ\text{C}$ ) before serial filtration ( $8.0, 0.45 \text{ } \& 0.22 \text{ } \mu\text{m}$ ). The OsHV-1 homogenate was confirmed to be free of culturable bacteria by plating  $100 \text{ } \mu\text{l}$  of homogenate on marine agar. Twenty-five juvenile oysters were injected with  $50 \text{ } \mu\text{l}$  of OsHV-1 homogenate in the adductor muscle through a notch filed in the oyster shell. Another 20 juvenile oysters were injected with  $50 \text{ } \mu\text{l}$  of autoclaved seawater (controls) and these two groups were placed in separate aquariums with filtered-seawater (aerated,  $22 \text{ }^\circ\text{C}$ ). At 3 days post injection, gill and mantle tissue from 4 moribund oysters (OsHV-1 homogenate) and 4 healthy oysters (seawater control) was excised using a sterile scalpel blade. Tissue from moribund and healthy oysters was used to make a fresh OsHV-1 and control homogenate as per the methods outlined above, respectively. The OsHV-1 genome copy number of the OsHV-1 and control homogenate was estimated by qPCR (see below) and these new homogenates were used for experimentation outlined below.

### 2.2. Experimental conditions

Prior to experimentation, 240 oysters had a notch filed in the side of their shell using an electric bench grinder to allow delivery of oyster homogenates and poly I:C. Oysters were distributed to twelve aerated aquariums ( $22 \pm 1 \text{ }^\circ\text{C}$ ) containing 20 L of filtered seawater (20 oysters per tank). Oysters were allowed to acclimatise to their research aquaria for 24 h.

At time 0 h, oysters were injected with  $100 \text{ } \mu\text{l}$  of one of four treatments into the adductor muscle using a 26-gauge needle attached to a multi-dispensing hand pipette. There were three replicate aquariums for each treatment. The four treatments consisted of (A) OsHV-1 homogenate, (B) OsHV-1 homogenate + poly I:C, (C) control homogenate, and (D) control homogenate + poly I:C. The concentration of OsHV-1 DNA in the virus homogenates was  $5.98 \times 10^4$  viral DNA copies  $\mu\text{l}^{-1}$ . Poly I:C (Sigma, cat #P0913) was resuspended in the OsHV-1 and control homogenates to a final concentration of  $1 \text{ mg ml}^{-1}$ , which resulted in each oyster being injected with  $100 \text{ } \mu\text{g}$  of poly I:C.

Two oysters were sampled from each aquarium at 0, 3, 9, 27 and

54 h post-injection. Cumulative mortality was assessed at 96 h for each treatment from the remaining 10 oysters in each tank. Sampling consisted of shucking each individual oyster and excising the gill tissue using a sterile scalpel blade. Gill tissue was homogenised and divided into three sub-samples: two sub-samples were snap-frozen at  $-80\text{ }^{\circ}\text{C}$  for RNA and DNA purification and the third sub-sample was resuspended in  $250\text{ }\mu\text{l}$  of  $0.01\text{ M}$  phosphate buffered saline (PBS, Sigma cat #P3813) for caspase activity assay and kept on ice. We chose to study the gill tissue because it is one of the main tissue compartments for detecting OshV-1 mRNAs by *in-situ* hybridisation [40]. The remaining oyster tissue was fixed in Seawater Davidson's fixative [41].

2.3. Nucleic acid extraction and qPCR

Total RNA was purified from gill tissue samples using bead-beating (Retsch MM 400) and Trizol (Invitrogen, cat #15596-026) according to the manufacturer's protocol. DNA was purified from gill tissue using a standard phenol:chloroform purification. Quantity and purity of nucleic acids was assessed by spectrophotometry (Thermo Scientific, ND-1000). RNA and DNA were resuspended in sterile water to a final concentration of  $100$  and  $20\text{ ng }\mu\text{l}^{-1}$ , respectively. First-strand synthesis was performed on  $500\text{ ng}$  of total RNA using  $250\text{ ng}$  of random hexamer primers (Invitrogen, cat #48190-011) and  $200$  units of M-MLV RT (Invitrogen, cat #28025-013). cDNA was diluted ten-fold in sterile water prior to use.

The detection and quantification of OshV-1 DNA was performed on individual oysters according to Pepin and colleagues [42] using a LightCycler 480 Real-Time Thermocycler (Roche). PCR reaction volumes were  $6\text{ }\mu\text{l}$  containing LightCycler 480 SYBR Green I Master Mix (Roche),  $100\text{ nM}$  of C9 and C10 primers [42] and  $20\text{ ng}$  of DNA. All PCR reactions were performed in duplicate and absolute quantification of OshV-1 DNA copies were estimated from a standard curve of the C9/C10 amplification ( $R^2 = 0.990$ ) product cloned into the pCR4-TOPO vector as per Green and Montagnani [22]. The linear dynamic range of the qPCR assay was assessed using ten-fold serial dilutions of the TOPO-C9/C10 plasmid, which revealed the upper and lower quantifiable limits to be  $10^9$  to  $10^1$  copies per ng of total DNA, respectively. The quantifiable lower limit measured in the current study is in agreement with Pepin and colleagues [42].

Host and virus gene expression was quantified in individual oysters using an Echo<sup>®</sup> 525 Liquid Handler (Labcyte) and LightCycler 480 Real-Time Thermocycler (Roche). PCR reaction volumes were  $1.5\text{ }\mu\text{l}$  containing LightCycler 480 SYBR Green I Master Mix (Roche),  $0.5\text{ nM}$  of gene specific primers (Table 1) and  $0.5\text{ }\mu\text{l}$  of cDNA in a LightCycler 480 Real-Time thermocycler (Roche) using an initial denaturation ( $95\text{ }^{\circ}\text{C}$  for  $5\text{ min}$ ) followed by  $40$  cycles of denaturation ( $95\text{ }^{\circ}\text{C}$ ,  $10\text{ s}$ ), hybridisation ( $60\text{ }^{\circ}\text{C}$ ,  $20\text{ s}$ ) and elongation ( $72\text{ }^{\circ}\text{C}$ ,  $25\text{ s}$ ). A subsequent melting temperature curve of the amplicon was performed and expression of *C. gigas* target genes were normalised with eEF1 $\alpha$  reference gene [43].

OshV-1 gene expression was reported as both quantitative and qualitative values. No gene has yet been identified in the OshV-1 genome that is uniformly expressed at the same level in all samples [44–47]. This prevents calculations involving a valid internal reference gene ( $2^{\Delta\text{Ct}}$ ). Quantitative values for OshV-1 gene expression were therefore normalised according to the method outlined by Segarra et al. [46], using the formula:  $F = \text{Log}_{10}((E + 1)^{40 - \text{Ct}}/N)$ , where E is qPCR efficiency of each viral gene, Ct (cycle threshold) corresponds to the PCR cycle number, N is the number of viral DNA copies  $\text{ng}^{-1}$  of total DNA determine by absolute qPCR for each individual and Ct =  $40$  is arbitrarily considered to 'no Ct' obtained by qPCR. Qualitative gene expression was reported as raw values (crossing-point, Cp) and a sample was considered to

Table 1  
Quantitative PCR primers used for measuring *Crassostrea gigas* and OshV-1 gene expression. The E-value and amino acid identity (identity %) are provided for OshV-1 ORFs that are homologous to *C. gigas* genes.

Function	Gene	Host ( <i>Crassostrea gigas</i> )		Virus (OshV-1)		% identity	E-value		
		Genbank	5' 3'	ORF	Genbank			5' 3'	
Reference	EFU	ABI22066	GAGCGTGAAGCTGGTATCAC	ACAGACAGCTAGCCTGTCA					
Apoptosis	TNF1	EKC35160	ACCCGTACTCACATAC	ACGTAGTGATACAGCGTTGTAG					
Apoptosis	TNF2	EKC39243	CTGGATTTACGACAGAGACATC	ACGACACCTGGCTGTAGAC					
Apoptosis	TNF_3	ADX31292	ATCTACCACCAACGTGCAC	GTCTTAAGGTCCGATTGGAG					
Apoptosis	TNFR_1	EKC31251	TATCGTCGCCCATCATC	TGACCTTGAATGACCTTGAC					
Apoptosis	TNFR_2	EKC31251	TATCGTCGCCCATCATC	TGACCTTGAATGACCTTGAC					
Apoptosis	Caspase-3	CU988427	ATCCACGGAAGGATCATGG	GTTCATCCGAAACCACTCG					
Apoptosis	Caspase-7	HO425703	ATTGCCACACAGACAACCG	TCGTCGCTTTTCAAGGGCTCC					
Apoptosis	IAP_1	EKC36433	CATCTTCTTCTATCGGCTTC	TCAGCTGTGAGGTGTGAC	ORF87	CACAGAGACATTTCCCAAA	AAAGCTGTTCCACATTTGGT	36	8.E-47
Apoptosis	IAP_2	EKC34022	CAGTAAAGAGGCCACGTAG	CTTACTGTAGGATAGCGTATG	ORF106	TCGTGGATCCAACTCCAAA	TCAGCCTATGACGAGCAATG	31	2.E-22
Apoptosis	CH25H	EKC31751	CTTTATTTGAATGGGACCCCAAG	GCTATTTCTGATGCTGAAC	ORF57	TTACAGCACCCGAGCAGGAT	TCGCCCTTTTATCCACAC	20	4.E-15
ISG	Viperin	EKC28205	TAAATCGGCTTCTGTITTC	CCATCTGAAGCTCTCTTTTC					
ISG	PKR	EKC34807	GAGCATCACAAGTGTTCAG	GTAGCACAGGAGATGTTTC					
ISG	ADAR-L	EKC20855	CTCAAAAGTGCACCTGCATC	TCACAGCCCTGTCTATCAC					
DNA Metab.	RNR	EKC28390	TCAAATCCATAGAGCATACAC	TCATCTCATCTGCCCAATC	ORF51	ACTAACGCAATGCTAGCCAG	ATCTGTCAGTATTGCCACAC	37	1.E-147
DNA Metab.	Helicase_1	EKC17722	GTCTGTCCAAATCCAAAGTC	CTTGTATTGTGTCCTCGTGAC	ORF67	GATGACGGCAITGGTGAGGT	CCTGTGTGCGGCTGTGTTA	23	2.E-16
DNA Metab.	Helicase_2	EKC17722	GTCTGTCCAAATCCAAAGTC	CTTGTATTGTGTCCTCGTGAC	ORF115	ACCACTAAGGCCCTTTCCAG	GTACAACTCTGCTGTTGAC	31	7.E-32

contain OsHV-1 RNA when the fluorescence of SYBR in the RTqPCR assay exceeded the background fluorescent threshold of 0.1 RFU.

#### 2.4. Histology & TUNEL assay

Oyster tissue fixed in Seawater Davidson's solution was dehydrated and embedded in Paraplast® (Sigma, cat #A6330) using standard histological techniques. Serial sections of 5 µm thickness were cut and one section was adhered to StarFrost® hydrophilic slide (Waldemar Knittel) for hematoxylin-eosin staining, while the companion section was adhered to a StarFrost® silane coated slide (Waldemar Knittel) for TUNEL assay. Histological sections were pre-treated with 10 µg ml<sup>-1</sup> of proteinase K (Roche, cat #03 115 887 001) for 15 min before being analysed with the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science, cat #12 156 792 910). Controls included the TUNEL reaction mixture without terminal transferase (negative control) and histology sections treated with DNase I (positive control). Sections were counterstained with 30 nM ml<sup>-1</sup> 4',6-diamidino-2'-phenylindole dihydrochloride (Roche Applied Science, cat #10236276001) for 5 min, and visualised with a fluorescent compound microscope (Leica DM5500 B) equipped with standard red (N3 λex: 546/12 nm, λem: 600/40 nm; TMR red) and blue (A4 λex: 360/40 nm, λem: 470 nm; DAPI) filter sets.

#### 2.5. Caspase-3/7 activity assay

Apoptosis was quantified fluorimetrically from caspase-3/7 activity at 0, 27 and 54 h post injection. Caspase activity was determined using the SensoLyte® Homogenous AFC Caspase-3/7 Assay Kit (AnaSpec Inc. cat #AS-71114) according to Rolland et al., [48]. Briefly, gill tissue (50 mg) was homogenised in 250 µl of PBS using a Potter-Elvehjem glass pestle and tube. In duplicate, 50 µl of gill homogenate was mixed with 50 µl of caspase reagent solution (50 µl caspases-3/7 substrate Ac-DEVD-AFC, 200 µl of DTT and 4.75 ml of assay buffer) in a black, v-bottom, 96-well plate. Caspase-3/7 mediated conversion of the substrate N-acetyl-Asp-Glu-Val-Asp-7 amino-4 trifluoromethyl coumarin was monitored every 5 min over a 60 min duration using a TECAN® microplate reader (λex: 380 nm, λem: 500 nm; TECAN Group, Switzerland). The protein concentration of gill tissue homogenates was estimated using the Quick Start™ Bradford 1 × Dye Reagent (BIO-RAD, cat #500-0205) in a microplate format with bovine serum albumin (BSA) as the standard. Caspase-3/7 activity was expressed in Relative Fluorescence Unit per min per mg of protein.

#### 2.6. Statistical analysis

Two-way analysis of variance (ANOVA) was performed to test the affect of the four treatments and five time-points on caspase-3/7 activity and gene expression of *C. gigas*/OsHV-1. Tukey's honest significance difference method for multiple comparisons was used to compare means if significant differences were found. Statistical analysis and graphs were produced using the computer package, SPSS version 20.0.0.2.

### 3. Results

#### 3.1. Viral load in gill tissue and oyster cumulative mortality

The cumulative mortality of *C. gigas* injected with the OsHV-1 homogenate was 13.4 ± 5.8%. The timing of mortality for *C. gigas* injected with the OsHV-1 homogenate occurred between 54 and 96 h post infection (p.i.). No mortality occurred in *C. gigas* injected with the OsHV-1 homogenate + poly I:C, control homogenate and

control homogenate + poly I:C.

The concentration of OsHV-1 DNA in gill tissue was quantified for each individual oyster during the experiment. The concentration of OsHV-1 DNA in *C. gigas* gill tissue was below 1.0 × 10<sup>1</sup> copies per ng of total DNA at 0, 3 and 9 h p.i. for all four treatments (Fig. 1). At 27 h, *C. gigas* injected with OsHV-1 homogenate had a mean OsHV-1 DNA concentration of 9.45 × 10<sup>5</sup> copies per ng of total DNA, which dropped to a mean concentration of 4.06 × 10<sup>3</sup> copies per ng of total DNA at 54 h. For *C. gigas* simultaneously injected with OsHV-1 + poly I:C, mean concentration of OsHV-1 DNA was 3.15 × 10<sup>2</sup> copies per ng of total DNA at 27 h and the mean concentration of OsHV-1 increased to 1.53 × 10<sup>3</sup> copies per ng of total DNA at 54 h. The concentration of OsHV-1 DNA was less than 1.0 × 10<sup>1</sup> copies per ng of total DNA for *C. gigas* injected with the control homogenates at all time points (Fig. 1).

#### 3.2. *Crassostrea gigas* gene expression in gill tissue

We measured the expression kinetics of 15 *C. gigas* genes in response to OsHV-1 infection and poly I:C stimulation. These target genes were involved in the apoptotic pathway (TNF\_1, TNF\_2, TNF\_3, TNFR\_1, TNFR\_2, IAP\_1, IAP\_2, caspase 3 & caspase 7), ISG response (PKR, ADAR-L, viperin, CH25H) and host cellular replication (RNR, Helicase\_1 & Helicase\_2). The expression of those target genes were normalised to elongation factor (eEF1α), which was stable in the current experiment (CV = 5.1%, p = 0.312). At 27 h, the mRNA levels of ISGs (viperin, PKR and ADAR) and TNF3 was significantly elevated in *C. gigas* injected with the OsHV-1 homogenate (Fig. 2, p < 0.001). The mRNA expression levels of these ISGs and TNF3 returned to normal by 54 h (p > 0.05). In contrast, only viperin mRNA levels was significantly elevated at 27 h in *C. gigas* injected simultaneously with OsHV-1 + poly I:C (Fig. 2A, p < 0.05). OsHV-1 infection did not alter the mRNA expression levels of the other target genes (TNF1, TNF2, TNFR1, TNFR2, IAP1, IAP2, caspase 3 & caspase 7) involved in the apoptotic pathway (p > 0.05). Likewise, injection of the control homogenate or control homogenate + poly I:C did not alter the expression levels of any of the target genes investigated (p > 0.05).

#### 3.3. OsHV-1 gene expression in gill tissue

OsHV-1 RNA was detected in *C. gigas* gill tissue using RT-qPCR

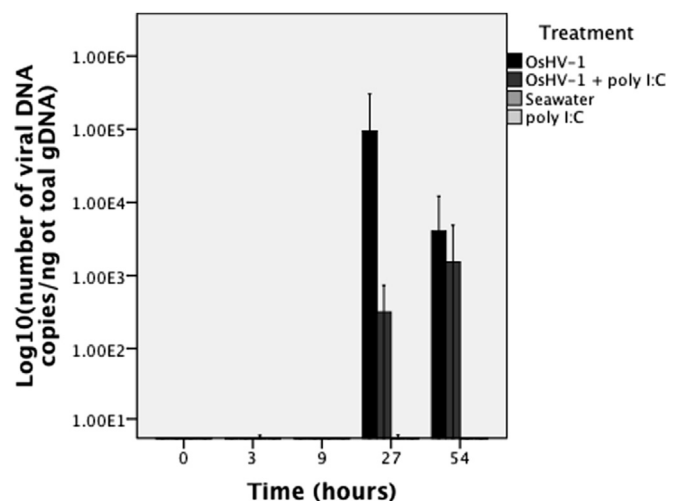
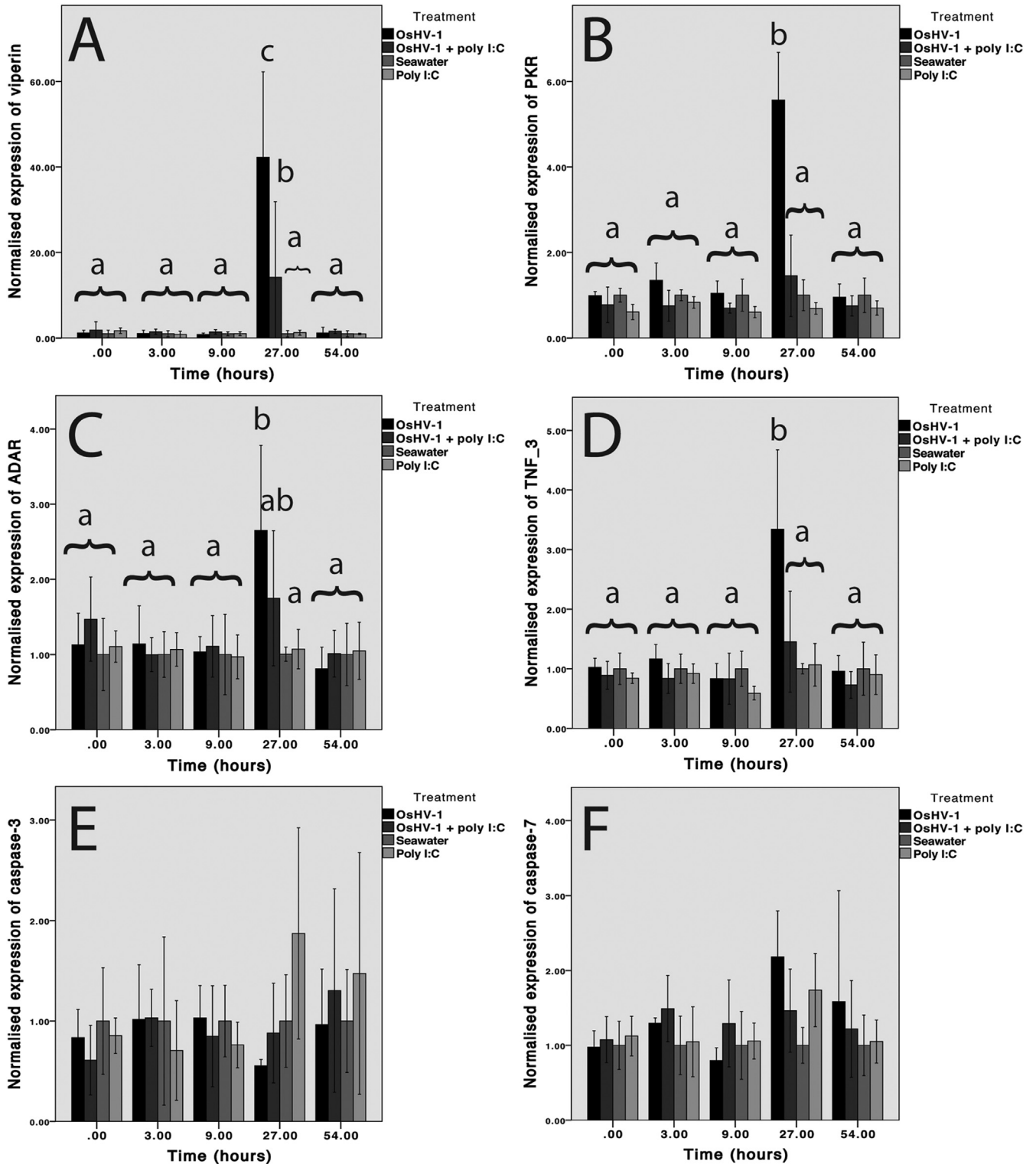


Fig. 1. Absolute quantification of OsHV-1 DNA in gill tissue of *Crassostrea gigas* by quantitative PCR according to Pepin et al. [42].



**Fig. 2.** Normalised gene expression of selected *Crassostrea gigas* immune genes (mean  $\pm$  SD). Among the 15 *C. gigas* target genes that were investigated, only four genes were differentially upregulated following OsHV-1 infection, viperin (A), PKR (B), ADAR (C) and TNF3 (D). The expression of *C. gigas* caspase-3 (E) and caspase-7 (F) were not up-regulated following OsHV-1 infection or poly I:C injection ( $p > 0.05$ ). Different letters indicate significant differences between treatments and time-points ( $p < 0.05$ , Tukey's HD).

and we report both qualitative and quantitative values for OsHV-1 gene expression. Qualitative detection determines whether a target RNA is present in a sample. We considered a sample to contain OsHV-1 RNA when SYBR fluorescence in the assay exceeded the threshold of 0.1 relative fluorescent units (RFU) within 40 cycles of

PCR. We first detected RNA transcripts corresponding to OsHV-1 ORF51 (Ribonuclease Reductase) and ORF67 (Helicase) at 3 h post inoculation (p.i.). Whereas, RNA transcripts corresponding to ORF87 (IAP), ORF106 (IAP), ORF57 (CH25H) and ORF115 (Helicase) were not detected by RT-qPCR in gill tissue until 27 h p.i.

OshV-1 gene expression was also normalised to the number of OshV-1 DNA genome copies within the gill tissue. Normalisation revealed these six OshV-1 RNA transcripts were all significantly expressed at 27 h in *C. gigas* inoculated with the OshV-1 homogenate (Fig. 3,  $p < 0.05$ ), but the normalised expression of these six RNA transcripts was significantly lower at 27 h in *C. gigas* simultaneously injected with OshV-1 + poly I:C (Fig. 3,  $p > 0.05$ ).

#### 3.4. Histopathology and apoptotic cell localisation

There were microscopic changes to the epithelium of *C. gigas* injected with OshV-1 poly at 27 and 54 h p.i. Examination of the epithelium tissue of *C. gigas* injected with the OshV-1 homogenate and OshV-1 homogenate + poly I:C revealed multifocal erosive lesions with underlying hemocyte infiltration at 27 and 54 h p.i. (Fig. 4A). These changes were not observed in *C. gigas* injected with the control homogenate or control homogenate + poly I:C (Fig. 4B).

*In-situ* detection of fragmented DNA (TUNEL assay) was undertaken to identify apoptotic cells in oyster tissues. The positive control (DNase I treated) showed extensive staining in all nuclei examined (data not shown). The TUNEL assay revealed apoptotic cells in *C. gigas* injected with both the OshV-1 and control homogenates. We did not observe a focal accumulation of apoptotic cells in any tissue type that was specific to oysters injected with the OshV-1 homogenate.

#### 3.5. Caspase-3/7 activity

OshV-1 and poly I:C did not alter the activity of caspase-3/7 at 0, 27 and 54 h post injection ( $p > 0.05$ , Fig. 5).

### 4. Discussion

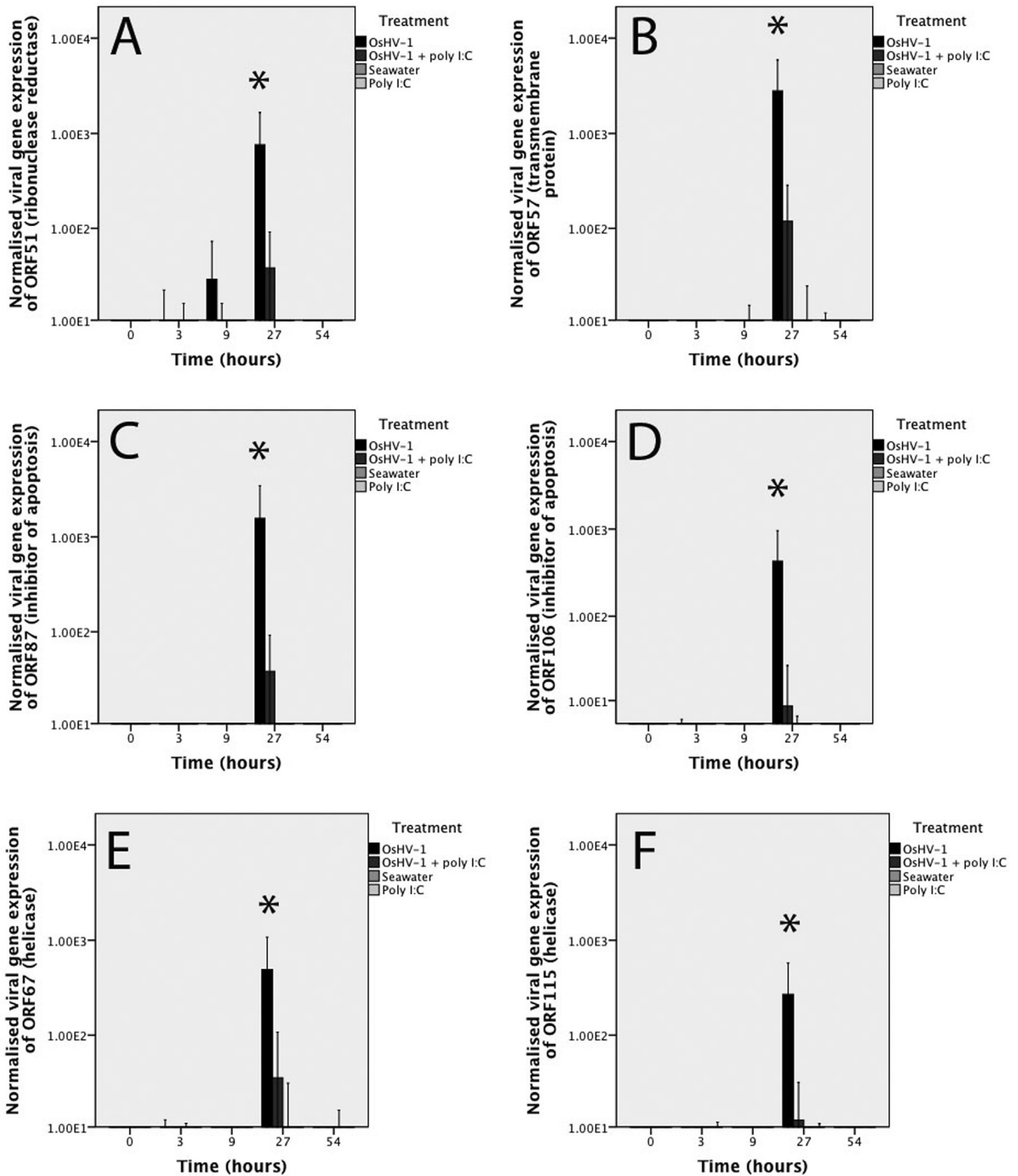
Vertebrates and model-invertebrates have evolved effective anti-viral responses [1]. The RNAi machinery can inhibit virus replication [49], whereas other evolutionary conserved anti-viral responses, such as apoptosis and the IFN-pathway, are capable of clearing viruses from the host [12,50]. In order for viruses to replicate and propagate, viruses also evolved strategies to counteract the host anti-viral response [36]. The first objective of this study was to investigate OshV-1 countermeasure to the oyster's anti-viral response involving apoptosis and ISG expression. These two anti-viral pathways were chosen based on OshV-1 ORFs that shared high homology to *C. gigas* inhibitors of apoptosis and CH25H. We also investigated OshV-1 ORFs that share high homology to *C. gigas* genes involved in DNA metabolism.

In the current study, OshV-1 was confirmed to replicate in the gill tissue of *C. gigas* upon infection. The amount of OshV-1 DNA in gill tissue of *C. gigas* injected with the OshV-1 homogenate was below the quantifiable lower limit at 0, 3 and 9 h p.i. and then rapidly increased to peak at 27 h p.i. (Fig. 1). This pattern of OshV-1 replication is similar to previous studies investigating OshV-1 replication in mantle tissue [44–46], however these studies first detected OshV-1 DNA at 2 h p.i. OshV-1 DNA was detected in some control oyster samples in the present study, but the amount of DNA was below the quantifiable lower limit. The presence of OshV-1 DNA in control animals is a recurrent problem [40,44,51] and may represent contamination or a persistent/latent infection. However, OshV-1 was not causing an active infection in control oysters because we failed to detect OshV-1 RNA by RT-qPCR (Section 3.3). OshV-1 mRNA corresponding to genes involved in DNA metabolism (ORF51 and ORF67) were first detected by RT-qPCR in the OshV-1 treatment at 3 h post-infection and OshV-1 mRNA corresponding to genes (ORF57, ORF87 and ORF106) that potentially modulate the host's immune response were not detected

until 27 h post-infection. This pattern of OshV-1 gene expression is similar to previous studies using *C. gigas* mantle tissue [45]. The corresponding *C. gigas* genes involved in DNA metabolism (ribonuclease reductase and DNA helicases), inhibitors of apoptosis (IAPs) and CH25H remained stable in response to OshV-1 infection.

Apoptosis is an important process to eliminate virus-infected cells that pose a threat to the host [50]. Electron microscopic examination of *C. gigas* larvae and spat infected with OshV-1 revealed hemocytes with condensed chromatin and extensive perinuclear fragmentation of chromatin, which suggest OshV-1 may induce apoptosis in oyster hemocytes [52]. The process of apoptosis is evolutionary conserved [53,54] and regulated by a specific set of genes [50]. Apoptotic signals are initiated and transduced via intrinsic (mitochondrial-mediated) or extrinsic (immune-mediated) pathways [50]. Extracellular signals can activate the extrinsic pathway through death receptors, resulting in the recruitment of FADD and caspase-8 and forming the death-inducing signalling complex (DISC) [55]. Activated caspase-8 then activates caspase-3, which plays a central role in the execution phase of apoptosis [55]. Ligands that activate the death receptors belong to the TNF superfamily of cytokines [55]. In the current study, we measured the mRNA expression levels of three TNF cytokines and two TNF-receptors in gill tissue of *C. gigas*. OshV-1 infection resulted in the up-regulation of a single TNF (GenBank #ADX31292, Fig. 2) at 27 h post-infection, but we did not observe evidence that apoptosis was induced in response to OshV-1 infection. We assessed apoptosis using three independent assays: biochemical assay to measure caspase-3,7 activity (Fig. 5), TUNEL reaction to localise apoptotic cells in histology sections and a molecular assay to measure gene expression of caspase 3 and 7 (Fig. 2). Some viruses have evolved distinct strategies to evade apoptosis to facilitate replication, spread and latency [50]. The genomes of mammalian herpesviruses encode immunomodulators that have host counterparts [reviewed by 50]. For example, Epstein–Barr virus (EBV) encodes an IAP (BHRF1) in the early stages of infection to prevent TNF $\alpha$ -induced apoptosis [56]. OshV-1 also encodes four IAPs with high homology to *C. gigas* IAPs (Table 1). In the current study, two OshV-1 IAPs (ORF87 & ORF106) were significantly up-regulated at 27 h post-infection (Fig. 3), suggesting OshV-1 IAPs might play an important role in OshV-1 replication via protecting infected *C. gigas* cells from TNF-mediated apoptosis, thus maximising viral particle numbers in the early stages of infection. Previous studies have also reported that OshV-1 ORF87 is expressed during the early stages (10 h p.i.) of OshV-1 infection [44–46].

Previous research has demonstrated that *C. gigas* injected with a high concentration of dsRNA (poly I:C, 5 mg ml<sup>-1</sup>) have elevated mRNA expression levels of ISGs at 27 h post-injection [24,25] and these oysters are resistant to subsequent infection with OshV-1 [22]. In the current experiment, *C. gigas* were injected with a lower concentration of poly I:C (1 mg ml<sup>-1</sup>) and this concentration of poly I:C was insufficient to induce ISG expression (Fig. 2). We observed *C. gigas* injected with a low concentration of poly I:C (OshV-1 homogenate + poly I:C) are still capable of inhibiting both OshV-1 replication (Fig. 1) and OshV-1 gene expression (Fig. 3) compared to *C. gigas* injected with only the OshV-1 homogenate. These observations suggest non-specific dsRNA induces both an ISG response and an additional anti-viral pathway in *C. gigas*. In other animal models, dsRNA not only activates the interferon-response, but it is also known to regulate different types of post-transcription gene processes that are collectively referred to as RNA interference (RNAi) [57]. RNAi can silence virus gene expression (virus-derived short interfering RNAs) [57]. The RNAi system is functional in oysters [reviewed by Refs. [58,59]], but the contribution of RNAi to oyster anti-viral immunity is unknown. Research from other marine invertebrates suggests there is cross-talk



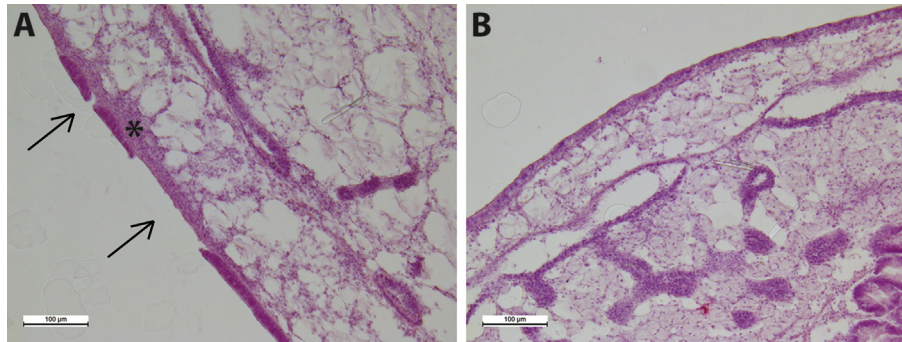
**Fig. 3.** Normalised viral gene expression of selected OsHV-1 open reading frames (ORFs). The expression of OsHV-1 ORFs were normalised according to Segarra et al. [44], and their expression (mean  $\pm$  SD) is present in individual graphs: (A) ORF51, (B) ORF57, (C) ORF87, (D) ORF106, (E) ORF67, and (F) ORF115. Asterisks indicate the ORF was significant up-regulated ( $p < 0.05$ , Tukey's HD).

between the RNAi-mediated anti-viral pathway and the transcriptomic response induced by non-specific dsRNA [60,61].

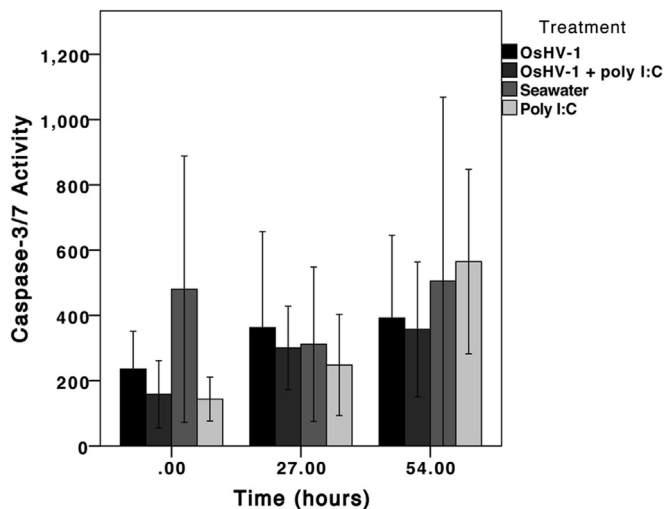
In the current study we observed a reduction in the concentration of OsHV-1 DNA between 27 and 54 h p.i. (Fig. 1), which

suggests *C. gigas* may have a mechanism to clear OsHV-1 from infected gill tissue. From our data, we speculate on two potential mechanisms to clear OsHV-1 from infected gill cells. The multifocal erosive lesions of epithelium tissue could be a host response to





**Fig. 4.** OsHV-1 infection of *Crassostrea gigas* was associated with multifocal ulceration the mantle and gill epidermis (arrows) with underlying inflammation (\*) at 27 and 54 h post-injection. (A) Mantle epithelium of *C. gigas* injected with OsHV-1 homogenate at 27 h p.i. (B) Mantle epithelium of *C. gigas* injected with control homogenate at 27 h post-injection. Scale bar corresponds to 100  $\mu\text{m}$  is indicated.



**Fig. 5.** Caspase-3/7 activity measurements in the gill tissue of *Crassostrea gigas* at 0, 27 and 54 h post-injection (mean  $\pm$  SD). OsHV-1 infection or poly I:C injection did not alter the activity of caspase-3/7 (two-way ANOVA,  $p > 0.05$ ).

shed virus-infected gill cells (Fig. 4). These histological observations are consistent with previous studies [62,63]. Cytopathological changes of scallop, *Chlamys farreri* infected with acute virus necrobiotic virus (AVNV, genotype of OsHV-1) include focal erosive lesions of epithelium cells and these cells were positive for AVNV using *in-situ* immunofluorescent detection and monoclonal antibodies raised against AVNV [63]. However, *in-situ* hybridisation of *C. gigas* reveals OsHV-1 DNA is mainly observed in connective tissue of mantle, gills, adductor muscle, labial palps and gonads [40,64] and we observed epithelium lesions in *C. gigas* injected with OsHV-1 homogenate + poly I:C, but no reduction in the concentration of OsHV-1 DNA in this group of *C. gigas* between 27 and 54 h p.i. (Fig. 1). The more likely alternative mechanism for *C. gigas* to clear OsHV-1 from infected gill tissue is the up-regulation of ISGs (*i.e.* viperin, PKR, ADAR) at 27 h p.i. (Fig. 2). In support of this mechanism is the lack of ISG induction in *C. gigas* simultaneously injected with OsHV-1 homogenate + poly I:C (Fig. 2) corresponding with no change in OsHV-1 DNA concentration in gill tissue between 27 and 54 h p.i. (Fig. 1).

## 5. Conclusion

Results from our study support the concept that IAPs encoded by the OsHV-1 genome can successfully inhibit apoptosis in gill

tissue of *C. gigas*. If experimental infection of *C. gigas* with OsHV-1 resulted in the initiation of the extrinsic apoptotic pathway (TNF $_3$  up-regulation), we didn't observe evidence of apoptosis using a combination of biochemical and molecular assays. Interestingly, we observed evidence that dsRNA induces an additional antiviral response in *C. gigas* that is capable of inhibiting OsHV-1 replication. Future research should focus on characterising the RNAi-mediated antiviral pathway in response to OsHV-1 infection and examine if cross-talk occurs between the IFN-like and RNAi response in *C. gigas*.

## Acknowledgements

The authors acknowledge the funding provided to T. Green by Macquarie University Research Fellowship Scheme (MQ Grant: 9201300681). The authors are grateful to Bruno Petton and Ifremer's Argenton facility staff for production of spat (Ifremer FINA action) and Max Nourry and Ifremer's Bouin facility staff for taking care and dispatch of spat between Ifremer's laboratories.

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