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RESEARCH ARTICLE

Long dsRNAs promote an anti-viral response in Pacific oyster hampering ostreid herpesvirus 1 replication

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ABSTRACT

Double-stranded RNA (dsRNA)-mediated genetic interference (RNAi) is a widely used reverse genetic tool for determining the loss-of-function phenotype of a gene. Here, the possible induction of an immune response by long dsRNA was tested in a marine bivalve (*Crassostrea gigas*), as well as the specific role of the subunit 2 of the nuclear factor κB inhibitor (*IκB2*). This gene is a candidate of particular interest for functional investigations in the context of oyster mass mortality events, as *Cg-IκB2* mRNA levels exhibited significant variation depending on the amount of ostreid herpesvirus 1 (OsHV-1) DNA detected. In the present study, dsRNAs targeting *Cg-IκB2* and green fluorescent protein genes were injected *in vivo* into oysters before being challenged by OsHV-1. Survival appeared close to 100% in both dsRNA-injected conditions associated with a low detection of viral DNA and a low expression of a panel of 39 OsHV-1 genes as compared with infected control. Long dsRNA molecules, both Cg-IκB2- and GFP-dsRNA, may have induced an anti-viral state controlling the OsHV-1 replication and precluding the understanding of the specific role of *Cg-IκB2*. Immune-related genes including *Cg-IκB1*, *Cg-Rel1*, *Cg-IFI44*, *Cg-PKR* and *Cg-IAP* appeared activated in the dsRNA-injected condition, potentially hampering viral replication and thus conferring a better resistance to OsHV-1 infection. We revealed that long dsRNA-mediated genetic interference triggered an anti-viral state in the oyster, emphasizing the need for new reverse genetics tools for assessing immune gene function and avoiding off-target effects in bivalves.

KEY WORDS: Anti-viral response, RNA interference, Inhibitor of NF-κB, Marine bivalve, Ostreid herpesvirus 1

INTRODUCTION

In invertebrates, the defense against infectious microorganisms is driven by an innate-type immune system, which relies on highly conserved mechanisms of pathogen recognition and activation of the immune response, identified throughout much of the animal kingdom (Hoffmann et al., 1999; Schmitt et al., 2011; Bachère et al., 2015; Escoubas et al., 2016). Studies revealed striking similarities in the signaling pathways ran in mammals and molluscs to activate their innate immune responses (Montagnani et al., 2004; Green and Barnes, 2009; Koutsogiannaki and Kaloyianni, 2010; De Zoysa et al., 2010; Renault et al., 2012; Green et al., 2015a). Among these pathways, the nuclear factor κB (NF-κB) pathway is known to play a pivotal role in innate immune responses in both vertebrates and invertebrates (Hoffmann et al., 1999). Certain types of infection lead to the induction of the NF-κB pathway, in which one of the most significant features is the role of the Rel/NF-κB family of transcriptional activator proteins. As concerns the Pacific oyster *Crassostrea gigas* (Thunberg 1793), several conserved genes from the Rel/NF-κB signaling pathway have been characterized (Green and Montagnani, 2013). Similarities of oyster Rel/NF-κB pathway with vertebrates and *Drosophila* were highlighted. For instance, it was demonstrated that two proteins, an IκB kinase-like (*oIKK*) and a Rel homolog (*Cg-Rel*), shared structural and functional properties with their mammalian and insect counterparts (Escoubas et al., 1999; Montagnani et al., 2004). Moreover, the presence of two IκB homologs (*Cg-IκB1* and *Cg-IκB2*) and their ability to inhibit NF-κB/Rel transcriptional activity were reported (Montagnani et al., 2008; Zhang et al., 2011). A third *Cg-IκB* has been recently identified and shown to play a role in host defense by inhibiting persistent over-activation of NF-κB/Rel, especially during the late stage of the immune response (Xu et al., 2015).

Besides NF-κB, there are other major transcription factors playing a key role in the innate response, such as the interferon regulatory factors (IRFs). Most viruses infecting vertebrates produce double-stranded RNA (dsRNA) during viral replication, and vertebrate cells recognize extracellular and endosomal dsRNA (viral or synthetic) via different sensors. They include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), cyclic GMP-AMP synthase (cGAS) and DICER proteins, which have been recently identified in molluscs and are potentially involved in virus recognition (Escoubas et al., 2016). Among the above-mentioned virus receptors, a major role is played by TLR3, which binds dsRNA, thus triggering a complex signal-transduction pathway resulting in the translocation of *NF-κB* and IRFs to the cell nucleus. The activation of this pathway leads to the upregulation of a number of genes, principally interferon. Interferon exerts an anti-viral state in neighboring cells by inducing the expression of interferon-stimulated genes (ISGs) and anti-viral proteins, such as protein kinase R (*PKR*), 2′,5′-oligoadenylate synthetase (*OAS*) and Myxovirus resistance protein (*Mx*) (Randall and Goodbourn, 2008). With regard to invertebrates, it was debated whether dsRNA can be recognized as a virus-associated molecular pattern inducing anti-viral protection, even in the absence of the interferon-signaling pathway. In Robalino et al. (2004), an anti-viral

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state was induced in the shrimp *Litopenaeus vannamei* by dsRNA injection, regardless of the sequence. This response was distinct from the sequence-specific dsRNA-mediated genetic interference (RNAi) phenomenon. Subsequently, mechanisms involved in dsRNA-induced immunity and RNAi were suggested to converge to mount an efficient anti-viral response in crustaceans (Labreuche et al., 2010).

Recently, the paradigm rejecting the absence of interferon-signaling pathway was reviewed by the detection of interferon-related genes in invertebrate sequences (Sodergren et al., 2006; Huang et al., 2008; Zhang et al., 2012; Philipp et al., 2012; Green et al., 2015b), and by the evidence in *C. gigas* of a polyinosinic: polycytidylic acid (poly I:C) mediated anti-viral protection associated with the upregulation of interferon-like response genes (Green and Montagnani, 2013). However, to date, interferons have never been detected and isolated in molluscs.

RNAi is a widely used reverse genetic tool for determining the loss-of-function phenotype of a gene (Fire et al., 1998). However, in molluscs such as marine bivalves, this technique is scarcely used and remains a technical challenge (Fabioux et al., 2009; Suzuki et al., 2009; Huvet et al., 2012; Li et al., 2016; Oyanedel et al., 2016; Zhao et al., 2016). The possible induction of an immune response by dsRNA should be taken into consideration for the interpretation of future RNAi studies. This is especially true for immune purposes, but more broadly for all physiological aspects, as long dsRNAs have been recently debated as agents threatening the physiological integrity and increasing maintenance costs of oyster aquaculture (Huvet et al., 2015).

Crassostrea gigas is an ecologically and economically important marine invertebrate that has been recently affected by large-scale mass mortality events in several countries, and is a major concern for aquaculture. Until 2008, it was generally accepted that the most important causative agents of *C. gigas* summer mortality were high temperature and reproduction stage, while some pathogens (e.g. *Vibrio* spp.) were considered as opportunistic (Samain and McCombie, 2008). Since 2008, mass mortality episodes have occurred on oyster spat. These mortality episodes were characterized by their more widespread geographic distribution (EFSA Panel on Animal Health and Welfare, 2010; Jolivel and Fleury, 2012) associated with the detection of a newly reported ostreid herpesvirus 1 (OsHV-1) variant, µVar (Segarra et al., 2010). Nevertheless, the identification of implicated factors still remains an open issue and the complexity of this phenomenon is attested by recent studies (Pernet et al., 2012; Renault, 2012; Petton et al., 2015a,b; Prado-Alvarez et al., 2016).

From investigations of the molecular reasons underlying the better survival of oysters reared in the field during the summer period (Fleury et al., 2010; Chaney and Gracey, 2011; De Lorgeril et al., 2011; Rosa et al., 2012), immune defense appeared to be one of the determinants of higher survival, for instance, through the NF-κB pathway (Renault et al., 2011; Fleury and Huvet, 2012; Normand et al., 2014). Among the genes involved in this pathway, the expression of the inhibitor 2, termed *Cg-IκB2* (Zhang et al., 2011), is a candidate of particular interest for functional investigation as *Cg-IκB2* mRNA levels exhibited highly significant variation depending on OsHV-1 DNA detection (Normand et al., 2014).

In the present study, a dsRNA targeting the *Cg-IκB2* candidate gene was injected *in vivo* into oyster spat before a challenge with OsHV-1 in order to evaluate the role of *Cg-IκB2* in response to this virus. Survival was measured as the main knock-down phenotype of interest. GFP-dsRNA was used as a mock control to discriminate between a specific activation of immune pathways by a dsRNA-mediated inhibition of *Cg-IκB2* and a general anti-viral response triggered by long dsRNA administration. The real-time PCR technique was employed to evaluate the mRNA levels of *Cg-IκB2* itself, the other subunit *IκB1*, *Rel1*, two homologues of vertebrate interferon stimulated genes (*IFI44* and *PKR*) and the endoribonuclease *DICER*. In addition, the gene expression of a suppressor of cytokines signaling (*SOCS*) and an apoptosis inhibitor (*IAP*) were assessed, as they are known to play crucial roles in regulating the host immune response to pathogens. Viral genes, based on 39 OsHV-1 open reading frames (ORFs) (Segarra et al., 2014a), were also assayed to evaluate OsHV-1 cycle in response to Cg-IκB2-dsRNA injection.

MATERIALS AND METHODS

Biological material and experiments

Healthy oysters (i.e. free of mortality, negative for OsHV-1 and showing very low *Vibrio* spp. concentration; Petton et al., 2013) were produced and reared at larval stage at the Ifremer hatchery in Argenton (Finistère, France) and spat were cultured at the Ifremer station in Bouin (Poitou-Charentes, France) under controlled conditions. Six-month-old oyster spat were transferred to Ifremer experimental facilities in Plouzané (Finistère, France) and placed in 20-µm-filtered seawater at 19°C before the experiments began. The whole study consisted of two separate experiments.

The first experiment (Exp1) aimed at evaluating whether the injection of Cg-IκB2-dsRNAs and exogenous GFP-dsRNAs may impact oyster mortality following OsHV-1 injection (Fig. 1). Four host genes were evaluated at 48 h post OsHV-1 injection (hpi) to estimate the RNA interference and the anti-viral response.

The second experiment (Exp2) investigated the transcriptional changes of a set of 39 viral and seven host genes (including three genes tested within Exp1) implicated in virus–host interactions in oysters injected with dsRNA, here targeting *Cg-IκB2* (Fig. 2).

dsRNA synthesis

The procedure was similar to that described by Fabioux et al. (2009). A 312 bp fragment from positions 144–456 of Cg-IκB2-cDNA (GenBank: HQ650768) was amplified by PCR using the following primers: forward: 5′-GGATTTGAACGACCTGGAAG-3′ and reverse: 5′-GCAGACGACTCGTTTTCATC-3′. For GFP cDNA, the fragment obtain by PCR was 318 bp, using forward 5′-CGTGCTGAAGTCAAGTTTGAAG-3′ and reverse 5′-CTTTTC-GTTGGGATCTTTCG-3′ primers. Both constructs were blasted against a reference *C. gigas* transcriptome (the GigaTON database; Riviere et al., 2015) using a script (B. Morga, unpublished) derived from the already published RNAit software (Redmond et al., 2003) preventing cross-talk between gene products and validating the specificity of the designed dsRNAs at the following level of similarity: a blast result returned with identity >70% for any sequence in the GigaTON database (except the targeted one), but <99% for the targeted sequence is scored as a potential conflict and therefore not conserved. The PCR fragments were then sub-cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced. Recombinant plasmids were purified using the Plasmid DNA Purification Kit NucleoBond®Xtra Midi (Macherey-Nagel, Düren, Germany), linearized with either *Not*I or *Spe*I enzymes (Promega, Madison, WI), phenol-chloroform extracted and finally ethanol-precipitated and suspended in RNAse-free water. The linearization products were verified by 1% agarose gel electrophoresis. Both strands of the purified plasmids were transcribed *in vitro* using T7 and T3 MEGAscript® Kits (Ambion, Austin, TX, USA) to produce *Cg-IκB2* and *GFP* sense and antisense single-stranded RNA (ssRNA). The two strands were phenol-chloroform extracted, ethanol-precipitated and resuspended in RNAse-free water obtaining a final concentration of 2 µg µl^{-1}. Equimolar amounts of sense and antisense ssRNA were heated

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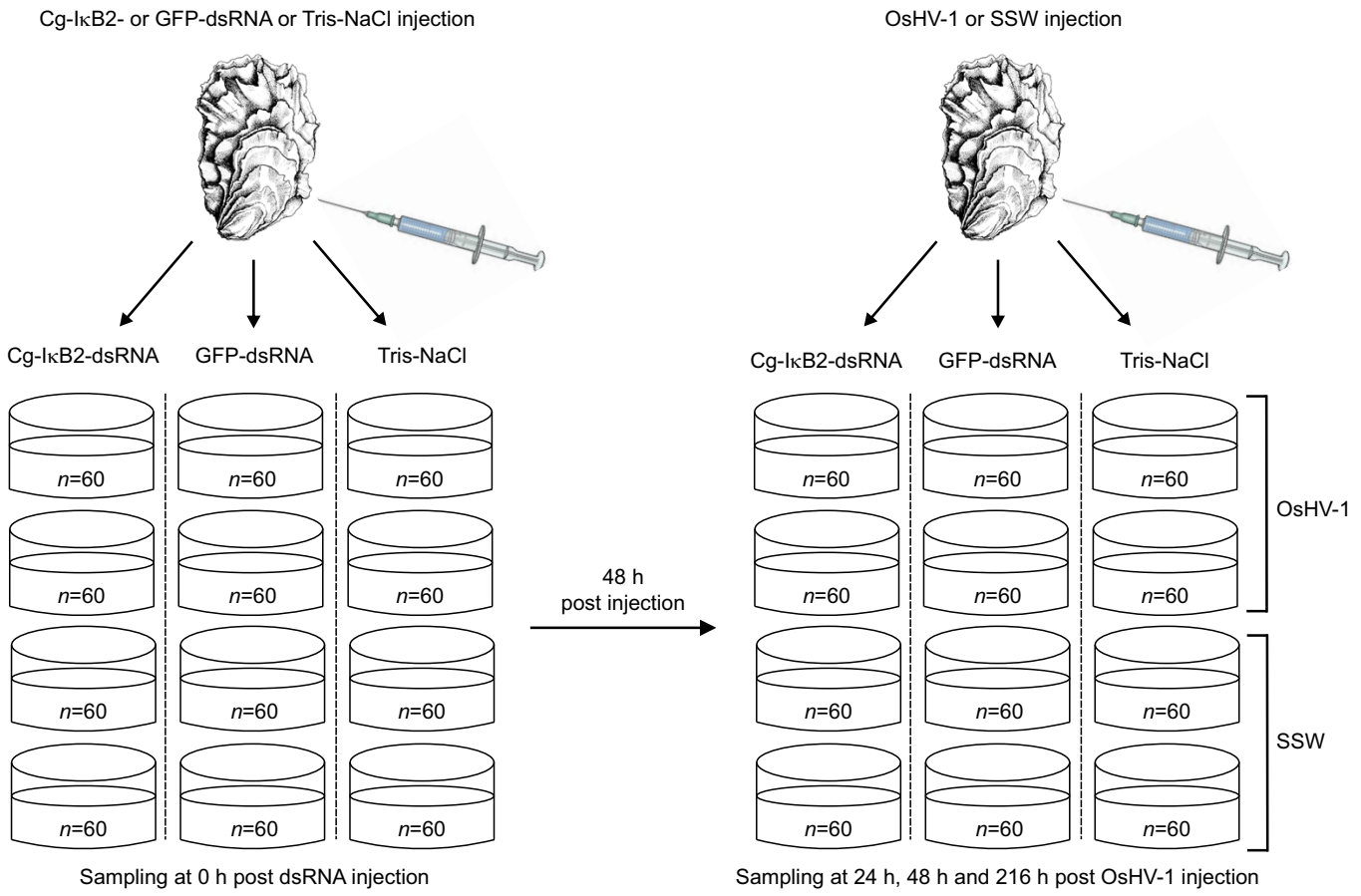

Fig. 1. Experimental protocol 1. The experiment began when oysters were injected with dsRNA or Tris-NaCl. The experiment corresponds to oysters injected with a herpesvirus OsHV-1 suspension or sterile seawater (SSW) 48 h post dsRNA injection.

to 100°C for 1 min and left to cool at room temperature for 10 h for annealing. Finally, 1 µg of each synthesized dsRNA was electrophoresed on 1% agarose gel confirming a single band of 312 bp and 318 bp for Cg-IκB2- and GFP-dsRNA, respectively.

DsRNA administration, challenges and sampling

Experiment 1

dsRNAs were suspended in RNAse-free saline solution (10 mmol l^{-1} Tris/10 mmol l^{-1} NaCl) to obtain a final concentration of 0.5 µg $µl^{-1}$. Oysters (average total mass of 6.6 g) were anesthetized in $MgCl_2$ solution (3/5 freshwater, 2/5 seawater and 50 g l^{-1} $MgCl_2$) for 5 h (Suquet et al., 2009). Oysters were injected with one of the following three solutions: (1) 100 µl of Cg-IκB2-dsRNA/Tris-NaCl solution (*n*=240), (2) 100 µl of GFP-dsRNA/AQ11 Tris-NaCl solution (*n*=240) or (3) the same volume of Tris-NaCl (*n*=240) (Fig. 1). Half of the solution volume (50 µl) was injected in the visceral mass (mantle-gonad tissue surrounding the digestive gland) and half in the adductor muscle sinus, where hemolymph circulates (Cheng, 1981). After injection, oysters were placed in four 20-liter tanks (60 oysters per tank) filled with 1-µm filtered seawater and maintained at 20°C with aeration and no seawater renewal. A solution containing 10^5 copies $µl^{-1}$ of OsHV-1 genome was prepared by dissecting and pooling tissues from infected oysters (Schikorski et al., 2011a). Forty-eight hours after the dsRNA or Tris-NaCl injection, half of the animals (*n*=60 per tank, two tanks per condition) were intramuscularly injected with 100 µl of the OsHV-1 suspension (Schikorski et al., 2011a), while the remaining oysters (*n*=60 per tank, two tanks per condition) were injected with 100 µl of sterile seawater (SSW) (Fig. 1). Following the standard operating procedure ('OsHV-1 detection and quantification by real time polymerase chain reaction using OsHV-1 DNA polymerase sequence'; www.eurl-mollusc.eu/SOPs), 10 oysters per condition were killed and mantle was collected. The quantitative PCR (qPCR) was performed for each condition before virus infection and at 24, 48 and 216 hpi in order to assay the viral DNA detection. For each sampled animal at 48 hpi, the gills were immediately dissected, frozen and stored in liquid nitrogen until total RNA extraction. Mortality was estimated by counting live and dead individuals two times per day until the 15th day after OsHV-1 injection. Dead oysters were removed from the tanks after counting, and were dissected for quantification of viral gene expression to confirm the viral infection as origin of death.

Experiment 2

As in Exp1, anesthetized oysters (average total mass of 4 g) were injected at T0, either with Cg-IκB2-dsRNA (*n*=186) or with the same volume of Tris-NaCl saline solution (*n*=186) (Fig. 2). Five (T1) and 24 h (T2) after dsRNA injection, 15 Cg-IκB2-dsRNA injected and 15 control animals were sampled (five replicates per tank). Twenty-eight hours after the dsRNA injection, all the animals were intramuscularly injected with either 100 µl of a suspension containing 10^5 copies of viral genome per microliter (*n*=252) or 100 µl of SSW (*n*=60; Fig. 2). Two additional samplings (*n*=15 in each of the two following conditions, Cg-IκB2-dsRNA+OsHV-1 and Tris-NaCl+OsHV-1) were performed at 20 (T3) and 44 hpi (T4), corresponding to 2 and 3 days after dsRNA injection, respectively. For each sampled animal,

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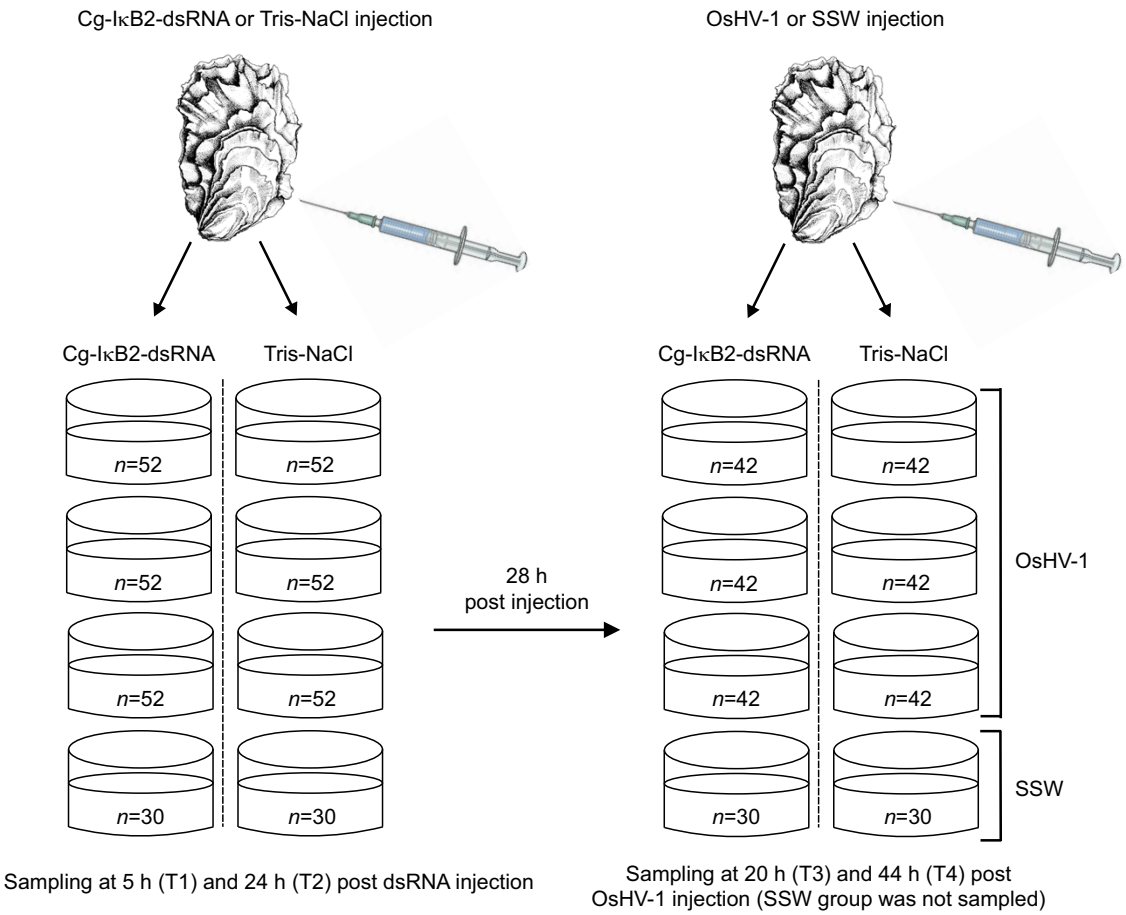

Fig. 2. Experimental protocol 2. The experiment began when oysters were injected with dsRNA or Tris-NaCl. Herpesvirus OsHV-1 or SSW injections were performed 28 h post dsRNA injection.

gonads and gills were immediately dissected, frozen and stored in liquid nitrogen until total RNA extraction. For each collected animal at T4, a piece of mantle was dissected and conserved in ethanol for further viral DNA quantification assay. Mortality and viral DNA amounts of dead oysters were monitored as in Exp1.

OsHV-1 DNA detection and quantification by qPCR

Total DNA was extracted from mantle tissues as described in Schikorski et al. (2011a). The detection and quantification of OsHV-1 DNA was carried out in duplicate using qPCR according to Pepin et al. (2008), with specific primers (Webb et al., 2007) amplifying a region of both 'reference' and µVar OsHV-1 genome predicted to encode a DNA polymerase catalytic subunit. Briefly, absolute quantification of OsHV-1 DNA copies was carried out by comparing the threshold cycle (C_t) values obtained for tested samples with a standard curve based on a 10-fold dilution of a stock solution of OsHV-1 genomic DNA (1×10^6 copies μl^{-1}) extracted from purified virus particles (Le Deuff and Renault, 1999). The results were expressed as virus DNA copy numbers per nanogram total DNA.

RNA isolation and cDNA synthesis

The gonad and gill tissues were individually crushed to a fine powder at −196°C with a Dangoumau mill and 30 mg of powder was added to 1.5 ml of Extract-all solution (Eurobio, Les Ulis, France). Total RNA of each sample was isolated according to the manufacturer's instructions. Total RNA extracted from gonads required an additional precipitation step with sodium acetate 3 mol l^{-1} in order to purify the RNA from contaminant substances adsorbing at 230 nm. Then, all samples were treated with RTS DNAse Kit (MO-BIO, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and quality were measured using a Nanodrop ND-1000 spectrophotometer and RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). In order to verify the absence of DNA carryover, RNA samples were 1:10 diluted and analyzed in real-time PCR using *elongation factor 1* primers (Fabioux et al., 2004) (Table 1). The first-stand synthesis was carried out following the Sigma MMLV (Moloney Murine Leukemia Virus) RT usage recommendations and using the DNAse-treated RNA as template and oligo-dT as primers (Fabioux et al., 2004).

Quantification of immune-related gene expression

Real-time PCR was performed in triplicate with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a MyIQ2 Bio-Rad thermal cycler to investigate the expression of immune-related genes in gonads and gills. The target genes in Exp2 were *Cg-IκB1*, *Cg-IκB2*, suppressor of cytokine signaling (*Cg-SOCS*), transcription factor Rel1 (*Cg-Rel1*), interferon-induced protein 44 (*Cg-IFI44*), inhibitor of apoptosis (*Cg-IAP*) and protein kinase R (*Cg-PKR*) (protocol in Huvet et al., 2004). Then, to validate Exp2 results and examine an anti-viral response, a subset of genes (*Cg-IκB2*, *Cg-IFI44* and *Cg-PKR*, already assayed in Exp2, plus the endoribonuclease *DICER*) was evaluated in Exp1. The PCR efficiency (E) for each primer pair was determined by performing

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Table 1. Real-time PCR primers

Primer	Sequence	Length (bp)	Efficiency (%)
Cg-IκB1 Forward	5′-GATATCGCCCTGATCTTGCT-3′	20	99.3
Cg-IκB1 Reverse	5′-AGGTTGGCTCCTGACATCAC-3′	20	
Cg-IκB2 Forward	5′-CAGCATTCACTGACGACGAT -3′	20	98.2
Cg-IκB2 Reverse	5′-TCTGCCTCAGTTTGTCGTTG-3′	20	
Cg-SOCS Forward	5′-ATCAGCCGATTCATCCTCAG-3′	20	100
Cg-SOCS Reverse	5′-TGCTGGAATGTGTAGGCAAC-3′	20	
Cg-Rel1 Forward	5′-GGTTAGACGAGACAAAGACAG-3′	21	98.9
Cg-Rel1 Reverse	5′-GCATCCAGTGAGGAAATGA-3′	19	
Cg-IFI44 Forward	5′-TGGTGGACTATGGACCGGACAGTG-3′	24	100.4
Cg-IFI44 Reverse	5′-GGTAGACAGTGGTGAGGCTGTGCT-3′	24	
Cg-IAP Forward	5′-CCCGAAAACGTAACCTCAGA-3′	20	99.3
Cg-IAP Reverse	5′-TTTCGTTTGCTGCTCATTTG-3′	20	
Cg-PKR Forward	5′-GAGCATCAGCAAAGTGTTGAG-3′	21	99
Cg-PKR Reverse	5′-GTAGCACCAGGAGATGGTTC-3′	20	
Cg-DICER Forward	5′- GATGACCGAGATCCAAAGGA-3′	20	95
Cg-DICER Reverse	5′- GCAATACAAGAGGCACACCA-3′	20	
Cg-EF1 Forward	5′-GATTGCCACACTGCTCACAT-3′	20	100
Cg-EF1 Reverse	5′-AGCATCTCCGTTCTTGATGC-3′	20	

standard curves from serial dilutions of the calibrator sample to ensure that E ranged from 95% to 105% (Table 1). Each run included a cDNA calibrator, negative controls (each total RNA sample with DNAse I treatment) and blank controls (water) analyzed for each primer pair. The calibrator used for the experiment was a pool of cDNA isolated from 30 gills or gonads randomly sampled over all analyzed conditions.

At each sampling point (T1–T4) of Exp2, host gene expression was measured in 15 Cg-IκB2-dsRNA-injected oysters and in 15 control animals. Thus, a total of 160 oysters were analyzed. In Exp1, host gene expression was measured in a total of 60 oysters corresponding to 10 oysters of each condition (Cg-IκB2-dsRNA +OsHV-1, Cg-IκB2-dsRNA+SSW, GFP-dsRNA+OsHV-1, GFP-dsRNA+SSW, Tris-NaCl+OsHV-1, Tris-NaCl+SSW) at 48 hpi (corresponding to 4 days after dsRNA injection).

Relative mRNA levels were calculated using the comparative C_t method (Livak and Schmittgen, 2001) and normalized to *Cg-EF1*, as no significant differences in C_t values were observed for *Cg-EF1* between conditions (Exp1: two-way ANOVA, $P>0.05$; CV=3.0%) or over time (Exp2: two-way ANOVA, $P>0.05$; CV=3.7%). All data are given in terms of relative mRNA expression level (relative quantification=RQ) as means±s.d.

Quantification of viral gene expression (Exp2)

The expression of 39 viral genes was studied in gills, as this is a main tissue, with mantle, for viral replication (Schikorski et al., 2011b; Martenot et al., 2016), from eight individuals at T3 (20 hpi) and T4 (44 hpi) in Cg-IκB2-dsRNA+OsHV-1 and Tris-NaCl +OsHV-1 conditions. A previous study has already shown that viral genes were not observed in the control condition (A. Segarra, unpublished data). Thirty-nine specific primer pairs were designed on a unique gene sense or antisense strand and a real-time PCR approach was performed following the previously described protocol with 5 µl of cDNA dilution (1/20) (Segarra et al., 2014b). Each viral gene expression was normalized using the viral DNA quantity with the formula $F=\log_{10}[(E+1)^{40-C_t}/N]$ from Segarra et al. (2014b), where E is the efficiency of each specific primer pair (Segarra et al., 2014a), C_t corresponds to the PCR cycle number obtained for each gene studied, and 40 is arbitrarily considered to correspond to 'no C_t' (absence of viral cDNA). Finally, N is obtained by subtraction between the maximal quantity of viral DNA observed in this study (10^6 viral DNA copies ng^{-1} total DNA) and the viral DNA quantity detected for each individual ($Y\times10^x$ viral DNA copies ng^{-1} total DNA), thus $N=(10^6-Y\times10^x)$.

Statistical analyses

To evaluate the survival rate, Kaplan–Meier survival curves were obtained with the statistical software GraphPad Prism v.6 (GraphPad Software, La Jolla, CA, USA).

Exp1 gene expression statistical analysis was performed using the Kruskal–Wallis test in RGui (www.r-project.org/). Messenger RNA relative values (RQ) were $\log_2$-transformed; normality and homoscedasticity were assessed using Shapiro–Wilk and Levene's tests, respectively. Significant levels for comparisons between groups were determined with Dunn *post hoc* test using RGui. Significance was set at $P\leq0.05$.

Target gene expression after the encounter with Cg-IκB2-dsRNA and OsHV-1 were assessed separately in Exp2. A two-way ANOVA was performed in RGui using a linear mixed model where 'time' and 'condition' are fixed effects and 'tank' is a random effect. Pairwise comparisons were performed using Tukey's HSD (95% confidence interval, $P<0.05$). Normality and homoscedasticity were assessed using quantile–quantile and residual plots, respectively, and skewed RQ values were log-transformed in order to meet the requirements for ANOVA.

General linear model (GLM) analyses were performed in order to compare the viral DNA quantity between each treatment (Cg-IκB2- and/or GFP-dsRNA or Tris-NaCl), time and condition (SSW or OsHV-1) for Exp1 and Exp2. Pairwise comparisons were performed using a *post hoc* test (Tukey's HSD, $P<0.05$). GLM analyses were performed using STATISTICA 7.0 software. A heatmap was generated in order to represent the viral gene expression using the 'pheatmap' package in R. Clustering of viral genes was performed using the Euclidean method.

RESULTS

Mortality

Experiment 1

Mortality was observed in the Tris-NaCl control group, 2 days after the viral injection (Fig. 3). Four days after viral injection, survival was approximately 60% for control oysters and went down to 41.7±5.9% at the end of the experiment. Conversely, in the experimental group injected with GFP- or Cg-IκB2-dsRNAs +OsHV-1, survival at the end of the experiment was nearly

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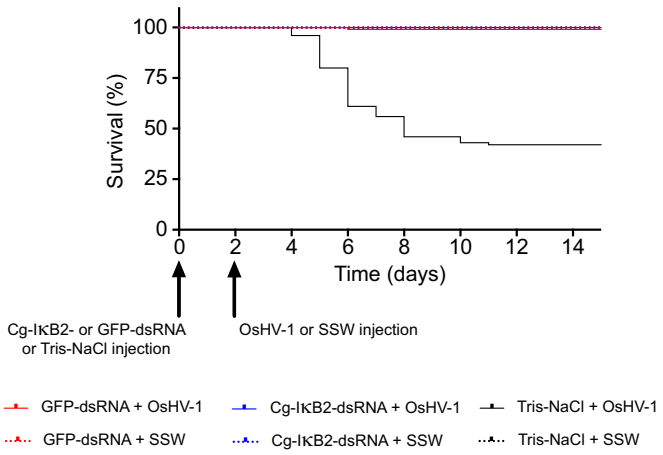

Fig. 3. Survival rates in Experiment 1. Kaplan–Meier survival curves obtained for the GFP-dsRNA (red lines), Cg-IκB2-dsRNA (blue lines) and Tris-NaCl (black lines) groups injected either with the herpesvirus OsHV-1 or SSW. On the *x*-axis, survival time is expressed in days after dsRNA injection.

98.8±1.5% and 100%, respectively (Fig. 3). Death was observed neither in dsRNA nor in Tris-NaCl oysters injected with SSW.

Experiment 2

Mortality of oysters injected with Tris-NaCl+OsHV-1 was observed 2 days after the viral infection, reaching a survival rate lower than 2% (Fig. 4) at the end of the experiment. Conversely, the final survival rate was nearly 94.1±10.3% in the Cg-IκB2-dsRNA +OsHV-1 oysters. In control animals injected with SSW after injection of Cg-IκB2-dsRNA or Tris-NaCl, a single mortality event occurred at 8 days post-OsHV-1 infection in one tank containing oysters injected with Tris-NaCl+SSW, with a final survival rate of 100% and 96.4±3.6%, respectively (Fig. 4).

Virus DNA quantity

Experiment 1

In dsRNA+OsHV-1 injected oysters, the median amounts of viral DNA were close to 0 $\log_{10}$ copies viral DNA ng^{-1} total DNA (Fig. 5) at 24, 48 and 216 hpi (Fig. 5). In Tris-NaCl+OsHV-1 injected oysters, the median of viral DNA quantities was 4.66 $\log_{10}$ copies ng^{-1} [interquartile range (IQR): 2.94 to 4.9], 5.16 $\log_{10}$ copies ng^{-1} (IQR: 4.19 to 5.4) and 1.45 $\log_{10}$ copies ng^{-1} (IQR: <0 to 2.25) at 24, 48 and 216 hpi, respectively (Fig. 5). Thus, the viral DNA quantity was significantly higher in Tris-NaCl+OsHV-1 injected oysters compared with those receiving GFP- or Cg-IκB2-dsRNA+OsHV-1 at 24 and 48 hpi (GLM with Tukey's *post hoc* HSD test, $P<0.000$; Fig. 5). Nevertheless, at 216 hpi, the viral DNA quantity was not significantly different between Tris-NaCl+OsHV-1 and Cg-IκB2-dsRNA+OsHV-1 injected oysters (GLM with Tukey's *post hoc* HSD test, $P>0.12$; Fig. 5). An interaction was demonstrated between time×dsRNA treatment×infection conditions (GLM, $P<0.000$). The medians obtained for Tris-NaCl and dsRNA groups injected with SSW were around or less than 0 $\log_{10}$ copies viral DNA ng^{-1} total DNA at 24, 48 and 216 hpi.

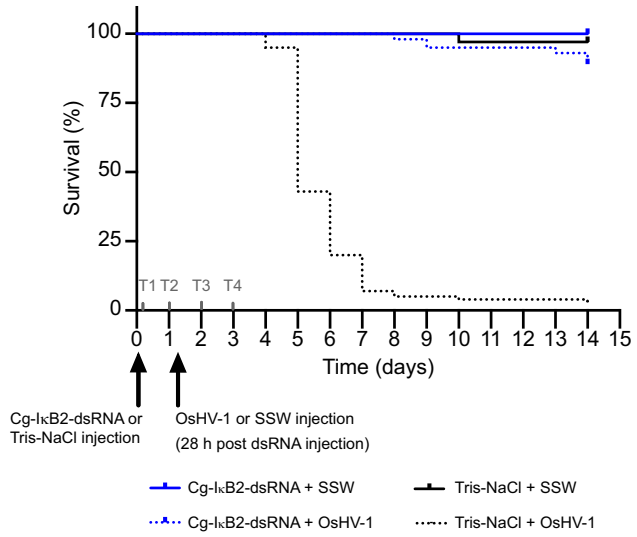

Fig. 4. Survival rates in Experiment 2. Kaplan–Meier survival curves obtained for the Cg-IκB2-dsRNA (blue lines) and Tris-NaCl (black lines) groups injected either with the herpesvirus OsHV-1 or SSW. On the *x*-axis, survival time is expressed in days after Cg-IκB2-dsRNA injection.

Experiment 2

At T3 (20 hpi), the median amounts of viral DNA were 4.93 (IQR: 4.39 to 5.53) and 0.45 $\log_{10}$ copies viral DNA ng^{-1} total DNA (IQR: 0.39 to 1.37) in oysters injected with Tris-NaCl (control) and Cg-IκB2-dsRNA, respectively, while at T4 (44 hpi), they were 6.1 (IQR: 6 to 6.26) and 1.66 $\log_{10}$ copies viral DNA ng^{-1} total DNA (IQR: 0.84 to 2.16) in control and Cg-IκB2-dsRNA-injected oysters, respectively (Fig. 6). The quantity of viral DNA at T3 (20 hpi) and T4 (44 hpi) in Tris-NaCl-treated oysters appeared significantly higher than in those receiving Cg-IκB2-dsRNA injection (GLM, Tukey's HSD test, $P<0.001$) (Fig. 6). A GLM demonstrated a highly significant difference in the function of time and treatment (dsRNA or control) ($P<0.0001$) but not the interaction time×treatment ($P>0.18$). Moreover, the viral DNA amount was also evaluated in dead animals (data not shown), demonstrating a log number of virus DNA copies significantly higher (mean fold change=54.8) in the mantle of dead control oysters in comparison with those reported in dead dsRNA injected oysters ($P<0.001$).

Host gene expression in response to Cg-IκB2-dsRNA (Exp2)

At T1 (5 h after dsRNA injection), the gonadal mRNA levels of *Cg-IκB2*, *Cg-Rel1* and *Cg-IAP* were all significantly higher in Cg-IκB2-dsRNA-injected than in Tris-NaCl-injected oysters, while *Cg-IκB1*, *Cg-SOCS*, *Cg-IFI44* and *Cg-PKR* transcriptional levels did not show significant differences between the two groups (Fig. 7A). Conversely, in gills, the sole gene showing a mild variation at T1 was *IκB2*, which was expressed at a significantly higher extent (mean fold change of 1.2) in Cg-IκB2-dsRNA-treated oysters compared with the control group (Fig. 7B).

At T2 (24 h after dsRNA injection), all the target genes except *SOCS* were significantly more expressed ($P<0.05$) in both gonads and gills of Cg-IκB2-dsRNA-treated oysters compared with controls. The most relevant variations were found for *Cg-IAP* in gonads and *Cg-IFI44* in both tissues. When compared with controls, gonadal mRNA levels of *Cg-IAP* and *Cg-IFI44* were several times higher in Cg-IκB2-dsRNA-injected oysters (mean fold change of 13.2 and 14.4, respectively; Fig. 7A). Likewise, in gills, the *Cg-IFI44* relative mRNA level was 30 times higher in the Cg-IκB2-dsRNA-injected group than in the Tris-NaCl injected group (Fig. 7B).

Host gene expression in cumulative response to both Cg-IκB2-dsRNA and OsHV-1 injections

Experiment 1

At 48 hpi, gills mRNA levels of *Cg-IκB2* and *Cg-DICER* were stable across all conditions (Cg-IκB2-dsRNA+OsHV-1, Cg-IκB2-

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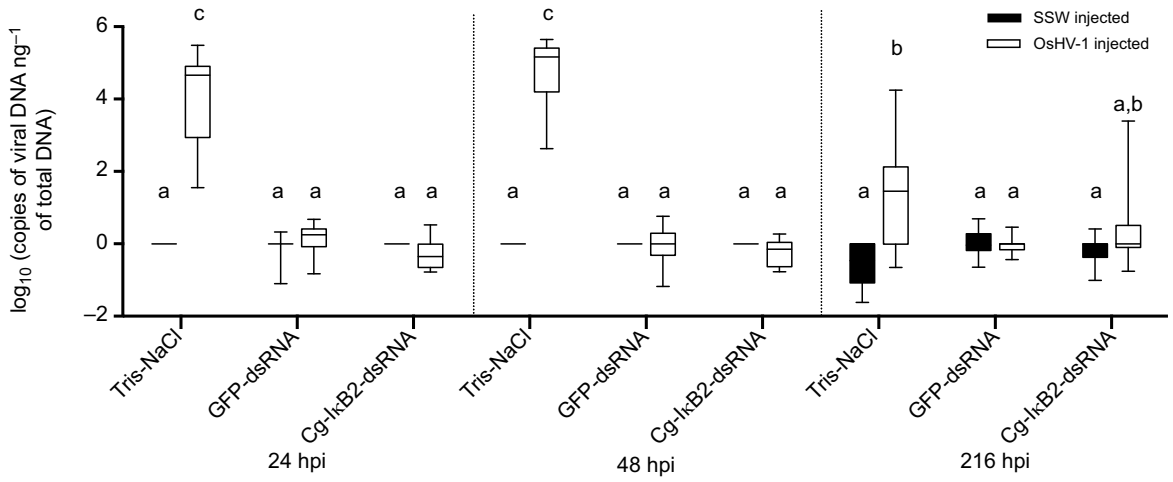

Fig. 5. Virus DNA detection and quantification in Experiment 1. Virus DNA detection by quantitative PCR in infected oysters from each condition (n=10) at 24, 48 and 216 h post OsHV-1 injection (hpi). Sampled oysters were injected with Tris-NaCl, GFP-dsRNA or Cg-IκB2-dsRNA and infected with OsHV-1 or SSW (control). Horizontal bar in boxplot represents median viral load after $\log_{10}$ transformation. Upper and lower limits of boxplot represent 75th and 25th percentiles, respectively. Whiskers represent 5–95% confidence intervals. For each boxplot, GLM was performed and pairwise comparisons were performed using a Tukey's HSD test (P<0.05).

dsRNA+SSW, GFP-dsRNA+OsHV-1, GFP-dsRNA+SSW, Tris-NaCl+OsHV-1 and Tris-NaCl+SSW; Fig. 8). Conversely, *Cg-IFI44* mRNA was less expressed in the Tris-NaCl+SSW group compared with all other conditions tested (Dunn test, P<0.05) at 48 hpi (corresponding to 4 days after dsRNA injection). Similarly to what was observed for *Cg-IFI44*, *Cg-PKR* transcript was less expressed in Tris-NaCl+SSW-treated oysters compared with all other conditions tested, except Cg-IκB2-dsRNA+SSW (Fig. 8).

Experiment 2

At T3 (20 hpi), the mRNA levels of *Cg-IκB1*, *Cg-IκB2* and *Cg-Rel1* were significantly higher in gonads collected in controls compared with gonads collected in *Cg-IκB2*-dsRNA-injected oysters (Fig. 7A). At T4 (44 hpi), these differences became greater, especially for the two *Cg-IκB* genes, whose mRNA level was 3.0 (*IκB1*) and 4.3 (*IκB2*) times higher in Tris-NaCl injected animals compared with Cg-IκB2-dsRNA-injected animals, similarly for the *IAP* mRNA level with a fold change (FC) equal to 2. Conversely, gonadal *Cg-IFI44* exhibited an mRNA level higher in Cg-IκB2-dsRNA-injected oysters than in control oysters (FC=4.3 at T3). However, at T4 this difference was a bit lower (mean FC of 2.2) and not significant (P=0.055). The mRNA levels of *Cg-SOCS* and *Cg-PKR* genes appeared not significantly affected by OsHV-1 injection (Fig. 7A).

In gills, *Cg-IκB1*, *Cg-IκB2*, *Cg-Rel1* and *Cg-IAP* genes showed patterns of mRNA levels similar to those observed in gonads: they were all more expressed in Tris-NaCl-injected oysters compared with Cg-IκB2-dsRNA-injected oysters (Fig. 7B). In particular, high fold changes were found at T3 for *Cg-IκB2* (FC=2.9) and at T4 for both *Cg-IκB2* and *Cg-IAP* (FC=3.1 and 8.2, respectively). The mRNA level of *Cg-IFI44* appeared to be similar to that revealed in gonads and its expression was significantly higher in Cg-IκB2-dsRNA-injected oysters than in controls at T3 (FC=2.8), as opposed to the other target genes. Small, but still significant differences between Cg-IκB2-dsRNA- and Tris-NaCl-injected oysters were reported for *Cg-SOCS* and *Cg-PKR* expression profiles in gills. The mRNA level of *Cg-SOCS* gene was higher in controls than in dsRNA-injected animals at both T3 and T4, while significant *Cg-PKR* variations were restricted to T4.

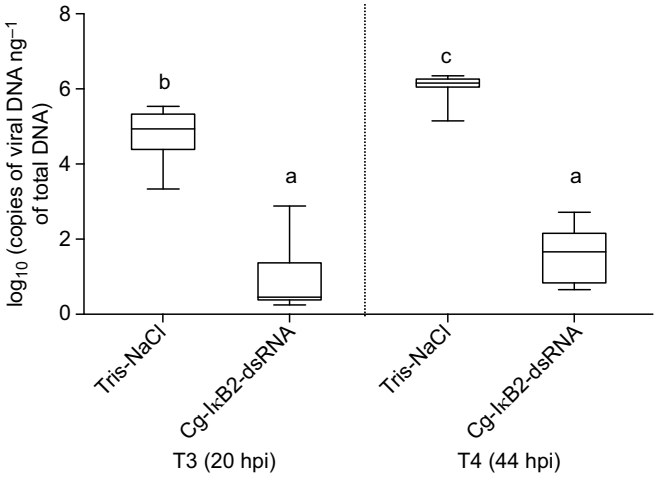

Fig. 6. Virus DNA detection and quantification in Experiment 2. Virus DNA detection by quantitative PCR in infected oysters at T3 (20 hpi) and T4 (44 hpi) (n=15). The box plot shows the median and 75th and 25th percentiles; whiskers extend to the upper and lower adjacent values with a confidence interval of 95%. For each boxplot, GLM was performed and pairwise comparisons were performed using a Tukey's HSD test (P<0.05).

Viral gene expression (Exp2)

All 39 ORFs were significantly over-expressed in gills (Mann–Whitney, P<0.05) of Tris-NaCl-injected oysters in comparison to Cg-IκB2-dsRNA injected oysters (FC=−3.1 and −5.3, respectively) at T3 (20 hpi) and T4 (44 hpi) (Fig. 9). A high inter-individual variability was noticed in the Cg-IκB2-dsRNA+OsHV-1 condition collected at identical time points, the extreme case being that some OsHV-1 genes were not detected (FC=−7, dark blue color).

Three clusters of high (cluster C1), medium (C2) and low (C3) viral gene expression levels were obtained for both conditions (Fig. 9). While an intra-cluster variability was observed (i.e. C1–

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C1), clustering comparison of Cg-IκB2-dsRNA and Tris-NaCl conditions revealed that 71.8% (28/39) of genes were allocated to the same clusters. Clusters 1, 2 and 3 contained 7, 26 and 6 genes for the Tris-NaCl condition and 11, 19, 9 genes for the Cg-IκB2-dsRNA condition, respectively. In cluster 1, three viral inhibitor apoptosis genes (vIAP) (ORF 42, ORF 87 and ORF 99), among the four studied, were found in the Cg-IκB2-dsRNA condition. Concerning the Tris-NaCl condition, two vIAP genes (ORF 42 and ORF 87) were observed in cluster 1 (Fig. 9).

DISCUSSION

The present work describes an RNAi experiment targeting *Cg-IκB2*, a candidate gene thought to play a pivotal role in the Pacific oyster defense, and testing its putative involvement in the response against OsHV-1 infection. At the same time, GFP-dsRNA was used as a control for testing the existence of a non-specific anti-viral response triggered by long dsRNA. This approach is clearly of interest for RNAi studies in marine molluscs, especially when targeting immune genes.

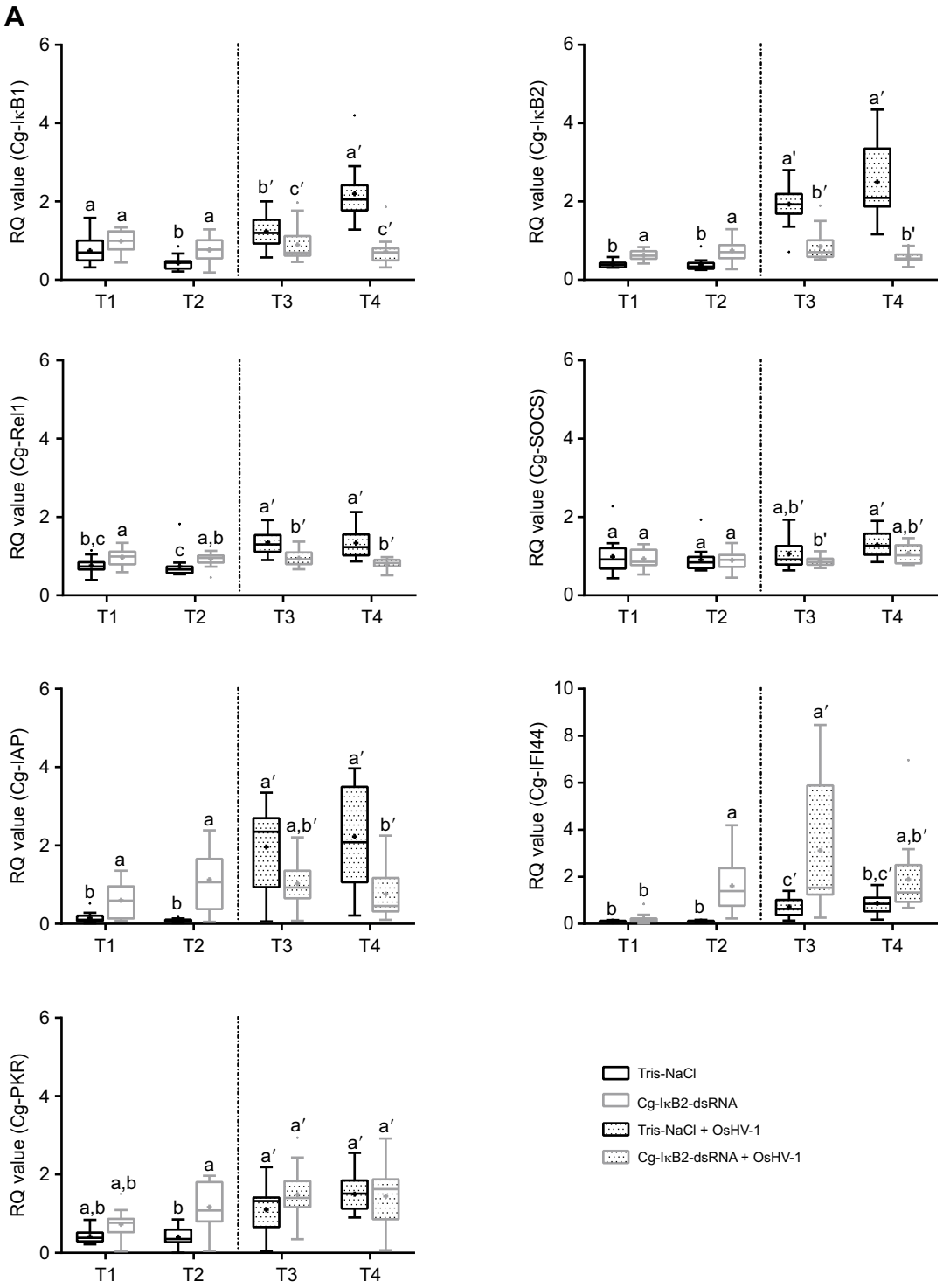

Fig. 7. See next page for legend.

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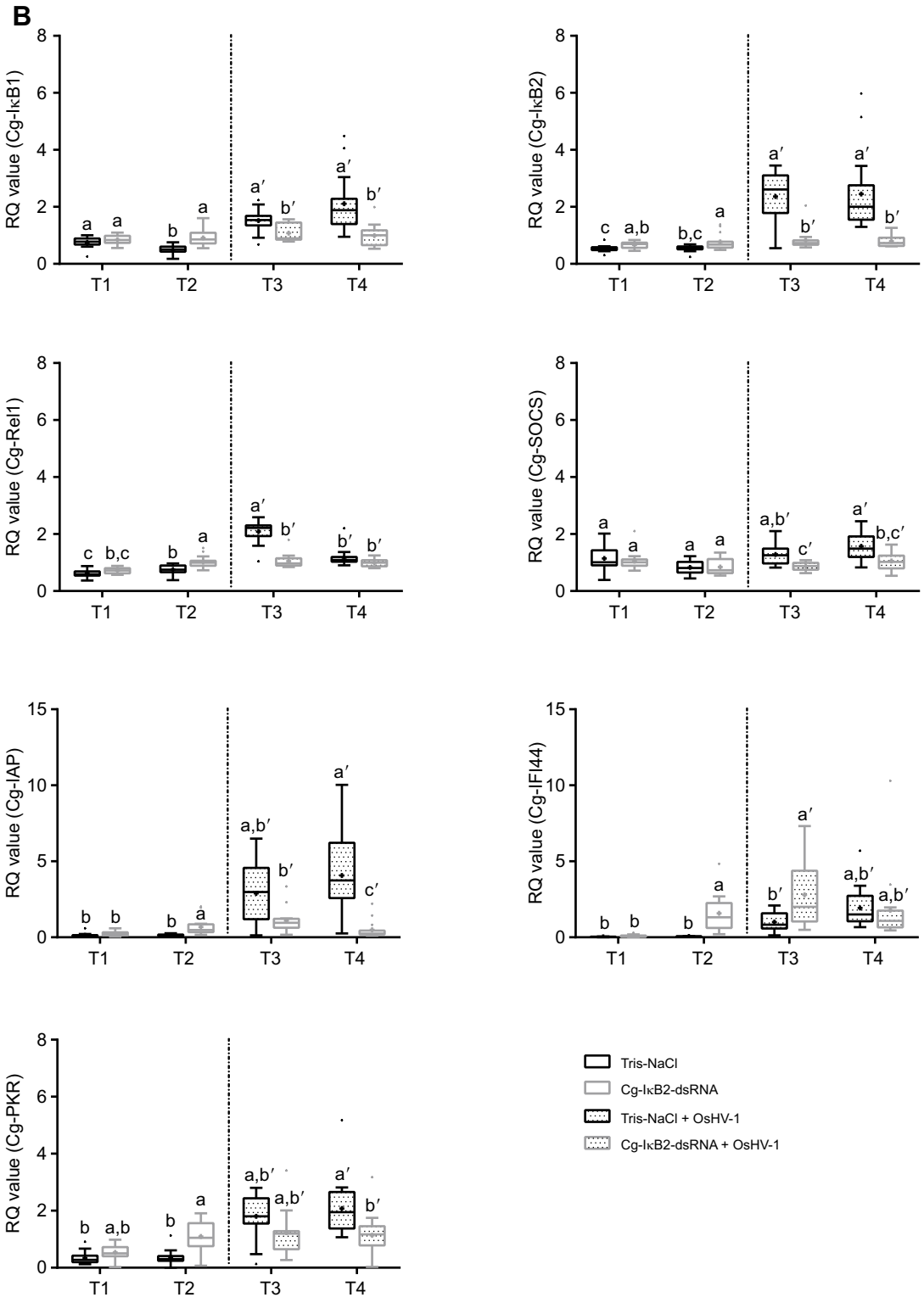

Fig. 7. Oyster gene expression pre and post OsHV-1 infection in gonads and gills (Experiment 2). Relative expression of Cg-IκB1, Cg-IκB2, Cg-Rel1, Cg-SOCS, Cg-IAP, Cg-IFI44 and Cg-PKR in gonad (A) or gill (B). For each condition, a total of 15 individual oysters were tested. Significance was evaluated through two-way mixed-model ANOVA and Tukey's HSD multi-comparison test. Black bars represent oysters injected with Tris-NaCl and gray bars represent oysters injected with dsRNA. Dotted pattern identifies the post-infection experimental phase. A common letter for two groups means that *post hoc* tests detected no significant differences for gene expression level between them. The vertical dotted line divides groups in pre and post OsHV-1 challenge (n=15). T1, 5 h post dsRNA injection; T2, 24 h post dsRNA injection; T3, 20 h post OsHV-1 infection; T4, 44 h post OsHV-1 infection.

Considering the role of *Cg-IκB2* in immunity (Zhang et al., 2011), we originally decided to inject dsRNA into the adductor muscle sinus, one of the most important sinuses in oysters (Cheng, 1981), expecting distribution of dsRNA into the hemolymphatic circulation and almost bathing tissues to target a large number of cells, e.g. hemocytes playing a key role in the immune defense

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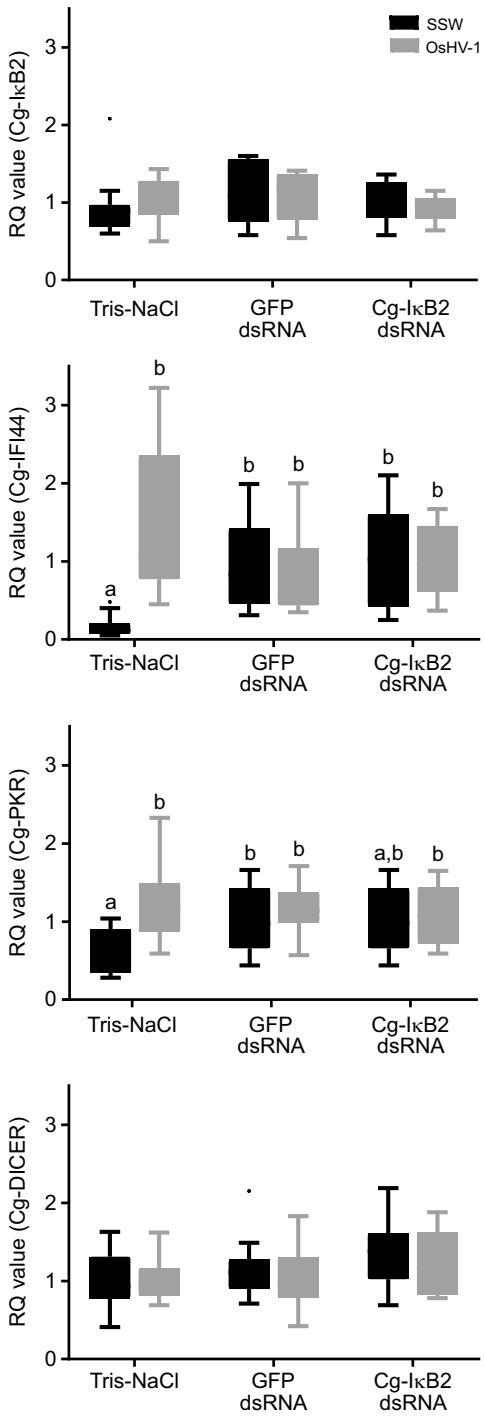

Fig. 8. Oyster gene expression in gills (Experiment 1). Relative expression of *Cg-IκB2*, *Cg-IFI44*, *Cg-PKR* and *Cg-Dicer* in gills at 48 hpi, namely 96 h post Cg-IκB2- or GFP-dsRNA injection. For each condition, a total of 10 individual oysters was tested. Significance was evaluated through Kruskal–Wallis test and Dunn's multi-comparison test. Black bars represent oysters injected with SSW and gray bars represent oysters injected with OsHV-1. A common letter for two groups means that *post hoc* tests detected no significant differences for gene expression level between them.

(Bachère et al., 2004; Le Foll et al., 2010; Venier et al., 2011). Gills, bathed by hemocytes, are often used as a good proxy of hemocyte response (Seo et al., 2005; Gonzalez et al., 2007), notably when experimental constraints exist such as insufficient amount of hemolymph for smaller oysters. Gills are also known to be one of the target organs for OsHV-1 replication (Schikorski et al., 2011a). As a result, gills have been sampled for gene expression analysis. We also targeted gonad, known to develop even in 6-month-old oysters (Degremont et al., 2005), to administrate Cg-IκB2- and GFP-dsRNA (Fabioux et al., 2009; Huvet et al., 2012) and evaluate gene expression levels because *Cg-IκB2* appeared to be differentially expressed in oyster gonad during a summer mortality event (Fleury and Huvet, 2012). Globally, an association between reproduction and higher survival during the summer period was previously suggested in adult oysters (Fleury et al., 2010; Huvet et al., 2010; Fleury and Huvet, 2012).

Long dsRNA molecules induce an anti-viral state preventing mortality related to OsHV-1 infection

The objective of this study was to evaluate the role of *Cg-IκB2* in response to OsHV-1 injection. In Exp2, the strong decrease of this transcript appeared at 48 and 72 hpi once OsHV-1 was injected (after 20 and 44 h, respectively). This decrease suggests that injection of gene-specific dsRNA led to substantial and specific depletion of the cognate mRNAs, as has been demonstrated in several RNAi experiments targeted oyster genes (Fabioux et al., 2009; Suzuki et al., 2009; Huvet et al., 2012, 2015; Li et al., 2016; Zhao et al., 2016). Conversely, the decrease of this mRNA level was not detected 96 h after dsRNA injection (Exp1), suggesting timing constraints and complex mechanisms of onset and duration of the RNAi (Mocellin and Provenzano, 2004). The high injected dose of dsRNA is expected to be sufficient to degrade newly synthesized mRNA, therefore in opposition to a rapid restoration of the targeted transcript expression through a feedback regulation mechanism, which is well established in the Rel/NF-κB pathway (Tieri et al., 2012).

We showed that GFP- and Cg-IκB2-dsRNA injection induced similar and maximal survival after OsHV-1 injection (close to 100%). Conversely, control oysters treated with the media solution Tris-NaCl experienced high mortality and showed high viral DNA amounts, indicating that death occurred as a consequence of the viral infection, as expected in laboratory challenges reproducing OsHV-1 infection (Schikorski et al., 2011a). Even if we cannot totally exclude off-target effects and indirect downstream secondary effects, as have been reported in the honey bee following dsRNA-GFP treatment (Nunes et al., 2013), the dsRNA-GFP construct was carefully designed with the aim of preventing off-target suppression. Thus, the observed survival rate in Exp1 would more likely be attributable to the injection of the long dsRNA molecule itself rather than sequence similarity between putative siRNAs generated from the dsRNA-GFP construct and oyster genes. This result emphasized a strong non-specific immune response, confirmed here in two independent experiments. An anti-viral response promoted by long dsRNAs was previously observed in shrimp, where a White Spot Syndrome Virus (WSSV) challenge showed that VP9- and GFP-dsRNA-treated shrimp had a survival rate of >70% compared with 0% in the control (Alenton et al., 2016). This anti-viral state precludes the understanding of the specific role of *Cg-IκB2*, while the same approach has been successful in shrimp for the voltage-dependent anion channel (VDAC) (Chen et al., 2011). Indeed, in *Penaeus japonicus* knocked-down for *VDAC* expression, WSSV-induced mortality and virion copy number were diminished cumulatively for the dsRNA VDAC group (cumulative mortality=47.5%) compared with the GFP-dsRNA (85%) and control (100%) groups (Chen et al., 2011).

Our present data support the existence of an interferon-like response in oysters as recently highlighted in response to poly I:C injection, a non-specific immuno-stimulant structurally similar to a

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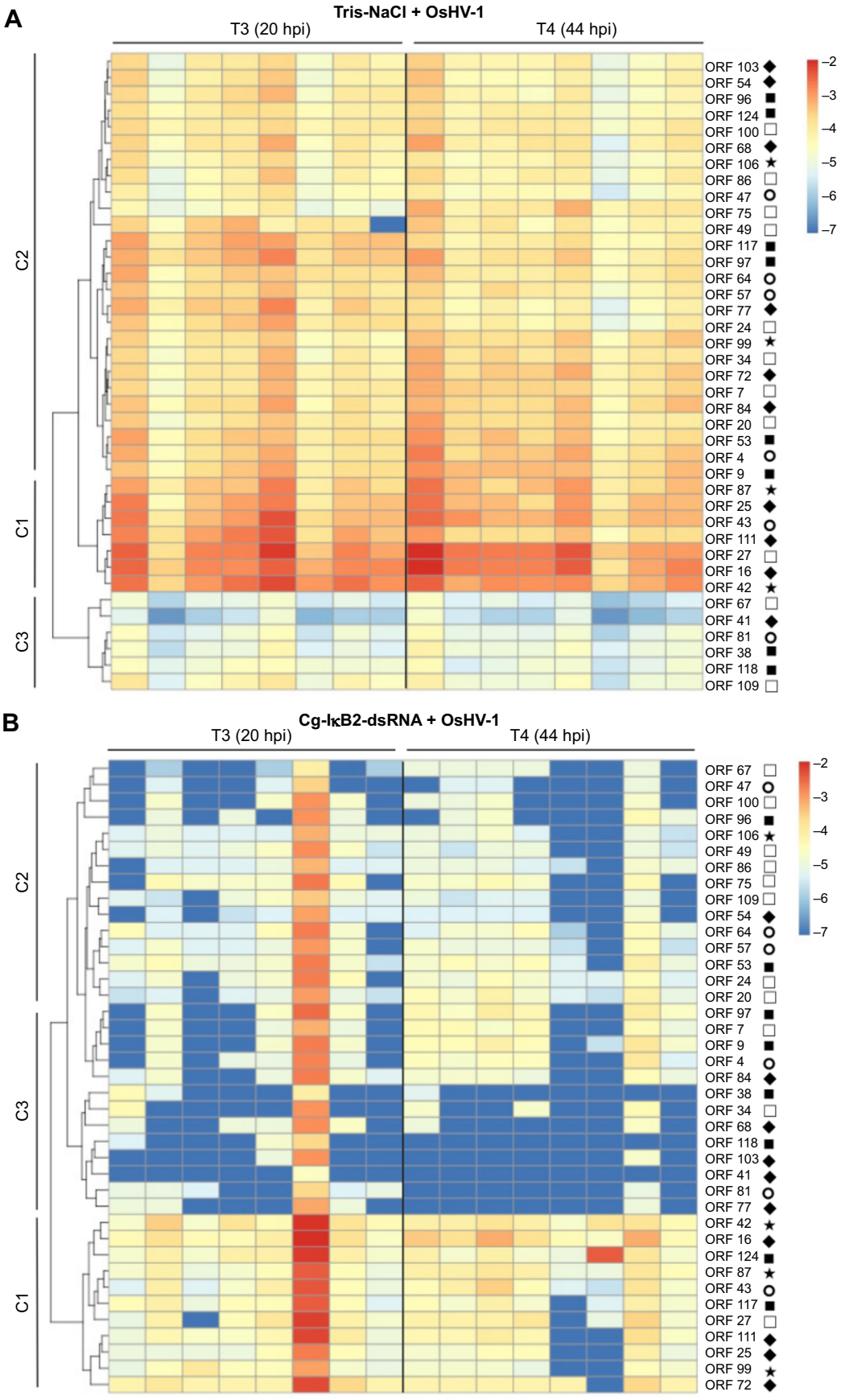

Fig. 9. See next page for legend.

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Fig. 9. Viral gene expression in Experiment 2. Heatmap illustrating the level of expression of 39 viral open reading frames (ORFs) for (A) Tris-NaCl+OsHV-1 and (B) Cg-IκB2-dsRNA+OsHV-1 conditions in oyster gills. T3, 20 h post OsHV-1 infection; T4, 44 h post OsHV-1 infection. Each row corresponds an ORF (viral gene) and each column represents an individual from Exp2 (*n*=8 per time and condition). Colors represent the fold change: blue (–7) represents the absence of viral gene detection, whereas red (–2) represents highly expressed genes. Symbols correspond to gene groups: circle, putative protein; open square, enzyme; filled square, RING finger protein; star, viral inhibitor apoptosis; and diamond, membrane protein. C1–C3, clusters 1–3.

dsRNA, that could induce *C. gigas* spat into an anti-viral state that provided protection against subsequent OsHV-1 infection (Green and Montagnani, 2013; Green et al., 2015b). Several striking similarities between the present study and that published by Green et al. (2015b) were highlighted. Indeed, following OsHV-1 injection, viral DNA amounts in poly I:C injected oysters were less abundant than in the control group, as demonstrated here for the dsRNA-injected animals. In accordance with our observations in gonads (at T1 and T2) and in gills (at T2) in response to dsRNA injection, the expression level of *Cg-Rel1* was found to be significantly upregulated 24 h after the poly I:C injection in the whole oyster tissues. Both poly I:C injection (Green et al., 2015b) and RNAi targeting *Cg-IκB2* employed in the present study, either directly or through the prior induction of interferon-like molecules, may have upregulated gene products that function individually and/or collectively to inhibit virus replication, as seen in *Ictalurus punctatus* (Milev-Milovanovic et al., 2009). Interestingly, the shrimp anti-viral immune system responds to dsRNA as a molecular pattern regardless of its sequence and base composition, except in the case of poly I:C, which failed to induce protection against WSSV (Robalino et al., 2004). Again in shrimp, the occurrence of a length-dependent anti-viral response triggered by non-specific dsRNA has suggested the existence of interferon-based immune mechanisms analogous, at least to some extent, to those described in vertebrates (Labreuche et al., 2010). Labreuche and colleagues hypothesized that non-specific activation of anti-viral immunity and induction of RNA interference engage the same pathway in *L. vannamei*. This assumption implies that mechanisms involved in dsRNA-induced immunity and RNAi might converge to boost the anti-viral response. Alternatively, the injection of RNAi might have triggered two distinct mechanisms. The first mechanism is mediated by the sequence-specific dsRNA and related to the RNAi phenomenon, involving genes such as endoribonuclease *DICER* and RNA-induced silencing complex (*RISC*). In the present study, the mRNA level of *Cg-DICER* appeared statistically similar in dsRNA- and Tris-NaCl-injected oysters, as reported in previous studies showing the absence of *Cg-DICER* modulation in response to OsHV-1 or poly I:C injections (Green et al., 2016; C.M., unpublished data). However, the dual function of *DICER*, i.e. its involvement in RNAi and in the induction of molecules controlling viral load (e.g. Deddouche et al., 2008), made gene expression analysis and interpretation difficult for this gene, as in our experimental design (Exp1) only one sampling was available at 48 hpi and 4 days after dsRNA injection. The second pathway is activated by the presence of non-specific dsRNA molecules and is driven by *Rel/NF-κB* and interferon-like molecules, here upregulated after Cg-IκB2-dsRNA injection. In oysters, additional studies are now needed to understand whether cross-talk and/or interaction occurs between RNAi and the sequence-independent anti-viral immunity observed following dsRNA injection.

The hypothesis that long dsRNA injection induces protection against OsHV-1 infection in oysters was confirmed by the upregulation in dsRNA-injected oysters at T1 and/or T2 (5 h post dsRNA injection, Exp2) of genes such as *Cg-IAP*, *Cg-PKR* and *Cg-IFI44*, all interpreted as involved in anti-viral defense. *Cg-IAP*s are key regulators of the innate anti-viral response owing to its capacity to inhibit caspase activity and facilitating immune responses. It has been already demonstrated in oysters that OsHV-1 infection induces expression of *IAP*s (Segarra et al., 2014a,b; Green et al., 2015b, 2016; He et al., 2015). Intriguingly, following the virus infection in the present study, the scenario completely changed: compared with the Cg-IκB2-dsRNA-injected group, Tris-NaCl-injected animals showed a higher expression of *Cg-IAP*, reaching statistical significance at T4 (44 hpi) in both tissues. Most likely, dsRNA-injected oysters that previously experienced an immune activation prevented a second *Cg-IAP* induction, while the control group encountering OsHV-1 showed a consistent upregulation of *Cg-IAP*, suggesting apoptosis inhibition and consequent virus spread.

Finally, the increase in mRNA expression of *Cg-PKR* and *Cg-IFI44*, homologues of vertebrate ISGs, supports the hypothesis that long dsRNAs induced an anti-viral state that most likely confers resistance to OsHV-1 infection. In Exp2, the experimental group with the highest expression of these anti-viral genes (i.e. Cg-IκB2-dsRNA injected oysters) experienced the lowest mortality rate. The virus also had an effect on the mRNA level of these two genes detected in Exp1 in the Tris-NaCl group. Notably, *Cg-PKR* and *Cg-IFI44* are known to be induced by OsHV-1 and to have direct anti-viral activity by inhibiting virus replication via targeting both transcription and translation of host and viral proteins in vertebrates (Schneider et al., 2014; Green et al., 2016). Moreover, the qPCR data obtained within Exp1 demonstrated that when a previous stimulation with a dsRNA molecule (both GFP- and Cg-IκB2-dsRNA) occurred, the transcription of *Cg-PKR* and *Cg-IFI44* was similar in SSW- and OsHV-1-treated oysters (Fig. 8), meaning that such anti-viral effectors were probably triggered by the dsRNA fragments, supporting the hypothesis of an anti-viral response triggered by both the injected dsRNA.

Virus–host interactions

The OsHV-1 cDNA study confirms a phase of active virus replication as demonstrated by the viral genes detected in the two conditions (Cg-IκB2-dsRNA+OsHV-1 and Tris-NaCl+OsHV-1). The 39 ORFs were indeed detected in the Tris-NaCl+OsHV-1 condition. Even in the Cg-IκB2-dsRNA+OsHV-1 treatment, where the mortality rate was low, 10 to 39 ORFs were expressed. Moreover, 71.8% of viral genes were allocated to the same clusters, highlighting that OsHV-1 was able to replicate in oyster gills, independently of the condition tested. A similar observation has been reported in the literature, showing that 82% viral genes had a similar expression profile in oyster families with different OsHV-1 susceptibility (Segarra et al., 2014a). These authors have shown an early and high expression for *vIAP* transcripts (ORF 42, 87 and 99). Interestingly, in our study, two (ORF 42 and 87) and three (ORF 42, 87 and 99) of four *vIAP* genes displayed early expression in the Tris-NaCl and Cg-IκB2-dsRNA conditions, respectively. Our results seem to confirm the hypothesis that OsHV-1 may actively manipulate host apoptosis using its *vIAP*s to multiply itself. Such a phenomenon may happen independently of the condition tested in the present study. Some herpesviruses (e.g. human herpes simplex virus, HSV-1 and HSV-2) have evolved various strategies to manipulate host cell death signaling pathways, particularly the apoptosis and necrosis pathways (Guo et al., 2015; Yu and He, 2016).

It is important to note the different sources of variability concerning the viral gene expression: (1) inter-condition, (2) intra-cluster

(i.e. C1–C1), (3) inter-individual and (4) inter-ORF (in a single individual). Inter-condition and intra-cluster sources of variability can be explained by a general immune response, induced by the Cg-IκB2-dsRNA injection, limiting OsHV-1 multiplication in this condition compared with Tris-NaCl. The inter-individual source of variability could be due to: (i) an unintentionally unequal dose of dsRNA and/or OsHV-1 injected inducing different effects in terms of host response and/or OsHV-1 replication between individuals, or (ii) a difference in RNAi response (e.g. Huvet et al., 2012) and/or in susceptibility to OsHV-1 infection between individuals. Finally, the inter-ORF variability depends on their function and time post-infection (e.g. viral IAPs). This could also reflect the cascade of viral immediate-early, early and late gene expression as described widely in vertebrate herpesviruses but never demonstrated in OsHV-1.

As far as gene expression of oyster genes belonging to Rel/NF-κB pathway, we demonstrated that OsHV-1 infection can also induce the activation of this pathway in the control group injected with Tris-NaCl solution, potentially to block apoptosis and prolong survival of the host cell in order to gain time for replication and increase the production of viral progeny (Roulston et al., 1999). OsHV-1 infection definitely had a great impact on the transcription of *Rel1*, *IκB2* and *IκB1*, in both gonads and gills. Despite *Cg-IκB1* and *Cg-IκB2* not having an identical pattern of expression, it has been demonstrated that their upregulation is likely involved in negative feedback regulation of NF-κB, which may be a mechanism for maintaining a balance between protecting the host and killing pathogens in oysters (Zhang et al., 2011). Noteworthy, evidence suggesting the involvement of IκBs in the innate immune response were provided by Fleury and Huvet (2012). The authors showed that less abundant *IκB1* and *IκB2* transcripts are associated with increased oyster survival during the summer period. As far as *Rel1*, we showed that this transcription factor is upregulated following OsHV-1 infection, thus being involved in the *C. gigas* anti-viral response, as previously demonstrated in this species (Green and Montagnani, 2013). An additional immune-relevant transcript that showed increased expression in oysters that died was *SOCS*, which is expected to limit *NF-κB* signaling following a classical negative feedback system that regulates cytokine signal transduction. *SOCS* is responsible for suppressing the JAK/STAT pathway, a signal transduction cascade regulating the expression of ISGs and activated by cytokines, interleukins and interferons (Yoshimura et al., 2007; Schneider et al., 2014). Taken together, these results suggest that the transcription of *Cg-Rel1*, *Cg-IκB1*, *Cg-IκB2* and *Cg-SOCS* has a prominent role in the oyster innate immune response and that the abundance of these transcripts may be associated with the survival competency.

New reverse genetic tools are needed in marine bivalves for assessing immune gene function

The existence of an interferon-like pathway playing a key role in the mollusc anti-viral response should be taken into consideration in RNAi-based knock-down experiments, as RNAi might induce an unintended stimulation of the innate immune response. Further, Robalino et al. (2005) have claimed that joint manipulation of RNAi and innate immune pathways can be a promising approach to the development of anti-viral therapeutics for the control of shrimp disease. This is of great relevance for further analyses investigating the loss-of-function phenotype of a gene, especially when targeting immune genes. Alternative molecular techniques should also be employed to investigate specific roles of immune genes and avoid a simultaneous non-specific innate immune response. For instance, a promising 'gene editing' strategy that seems to be exceptionally precise and efficient, and thus could be tested in *C. gigas* immune studies, is CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) (Sander and Joung, 2014).

dsRNA-mediated genetic interference is a widely used reverse genetic tool in many invertebrate models. The induction of a general immune response by dsRNA should be taken into consideration when interpreting studies using RNAi, especially when targeting immune genes whose role in functional development still needs to be explored. The involvement of the Rel/NF-κB pathway in the Pacific oyster anti-viral response is one promising starting point. These findings point out some important issues concerning the bivalve innate immunity and confirm the possibility, recently proposed, of testing dsRNAs in these species as therapeutic agents against viruses that cause severe economic losses in aquaculture (Lima et al., 2013). Nonetheless, the possibility of using dsRNAs in aquaculture should be carefully assessed considering also the regulatory limitations imposed by both ecological reasons and food safety. Despite the enormous potential of this approach, more studies are needed and many obstacles must still be overcome before this technique finds practical application in aquaculture.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.H.; Methodology: M.P., A.S., V.Q., N.F., J.L.G., P.M., B.P., Y.L., E.F., C.F., A.H.; Software: M.P., A.S.; Formal analysis: M.P., A.S., V.Q., N.F., Y.L., C.F.; Investigation: M.P., A.S., C.M., C.F., L.B., T.R., A.H.; Data curation: M.P., A.S.; Writing - original draft: M.P., A.S., A.H.; Writing - review & editing: A.S., C.M., C.F., L.B., T.R.; Supervision: C.F., T.R.

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