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### ► To cite this version:

Lizenn Delisle, Marianna Pauletto, Jeremie Vidal-Dupiol, Bruno Petton, Luca Bargelloni, et al.. High temperature induces transcriptomic changes in *Crassostrea gigas* that hinder progress of ostreid herpesvirus (OsHV-1) and promote survival. *Journal of Experimental Biology*, 2020, 223, pp.jeb226233. 10.1242/jeb.226233 . hal-03049914

**HAL Id: hal-03049914**

**<https://hal.science/hal-03049914>**

Submitted on 10 Dec 2020

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## High temperature induces transcriptomic changes in *Crassostrea gigas* that hinders progress of Ostreid herpesvirus (OsHV-1) and promotes survival.

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### Abstract

Among all the environmental factors, seawater temperature plays a decisive role in triggering marine diseases. Like fever in vertebrates, high seawater temperature could modulate the host response to the pathogens in ectothermic animals. In France, massive mortality of Pacific oysters *Crassostrea gigas* caused by the ostreid herpesvirus 1 (OsHV-1) is markedly reduced when temperatures exceed 24°C in the field. In the present study we assess how high temperature influences the host response to the pathogen by comparing transcriptomes (RNA-sequencing) during the course of experimental infection at 21°C (reference) and 29°C. We show that high temperature induced host physiological processes that are unfavorable to the viral infection. Temperature influenced the expression of transcripts related to the immune process and increased the transcription of genes related to apoptotic process, synaptic signaling, and protein processes at 29°C. Concomitantly, the expression of genes associated to catabolism, metabolites transport, macromolecules synthesis and cell growth remained low since the first stage of infection at 29°C. Moreover, viral entry into the host might have been limited at 29°C by changes in extracellular matrix composition and protein abundance. Overall, these results provide new insights into how environmental factors modulate the host-pathogen interactions.

## Introduction

Mortality outbreaks in the Pacific oyster, *Crassostrea gigas* associated with infection by viral and bacterial pathogens have increased during the last 10 years worldwide (Barbosa Solomieu et al., 2015; EFSA, 2010; Pernet et al., 2016). The most striking example is the massive mortality of less than one-year old individuals, which can decimate up to 100% of the farmed oysters during the warm season. These mortalities coincided with the recurrent detection of ostreid herpesvirus 1 (OsHV-1) variants (Jenkins et al., 2013; Lynch et al., 2012; Mortensen et al., 2016; Segarra et al., 2010). The virus induces an immune-compromised state of oysters evolving towards subsequent bacteremia by opportunistic bacterial pathogens leading to oyster death (De Lorgeril et al., 2018). Concomitantly, like other herpesviruses, OsHV-1 uses the host cell machinery to replicate (Jouaux et al., 2013; Renault and Novoa, 2004; Segarra et al., 2014a) and alters its metabolism (Corporeau et al., 2014; Pernet et al., 2019; Young et al., 2017).

Seawater temperature is a major trigger of marine disease by influencing the host and the pathogen (Burge et al., 2014; Harvell et al., 2002; Rahman et al., 2006). Temperature modulates the host physiology by altering the velocity of chemical and enzymatic reactions, rates of diffusion, membrane fluidity and protein structure (Hochachka and Somero, 2002; Pernet et al., 2007). Previous studies showed that thermal stress induces a beneficial expression of immune-related genes in oysters (Green et al., 2014; Zhang et al., 2015). For instance, nine months old oysters respond more vigorously to a virus-associated molecular pattern at 22°C compared to 12°C, increasing the levels of TLR (Toll Like Receptor), MyD88 (Myeloid differentiation response 88), Rel (C-REL), MDA5 (Melanoma Differentiation-Associated protein 5), IRF (Interferon Regulatory Factor) , and viperin in their hemocytes (Green et al., 2014).

Regarding OsHV-1, the optimal seawater temperature in Europe for disease transmission and subsequent mortalities is between 16°C and 24°C (Pernet et al., 2012; Renault et al., 2014). In a previous study, we found that survival of oysters challenged with OsHV-1 at 29°C was markedly higher (85.7%) than at 21°C (52.4%) whereas virus infectivity and virulence were unaltered (Delisle et al., 2018). We therefore hypothesized that the key factor affecting survival rate was the host response to the pathogen rather than the virus itself. To test this hypothesis, we characterized the physiological condition of oysters at 21°C and 29°C, assessed the amount of OsHV-1 in oysters and then compared their transcriptomes during the course of infection. In contrast to previous studies which describe the mechanisms of

infection under permissive conditions by comparing healthy vs. infected (Jouaux et al., 2013; Rosani et al., 2015) or resistant vs. susceptible populations (De Lorgeril et al., 2018; Segarra et al., 2014a), we investigate the physiological mechanisms that modulate the severity of the disease by infecting susceptible oysters at two temperatures that are more or less permissive.

## Material and methods

### Experimental design

Details about the rearing procedures experimental design and samples are presented in a previous paper (Delisle et al., 2018). Briefly, specific pathogen-free oysters were produced in a hatchery at the Ifremer facilities in Argenton according to Petton et al., 2015. Prior to starting the experiments, young oysters were maintained in a 500L open flow tank under controlled conditions (21°C, 35.2‰ salinity, O<sub>2</sub> > 85%), and fed *ad libitum*. At the onset of the experiment, oysters were 8 months old with a mean weight of 1.48±0.2 g. On 9 May 2016, oysters were divided in two groups, oysters for injection that would be injected with a suspension of OsHV-1 (*i.e.* pathogen donors) or oysters for cohabitation with pathogen donors (*i.e.* pathogen recipients). Pathogen donors were left at 21°C (control temperature) in 500L tank while recipient oysters were transferred to 45L tanks either left at 21°C or gradually increased to 29°C at 2°C day<sup>-1</sup> (n=3 replicate tanks per temperature). On 19 May, oysters for injection were myorelaxed in hexahydrate MgCl<sub>2</sub> (30 g.l<sup>-1</sup>) as describe in Suquet et al., 2009. Oysters were injected in the adductor muscle with 100 µl of viral suspension containing 6.9 × 10<sup>6</sup> copies of OsHV-1 µVar (Schikorski et al., 2011) and incubated at 21°C for 5 hours. Then, injected oysters (now pathogen donors) were transferred into the 45L tanks to cohabit with the recipients acclimated at 21°C or 29°C. Survival of recipients was followed every day for 14 days (Delisle et al., 2018), and 15 recipients per tank were sampled at 0, 12, 24, 48, 96 hours post cohabitation (hpc). Whole oyster tissues were removed from the shell, frozen in liquid nitrogen and individually ground in liquid nitrogen with a MM400 homogenizer (Retsch, Eragny, France). One individual oyster per tank was used for both transcriptomic analyses and OsHV-1 DNA quantification (n=3 replicate tank for each temperature) and one pool of nine oysters was used for biochemical analysis.

### **OsHV-1 DNA quantification.**

Level of OsHV-1 DNA was quantified in one whole oyster per tank (n=3 replicate tank for each temperature), sampled at 0, 12, 24, and 48 hpc. These analyses were conducted by Laboceca (Brest, France) using oyster powder homogenized in sterile artificial seawater (Pepin et al., 2008). Total DNA was then extracted with a QIAamp tissue mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extract was stored at  $-20^{\circ}\text{C}$  before detection and quantification according to a real-time PCR protocol based on SYBR Green chemistry (Pepin et al., 2008) with specific primers previously validated (Webb et al., 2007). The results were expressed as the log of OsHV-1 DNA copies per mg of wet tissue.

### **RNA extraction and RNA sequencing**

Total RNA was isolated using the Direct-Zol RNA Miniprep kit (Proteogene, Saint Marcel, France) according to the manufacturer's protocol. Total RNA was treated with Turbo<sup>TM</sup> DNase (Ambion®, Austin, Texas, USA) to remove genomic DNA. The RNA quality and quantity were determined using NanoDrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). All samples complied with purity ( $\text{OD}_{260}/\text{OD}_{280}$  and  $\text{OD}_{260}/\text{OD}_{230} > 1.8$ ) and quality criteria (RNA Integrity Number  $> 8$ ) required for cDNA library preparation. A total of 30 cDNA libraries were synthesized and sequenced (2 temperatures, 5 sampling times, in triplicate).

RNA-seq library construction and sequencing were performed by Eurofins Genomics (Nantes, France). Directional cDNA libraries were constructed using a TruSeq mRNA Stranded kit (Illumina, California, USA) and sequenced on a HiSeq4000 in paired end reads of  $2 \times 125$  bp.

### ***Reads processing***

Initial quality control was carried out with the FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) version 0.11.5 (Andrews, 2010). In order to filter out any remaining post-sequencing ribosomal RNA, the local sequence alignment tool SortMeRna 2.0 (Kopylova et al., 2012) was applied against different public databases (Rfam 5.8S; Rfam 5S; Silva 16S archaeal, bacterial; Silva 18S eukaryote; Silva 23S archaeal, bacterial; Silva 28S eukaryote) and a custom database including *C. gigas* ribosomal RNAs. Raw reads were then trimmed for low quality bases using CLC Genomics Workbench v 11.0 as follows: 1) Illumina adapters were removed; 2) only reads with less than 2

ambiguous nucleotides were allowed; 3) reads with >5% nucleotide with PHRED scores <20 were filtered out; 4) broken pairs were discarded.

### ***Reads mapping***

Reads were mapped against the Ensembl *C. gigas* reference genome v 9.38 by means of the STAR aligner and following the two-pass mapping mode (Dobin and Gingeras, 2015). The maximum number of mismatches allowed was set to 20 and only uniquely mapped reads were counted. Read counts for each sample, at the gene level, were extracted by setting the “GeneCounts” quantification while running STAR. Summary of metrics of the reads obtained were presented in Table S1.

### ***Differential expression analysis***

Extracted read counts were used for the analysis of differential gene expression, that was conducted using the Bioconductor package edgeR version 3.10.0 (Robinson et al., 2010) in the R environment (version 3.2.2). Samples were grouped according to condition, temperature and time. The edgeR “calcNormFactors” normalization function was used to find a set of scaling factors for the library sizes that minimized the log-fold changes between samples. The scale factors were computed using a trimmed mean of M-values (TMM) between samples (Robinson et al., 2010). After estimating dispersions, the “glmLRT” test provided in edgeR was used to assess differential expressed genes (DEGs) between experimental conditions, with a threshold for a significant false discovery rate (FDR) set to < 0.05.

### ***Go term enrichment analysis***

A functional interpretation of the lists of significant genes was obtained through enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software. “Biological process” (BP) annotation category (BP\_FAT) was used by setting the gene count equal to 3 and the ease value equal to 0.05. Since DAVID database contains functional annotation data for a limited number of species, it was necessary to link the *C. gigas* genes with sequence identifiers that could be recognized in DAVID. This process was accomplished using Uniprot/Swiss-prot accession IDs corresponding to each contig. These identifiers were used to define different “gene lists” of DA transcripts (up and down-regulated separately) and a “background” (all the expressed genes) in the bioinformatic tool DAVID. Heatmaps were constructed using Multiple experiment Viewer software (Saeed et al., 2003).

## **Biochemical analysis**

### ***Lipid class determination***

Lipid class determination was obtained using 300 mg of oyster powder homogenized in 6 ml chloroform-methanol (2:1, v/v) according to Folch, (Folch et al., 1957). Neutral and polar lipid class determinations were performed using a CAMAG automatic sampler (CAMAG, Switzerland) as described in Da Costa et al., 2016. These methods allowed the different lipid classes to separate into sterols (ST), alcohols (AL), alkenones 1 and 2 (ALK 1 and 2), free fatty acids (FFA), triacylglycerol (TAG), glyceryl ethers (GE) and sterol esters (StE) for neutral lipids and lysophosphatidyl-choline (LPC), sphingomyelin (Sm), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cardiolipin (Ca) for phospholipids.

### ***Fatty acid composition***

Fatty acid composition was analyzed in neutral and polar lipids of oysters at 21°C or 29°C at 0 hpc, 12 hpc, 24 hpc and 48 hpc. Neutral and polar lipids were separated using a silica gel micro-column as described in Marty and collaborators (Marty et al., 1992). Each lipid fraction was transesterified (Metcalf and Schmitz, 1961) and analyzed in a gas chromatograph with an on-column injector, DB-Wax capillary column and a flame ionization detector. Fatty acids were then identified by comparison of retention times with standards.

### ***Carbohydrates***

Samples of 50 mg of oyster powder were homogenized in 2 ml of nanopure water using a Polytron® PT 2500 E (Kinematica, Luzernerstrasse, Switzerland) and diluted 10 times. Carbohydrate concentrations were determined by the colorimetric method according to (DuBois et al., 1956). Samples (250 µl) were mixed with phenol (0.5 ml, 5% m/v), and incubated for 20 min. Sulfuric acid (2.5 ml, 96 %) was added in samples. Absorbance was read at 490 nm and at 600 nm with a UV 941 spectrophotometer (Kontron instruments, San Diego, California, USA). Carbohydrate concentrations were determined with the following formula:  $[Abs = ABS_{490nm} - 1.5 \times (ABS_{600nm} - 0.003)]$  and using a standard calibration curve. Concentration was expressed as mg of carbohydrates per g of dry weight.



### ***Protein and Citrate synthase activity***

Total protein extraction was performed as described in (Guévelou et al., 2013) using 600 mg of oyster powder. Total protein concentration of each lysate was determined using the DC protein assay (Bio-Rad, Hercules, California, USA). The resulting lysates were divided in aliquots and stored at -80°C until enzymatic assays.

All the assays were performed in triplicate at 21°C. Enzyme activity was measured using Nunc TM 96-well microplates (Thermo Fisher Scientific, Waltham, Massachusetts, USA), a synergy HT microplate reader and the software Gen5, both from Biotek (Winooski, Vermont, USA). Enzymatic activity was measured and related to the total protein concentration of each sample.

Enzymatic activity of citrate synthase (CS; EC 2.3.3.1) was measured using 20 µL of total protein lysates as described in (Epelboin et al., 2015; Fuhrmann et al., 2018).

### **Statistical analyses**

Mixed-design ANOVAs were performed to assess differences in (i) OsHV-1 DNA load in oysters depending on temperature (2 levels, main plot) and time (4 levels, subplot) and (ii) the proximate composition of oysters, activity of citrate synthase and fatty acid composition of polar lipids depending on temperature (2 levels, main plot) at 48 hpc. The replication unit was the tank in which the temperature treatments were applied. All mutual interactions among factors were tested, and Tukey's HSD was used as a post hoc test. The normality of residuals and homogeneity of variances were graphically checked, and the data was log (x+1) transformed where necessary. Statistical analyses were performed in R version 3.4.3 (2017-11-30, R, Vienna, Austria. <https://www.R-project.org/>).

## **Results**

### ***Survival***

The recipient oysters showed significant mortalities 72 hours post cohabitation with donors (hpc) at both tested temperatures. At this time survival rates were 98.3%±0.98 at 21°C and 96.4%±1.8 at 29°C. At the end of cohabitation trial (14 days), the survival of oysters acclimated at 29°C was higher (85.7±2.0%) than at 21°C (52.4±3.1%, Fig.1A).



### *Virus load through infection course.*

At the onset of the experiment (0 hpc), low levels of OsHV-1 DNA were occasionally detected in recipient oysters ( $<10^3$  cp.mg<sup>-1</sup> wet tissues, Fig. 1B). After infection, the level of OsHV-1 DNA increased above  $10^4$  cp.mg<sup>-1</sup> in oysters irrespective of temperature, but rates of increase were the highest at 21°C. At this temperature, the level of OsHV-1 amount exceeded  $10^7$  cp.mg<sup>-1</sup> in one individual out of three at 24 hpc and in all oysters sampled at 48 hpc. This high value was reached 48 hpc at 29°C in only one individual out of three (Fig. 1B).

### *Biochemical indicators of oyster physiology.*

We first characterized the initial physiological condition (0 hpc) of oysters at 21°C and 29°C by means of biochemical analyses. Proximate composition (protein, lipid and carbohydrate) and citrate synthase activity, a proxy of tricarboxylic acid cycle activity and physiological condition (Dahlhoff, 2004) of oysters acclimated at 21°C and 29°C were remarkably similar (Table S2). In contrast, unsaturation index of polar lipids, an indicator of thermal adaptation of biological membranes, was higher at 21°C than at 29°C, mostly reflecting variations in 20:5n-3. Finally, the ratio of the fatty acids 20:4n-6 to 20:5n-3 increased with temperature.

### *Sequencing and mapping*

An average number of  $15,699,960 \pm 4,059,920$  millions of reads per library were obtained. All samples passed quality control measures for raw and trimmed sequence reads. After rRNA removal and trimming, about 76% of the reads mapped against the *C. gigas* reference genome. Numbers of raw reads, reads passing the quality filters, and uniquely mapping reads, are provided for each library in supplementary Table S1.

### *Transcriptomic responses among permissive and non-permissive interaction.*

We then investigated the temporal transcriptomic response of oysters at 21°C and 29°C using 0 hpc as a reference point for each temperature. We found that the number of differentially expressed genes (DEGs) increased from 38 to 1,413 between 12 and 48 hpc in oysters at 21°C while it remained low in oysters at 29°C (39 and 271 at 12 and 48 hpc respectively, Fig. 2).

At 21°C 12 hpc, the functional enrichment analysis revealed that up-regulated genes were associated to 9 biological processes (BPs) all related to innate immune response (Table S3). Then, at 24 hpc, 46% of the enriched BPs were related to immune response (*e.g.* cytoplasmic pattern recognition receptor signaling pathway in response to virus, cellular response to virus, positive regulation of interferon-alpha production, Table S4). Concomitantly, BPs related to negative regulation of necrosis, cell death, negative regulation of protein maturation, and inhibition of G2/M transition of mitotic cell cycle were enriched in up-regulated genes (Table S4). Among up-regulated genes at 48 hpc, a significant enrichment of gene related to innate immune response, negative regulation of cell death and negative regulation of protein maturation processes was observed. When analyzing down-regulated genes, BPs related to growth, metabolic processes and regulation of cardiac muscle contraction were detected (Table S5).

At 29°C 12 hpc, a significant number of up-regulated genes were associated to 19 BPs related to innate immune response (Table S3). At 24 hpc, down-regulated genes were enriched in BPs related to growth processes, cell development, and transmembrane transport (Table S4). In addition, genes encoding apoptotic processes such as “lymphocyte apoptotic process”, “regulation of extrinsic apoptotic signaling pathway” and “cell death” were less abundant. The enrichment in genes involved in metabolic processes was confirmed at 48 hpc. BPs like “system process”, “endothelial cell development”, “regulation of anatomical structure morphogenesis”, “divalent metal ion transport” were overrepresented among down-regulated genes, whereas those BPs related to innate immunity, “defense response to virus” and “cellular homeostasis” were overrepresented among up-regulated genes (Table S5).

#### *Transcriptomic responses between permissive and non-permissive interaction.*

We then compared the transcriptome of oysters infected at 29°C and 21°C (reference) at each time point to identify key transcriptional events required for the repression of infection at 29°C (Fig. 3, Table S6). We found that oyster transcriptional profiles at 29°C and 21°C showed only minor differences before infection as only 9 DEGs came out. However, the number of DEGs increased to 1,189 at 12 hpc and decreased to 298 at 24, 93 at 48 hpc and to 66 at 96 hpc. Therefore, the effect of temperature on the transcriptional response of infected oysters appeared to be particularly strong 12 hpc. For this reason, we focused the functional enrichment analysis on this point.

The functional enrichment analysis revealed an enrichment of functional categories related to immunity at both temperatures (Fig. 3B, and Fig 4). At 29°C, eleven functional categories related to immune response (*e.g.* positive regulation of T cell activation, positive regulation of leukocyte differentiation, positive regulation of immune system process, regulation of immune response, innate immune response) were enriched at 12hpc compared to only five enriched in reference condition (21°C, negative regulation of NF- $\kappa$ B import into nucleus, cellular response to interleukin 4, immune response, cellular response to lipopolysaccharide, and response to virus; Fig.4). Among the enriched functional categories related to immunity at 29°C (12hpc), we identified some up-regulated genes that could be implicated in the OsHV-1 immune response like some Pattern Recognition Receptors, intracellular signal transduction-TLR adaptor molecules, macrophage scavenger receptor and  $\alpha$ 2-macroglobulin molecule like CD109 (Table S7). However, genes coding lectins, intracellular transduction TLR adaptor molecules, complement system component, intercellular signaling -TNF pathway or immune effectors were down-regulated at 29°C compare to the reference condition (21°C).

The functional enrichment analysis also showed that genes related to cell death, cell-signaling, and protein processes were up-regulated in oysters at 29°C compare to 21°C whereas it was the opposite for those related to metabolism, growth and cell development (Fig. 3B, Table S6).

Up-regulated genes at 29°C related to cell death coded for eleven proteins involved in cell death *per se*, one autophagy protein and five apoptosis inhibitor proteins (Table S8). The fourteen up-regulated genes related to cell signaling are mainly related to synaptic signaling and coded for neurotransmitter synaptic receptors such as acetylcholine, GABA, glycine, serotonin, thyrotropin releasing hormone, and members of glutamate pathway, neuropeptide receptor and G protein subunit alpha (Table S9). Finally, up-regulated genes at 29°C related to protein process coded for ubiquitin conjugating enzyme E2, E3 ubiquitin ligases proteins in charge of the substrate specificity (24 DEGs), and 3 deubiquitinase proteins were identified as regulators of this process (Table S10). Genes E3 are involved in regulation of apoptosis, protein translation arrest via EIF4E2 ubiquitination, degradation of misfolded proteins, regulation of immune response and major histocompatibility complex (MHC).

Down-regulated genes at 29°C related to metabolic processes coded for several proteins involved in catabolism of carbohydrate (4), amino acid (5) and triglyceride (4) and synthesis of fatty acids and phospholipid (3) and amino acids (3) (Fig. 5, Table S11). Genes coding for proteins involved in energy production pathways such as glycolysis (5), neoglucogenesis (1),

pentose-phosphate (4), and transport of monocarboxylate metabolites (5) were also down-regulated at 29°C. Finally, some down-regulated genes at 29°C were related to cholesterol metabolic process (5), retinol metabolism (5), and transcription/translation processes (11).

Thirty down-regulated genes at 29°C were also associated to processes like “regulation of growth”, and “cell adhesion”. These genes coded regulators of tissue growth (MEGF10, NOTCH1, CD63 antigen), and particularly for extracellular matrix components (Collagen alpha chain, Chondroitin Sulfate Proteoglycan, Uromodulin, Table S12). Interestingly, the chondroitin sulfate proteoglycan was strongly down-expressed at 29°C (Log2 fold change: -11.09, p value=1.37.10<sup>-18</sup>) like five members of the Tetraspanin family, likely reflecting that these genes are associated to proteins that are involved in the entry of OsHV-1 into host cell. Other genes were involved in cell-cell adhesion (SVEP 1, contracted associated protein, Neurexin-2-alpha), wounding repairs and inflammatory responses (Thrombospondin-1 and 2, Tenascin, and Ninjurin-1). Most of these genes are associated to membrane glycoproteins that regulate numerous cellular functions such as cell extracellular matrix attachment, immunity, and cell migration.

## Discussion

In a previous paper, we found that survival of oysters challenged with OsHV-1 at 29°C was markedly higher than at 21°C whereas virus infectivity and virulence were unaltered (Delisle et al., 2018). Here we investigate how temperature influenced the host response to the pathogen by comparing transcriptional profiles by means of RNA-sequencing during the course of infection at 21°C and 29°C. We found that temperature affected the expression of genes related to immune processes, cell death, synaptic signaling, protein processes, metabolism, growth and cell development (Fig. 3). These processes occur in all stages of infection (from 12 to 96 hpc) and are summarized in Fig. 6.

Prior to infection, oysters at 21°C and 29°C showed only 9 differentially expressed genes (DEGs), suggesting that they were fully acclimated after 10 days. This is consistent with the fact that filtration rates of oysters (Delisle et al., 2018) and their proximate composition and aerobic metabolism (citrate synthase activity) were similar at both temperatures. Also, the unsaturation index of polar lipids, an indicator of thermal adaptation of biological membranes, varied consistently with temperature (Hochachka and Somero, 2002; Pernet et al., 2007).

The number of DEGs varied from 38 at 12 hpc to 1,413 to at 48 hpc depending on temperature and time. In previous studies investigating the response of oysters to OsHV-1 infection, the number of DEGs varied from 250 to 9,400 (De Lorgeril et al., 2018; Jouaux et al., 2013; Rosani et al., 2015). The relatively low number of DEGs obtained here probably reflects that we compared transcriptomes of both susceptible and infected oysters at two temperatures that exhibited subtle differences in disease severity.

The number of DEGs in oysters infected at 21°C, compared to relative controls, increased markedly throughout the duration of the study whereas it remained low and stable at 29°C. A similar trend was reported in literature when comparing transcriptomes of oyster families with contrasted resistance phenotypes with regards to the disease (De Lorgeril et al., 2018). For instance, the number of DEGs increases markedly during the time course of infection in susceptible oyster family whereas it remains low and stable in resistant family (De Lorgeril et al., 2018). Therefore, from a quantitative point of view, the temporal transcriptomic response of oysters infected at 21°C and 29°C resembles that of susceptible and resistant families, respectively.

Our results suggest that seawater temperature could modulate the entry of OsHV-1 into the host cell by modulating the composition of the cell matrix and the attachment of the virus in a way consistent with a reduced susceptibility of the host to the virus at 29°C. For instance, the gene coding for chondroitin sulfate proteoglycans (CSPGs) was down-regulated at 29°C compare to 21°C, 12 hpc. This protein is a major component of the extracellular matrix of vertebrates, which, among other things, participates in the attachment of the herpes simplex virus 1 to the host cell (Mårdberg et al., 2002). In *C gigas*, CSPGs are particularly abundant in gills (Zhang et al., 2012), which is the entry portal of pathogens like OsHV-1 (Martenot et al., 2016; Segarra et al., 2016). Previous studies suggested that heparan sulfate, another extracellular matrix component, could be implicated in OsHV-1 entry in oysters cells (Jouaux et al., 2013; Segarra et al., 2014a). We also found that five members of Tetraspanins, a family of transmembrane proteins, were less abundant at 29°C. Notably, among these proteins, the CD63 gene codes for an antigen involved in the entry of several types of viruses into host cells (Spoden et al., 2008; Van Sproel and Figdor, 2010).

Although viral loads reached values above  $10^7$  cp.mg<sup>-1</sup> 24-48 h after the onset of infection at both 21°C and 29°C, expressions of some viral genes were lowered and viral infection failed to induce mortality at 29°C (Delisle et al., 2018). We found that temperature modulates oyster immunity as reported in Meistertzheim et al., 2007. For instance, at 29°C, eleven functional categories related to immune response were enriched and genes coding for pattern recognition receptors were up-regulated. Temperature increases the expression of genes related to immunity such as Toll like receptors, RIG-I, and Tumor necrosis factors in healthy oysters (Meistertzheim et al., 2007; Zhang et al., 2015, Lafont et al., 2020).

In accordance with the RNA-sequencing the ratio of the fatty acids 20:4n-6 to 20:5n-3 increased with temperature, suggesting that the immune status of oysters was more favorable at 29°C. Indeed, these two fatty acids are involved in eicosanoid production which is associated with stimulation of immune function in invertebrates (Howard and Stanley, 1999). However, eicosanoids produced from 20:4n-6 are generally more active than those produced from 20:5n-3, and the replacement of 20:5n-3 by 20:4n-6 in bivalves decreases immune parameters of hemocytes (Delaporte, 2003; Delaporte et al., 2006; Delaporte et al., 2007). Despite these results, up-regulation of genes known to be involved in antiviral defense against OsHV-1 were not highlighted at 29°C. This might be consistent with the hypothesis that efficient antiviral response at 29°C has occurred earlier than 12 hpc as suggested by De Lorgeril et al, (2018) where strong antiviral response arose 6 hours post infection in resilient oysters. Besides, these results might also suggest that high survival of oysters infected at 29°C could be based on other processes such as apoptosis.

We found that temperature increased the expression of pro-apoptotic genes in oysters at 12 hpc. Apoptosis is one of the major mechanisms of antiviral response inducing the abortion of viral multiplication and the elimination of viral progeny by premature lysis of infected cells (Pilder et al., 1984; Segarra et al., 2014b). Therefore, up-regulation of some pro-apoptotic genes at 29°C may have limited virus proliferation, as previously reported in shrimps exposed to high temperature treatment during a viral infection (Granja et al., 2003). At the same time, some genes coding for apoptosis inhibitors were also up-regulated at both 21°C and 29°C. The role of apoptosis during OsHV-1 infection is still unclear (De Lorgeril et al., 2018; Rosani et al., 2015; Segarra et al., 2014a; Wang et al., 2018). However, the outcome of the disease seem to rely on a fine tuned balance between apoptotic and anti-apoptotic effects sometimes even triggered by the virus (Green et al., 2015).

Temperature also influenced the ubiquitylation/proteasome system of oysters. For instance, the number of DEGs coding for the ubiquitylation/proteasome system increased at 29°C 12 hpc. Similarly, previous studies showed that the number of DEGs related to the ubiquitylation process increased during the first 6-12 h post-infection and was higher in resistant than in susceptible oysters (De Lorgeril et al., 2018; Lorgeril et al., 2020). Moreover, the amount of protein related to the ubiquitination process was altered in infected oysters (Corporeau et al., 2014). In vertebrates, ubiquitylation and proteolysis are involved in protein recycling and regulate the stability, activity and localization of target proteins (Bhoj and Chen, 2009). Several up-regulated genes identified in our study belong to protein E3 which is involved in major histocompatibility complex, apoptosis, cell cycle and the protein translation arrest. These processes are crucial for mounting an adequate immune response and likely to fight against bacterial pathogens (Cheng et al., 2016; Seo et al., 2013). It is therefore likely that over-stimulation of the proteasome system in oysters at 29°C could limit the bacteremia that normally follows OsHV-1 infection and lead to oyster death (De Lorgeril et al., 2018).

We found that genes related to several neurotransmitter synaptic receptors were up-regulated in oysters infected at 29°C 12 hpc. These genes coded for receptor for acetylcholine, GABA, glutamate, serotonin, thyrotropin-releasing hormone, and gonadotropin-releasing hormone. Oyster hemocytes generally have receptors for several of the previously listed neurotransmitters. Hemocytes regulate receptors abundance during immune response to influence apoptosis and phagocytosis, indicating that, once the host recognized an invader, some neurotransmitters might be released to optimize immune responses (Li et al., 2016; Liu et al., 2017). In oysters, the release of GABA and acetylcholine seems to be activated by immune stimulation. At long-time scale, they tend to down-regulate the immune response avoiding the excess of immune reactions in order to maintain the immune homeostasis (Li et al., 2016; Liu et al., 2017; Wang et al., 2018). At 29°C, over-expression of neuroendocrine receptors could optimize immune activities helping to eliminate OsHV-1 and to restore homeostasis after viral elimination.



In response to viral infection at 29°C, genes related to catabolism, metabolites transport, amino acids, nucleotides and fatty acids synthesis, transcription and translation were down-regulated at 29°C than at 21°C in our study. This probably reflects that OsHV-1, like other herpesvirus, alters host cell metabolic pathways to provide an optimal environment for its replication and spread (De la Re Vega et al., 2017; Thaker et al., 2019). More particularly, viruses increase *i*) nucleotide synthesis to supply rapid viral genome replication, *ii*) amino acid production used for virion assembly, *iii*) lipid availability to provide additional membrane material for envelopment of viral particles and *iv*) ATP flux for supporting the energy cost of genome replication and packaging (Sanchez and Lagunoff, 2015; Yu et al., 2011). More specifically, OsHV-1 increases glycolysis, TCA cycle, and fatty acid synthesis and triglyceride catabolism, indicative of host metabolic changes related to viral infection (Corporeau et al., 2014; Young et al., 2017). Other studies report depletions of host carbohydrate and triglyceride reserves during OsHV-1 infection (Pernet et al., 2019). Here we showed that potential hijacking of the host cell machinery by OsHV-1 was altered at 29°C.

## **Conclusion**

To conclude, this study provides new insights into the mechanisms underlying the high survival of oysters infected with OsHV-1 at high temperature (29°C) as demonstrated previously (Delisle et al., 2018). As summarized in Figure 6, temperature induces alterations in the membrane composition limiting the entry of OsHV-1. At 29°C, immune response and up-regulation of genes encoding for apoptosis result in the limitation of early viral replication. At the same time, down-regulation of genes encoding for catabolism, metabolite transport, macromolecule synthesis and cell growth have reduced the availability of cellular components essential to the viral particle synthesis. Finally, up-regulation of genes encoding proteasome processes and synaptic signaling associated with the limitation of viral replication seems to have prevented bacteremia and limited oyster mortality. Taken together, these transcriptomic responses can explain the increased robustness of infected oysters at 29°C.

## **Acknowledgment**

The authors warmly thank Claudie Quéré and Valerian le Roy for biochemical analyses and samples preparation. The authors are grateful to Benjamin Morga and Nicole Faury from the Laboratoire de génétique et Pathologie des Mollusques Marins (LGPMM), Ifremer, La Tremblade, France for RNA extraction and in libraries preparation, to Guillaume Mitta for revising the manuscript and Joanne Mulvena for her help with English editing. This study is set within the framework of the "Laboratoires d'Excellences (LABEX)" TULIP (ANR-10-LABX-41). Results presented in this paper are reproduced from the PhD thesis of Lizenn Delisle (University of Western Brittany (Université de Bretagne Occidentale), France, 2018).

## **Competing interest**

The authors declare no competing or financial interests.

## **Funding**

The present study was supported by the EU funded project VIVALDI (H2020 program, n°678589) and TEMPO project was funded by the French Ministry of Environment, Energy and Sea (Convention DPMA 2016 16/1212569).

## **Data availability**

RNA-seq data are available under SRA accessions PRJNA593309. Other data analyzed during this study are available as supplementary information files under DRYAD accession ([https://datadryad.org/stash/share/rGWJYSIPGLop\\_JyG4tOzkl7C1KdDOMQ0W30ozfDIQU](https://datadryad.org/stash/share/rGWJYSIPGLop_JyG4tOzkl7C1KdDOMQ0W30ozfDIQU)).

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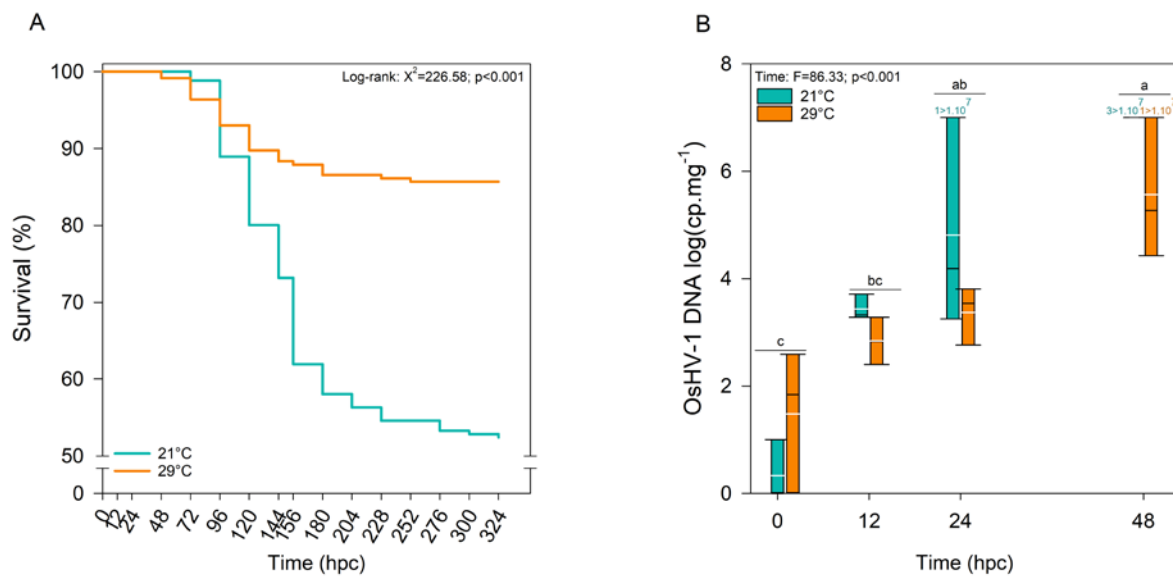


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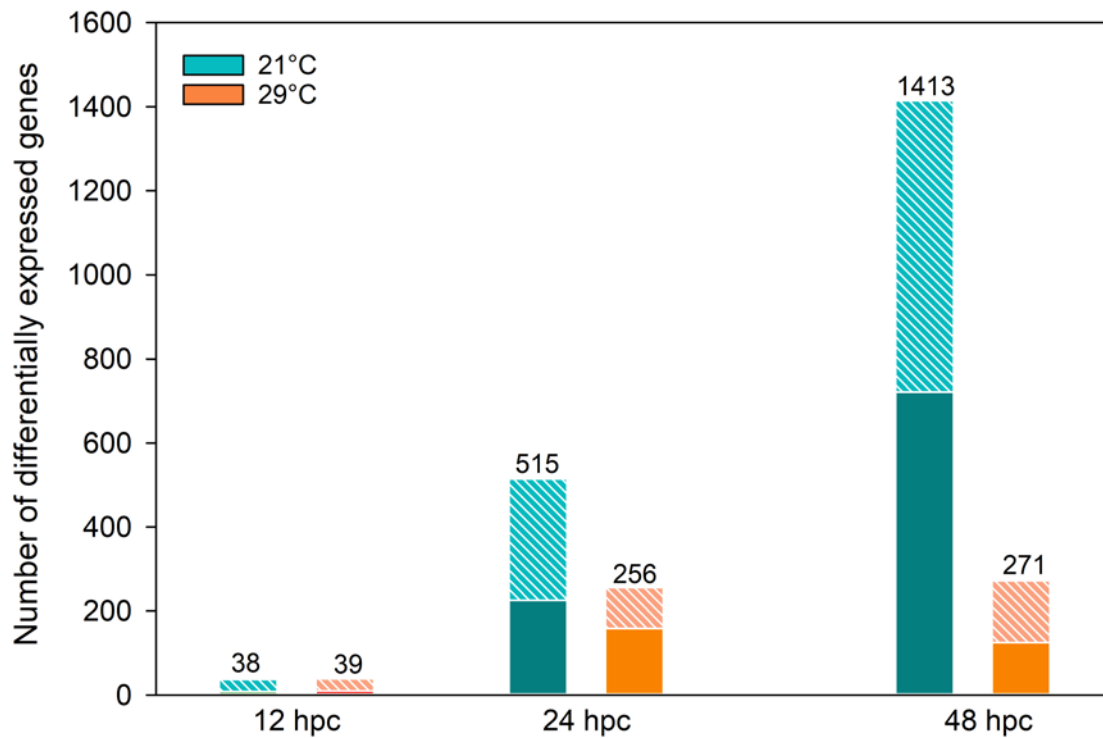
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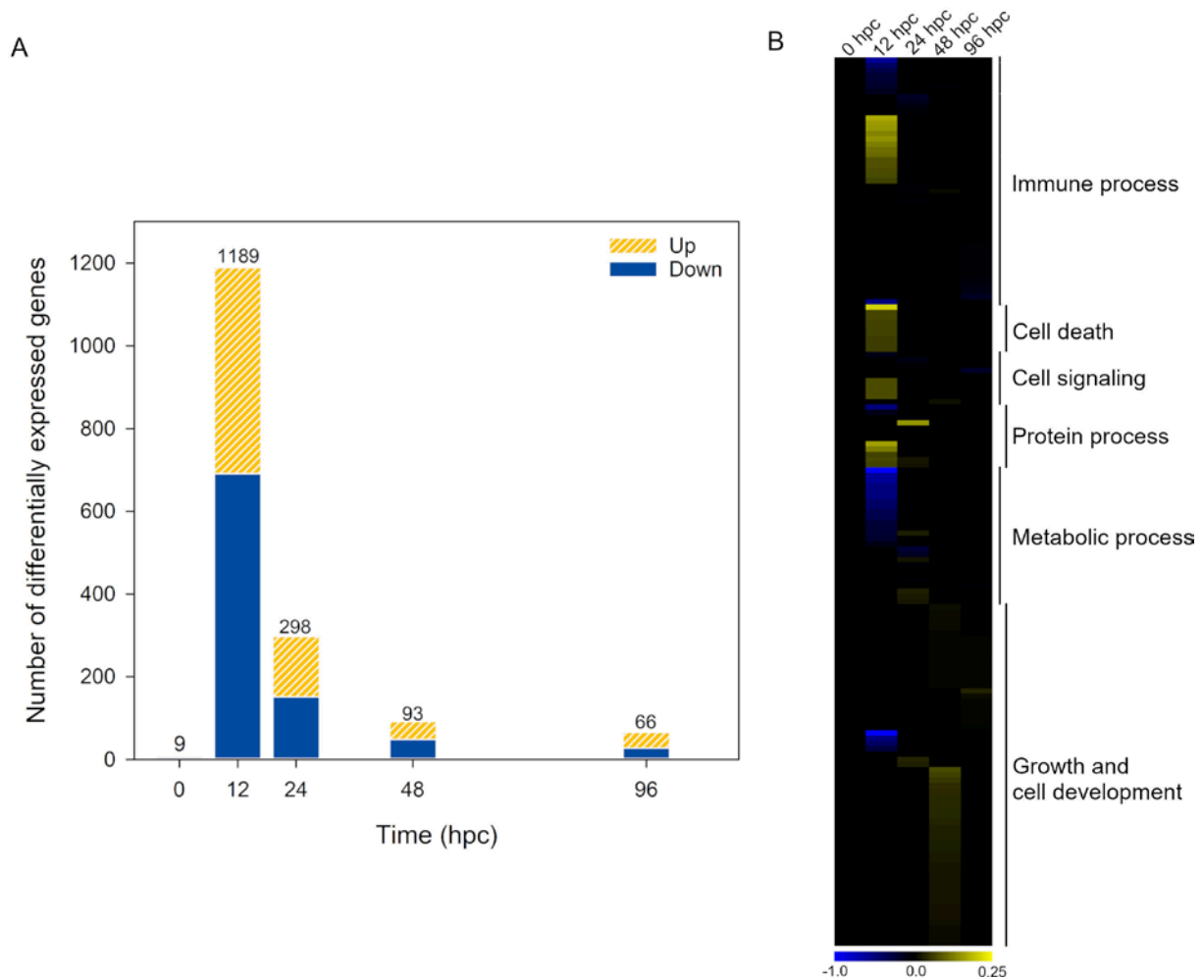
## Figures



**Figure 1: Survival of oysters infected with OsHV-1 at 21°C and 29°C (A).** Survival time was measured in hour post-cohabitation (hpc) with pathogen donors. **Quantification of OsHV-1 DNA in oysters as a function of time at 21°C and 29°C (B).** Data were  $\log(x+1)$  transformed, and letters indicate significant differences. White and black lines represent the mean and median values respectively. Numbers “1” for 24 hpc and “3” for 48 hpc indicate the number of oysters per condition that displayed OsHV-1 DNA value higher than  $1.10^7$  copies per mg.



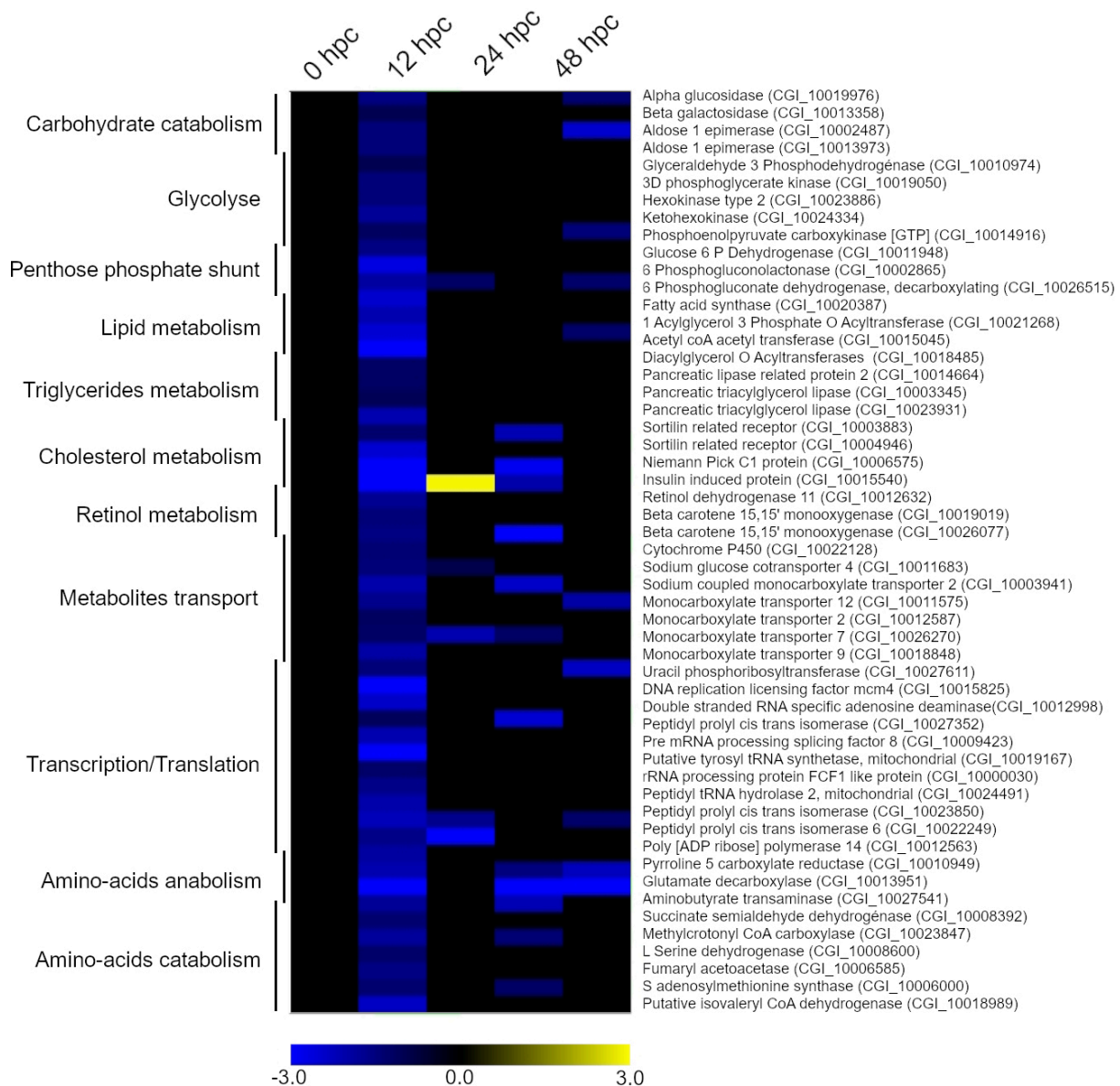
**Figure 2: Number of genes that are differentially expressed during OsHV-1 infection at 21°C and 29°C compared to 0 hpc (reference).** The total number of differentially expressed genes is indicated above each bar. Up-regulated genes are represented as hatched bars and down-regulated genes as filled colored bars.



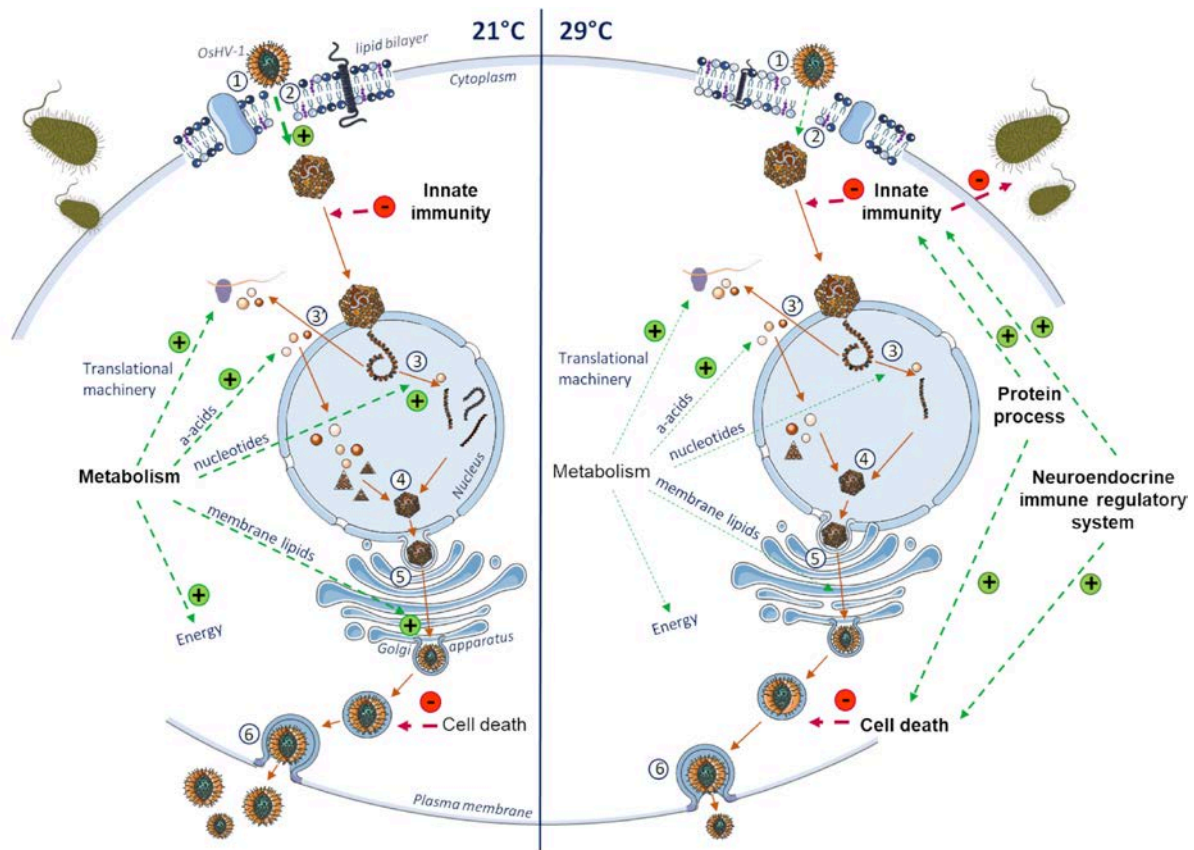
**Figure 3: Panel presenting the transcriptomic response of oysters infected at 29°C compared to their counterparts at 21°C. (A).** Number of genes that are differentially expressed at 29°C compared to 21°C (reference) over the time course of infection. The total number of differentially expressed genes is indicated above each bar. Up-regulated genes are represented in yellow filled colored bars and down-regulated appeared in blue. **Heatmap of the 169 significantly enriched GO categories (p-value <0.05).** (B). The enrichment intensity was expressed as the ratio between the number of genes that were significantly up- (yellow heat) or down-regulated (blue heat) in the category compared with the total number of genes in the category. If there is no significant difference in GO categories enrichment between the tested condition, the intensity was equal to zero (black heat). Details of the result (GO term, enrichment values) are presented in Supplementary table 13.







**Figure 5: Heatmap focusing on the 55 genes related to metabolic processes that were significantly down-regulated at 29°C, 12 hours post cohabitation.** The intensities of the colors indicate the magnitude of the differential expression (log<sub>2</sub> fold change). The fold changes were calculated at each time point comparing the expression of each gene at 29°C to its expression at 21°C. Details of the result (GO term, enrichment values) are presented in Supplementary table 13.



**Figure 6: Schematic representation of physiological responses occurring in oysters infected at 21°C and 29°C 12 hpc.** This picture is a data compilation of our results and the life cycle of OsHV-1 proposed by Jouaux et al. (2013). Font size increases for up-regulated processes and decreases for those that were down-regulated for the two tested temperatures. The green + and the red - arrows indicate the positive and negative effect of each process on oyster disease. Numbers refer to the different steps of OsHV-1 cycle: 1: viral attachment, 2: membrane fusion, 3: viral genome replication, 3': transcription and translation of viral DNA, 4: capsid assembly, 5: envelopment of viral particle and releasing of mature virion. Abbreviations: a-acids stand for amino-acids.