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Transgenerational plasticity and antiviral immunity in the Pacific oyster (*Crassostrea gigas*) against Ostreid herpesvirus 1 (OsHV-1)

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Abstract :

The oyster's immune system is capable of adapting upon exposure to a pathogen-associated molecular pattern (PAMP) to have an enhanced secondary response against the same type of pathogen. This has been demonstrated using poly(I:C) to elicit an antiviral response in the Pacific oyster (*Crassostrea gigas*) against Ostreid herpesvirus (OsHV-1). Improved survival following exposure to poly(I:C) has been found in later life stages (within-generational immune priming) and in the next generation (transgenerational immune priming). The mechanism that the oyster uses to transfer immunity to the next generation is unknown. Here we show that oyster larvae have higher survival to OsHV-1 when their mothers, but not their fathers, are exposed to poly(I:C) prior to spawning. RNA-seq provided no evidence to suggest that parental exposure to poly(I:C) reconfigures antiviral gene expression in unchallenged larvae. We conclude that the improved survival of larvae might occur *via* maternal provisioning of antiviral compounds in the eggs.

Highlights

► The molecular mechanism involved in transgenerational immune priming was investigated in the oyster, *Crassostrea gigas.*► *Crassostrea gigas* larvae have higher survival to OsHV-1 when their mothers, but not their fathers, are exposed to poly(I:C) prior to spawning. ► RNA-seq provided no evidence to suggest that parental exposure to poly(I:C) reconfigures antiviral gene expression in unchallenged larvae. ► Improved survival of *C. gigas* larvae might occur *via* maternal provisioning of antiviral compounds in the eggs.

Keywords : *Crassostrea gigas*, OsHV-1, transgenerational immune priming, Trained immunity, RNA-seq

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1. Introduction

Invertebrates can mount sophisticated immune responses with the potential to exhibit a form of innate immune memory (Chang et al., 2018; Contreras-Garduno et al., 2016; Milutinovic and Kurtz, 2016). After exposure to certain stimuli or parasite infections, the immune system of some invertebrate species can be primed to respond more vigorously upon a secondary infection caused by the same type of parasite (Contreras-Garduno et al., 2016). This long-lasting, group-specific immune response in invertebrates is called 'immune-priming' (Kurtz and Franz, 2003). In some incidences, immune priming occurs not only within a generation, but also across generations with offspring from primed parents also having improved survival to parasite exposure, a state called 'transgenerational immune-priming' (Little et al., 2003; Sadd et al., 2005). Immune priming has been reported in different groups of invertebrates, including ctenophores, sponges, mollusks and arthropods (Milutinovic and Kurtz, 2016). Studies investigating the phenomenon of immune-priming in invertebrates are quite heterogeneous and largely differ in terms of host-parasite combination, experimental design, elicitors used for priming (*i.e.* non-lethal dose of parasite or PAMP) and route of priming (*i.e.* oral or injection) (reviewed by Contreras-Garduno et al., 2016). This heterogeneous array of experiments makes it complicated to provide a mechanistic explanation for this phenomenon.

Ostreid herpesvirus 1 (OsHV-1) is responsible for serious economic losses of the edible Pacific oyster, *Crassostrea gigas* (Burge et al., 2006; Jenkins et al., 2013; Keeling et al., 2014; Renault et al., 1994; Segarra et al., 2010). OsHV-1 can cause 100 % mortality rate of *C. gigas* in less than one week (Paul-Pont et al., 2014), with the commercial production of *C. gigas* having now ceased entirely in several affected estuaries within Australia (Whittington et al., 2015). Urgency to mitigate the impacts of OsHV-1 has led to a closer examination of the antiviral responses of *C. gigas* to OsHV-1 infection (reviewed by Green and Speck, 2018). Observational studies suggest *C. gigas* are capable of adapting to OsHV-1 infection. *C. gigas* that have survived a mortality event appeared to be more resistant later in life to OsHV-1 (Evans et al., 2017; Pernet et al., 2012) and female *C. gigas* infected with OsHV-1 can transfer this protection to their offspring (Barbosa-Solomieu et al., 2005).

Experimental studies using a heterologous immune-priming model provide evidence of immune plasticity in *C. gigas* to OsHV-1 infection (Green and Montagnani, 2013; Green et al., 2015b; Lafont et al., 2017; Pauletto et al., 2017). The immune system of *C. gigas* can be primed with synthetic virus-associated molecular patterns (*i.e.* poly(I:C)) to induce an antiviral response that hampers subsequent infection with OsHV-1 (Green and Montagnani, 2013; Green et al., 2014b). This protection to OsHV-1 can be long-lasting, persisting for at

least 5 months (Lafont et al., 2017). Furthermore, this protection appears to be transmitted to offspring. *C. gigas* larvae produced from parents stimulated with poly(I:C) have improved survival to OsHV-1 infection (Green et al., 2016). Discovering the mechanism used by *C. gigas* to transmit antiviral immunity to the next generation would be highly beneficial to the aquaculture industry. This knowledge could motivate the development of practical and cost-effective treatments for improving oyster health (Contreras-Garduno et al., 2016; Wang et al., 2015).

Transgenerational immune priming in invertebrates can arise from both maternal and paternal sources (McNamara et al., 2014; Roth et al., 2010; Zanchi et al., 2011). Maternal immune priming appears to be mediated by at least three non-exclusive mechanisms. Mothers exposed to pathogens or certain stimuli may provision their eggs with antimicrobial compounds (Yue et al., 2013). This antimicrobial activity declines as the antimicrobial compounds are metabolized in the developing embryos (Benkendorff et al., 2001). Mothers may also transmit signals to enhance the immune response of their offspring (Barribeau et al., 2016; Hernandez Lopez et al., 2014; Zanchi et al., 2011). These signals include the transfer of microbial degradation products (*i.e.* PAMPs) to their developing embryos to prime their immune response (Freitak et al., 2014), or *via* epigenetic mechanisms, such as DNA chromatin modification or small RNA, to elevate the constitutive expression of immune effector genes (Castro-Vargas et al., 2017; Norouzitallab et al., 2016). Paternal immune priming can also be transmitted by epigenetic mechanisms *via* the sperm or by compounds transferred with the seminal fluid (Eggert et al., 2014).

Here, we investigated the mechanisms that underpin transgenerational immune priming in *C. gigas* against OsHV-1, using the immune elicitor poly(I:C). This study aimed to (i) investigate the effect of time between parental exposure to poly(I:C) and spawning on the resistance of larvae to OsHV-1 infection, (ii) quantify the contribution of maternal and paternal provisioning to offspring response, and (iii) determine if parental exposure to poly(I:C) reconfigures the constitutive expression of immune-related genes in unchallenged *C. gigas* larvae.

2. Materials and Methods

2.1 Oysters, Immune Challenge & Mating Trials

Adult *Crassostrea gigas* were collected from Port Stephens estuary (NSW, Australia) and held in conditioning tanks at the Sydney Institute of Marine Science (Sydney, Australia). OsHV-1 DNA has not been detected in *C. gigas* cultivated in Port Stephens estuary (Go et

al., 2017). Adult *C. gigas* were held in 60 L tanks at 21 ºC with supplemental feeding with live microalgae (*Isochrysis galbana*, *Chaetoceros muelleri*) and microalgae concentrate (Shellfish Diet 1800, Reed Mariculture). Prior to spawning, *C. gigas* had a notch filed in their shells and were injected with 100 µl of poly(I:C) (Sigma, 5 $mg.ml^{-1}$ in seawater) or sterile seawater (control) in the adductor muscle. *C. gigas* were stripped spawned by making small incisions in the gonad with a scalpel blade, and washing gametes into a plastic beaker with 1 µm filtered seawater. Gametes from each parent were kept separate until fertilization. Fertilization strategies are outlined below. Fertilized eggs were transferred to individual 20 L tanks filled with 5 µm filtered seawater (temperature 21 ºC, salinity 35 ppt) and D-veliger larvae were harvested at 24 h post-fertilization by draining tanks through a 40 µm nylon sieve.

Experimental infection of *C. gigas* D-larvae with OsHV-1 was conducted according to the protocol outlined in Burge and Friedman (2012). Experiments involving OsHV-1 were conducted in a physical containment level 2 (PC2) facility at the Sydney Institute of Marine and all waste arising from experiments was decontaminated by heat sterilization (autoclaved at 121 ^{o}C for 15 min (Hick et al., 2016). Briefly, gill and mantle tissue from OsHV-1 infected and non-infected adult *C. gigas* was homogenized in 10-volumes of sterile seawater containing 1000 $units.ml^{-1}$ of penicillin and 1 $mg.ml^{-1}$ of streptomycin, clarified by centrifugation and 0.2 µm filtered to prepare OsHV-1 and control homogenates, respectively (Burge and Friedman, 2012; Renault et al., 2011). D-veliger larvae (24 h post-fertilization) from each family were placed in duplicate 500 ml Erlenmeyer flasks containing 200 ml of sterile seawater, 100 $units.ml^{-1}$ of penicillin and 0.1 $mg.ml^{-1}$ of streptomycin. Larvae density was 30 $larvae.ml^{-1}$ and cultures were fed daily with 10^7 cells of live *I. galbana*. For each family, one Erlenmeyer flask was inoculated with OsHV-1 homogenate and the other flask was inoculated with the control tissue homogenate (Burge and Friedman, 2012; Renault et al., 2011). Flasks inoculated with OsHV-1 received the equivalent of 10^9 OsHV-1 genome copies. Cultures were sampled at 48 h post inoculation and the assessment of live/dead larvae was performed using a compound microscope and Sedgewick rafter slide. Aliquots of 10^3 larvae from each culture were pelleted by centrifugation (1000 g, 5 min) and stored at -80 ºC for subsequent nucleic acid extraction.

To address the specific aims of this study, different fertilization strategies were adopted, as follows:

(i) Experiment 1: effect of time between parental immune stimulation and spawning on offspring immunity.

Two cohorts of adult oysters (both males and females) were injected with poly(I:C) at 10 or 3 days prior to spawning. A third cohort of adult oysters were injected with sterile seawater at 3 days prior to spawning as a control. Up to six pair-mated families were produced for each treatment (Figure 1). Offspring from the 11 pair-mated families were challenged with OsHV-1 as described above. In addition to the assessment of survival rates following OsHV-1 inoculation, unchallenged offspring from the pair-mated families generated in this experiment was analyzed by RNA-seq and qPCR (sections 2.4 and 2.5). Molecular analyses were performed in order to investigate whether poly(I:C) treatment alters offspring transcriptional responses.

(ii) Experiment 2: contribution of maternal and paternal immune stimulation to offspring performance.

The role of maternal immune stimulation on offspring immunity to OsHV-1 was examined by mating a single male oyster with five poly(I:C)-treated females and five control (seawater-injected) females to produce 10-half sibling families that share the same father (Figure 2). Likewise, the role of paternal immune stimulation on offspring immunity was tested by mating a single female oyster with five poly(I:C)-treated males and five seawater-injected males to produce 10 families that share the same mother (Figure 2). Offspring from each family was challenged with OsHV-1 as detailed above.

2.2 Nucleic acid purification

Total RNA and DNA was isolated using TriReagent® (Sigma-Aldrich) and Isolate II Genomic DNA Kit (Bioline), respectively. Purity and yield of nucleic acids were evaluated using NanoDrop™ 2000 Spectrophometer (Thermo Scientific™). Total RNA (0.5 µg) was reverse transcribed using the Tetro cDNA synthesis kit (Bioline) with random hexamers.

2.3. OsHV-1 DNA detection and quantification

Absolute quantification of OsHV-1 DNA was determined by quantitative polymerase chain reaction (qPCR) according to Pepin et al. (2008) using SensiFAST™ SYBR® No-ROX (Bioline) and the C9/C10 primer pair (Table 1). The concentration of OsHV-1 DNA was estimated from a standard curve generated from the C9/C10 amplicon product cloned into the pCR4-TOPO vector (Thermo Scientific™) according to the protocols and calculations outlined in the Applied Biosystems manual of absolute real-time RT-PCR quantification (Applied_Biosystems, 2003). The plasmid was diluted in distilled water (Standard Curve:

PCR efficiency = 95 %, R^2 = 0.995) and the dynamic range of the qPCR assay was 10^8 to 10^3 copies.

2.4 Transcriptome sequencing and differential gene expression

Six larval RNA samples were selected for high-throughput mRNA sequencing (RNA-seq). These samples were from experiment 1 and consisted of three pools (families) of larvae produced from *C. gigas* treated with poly(I:C) and three pools of control larvae. These pools of larvae had not been inoculated with OsHV-1 as we were trying to determine whether unchallenged larvae from poly(I:C)-stimulated parents have a different transcriptome to control larvae. The TruSeq protocol (Illumina), including a poly(A) isolation step, was followed for cDNA synthesis and library preparation. Samples were barcoded and sequenced by the Australian Genome Research Facility (AGRF, Brisbane) on a HiSeq2500 instrument (Illumina) using a 50 bp single end, strand-specific run. The raw sequence reads are available at the National Center for Biotechnology Information (NCBI) Short Read Archive (Accession no. SRP??????).

Nucleotide reads were quality filtered using Trimmomatic (version 0.32) (Bolger, Lohse & Usadel, 2014) and the quality of the trimmed reads was visualized using fastQC (version 0.10.1). Processed reads from each library were individually mapped back to the GigaTON reference transcriptome for *C. gigas* (Riviere et al., 2015). The GigaTON transcriptome assembly encompasses a total of 56,621 contigs (median length = 1,659; N50 = 2,238) generated by the combination of 114 RNA-seq libraries, which include an extensive range of developmental stages (*e.g.* unfertilized eggs, two-cell embryos to two-year-old adults), tissues (*e.g.* whole-embryos, whole-larvae, whole-spat, gill, hemocyte, mantle, adductor muscle, gonad, digestive tract and labial palp) and physiological conditions (*e.g.* oysters at ambient conditions and exposed to disturbed temperature, salinity and heavy metals). In addition, mapping of our sequencing reads to the GigaTON transcriptome produced higher alignment rates than the mapping against the *C. gigas* genome (version 101; 79.4 vs. 66.4% overall mapping, respectively). The draft genome sequence for *C. gigas* is known to have a number of assembly errors in genome scaffolds (Hedgecock et al., 2015). Therefore, given its complexity and broad genetic diversity, the GigaTON assembly was used as a reference transcriptome in the current study. Mapping of processed reads to the GigaTON assembly was performed using Bowtie2, with strict parameters (-score-minL,-0.1,-0.1, -no-mixed, -no-discordant, -fr -nofw). Assembled contigs were then clustered based on the proportion of shared reads and expression patterns using Corset (default settings)

(Davidson & Oshlack, 2014). The cluster-level count data were processed using the edgeR Bioconductor package (Robinson, McCarthy & Smyth, 2010), testing for differences in gene expression between larvae produced from *C. gigas* treated with poly(I:C) or seawater. Clusters with non-zero counts in at least half of the analyzed samples (3 out of 6 samples) were kept for downstream analyses. Data were normalized for sequencing depth (library size) and RNA composition (TMM normalization). Differential expression was calculated using the quantile-adjusted conditional maximum-likelihood method, followed by the exact test. Contigs were considered to be differentially expressed at $p < 0.05$ with false detection rate (FDR) lower than 5%. Differentially expressed genes were annotated using BlastX and the molluca non-redundant (nr) database.

2.5 RT-qPCR

To validate the transcriptome data, we evaluated the relative expression profiles of 10 genes identified as differentially expressed by RNA-seq (section 2.4). Annotation could not be assigned to these 10 differentially expressed genes because they matched uncharacterized proteins in the NCBI databases. Genes were chosen for RT-qPCR based on fold change (up- or down-regulated) and function (known or unknown). Another 3 antiviral genes (*IRF*, *Viperin* and *ADAR-L*) were included in this analysis. These genes were not identified to be differentially expressed by RNA-seq, but their expression has been shown to be altered by poly(I:C) treatment and OsHV-1 infection (Green et al., 2015a). The relative expression of these 13 target genes were quantified in cDNA samples from unchallenged eggs and larvae produced from parents stimulated with poly(I:C) or seawater. RT-qPCR was performed in a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD), as described previously by Green et al. (2016), using the primers in Table 1, which included the internal reference gene eEF1α. Amplification efficiency of each primer pair was validated using a serial dilution of cDNA.

2.6 Statistical analysis

Survival and qPCR data were analyzed for statistical differences using analysis of variance (ANOVA) in the SPSS (IBM) version 22.0. Tukey's method for multiple comparisons was used to compare means if significant differences were found ($p < 0.05$). Data that did not meet Levene's test of equal variances was arcsine transformed. Data are presented as mean ± standard deviation.

3.0 Results

3.1 Effect of time between parental immune stimulation and spawning on offspring immunity

The time between parental immune stimulation and spawning had a significant effect on the survival of offspring to OsHV-1 inoculation ($F_{5,28} = 10.99$, $p < 0.01$). At 48 h post-inoculation with OsHV-1, average cumulative mortality of larvae produced from parents stimulated with poly(I:C) at 3 and 10 days prior to spawning was 14.4 ± 7.2 % and 37.5 ± 12.2 %, respectively. Larvae produced from control parents (injected with seawater at 3 days pre-spawning) had a cumulative mortality of 45.3 ± 21.7 % at 48 h post-inoculation with OsHV-1 (Figure 1). Priming parents with poly(I:C) at 3 days prior to spawning did significantly reduce mortality of larvae compared to controls ($p < 0.05$), but no improvement was observed in larvae generated from parents treated with poly(I:C) at day 10 prior to spawning ($p > 0.05$). Priming parents with poly(I:C) had no effect on OsHV-1 replication in larvae. No difference in the amount of OsHV-1 DNA in the tissue of larvae was observed between the three treatments ($p > 0.05$). The mean concentration of OsHV-1 DNA in larvae was 1.2 x 10^5 and 1.61 x 10^5 genome copies.larva^{-1} at 48 h post-inoculation for larvae produced from parents primed with poly(I:C) at 3 and 10 days prior spawning, respectively. Control larvae had 1.1 x 10^5 genome copies.larva^{-1}.

We also investigated whether poly(I:C) stimulation of parents had an effect on survival of non-challenged (NC) larvae. Parental immune stimulation prior to spawning did not effect survival of offspring (Figure 1, $p > 0.05$). The cumulative mortality of larvae at 48 h post inoculation with the control homogenate was 9.7 ± 6.9 % and 7.9 ± 5.3 % for larvae produced from parents stimulated with poly(I:C) at 10 and 3 days prior to spawning, respectively. The cumulative mortality of control larvae was 14.8 ± 7.5 % at 48 h post inoculation with the control homogenate. No OsHV-1 DNA was detected in the tissue of larvae exposed to the control homogenate.

3.2 Effect of maternal and paternal immune stimulation on offspring immunity to OsHV-1

Maternal immune stimulation prior to spawning resulted in *C. gigas* offspring (larvae) with improved immunity to OsHV-1 infection ($p < 0.05$, Figure 2). The average cumulative mortality of larvae produced from poly(I:C) stimulated mothers was 8.3 ± 5.3 %, whereas control larvae had a cumulative mortality 2.3 times higher (18.9 ± 6.3%) at 48 h post-inoculation with OsHV-1. Larvae produced from poly(I:C) stimulated mothers also had significantly lower amounts of OsHV-1 DNA in their tissues compared to control larvae ($p < 0.05$). The average concentration of OsHV-1 DNA in larvae produced from poly(I:C)

stimulated mothers was 4.0 x 10^4 genome copies per larva, while control larvae had 2.3-fold more OsHV-1 (9.3 x 10^4 copies.larva^{-1}) at 48 h post inoculation.

Paternal immune stimulation prior to spawning had no significant effect on the survival of *C. gigas* offspring to OsHV-1 ($p > 0.05$, Figure 2). The average cumulative mortality of larvae produced from poly(I:C) stimulated fathers was 20.6 ± 4.6 %, whereas control larvae had a cumulative mortality of 31.5 ± 3.4 % at 48 h post-inoculation with OsHV-1. There was also no difference in the amount of OsHV-1 DNA in the tissue of D-larvae produced from poly(I:C) or control stimulated fathers ($p > 0.05$).

Paternal or maternal immune stimulation with poly(I:C) prior to spawning did not affect the survival of non-challenged larvae (*i.e.* offspring exposed to the control inoculum) ($p > 0.05$, Figure 2). Average mortality of larvae in these control treatments was lower than 4% and OsHV-1 DNA was not detected in their tissue.

3.3 Differential gene expression in unchallenged larvae following parental immune challenge

RNA-seq was used to investigate whether parental immune stimulation alters the transcription of immune-related genes in offspring. Six cDNA libraries were generated with mRNA from unchallenged D-larvae (24 h post-fertilization) produced from parents stimulated with poly(I:C) (N = 3, 3 days prior to spawning) or seawater (N = 3). Illumina sequencing yielded more than 203 million reads with an average PHRED quality of 37. The average number of reads was 34 M per library (SD = 3.3 M; max = 37 M; min = 28 M). Overall, 79.4 % of the total output reads mapped to the GigaTON reference transcriptome (SD = 7.2%; max = 87.8%; min = 70.8%). Reads mapped to contigs were clustered into 22,450 gene clusters using Corset. A total of 14,479 (64.5 %) of these gene clusters were present in at least half of the samples sequenced (3 out of 6 RNA-seq libraries) and were used for downstream analysis.

Analysis of expression levels for the 14,479 gene clusters revealed that larvae produced from immune-stimulated parents have very similar gene expression profiles to control larvae (Figure 3a). Of the 14,479 gene clusters, only 47 were putatively identified to be differentially expressed (FDR-adjusted, $p < 0.05$) in D-larvae produced by immune-stimulated parents. D-larvae produced from immune-stimulated parents exhibited 22 up-regulated genes (fold-change between 2.7 and 302.6) and 25 down-regulated genes (fold-change between -2.9 and -153.1). Blast analysis showed that 87 % of the differentially expressed genes (DEGs; 41 gene clusters) had a significant match against the Mollusca non-redundant (nr) and/or the NCBI nucleotide (nt) databases (e-value < 1^{-10}). Of the DEGs with

Blast hits, only 39 % were annotated with a putative gene function. Differentially expressed genes were found to be associated with immune and stress responses (*e.g.* caspase-14, sacsin, zinc metalloproteinase, F-box only protein 22), cellular signaling and communication (*e.g.* regulator of G-protein signaling protein, integrin beta pat-3, tenascin and fibrocystin-L), regulation of the cell cycle and cellular division (*e.g.* nibrin and baculoviral IAP repeat-containing protein 2) (Figure 3b). We have also identified a number of differentially expressed genes encoding uncharacterized proteins. A few of these uncharacterized proteins exhibited conserved domains, including integrase, reverse transcriptase and recombinase motifs (Table 2).

3.4 Validation and expression pattern analysis

A total of 47 DEGs were identified by RNA-seq to be differentially expressed between larvae produced from parents treated with poly(I:C) or seawater (controls) prior to spawning. To validate the RNA-seq analysis, we chose 10 candidate DEGs (25 %) for RT-qPCR analysis. The expression of three known antiviral genes (*interferon regulatory factor*, *Viperin* and *adenosine deaminase RNA-specific*) were also evaluated. RT-qPCR did not identify differences in the expression level of these ten DEGS in groups of larvae produced from parents treated with poly(I:C) or seawater. These samples analysed by RT-qPCR included cDNA sequenced by RNA-seq (experiment I) and cDNA samples from experiment II. Based on these results, we were unable to validate the RNA-seq analysis by RT-qPCR.

The antiviral genes of IRF, viperin and ADAR-L had higher relative expression in eggs from mothers stimulated with poly(I:C) at 3 or 10 days prior to spawning ($p < 0.05$, Figure 4a). Higher expression of these genes were also observed in eggs produced by mothers treated with poly(I:C) at 3 days prior to spawning ($p < 0.05$, Figure 4b) but no difference was observed in unchallenged D-larvae produced from parents stimulated with poly(I:C) or seawater ($p > 0.05$).

Discussion

Heterogeneous immune priming experiments showed that *Crassostrea gigas* primed with poly(I:C) have improved survival to OsHV-1 infection (Green and Montagnani, 2013; Green et al., 2015b; Lafont et al., 2017), and this protection can be passed to the next generation (Green et al., 2016). Despite the physiological, metabolic and immunological response of *C. gigas* to OsHV-1 infection being well characterized (Corporeau et al., 2014; Green and Speck, 2018; Martenot et al., 2017; Tamayo et al., 2014; Young et al., 2017), relatively little

342 is known regarding the molecular mechanisms underpinning the phenomenon of immune
343 priming. A core set of genes expressed in response to OsHV-1 infection has been identified
344 in adults (He et al., 2015; Rosani et al., 2015) and larvae (Zhang et al., 2015), and differences
345 in antiviral gene expression in *C. gigas* primed with poly(I:C) (Green and Montagnani, 2013;
346 Green et al., 2015b), and their progeny (Green et al., 2016) following inoculation with
347 OsHV-1 have been characterized. Stimulation with double-stranded RNA, such as poly(I:C),
348 induces the expression of antiviral effector genes (Green et al., 2014b), for at least seven days
349 (Green et al., 2014a), and this response appears to inhibit OsHV-1 replication (Lafont et al.,
350 2017; Pauletto et al., 2017). Do *C. gigas* offspring produced from parents stimulated with
351 poly(I:C) also have elevated expression of antiviral effector genes?

352 In the current study, oyster larvae had higher survival to OsHV-1 when their mothers,
353 but not their fathers, were administered poly(I:C) prior to spawning (Figure 2). This
354 improved survival could not be explained by reconfiguration of the constitutive expression of
355 antiviral genes in unchallenged larvae. Transcriptomic analysis revealed that non-challenged
356 larvae produced from parents primed with polyI:C have similar transcriptional profiles to
357 control larvae. Despite this overall similarity in transcriptome response, a small subset of 47
358 genes was found to be differentially regulated between offspring of polyI:C-treated and non-
359 treated parents (Figure 3). However, validation of RNA-seq data by RT-qPCR did not
360 identify any differentially expressed genes between the two offspring cohorts (21% of the
361 genes identified by RNA-seq were tested by RT-qPCR). This discrepancy might be caused by
362 the small sample sizes (N = 3) used in the current study. Alternatively, our results imply that
363 the improved survival of *C. gigas* larvae might occur *via* maternal provisioning of antiviral
364 compounds in the eggs. In support of maternal provisioning is the up-regulation of antiviral
365 effector genes, including viperin, in the eggs of *C. gigas* following stimulation with poly(I:C)
366 (Figure 4). Viperin has been shown to be induced by poly(I:C) treatment via a hemolymph
367 cytokine and to play a direct role in oyster antiviral defense (Green et al., 2015c). Maternal
368 provisioning is consistent with a previous study on the scallop *Chlamys farreri*, where
369 mothers stimulated with heat-killed *Vibrio* transfer antibacterial proteins to their offspring *via*
370 the egg (Yue et al., 2013). Crosses involving OsHV-1 infected *C. gigas* mothers produce
371 progengy (larvae and spat developmental life-stages) have survival rates statistically higher
372 than other types of crosses, suggesting OsHV-1 infection mothers transmit some form of
373 protection to their offspring (Barbosa-Solomieu et al., 2005).

374 Maternal provisioning of antiviral compounds to *C. gigas* larvae has the potential
375 advantage that it is the mother, not the developing embryo, who invests resources into its

offspring's antiviral defense. Immunity is a life-history trait that can be expected to be traded off with other physiological processes, such as growth, reproduction and self-maintenance (Rauw, 2012). Immune activation entails a significant energetic cost, revealed by raised metabolic rates between 8-28% in a range of terrestrial insects following PAMP inoculation or injury (Ardia et al., 2012; Freitak et al., 2003). The metabolic requirement of immunity has not been quantified for marine bivalves, but it is likely to have a similar high cost. During early larval development of *C. gigas*, activating an immune response can compromise other physiological processes. In ideal environmental conditions, *C. gigas* larvae allocate ~75% of their total metabolic energy budget to protein synthesis (~55%) and ion transport (~20%) (Lee et al., 2016; Pan et al., 2016). Thus, maternal provisioning of immunity represents a beneficial investment from mother to offspring, reducing the cost of producing and maintaining an expensive antiviral response. It now needs to be determined if this antiviral immunity persists or declines during *C. gigas* embryonic developments, as the antiviral compounds are metabolized.

Selective breeding programs in many countries have developed improved *C. gigas* stocks, which are better suited for aquaculture purposes (de Melo et al., 2016; Degremont et al., 2015a; Swan et al., 2007). Indeed, survival to OsHV-1 infection is a trait that has received considerable scientific attention given the benefits it could provide to oyster production worldwide (Camara et al., 2017; Dégremont, 2011; Degremont et al., 2015b). To access genetically improved *C. gigas* stocks, shellfish farms have to source spat from aquaculture hatcheries (Robert and Gerard, 1999). However, aquaculture hatchery supply of *C. gigas* spat is inconsistent due to larval mortality events (Robert and Gerard, 1999), which can be caused by OsHV-1 (Barbosa-Solomieu et al., 2005; Hine et al., 1992; Renault et al., 2000). Our results demonstrate that it is possible to utilize transgenerational immune priming to reduce mortalities in hatchery production of *C. gigas*, thus challenging the concept that such therapies cannot be adapted for oyster aquaculture (Pernet et al., 2016; Rodgers et al., 2018). This study also showed that transgenerational immune priming against OsHV-1 is consistently reproducible, alleviating the concerns surrounding the existence of immune priming in shellfish (Hauton and Smith, 2007). Future research should focus on characterizing the transfer of antiviral compounds from mother to offspring. The application of this knowledge can have great potential for improving oyster health and welfare in aquaculture.

Competing interests

The authors declare no competing interests.

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618 **Tables**

619 Table 1: Primer pairs used for qPCR analysis. Genes in bold are known internal reference
620 genes or antiviral proteins selected from the scientific literature. The closest match in the
621 NCBI mollucan database (BlastX) and its annotation is provided.

Cluster	Genbank Match	Sense Primer	Antisense Primer	Annotation
EFU	ABI22066	GAGCGTGAACGTGGTATCAC	ACA GCA CAG TCA GCC TGT GA	Internal Reference Gene
Cluster-9448.0	XP_01142697	ATC GAA TGT AAA TGT ATG ACC AC	TTG ATC GGT GCA GTG TCT G	
Cluster-14532.0	XP_011427757	ACT GCA CTC GAT CCA AGA TG	AGA TAC ATT CAT CAT ACG GAC TG	
Cluster-5145.0	XP_011422960	AGA GCC AAT GAT ATC ACA TGA G	TGT AGC AGC TTT CCC ATC TG	
Cluster-162.2748	EKC32605	GTC TAT TTA CGG ACT TGC TAA C	GAG TCG TCG CAT CGT TAC	
Cluster-10608.0	XP_011451618	ATC CAG GAC GCG GTA GAG	TCT CTT AGC ACA GTC ACT AG	
Cluster-3884.0	XP_011441565	ACA GCA AAC ATG TGT CCA AG	TCT GGT GAC GAA GCT GGT G	
Cluster-7150.0	NO ID	TGC TGT AAA GTT CTT GCA TC	TGC TTT GGT GTG CGC AAG	
Cluster-13785.3	NO ID	GTA GGA TGG TAA AGT GCA CAG	CAT TCA CCA CAC TCC ATT CTG	
Cluster-4797.0	XP_011445587	ACC TGT TTG GAG CAG TGT C	AGT AGC GAC CGA CTT CAG TC	
Cluster-162.3841	XP_011450578	GTC ACT TTT GCT CAG CTG ATG	GAT AAC TCT TCC TTC CCA GAC	
IRF	EKC43155	CGA AAC GCA GAA ACT GTT C	ATT TGC CTT CCA TCT TTT GG	Antiviral Signaling
Viperin	EKC28205	GCT TTG ACC CGG AAA CCA AC	TGA CAC CAA TCC CGA ACT CG	Antiviral Effector
ADAR	EKC20855	CTC AAA CAG TGC AAC TGC ATC	TCA CAA GCC CTG CTA TCA C	Antiviral Effector
OsHV-1 C9/C10		GAG GGA AAT TTG CGA GAG AA	ATC ACC GGC AGA CGT AGG	OsHV-1 quantification

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624 Table 2: Genes identified by RNA-seq to be differentially expressed between larvae produced
625 from broodstock stimulated with poly(I:C) or seawater. Genes highlighted in bold were
626 investigated by RT-qPCR.

Cluster ID	FC	PValue	FDR	BlastX	Annotation
Cluster-3884.0	**302.62**	**3.28781E-29**	**4.76042E-25**	**XP_011441565**	**Uncharaterized Protein**
Cluster-14532.0	**70.02**	**7.1539E-19**	**3.45271E-15**	**XP_011427757**	**Caspase-14-like protein**
Cluster-162.709	42.48	8.86638E-11	1.60E-07	XP_011433740	Integrin beta containing protein
Cluster-5194.0	33.92	7.85977E-15	2.85E-11	XP_011431106	Uncharaterized Protein
Cluster-9448.0	**24.53**	**4.4828E-19**	**3.25E-15**	**XP_011426297**	**Uncharaterized Protein**
Cluster-26.1	17.06	8.98332E-09	1.08391E-05	XP_019927010	sacsin-like protein
Cluster-8341.0	15.81	5.77909E-09	7.61E-06	No Match	Hypothetical Protein
Cluster-2997.1	10.58	2.27381E-09	3.65806E-06	XP_019921756	E3 ubiquitin-protein ligase
Cluster-9682.0	8.77	2.88696E-06	0.001990491	No Match	Hypothetical Protein
Cluster-806.0	8.18	6.64977E-05	2.29E-02	No Match	Hypothetical Protein
Cluster-162.3691	8.14	2.81483E-09	4.08E-06	EKC28297	Hypothetical Protein
Cluster-1784.1	7.11	1.88997E-05	8.83E-03	XP_011415777	Uncharaterized Protein
Cluster-4559.0	6.94	4.33946E-05	1.65E-02	EKC27857	EGF-like domain containing protein
Cluster-13205.0	6.51	2.96782E-05	1.19E-02	XP_019919735	Uncharaterized Protein
Cluster-10168.0	5.99	1.18908E-12	2.46E-09	EKC37705	Hypothetical Protein
Cluster-71.0	5.99	1.29584E-06	0.001103675	XP_011447098	Zinc finger containing protein
Cluster-6742.0	5.67	9.20341E-06	5.13E-03	EKC22504	Hypothetical Protein
Cluster-8759.0	4.26	0.000152333	0.046928379	XP_011425705	Uncharaterized Protein
Cluster-9418.0	3.21	5.42253E-05	0.020131487	EKC21305	Hypothetical Protein
Cluster-2485.0	3.07	9.38279E-05	0.030875788	XP_011416425	Perlucin-like protein
Cluster-4860.0	3.03	0.000139626	4.39E-02	EKC35803	Hypothetical Protein
Cluster-6877.3	2.74	2.50613E-05	1.07E-02	XP_011456604	Ribonucleoside diphosphate reductase
Cluster-162.3562	0.34	7.82385E-05	2.63E-02	XP_019929668	Uncharaterized Protein
Cluster-13785.3	**0.27**	**2.48153E-06**	**1.80E-03**	**No Match**	**Hypothetical Protein**
Cluster-1470.0	0.27	5.68381E-05	2.06E-02	EKC20054	Cleavage Stimulation Factor 77kDa
Cluster-10608.0	**0.20**	**1.39379E-05**	**7.21E-03**	**XP_011451618**	**Uncharaterized Protein**
Cluster-162.2025	0.20	2.13317E-05	0.009651932	XP_011437445	Baculoviral IAP repeat protein
Cluster-162.946	0.19	0.000115807	0.037261697	EKC27582	Tripartite motif protein 2
Cluster-162.639	0.18	2.10392E-06	0.001603299	XP_019922312	Nibrin-like protein
Cluster-162.3841	**0.17**	**2.4699E-05**	**1.07E-02**	**XP_011450578**	**Uncharaterized Protein**
Cluster-4685.0	0.16	6.68718E-06	4.03E-03	XP_011446049	Fibrocystin-L-like protein
Cluster-4797.0	**0.15**	**3.23101E-06**	**0.002126448**	**XP_011445587**	**Uncharaterized Protein**
Cluster-2950.0	0.14	1.53162E-05	7.44E-03	EKC39917	Fibrocystin-L-like protein
Cluster-7150.0	**0.13**	**1.00793E-06**	**9.12E-04**	**No Match**	**Hypothetical Protein**
Cluster-5881.0	0.13	1.03442E-05	0.005547163	XP_019928976	Uncharaterized Protein
Cluster-162.2272	0.12	6.47005E-05	2.28E-02	EKC37905	EGF-like domain containing protein
Cluster-6413.0	0.10	4.4625E-08	4.61518E-05	XP_011413924	Uncharaterized Protein
Cluster-162.1461	0.10	3.21255E-05	1.26E-02	EKC20083	F-box only protein 22
Cluster-8476.1	0.09	1.85335E-07	0.000178897	XP_011433732	Zinc finger containing protein
Cluster-13153.0	0.08	2.76871E-05	1.15E-02	EKC33341	Hypothetical Protein
Cluster-13573.0	0.07	1.57403E-14	4.56E-11	EKC40398	SEC7 domain-containing protein
Cluster-3702.0	0.07	7.75681E-06	4.49E-03	XP_011447229	Zinc metalloproteinase
Cluster-5145.0	**0.04**	**5.21847E-13**	**1.2593E-09**	**XP_011422960**	**Uncharaterized Protein**
Cluster-162.2749	0.04	4.12664E-06	2.60E-03	XP_011453448	Uncharaterized Protein

Cluster-162.2748	**0.04**	**2.05084E-06**	**1.60E-03**	**EKC32605**	**Tenascin-N**
Cluster-14227.0	0.02	2.367E-08	2.64E-05	XP_011413410	G-protein
Cluster-162.3084	0.01	1.54057E-05	7.44E-03	No Match	Hypothetical Protein

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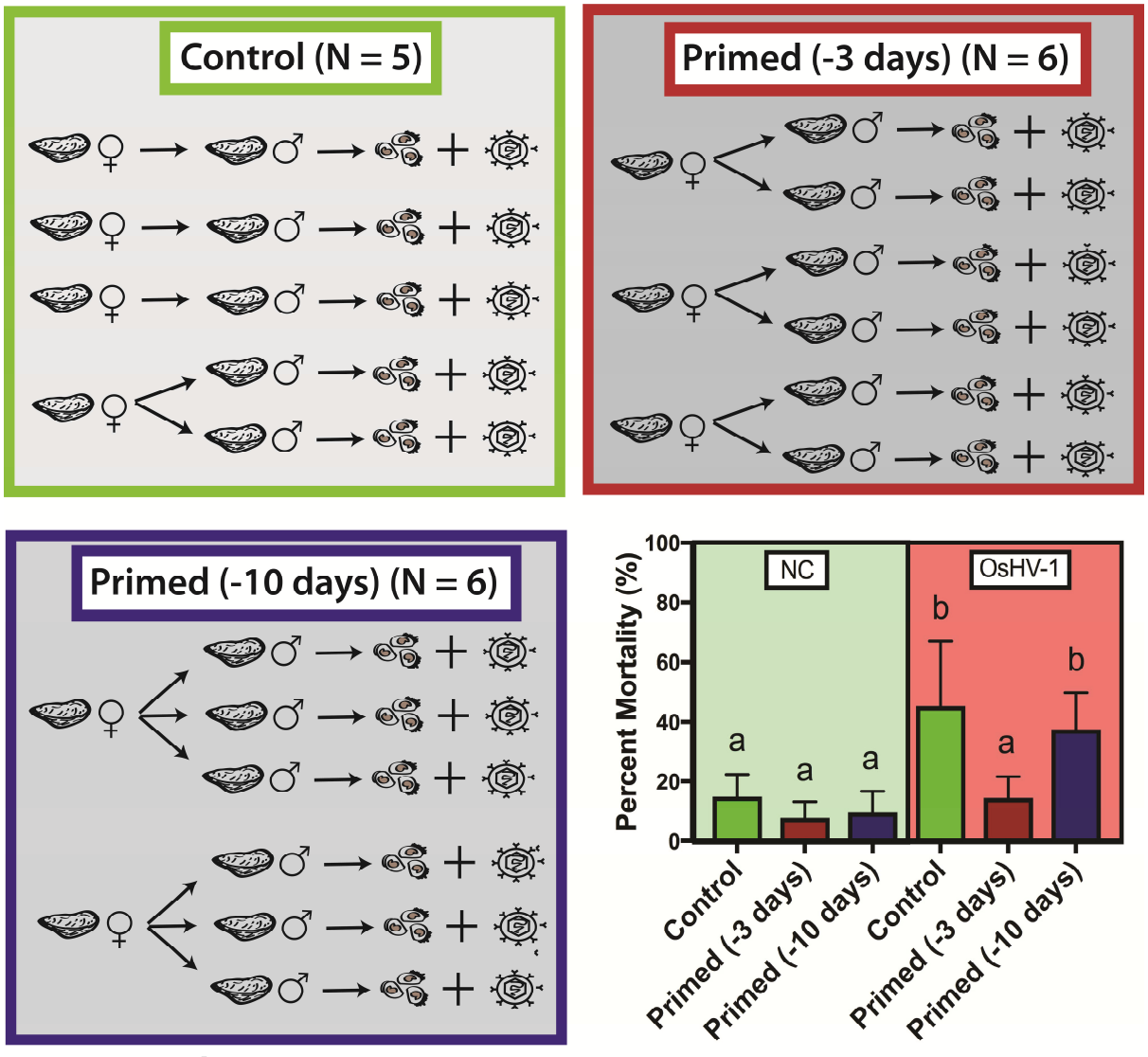
629 **Figure Legends**



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631 Figure 1: Schematic diagram of experimental design for investigating the effect of time
632 between parental immune stimulation and spawning on offspring immunity to OsHV-1. Two
633 cohorts of adult *Crassostrea gigas* were injected with poly(I:C) at 3 and 10 days prior to
634 spawning. A third cohort of *C. gigas* were injected with seawater. Up to 6 pair mated families
635 were produced for each treatment. D-larvae from each family were inoculated with OsHV-1
636 or control homogenate (NC). Cumulative mortality for each treatment was determined at 48
637 hours post-inoculation (mean ± standard deviation). Different letters indicate significant
638 differences ($p < 0.05$) between treatments.

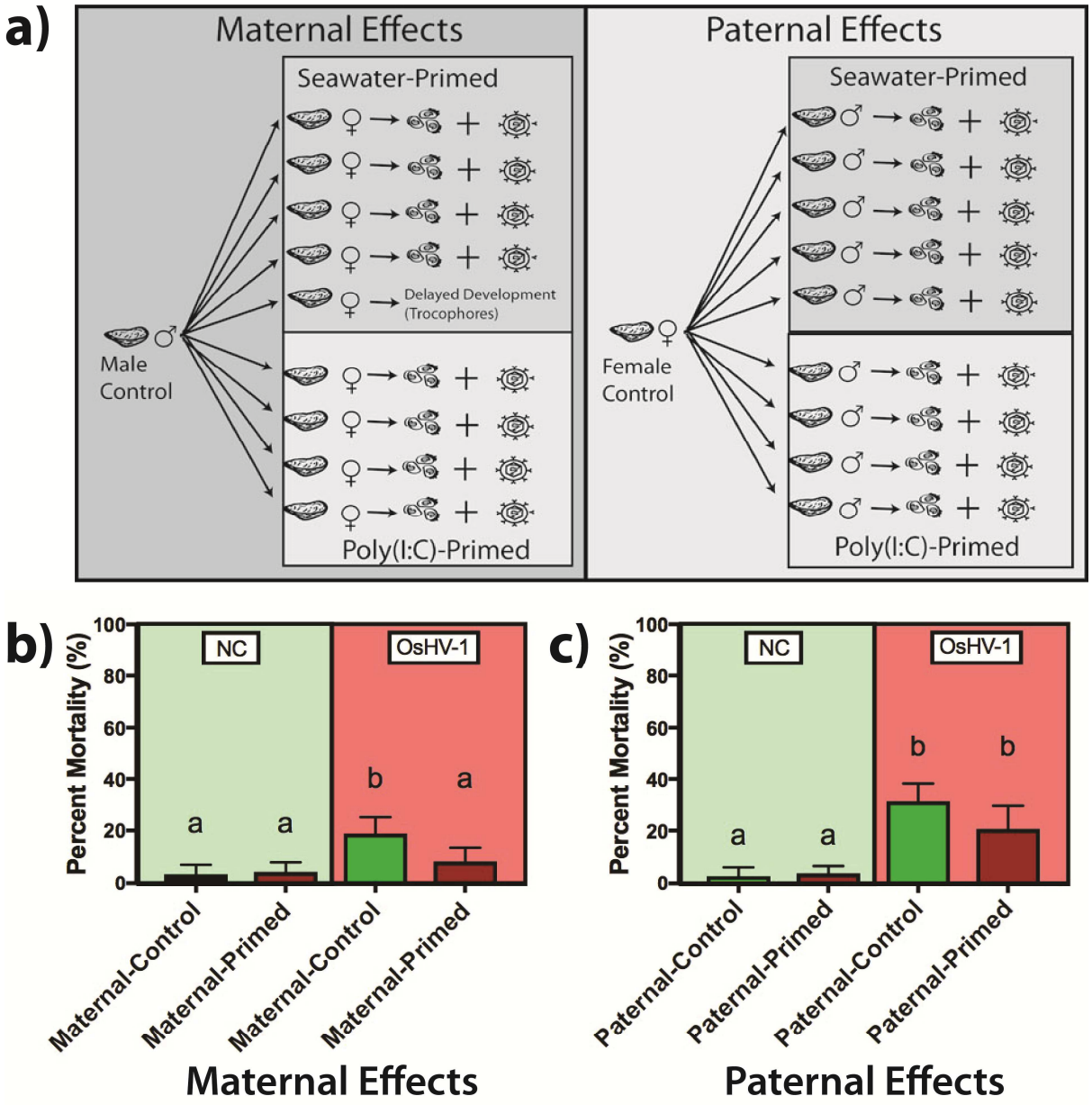

Figure 2: Schematic diagram of experimental design for investigating the contribution of maternal and paternal immune stimulation to offspring performance. D-larvae from each family were inoculated with OsHV-1 or control homogenate (NC). Cumulative mortality for each treatment was determined at 48 hours post-inoculation (mean ± standard deviation). Different letters indicate significant differences ($p < 0.05$) between treatments.

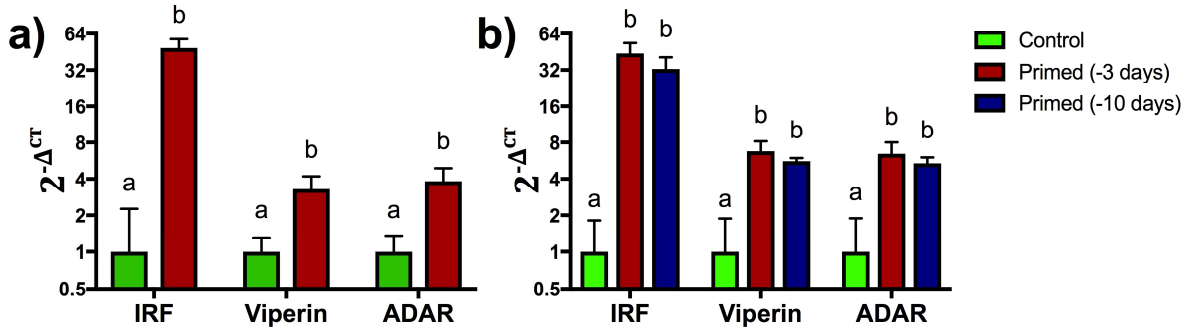

Figure 3: The expression of interferon regulatory factor (IRF), viperin and double-stranded RNA-specific adenosine deaminase (ADAR) in unfertilized eggs of *Crassostrea gigas* that were stimulated with poly(I:C) or seawater (control). a) experiment i: effect of time between parental immune stimulation and spawning on offspring immunity to OsHV-1. b) experiment ii: maternal effect on offspring immunity to OsHV-1. Expression is presented as the mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$) between treatments.

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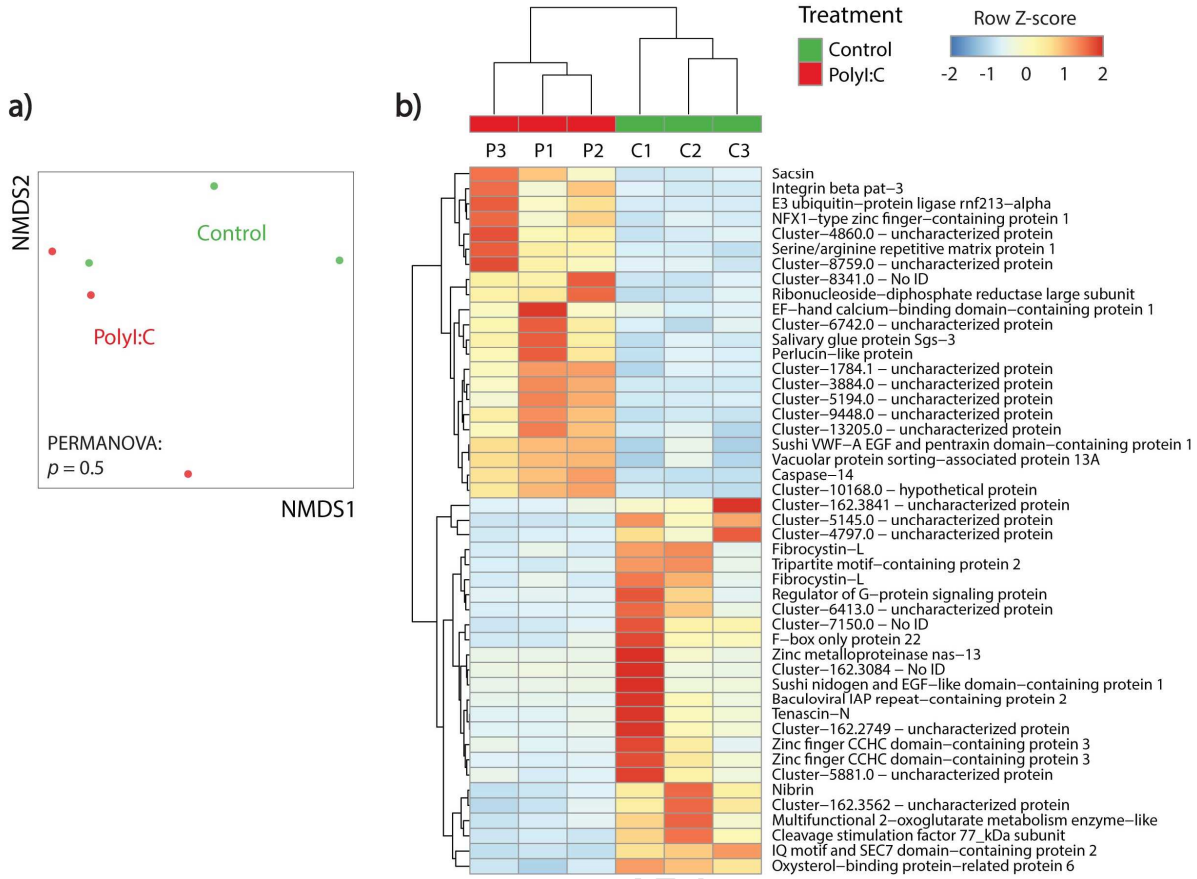

Figure 4: Transcriptional response of unchallenged D-larvae of *C. gigas* produced from parents stimulated with poly(I:C) or seawater (control) prior to spawning. (a) Non-metric multidimensional scaling (NMDS) plot summarizing the expression level of 14,479 gene clusters identified by Trinity and Corset in pooled D-larvae samples produced from pair-mated families. (b) Heat map of gene identified to be differentially expressed by RNA-seq between unchallenged D-larvae.

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- The molecular mechanism involved in transgenerational immune priming was investigated in the oyster, *Crassostrea gigas*.
- *Crassostrea gigas* larvae have higher survival to OsHV-1 when their mothers, but not their fathers, are exposed to poly(I:C) prior to spawning.
- RNA-seq provided no evidence to suggest that parental exposure to poly(I:C) reconfigures antiviral gene expression in unchallenged larvae.
- Improved survival of *C. gigas* larvae might occur *via* maternal provisioning of antiviral compounds in the eggs.

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