Fish & Shellfish Immunology
January 2014, Volume 36, Issue 1, Pages 151–157
http://dx.doi.org/10.1016/j.fsi.2013.10.026

Ontogeny and water temperature influences the antiviral response of the Pacific oyster, *Crassostrea gigas*

Timothy J. Green[a, *], Caroline Montagnani[b], Kirsten Benkendorff[c], Nick Robinson[a, d], Peter Speck[a]

[a] School of Biological Sciences and Australian Seafood Cooperative Research Centre, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia
[b] Ifremer, UMR 5119 Ecology of Coastal Marine Systems, Université Montpellier 2, Place Eugène Bataillon, CC80, 30495 Montpellier cedex 05, France
[c] Marine Ecology Research Centre, Southern Cross University, P.O. Box 157, Lismore, NSW 2480, Australia
[d] Nofima, P.O. Box 210, N-1431 Ås, Norway

*: Corresponding author : Timothy J. Green, email address : tim.green@flinders.edu.au

Abstract:

Disease is caused by a complex interaction between the pathogen, environment, and the physiological status of the host. Determining how host ontogeny interacts with water temperature to influence the antiviral response of the Pacific oysters, *Crassostrea gigas*, is a major goal in understanding why juvenile Pacific oysters are dying during summer as a result of the global emergence of a new genotype of the Ostreid herpesvirus, termed OsHV-1 µvar. We measured the effect of temperature (12 vs 22 °C) on the antiviral response of adult and juvenile *C. gigas* injected with poly I:C. Poly I:C up-regulated the expression of numerous immune genes, including TLR, MyD88, IκB-1, Rel, IRF, MDA5, STING, SOC, PKR, Viperin and Mpeg1. At 22 °C, these immune genes showed significant up-regulation in juvenile and adult oysters, but the majority of these genes were up-regulated 12 h post-injection for juveniles compared to 26 h for adults. At 12 °C, the response of these genes was completely inhibited in juveniles and delayed in adults. Temperature and age had no effect on hemolymph antiviral activity against herpes simplex virus (HSV-1). These results suggest that oysters rely on a cellular response to minimise viral replication, involving recognition of virus-associated molecular patterns to induce host cells into an antiviral state, as opposed to producing broad-spectrum antiviral compounds. This cellular response, measured by antiviral gene expression of circulating hemocytes, was influenced by temperature and oyster age. We speculate whether the vigorous antiviral response of juveniles at 22 °C results in an immune-mediated disorder causing mortality.

Highlights

► We investigate the immune response of *Crassostrea gigas* against poly I:C. ► Poly I:C induces expression of TLR, MDA-5, Rel, IRF, STING, PKR, Viperin. ► Hemolymph anti-HSV-1 activity is not induced by poly I:C. ► Temperature and host ontogeny influence the oyster's antiviral response. ► Juvenile oysters at 22 °C respond vigorously to poly I:C.

Keywords : Crassostrea ; OsHV-1 ; Water temperature ; Antiviral response ; Herpesvirus

1.0 Introduction

Aquatic diseases involve complex interactions between pathogen, the prevailing environment, and host physiological status. Despite this, many scientific studies on aquatic diseases only investigate the effect of one or two simultaneous factors [1]. This creates problems in interpreting the causes of multifactorial diseases, such as Pacific oyster mortality syndrome (POMS), a worldwide and complex phenomenon affecting the Pacific oyster, *Crassostrea gigas* [2-4]. This disease has coincided with the global emergence of a new genotype of the Ostreid herpesvirus, termed OsHV-1 µvar [2, 3, 5], and the disease has been attributed to the "massive mortalities" that have occurred in France and "juvenile oyster mortality (JOM)" in New Zealand. Epidemiological studies demonstrated that younger oysters (spat > juveniles > adults) are more susceptible to OsHV-1 µvar [6-8], and that the onset of disease occurs when seawater temperatures exceed 17^{o}C [4, 7, 8]. Aquaculture selection programs have shown differential survival of *C. gigas* family lines exposed to OsHV-1 µvar [9], establishing that there is a unidentified genetic component(s) affecting resistance to the disease.

It is unknown why younger developmental stages of *C. gigas* are more susceptible to OsHV-1 µvar. One possibility is they have an immature immune system [2, 10]. Alternatively, replication of OsHV-1 µvar is reliant on host cell replication machinery and the higher cell replication rates of immature oysters could favour the virus. Seawater temperature above 16^{o}C is a risk factor [7, 8, 11, 12], and it is presumed that OsHV-1 causes asymptomatic (latent) infections below this temperature [10]. This presumption is based on observations of herpesvirus infection in fish. For example, carp exposed to Koi herpesvirus (KHV) at 11^{o}C do not succumb to the disease until water temperature is elevated to 23^{o}C, and these fish do not shed the virus and infect naïve fish during co-habitation experiments unless temperatures are above 20^{o}C, suggesting the virus is in an altered transcriptional state below 20^{o}C [13]. Seawater temperature would not only influence the pathogen, but would also increase the metabolism of the host [1, 14]. A trade-off for increasing the host's metabolic requirements is less energy and resources available for maintaining the host's immunological status [14]. It is therefore not surprising that *C. gigas* also suffer mortalities from other pathogens, such as *Vibrio* bacteria, when the seawater temperature rises above 18^{o}C [15].

Oysters have no acquired immune system, but they have an efficient defence mechanism for eliminating invading agents, based on innate immunity. Host-pathogen

interactions between *C. gigas* and pathogenic bacterial strains belonging to *Vibrio aestuarianus* and *V. splendidus* are well documented in the literature [reviewed by 16]. In contrast, there is a lack of information about mollusc antiviral immunity in the aetiology of "POMS". Oysters injected with poly I:C are induced into an antiviral state preventing subsequent OsHV-1 µvar infection [17]. Poly I:C is a synthetic dsRNA compound that mimics a virus because nearly all viruses produce dsRNA at some point during their replication [18]. Oysters have therefore been shown to recognise these virus-associated molecular patterns [19] and to induce an immune response that either inhibits virus entry or replication [17]. Previous research has shown that *C. gigas* hemolymph contains a compound(s) that inhibits herpes simplex virus type 1 (HSV-1) replication in Vero cell monolayers [20, 21] and that OsHV-1 infection up-regulates expression of immune genes in hemocytes collected from adult *C. gigas* [22]. To date, investigations into the influence of water temperature and host ontogeny on the antiviral response of *C. gigas* have not been undertaken.

Here, we address the effect of temperature and host ontogeny on the antiviral response of *C. gigas*. Specifically, we hypothesize that (i) the oyster's antiviral response is compromised at elevated water temperatures and (ii) the antiviral response of adults and juvenile oysters is comparable. We test these hypotheses using a multifactorial experimental design by acclimating juvenile and adult *C. gigas* to 12 and 22^{o}C and measuring their immune response to poly I:C in comparison to sterile seawater controls. These temperatures were chosen because OsHV-1 causes mortality in France, USA and Australia between 16-25^{o}C [2, 8, 11], whereas OsHV-1 µvar is unable to be transmitted from positive to naïve oysters at temperatures below 13^{o}C [12]. Our results support the hypothesis that the physiology of juvenile oysters contributes to the aetiology of Pacific oyster mortality syndrome.

2.0 Materials and Methods

2.1 Animals and experimental conditions

Adult (1.5 years) and juvenile (0.8 years) Pacific oysters (wet weight: 11.45 ± 0.64 & 6.23 ± 1.1 grams, respectively) were kindly donated by Zippel Enterprises Pty Ltd, Smoky Bay, South Australia. Adult and juvenile oysters originated from the same family line spawned in January and September of 2012, respectively. Oysters were raised using standard commercial farming techniques. Oysters were delivered to Flinders

University in June 2013 by overnight courier and immediately placed in seawater recirculation system (salinity 35 ppt, temperature 16^{o}C). Prior to experimentation, a notch was filed in the shell adjacent to adductor muscle of each oyster using an electric bench grinder. Oysters were then returned to their recirculation system to recover for 24 hours. Next, oysters were transferred to aerated aquariums (40 L) maintained at either 12 or 22^{o}C and allowed to acclimatize for one week before experimentation. Water quality was assessed daily and oysters were not fed during this period.

At time 0 hours (prior to injection), juvenile and adult oysters maintained in aquaria at either 12 or 22^{o}C were injected in the adductor muscle with either poly I:C (Sigma, Cat# P0913, 5 mg.mL^{-1} in seawater) or sterile seawater (control) using a 25-gauge needle attached to a multi-dispensing pipette. Juvenile and adult oysters were injected with either 50 or 100 µl of poly I:C or seawater, respectively. Hemolymph samples were taken, using a sterile 23-gauge needle attached to a 1 mL syringe, from the adductor muscle of four individual oysters from each group at 0, 12 and 26 hours post-injection. Hemolymph was immediately centrifuged, and hemocyte cell pellets and cell-free hemolymph was snap frozen in liquid nitrogen and stored at -80^{o}C.

2.2 Total RNA extraction, first-strand synthesis and qPCR.

Total RNA was extracted from hemocyte pellets using the Isolate II RNA mini kit (Bioline) with on-column DNAse treatment following the manufacturer's protocol. The purity and quantity of purified RNA was estimated by spectrophometry (Thermo Scientific, ND-1000). First-strand synthesis was performed on 150 ng of total RNA using the Tetro cDNA synthesis kit (Bioline) following the manufacturers protocol. cDNA was diluted in ten-volumes of sterile water prior to use.

Twenty putative antiviral genes were selected for RT-qPCR analysis using the gene specific primers listed in Table 1. The PCR reaction volume was 6 µl and contained SensiFAST™ SYBR Lo-ROX master mix (Bioline), 100 nM of each specific primer and 1.5 ng of cDNA in a ViiA7™ thermocycler (Applied Biosystems) using an initial denaturation (95^{o}C for 2 min) followed by 40 cycles of denaturation (95^{o}C, 5 sec) and hybridisation-elongation step (60^{o}C, 30 sec). A subsequent melting temperature curve of the amplicon was performed. Expression of target genes were normalised with the elongation factor 1-alpha reference gene [23], which was stable in the current study ($p > 0.05$, CV = 2.2 %).

The relative expression of target genes was calculated using the ΔCt method using the formula: $2^{Ct(target)-Ct(reference)}$, with the cycle threshold (Ct) set at 1.0 for all genes.

2.3 Antiviral activity of hemolymph against HSV-1

Antiviral activity of cell-free hemolymph against herpes simplex virus type 1 (HSV-1) was determined by plaque assay, as described [20, 24]. Briefly, Vero cell monolayers in 24-well plates were infected in duplicate with 50 plaque forming units (PFU), in a total volume of 0.56 ml, in the presence of hemolymph (8 % v/v). Hemolymph was pre-incubated with the cell monolayers for 15 minutes prior to the addition of HSV-1 and the hemolymph was included for the duration of the assay. Cells were incubated for 2 days at 37°C before monolayers were fixed with 37% formaldehyde, stained with 2% toluidine blue and plaques counted using an inverted light microscope (Olympus CK2). Antiviral activity was expressed as percentage reduction in plaque numbers.

2.4 Statistical analysis

To determine statistical differences, univariate data was analysed using the computer software package, SPSS v.20 and differences were considered significant when $p < 0.05$. Four-way analysis of variance (ANOVA) was performed to test differences in gene expression and antiviral activity of the hemolymph against HSV-1. The four factors analysed were 'TREATMENT' with the levels 'poly I:C' and 'control', 'HOST ONTOGENY' with levels of 'adults' and 'juveniles', 'WATER TEMPERATURE' with levels of '12°C' and '22°C' and 'TIME-POINT' with three levels (0, 12 & 26 hours). Pairwise comparisons were performed using Tukey's HSD test when significant interactions between factors were identified. Normality and homogeneity of equal variances was assessed using Levene's test of equal variances and residual plots. Gene expression data that was skewed was log-transformed in order to meet the requirements for ANOVA.

To test the overall immune gene response, permutational multivariate analysis of variance (PERMANOVA) and principle component ordination (PCO) analyses were conducted using PRIMER V.6 + PERMONANOVA (Plymouth Marine Laboratory, UK) [25]. Similarity matrices were prepared using Euclidean distance and 9999 permutations of residuals were performed under a full model. Pair-wise tests were performed on significant interaction terms ($p < 0.05$). PCO was used to produce scatter plots to visually represent the multivariate differences among the significant factors. Vectors

overlaid on the graphs were based on variables (genes) with >0.8 correlations to the two primary PCO axes.

3.0 Results

3.1 Gene expression

The experimental design allowed us to investigate the influence of water temperature, age and the interaction of water temperature and age on the antiviral response of *C. gigas*. We measured the expression kinetics of 20 putative antiviral genes, which were normalised to the expression of elongation factor 1-alpha [23]. Elongation factor 1-alpha was found to be stable in the current study (CV = 2.2 %, $p > 0.05$). Injection of seawater (control) into the adductor muscle of *C. gigas* did not alter the mRNA levels of any of the target genes investigated. Poly I:C did increase the hemocyte mRNA levels of TLR, MyD88, IκB-1, Rel, MDA5, STING, IRF, SOC, PKR, viperin and Mpeg1 (Figure 1, $p < 0.01$). Temperature and age were both found to influence the response of these genes to poly I:C injection. At 22^{o}C, hemocytes from juvenile oysters had increased mRNA levels of TLR, MyD88, Rel, MDA5, IRF, SOC, PKR, viperin and Mpeg1 at 12 hours post-injection (Figure 1, $p < 0.05$). In contrast, the response of adult oysters at 22^{o}C was delayed, with increased mRNA levels of TLR, IRF, MDA5, STING, viperin and Mpeg1 occurring at 26 hours post-injection (Figure 1, $p < 0.05$). In adults, no up-regulation of individual target genes was observed at 12 hours post-injection ($p > 0.05$). At 12^{o}C, the antiviral response of juvenile oysters was inhibited with no significant change in mRNA levels at 12 or 26 hours post-injection (Figure 1, $p > 0.05$). Temperature also delayed the antiviral response of adult oysters at 12^{o}C with only IRF and SOC being up-regulated at 26 hours post-injection (Figure 1, $p < 0.05$).

Poly I:C, temperature, age and their respective interactions had no significant effect on mRNA levels of IκB-2, IK cytokine, cytokine receptors 1 and 2, IL-17D, IL-17D receptor, NOS, and multi-copper oxidase (Figure 1, $p > 0.05$). Temperature influenced mRNA levels of MPO ($p < 0.01$), with a 0.54-fold decrease in the expression of MPO at 22^{o}C compared to 12^{o}C.

Multivariate PERMANOVA revealed no significant four-way interactions between treatment, ontogeny, temperature and time (pseudo F = 0.243, $p = 0.845$). However, there were significant three-way interactions between injection x ontogeny x temperature (pseudo F = 3.346, $p = 0.044$), as well as between treatment x temperature

x time (pseudo F = 5.286, p = 0.01). Pairwise tests confirmed the finding above by revealing that the effects of poly I:C injection were significant at both temperatures for adult oysters ($p < 0.005$), whereas juvenile only showed a significant response to poly I:C injection at 22°C ($p < 0.0001$), but not at 12°C ($p = 0.061$).

PCO was able to account for 87.4% of the variation in the data on two axes (Figure 2). The injection controls were found to group tightly towards the right hand side of the plot, whereas the poly I:C injected oysters showed much greater variability (Figure 2A). The difference between controls and poly I:C was driven by a large number of genes, including SOC1, IRF, Viperin, TLR, Rel, PKR, MDA5 and Mpeg1 (Figure 2). The effects of temperature appear more visible along the second PCO Y axis, with oysters exposed to 12°C separating out higher on the plot compared to those at 22°C (Figure 2A & 2B). The temperature effect is largely driven by difference in Cytk-R2, which is negatively correlated to IκB-1 expression levels (Figure 2A). Adults and juveniles showed a similar spread in the data points, with the effects of temperature (Figure 2B) and injection treatment apparent at both developmental stages. The interaction between time and treatment can be seen along PCO1 in Figure 2C, with the data taken at time 0 hours clustering with all the controls towards the right of the plot, and much more variation in the poly I:C injected oysters after 12 and 26 hours (Figure 2C).

3.2 Humoral factors

The hemolymph of *C. gigas* exhibited anti-HSV-1 activity. Addition of cell-free hemolymph to Vero cell monolayers (8 % v/v) reduced the number of HSV-1 plaques by 62.2 %. Treatment, age, temperature and their interactions did not alter the anti-HSV-1 activity of the hemolymph ($p > 0.05$, data not presented).

4.0 Discussion

Herpesviruses pose a large threat to worldwide production of Pacific oysters. Field and laboratory studies have revealed younger developmental stages of *C. gigas* are more sensitive to infection by OsHV-1 µvar [2, 7, 8], and mortality coincides with water temperatures between 17 to 24°C [7, 8]. It has often been suggested that the increased mortality of younger *C. gigas* reflects an immature immune system [2, 10, 26]. High water temperatures and associated low dissolved oxygen may further compromise their

248 immune system. Our study showed that juvenile oysters at 22°C respond vigorously to a
249 virus-associated molecular pattern.
250 To date, two studies have investigated the antiviral response of *C. gigas* against
251 OsHV-1 and both these studies have demonstrated that the immune system of *C. gigas* is
252 not ignorant of virus infection with increased expression of genes involved in virus
253 recognition, signalling and immunity [17, 22]. Oysters can recognise different virus-
254 associated molecular patterns and can respond in a specific manner at the
255 transcriptional level [19]. Numerous studies have demonstrated that the hemolymph of
256 *C. gigas* contains a compound(s) with broad-spectrum antiviral activity [20, 21, 27, 28].
257 These compounds are yet to be identified, but display virucidal activity against HSV-1
258 and also inhibit HSV-1 replication [28]. In the current study, we measured cellular and
259 humoral responses to a virus-associated molecular pattern. We chose poly I:C because
260 previous research has demonstrated it induces the same antiviral gene expression
261 profiles as experimental infection with OsHV-1 µvar [17]. In the current study, poly I:C
262 appears to induce a cellular response targeted at preventing virus transcription and
263 replication as opposed to inducing the production and/or release of humoral antiviral
264 (anti-HSV-1) compounds. This observation is consistent with a previous study
265 measuring the antiviral activity of the hemolymph of hybrid abalone, *Haliotis rubra* x
266 *laevigata* infected with abalone herpesvirus (AbHV-1) [29]. Seasonal variations in the
267 anti-HSV-1 activity of the hemolymph have been reported for *C. gigas* and *H. rubra* with
268 both species displaying higher anti-HSV-1 activity during summer [20, 30]. In contrast,
269 we observed that temperature and oyster age had no effect on anti-HSV-1 activity of the
270 hemolymph of *C. gigas*, suggesting the antiviral compounds are constitutively expressed.
271 Double-stranded RNA, either viral or synthetic, is an efficient inducer of
272 interferon signalling pathways in vertebrates [18]. The antiviral action of interferon is
273 mediated by a two-step pathway, which has been characterised in detail and reviewed
274 comprehensively [18, 31]. Typically, transcription of the interferon gene occurs when
275 virus-infected cells recognise dsRNA produced during virus replication [18]. Vertebrate
276 cells recognise dsRNA using an evolutionarily conserved set of pattern recognition
277 receptors, including toll-like receptor 3 (TLR3) and members of retinoic acid inducible
278 gene I (RIG-1) family [18]. Upon recognition of dsRNA, these receptors initiate complex
279 signalling pathways resulting in IRF-3 (interferon regulatory factor 3) and NF-κB
280 assembling on the promoter region of the interferon gene [18]. Interferon is then

281 secreted by virus-infected cells [31]. Interferon exerts an antiviral effect by binding to
282 interferon receptors on neighbouring cells and triggers signal transduction through the
283 JAK (Janus activated kinase)-STAT (signal transducer and activation of transcription)
284 pathway resulting in up-regulation of several hundred interferon-stimulated genes
285 (ISGs), including protein kinase R (PKR), 2'5'-oligoadenylate synthetase (OAS), Mx and
286 viperin [18, 31].

287 No invertebrate has been shown to have an obvious homolog of interferon [32].
288 However, an equivalent pathway is likely present in the oyster (Figure 3) [17]. In the
289 current study, poly I:C induced expression of putative dsRNA recognition receptors (TLR
290 & MDA5), downstream signalling molecules (MyD88, IκB-1, Rel, STING, IRF & SOC-1)
291 and several interferon-stimulated genes (PKR & viperin) (Figure 1), which suggests at
292 least two dsRNA-signalling pathways (TLR-dependent & MDA5-dependent) are present
293 in the oyster (see Figure 3). PCO revealed positive correlatons in the expression of most
294 of these receptor and signalling genes in response to poly I:C injection (Figure 2),
295 suggesting that these two pathway are initiated simultaneously. The purpose of the
296 vertebrate interferon pathway is to induce ISGs that limit synthesis of viral proteins and
297 induce programmed cell death (autophagy and apoptosis) of virus-infected cells [18,
298 33]. Data in Genbank suggests the oyster possesses several classic ISGs, including PKR,
299 OAS, Mx, ADAR and viperin. PKR has been studied intensively and in response to its
300 cofactor, dsRNA, it undergoes activation to inhibit viral and cellular protein synthesis by
301 phosphorylating eukaryotic initiation factor 2 (eIF2) [18]. PKR is also required for viral
302 induced autophagy and apoptosis [34]. Viperin's mode of action is unclear, but it may
303 impair virus budding [33].

304 Relatively few studies have investigated the influence of temperature or host
305 ontogeny on the immunological response of *C. gigas* [35-38]. *In vivo* and *vitro* studies
306 suggest *C. gigas* is immunocompromised at elevated seawater temperature [35, 36]. *C.*
307 *gigas* at 21^{o}C have reduced numbers of hemocytes.mL^{-1} and decreased phagocytosis
308 activity in the hemolymph compared to oysters at 12^{o}C [36]. Our results provide the
309 first evidence that temperature and host ontogeny influence the antiviral response of *C.*
310 *gigas* (Figure 1). Complex interactions appear to occur with temperature over time, and
311 whilst some genes such as Cytk-R2 appear to suppressed at 22^{o}C, others such as Cytk-R1
312 and IκB-1 were elevated in response to the higher temperature (Figure 1 & 2). Similarly,
313 trade-offs between antibacterial and antiviral immune responses have been reported for

abalone under temperature stress [30]. However, in contrast to previous studies investigating the immunological response to bacteria infections [35, 36], 12°C delayed the antiviral response of adults and inhibited the response of juveniles. At 22°C, juvenile oysters responded vigorously to poly I:C injection (Figure 1). Experimental trials show mortality of juvenile *C. gigas* peaks within three days after infection with OsHV-1 µvar [26, 39] and peak mortality of adults occurs after juveniles (authors personal observations). We speculate whether the vigorous response of juvenile oysters results in a "cytokine storm" leading to tissue dysfunction and death of the oyster. The antiviral response observed would prevent transcription and translation of host and virus proteins (program cell death) and may explain the multifocal and extensive ulcerative lesions observed in epithelium tissue of *C. gigas* infected with OsHV-1 µvar [2].

The antiviral response of *C. gigas* against poly I:C may show differences to the response against OsHV-1 µvar. All viruses have evolved mechanisms to circumvent and modulate the host immune response [18]. In the present study, a high proportion of the inducible genes (TLR, IRF, Rel, PKR & Mpeg1) are also up-regulated in *C. gigas* in response to experimental OsHV-1 infection [17, 22]. We were surprised that IK cytokine, myeloperoxidase (MPO) and the multi-copper oxidase were not induced in this study (Figure 1). Experimental herpesvirus infections in the scallop, *Chlamys farreri* with acute viral necrobiotic virus (AVNV) have found increased plasma enzyme activities for MPO and phenoloxidase [40] and increased hemocyte mRNA transcript levels for a multicopper oxidase (phenoloxidase) [41]. Likewise, *C. gigas* up-regulates a multicopper oxidase and IK cytokine in response to OsHV-1 [22]. The failure of poly I:C to induce the expression of MPO and multicopper oxidase (Figure 1) suggests these genes are induced by other virus-associated molecular patterns, damage-associated molecular patterns (DAMPs) or microbial components associated with herpesvirus infection (i.e. *Vibrio* sp.). Previous studies have demonstrated IK cytokine is up-regulated in the Sydney rock oyster, *Saccostrea glomerata* in response to poly I:C [19].

Conclusion

Presence of OsHV-1 µvar is insufficient on its own to cause significant mortality of juvenile *C. gigas* [4]. Host ontogeny and water temperature are causal factors in the aetiology of Pacific oyster mortality syndrome. Our results demonstrate *C. gigas* relies on a cellular response to prevent virus replication and this antiviral response is

influenced both by seawater temperature and host ontogeny. Whether the vigorous antiviral response of juvenile oysters at 22°C results in an immune mediated disorder leading to higher mortality is worthy of further investigation. Future studies should focus on comparing the antiviral response of adult and juvenile *C. gigas* against OsHV-1 µvar.

Acknowledgements

The authors acknowledge the donation of oysters from Gary Zippel, laboratory assistance from David White and research funds provided by the Australian Seafood Cooperative Research Centre (Grant: 2011/758).

5.0 References

1. Wendling CC, Wegner KM. Relative contribution of reproductive investment, thermal stress and *Vibrio* infection to summer mortality phenomena in Pacific oysters. Aquaculture. 2013 412:88-96.
2. Jenkins C, Hick P, Gabor M, Spiers Z, Fell SA, Gu X, et al. Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 u-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis Aquat Org. 2013 105:109-26.
3. Segarra A, Pepin JF, Arzul I, Morga B, Faury N, Renault T. Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Res. 2010 153:92-9.
4. Paul-Pont I, Dhand NK, Whittington RJ. Spatial distribution of mortality in Pacific oyster *Crassostrea gigas*: reflection on mechanisms of OsHV-1 transmission. Dis Aquat Org. 2013.
5. Renault T, Moreau P, Faury N, Pepin J-F, Segarra A, Webb S. Analysis of clinical ostreid herpesvirus 1 (*Malacoherpesviridae*) specimens by sequencing amplified frangments from three virus genome areas. Journal of Virology. 2012 86:5942-7.
6. Paul-Pont I, Dhand NK, Whittington RJ. Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. Aquaculture. 2013 412-413:202-14.
7. Peeler EJ, Allan Reese R, Cheslett D, Geoghegan F, Power A, Thrush MA. Investigation of mortality in Pacific oysters associated with *Ostreid herpevirus-1 uvar* in the Republic of Ireland in 20089. Prev Vet Med. 2012 105:136-43.
8. Pernet F, Barret J, Le Gall P, Corporeau C, LDegremont L, Lagarde F, et al. Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquaculture Environ Interact. 2012 2:215-37.
9. Dégremont L. Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. Aquaculture. 2011 317:94-8.

10. Garcia D, Thebault A, Degremont L, Arzul I, Miossec L, Robert M, et al. Ostreid herpesvirus 1 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. Vet Res. 2011 42:73-84.
11. Burge CA, Griffin FJ, Friedman CS. Mortality and herpesvirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA. Dis Aquat Org. 2006 72:31-43.
12. Petton B, Pernet F, Robert R, Boudry P. Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. Aquacult Environ Interact. 2013 3:257-73.
13. St-Hilaire S, Beevers N, Way K, Le Deuff RM, Martin P, Joiner C. Reactivation of koi herpesvirus infection in common carp (*Cyprinus carpio*). Dis Aquat Org. 2005 67:15-23.
14. Wang X, Wang L, Zhang H, Ji Q, Song L, Qiu L, et al. Immune response and energy metabolism of *Chlamys farreri* under anguillarum challenge and high temperature exposure. Fish Shellfish Immunol. 2012 33:1016-26.
15. Beaz-Hidalgo R, Balboa S, Romalde JL, Figueras MJ. Diversity and pathogenecity of *Vibrio* species in cultured bivalve molluscs. Environ Microbiol Rep. 2010 2:34-43.
16. Schmitt P, Duperthuy M, Montagnani C, Bachere E, Destoumieux-Garzon D. Immune responses in the Pacific oyster *Crassostrea gigas*: an overview with focus on summer mortalities. In: Qin JG, editor. Oysters: physiology, ecological distribution and mortality: Nova Science Publishers; 2011, p. 311.
17. Green TJ, Montagnani C. Poly I:C induces a protective antiviral immune response in the Pacific oyster (*Crassostrea gigas*) against subsequent challenge with Ostreid *herpesvirus* (OsHV-1 µvar). Fish Shellfish Immunol. 2013 35:382-8.
18. Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and viral countermeasures. J Gen Virol. 2008 89:1-47.
19. Green TJ, Barnes AC. Inhibitor of REL/NF-kB is regulated in Sydney rock oysters in response to specific double-stranded RNA and *Vibrio alginolyticus*, but the major immune anti-oxidants EcSOD and Prx6 are non-inducible. Fish Shellfish Immunol. 2009 27:260-5.
20. Olicard C, Didier Y, Marty C, Bourgougnon N, Renault T. *In vitro* research of anti-HSV-1 activity in different extracts from Pacific oysters *Crassostrea gigas*. Dis Aquat Org. 2005 67:141-7.
21. Olicard C, Renault T, Torhy C, Benmansour A, Bourgougnon N. Putative antiviral activity in hemolymph from adult Pacific oysters, *Crassostrea gigas*. Antiviral Res. 2005 66:147-52.
22. Renault T, Faury N, Barbosa-Solomieu V, Moreau K. Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, *Crassostrea gigas*, challenged with Ostreid herpesvirus 1. Dev Comp Immunol. 2011 35:725-35.
23. de Lorgeril J, Zenagui R, Rosa R, Piquemal D, Bachere E. Whole transcriptome profiling of successful immune response to *Vibrio* infections in the oyster *Crassostrea gigas* by digital gene expression analysis. Plos One. 2011 6:e23142.
24. Dang VT, Speck P, Doroudi M, Smith B, Benkendorff K. Variation in the antiviral and antibacterial activity of abalone *Haliotis laevigata, H. rubra* and their hybrid in South Australia. Aquaculture. 2011 315:242-9.
25. Anderson MJ, Gorley RN, Clarke KR. PERMANOVA+ for PRIMER: Guide to software and statistical methods.: Plymouth Marine Laboratory; 2008.

26. Schikorski D, Renault T, Saulnier D, Faury N, Moreau P, Pepin JF. Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. Vet Res. 2011 42:27.
27. Zeng M, CUI W, Zhao Y, Liu Z, Dong S, Guo Y. Antiviral active peptide from oyster. Chinese Journal of Oceanology and Limnology. 2008 26:307-12.
28. Carriel-Gomes MC, Kratz JM, Muller VDM, Barardi CRM, Simoes CMO. Evaluation of antiviral activity in the hemolymph from oysters *Crassostrea rhizophorae* and *Crassostreea gigas*. Aquat Living Resour. 2006 19:189-93.
29. Dang VT, Benkendorff K, Corbeil S, Williams LM, Hoad J, Crane MSJ, et al. Immunological changes in response to herpesvirus infection in abalone *Haliotis laevigata* and *Haliotis rubra* hybrids. Fish Shellfish Immunol. 2013 34:688-91.
30. Dang VT, Speck P, Benkendorff K. Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*. Fish Shellfish Immunol. 2012 32:732-40.
31. Robertsen B. The interferon system of teleost fish. Fish Shellfish Immunol. 2006 20:172-91.
32. Loker ES, Adema CM, Zhang S-M, Kepler TB. Invertebrate immune systems - not homogeneous, not simple, not well understood. Immunolog Rev. 2004 198:10-24.
33. Mattijseen S, Pruijn GJM. Viperin, a key player in the antiviral response. Microbes Infect. 2012 14:419-26.
34. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009 43:67-93.
35. Gagnaire B, Frouin H, Moreau K, Thomas-Guyon H, Renault T. Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish Shellfish Immunol. 2006 20:536-47.
36. Malham SK, Cotter E, O'Keeffe S, Lynch S, Culloty SC, King JW, et al. Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: The influence of temperature and nutrients on health and survival. Aquaculture. 2009 287:128-38.
37. Tirape´ A, Bacque C, Brizard R, Vandenbulcke F, Boulo V. Expression of immune-related genes in the oyster Crassostrea gigas during ontogenesis. Dev Comp Immunol. 2007 31:859-73.
38. Montagnani C, Tirape´ A, Boulo V, Escoubas JM. The two Cg-timp mRNAs expressed in oyster hemocytes are generated by two gene families and differentially expressed during ontogenesis. Dev Comp Immunol. 2005 29:831-9.
39. Schikorski D, Faury N, Pepin JF, Saulnier D, Tourbiez D, Renault T. Experimental ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas*: Kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. Virus Res. 2011 155:28-34.
40. Xing J, Lin T, Zhan W. Variations of enzyme activities in the haemocytes of scallop *Chlamys farreri* after infection with the acute virus necrobiotic virus (AVNV). Fish Shellfish Immunol. 2008 25:847-52.
41. Chen G, Wang C, Zhang C, Wang Y, Xu Z, Wang C. A preliminary study of differentially expressed genes of the scallop *Chlamys farreri* against acute viral necrobiotic virus (AVNV). Fish Shellfish Immunol. 2013 34:1619-27.
42. Zhang Y, He X, Yu Z. Two homologues of inhibitor of NF-Kappa B (IkB) are involved in the immune defense of the Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol. 2011 30:1354-61.
43. Montagnani C, Kappler C, Reichhart JM, Escoubas JM. Cg-Rel, the first Rel/NF-kB homolog characterized in a mollusk, the Pacific oyster *Crassostrea gigas*. FEBS Lett. 2004 561:75-82.

485 44. Roberts S, Gueguen Y, Lorgeril Jd, Goetz F. Rapid accumulation of an interleukin
486 17 homolog transcript in *Crassostrea gigas* hemocytes following bacterial exposure. Dev
487 Comp Immunol. 2008 32:1099-104.
488
489
490

Table Legend

Table 1: Primer pairs used in RT qPCR expression analysis. The Genbank accession number and gene function is provided for each gene. Previously published primer pairs are referenced.

Figure Legends

Figure 1: Expression heat map (**A**) illustrating the average relative expression levels ($2^{\Delta CT}$) for all target genes according to treatment, host ontogeny, water temperature and time. For each gene, the largest expression values are displayed in red and the smallest values in black. Relative expression for TLR (**B**), MDA-5 (**C**), STING (**D**), IRF (E), PKR (**F**) and Viperin (**G**) are presented in individual graphs. Gene expression is presented relative to the EFU house-keeping gene ($2^{\Delta CT}$, mean ± SE). Differences in relative gene expression was determined using four-way analysis of variance (ANOVA). Asterisks denote significant differences between poly I:C and control group ($p < 0.05$, Tukey's HSD).

Figure 2: Principle component ordination (PCO) for the multivariate gene expression in response to (**A**) temperature (12 and 22^{o}C labels) and injection with sterile seawater (black filled upwards triangles) or poly I:C (grey filled downward triangles); (**B**) temperature and ontogeny (juveniles = black filled downward triangles and adults = grey filled upward triangles); and (**C**) time (0, 12 & 26 hour labels) and injection with sterile seawater (black filled upwards triangles) or poly I:C (grey filled downward triangles). Vector overlays indicate genes with correlation coefficients > 0.8.

Figure 3: Conceptual diagram of the inducible antiviral response of *Crassotrea gigas*. We hypothesize *C. gigas* recognises dsRNA using evolutionarily conserved dsRNA recognition receptors (TLR & MDA5). Upon recognistion, these receptors instigate TLR-dependent and MDA5-dependent signalling resulting in the transcription factors, Rel and IRF, to trans-locate to the cell nucleus resulting in the induction of cytokines and antimicrobial peptides. Cytokines are secreted from viral infected cells. Cytokines signal through the Jak-STAT pathway resulting in the induction of interferon stimulated genes (ISGs), such as protein kinase R (PKR) and viperin.

Table 1
Primer pairs used in RT qPCR expression analysis. The Genbank accession number and gene function is provided for each gene. Previously published primer pairs are referenced.

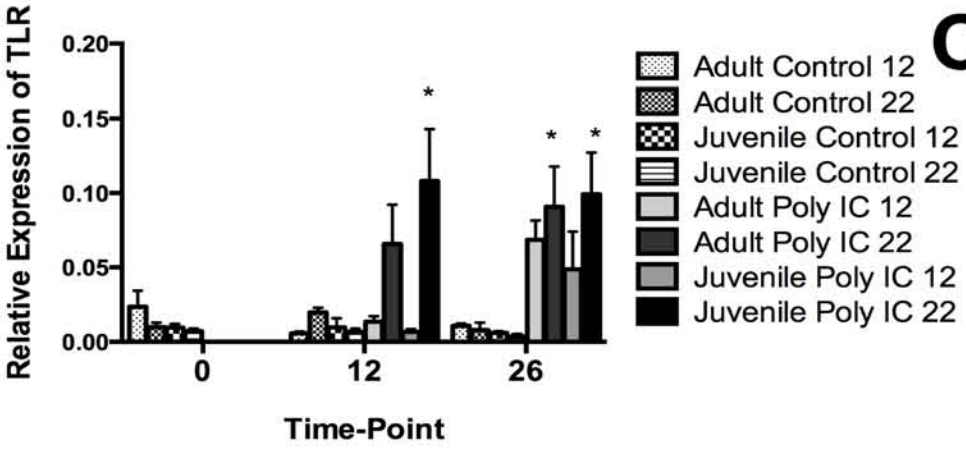
Gene	Function	Accession#	Sense primer	Antisense primer	Reference
EFU	Reference Gene	ABI22066	GAGCGTGAACGTGGTATCAC	ACAGCACAGTCAGCCTGTGA	[23]
TLR	Pathogen Recognition Receptor		GCAGGACTCCACTTTCTCAC	GTTGGCACCCAGGTAAAGG	[17]
MyD88	Cell-Signalling	EKC40070	GTGACTACACCAAGCAGGAC	GTACTGACCCTGAGTTCTGC	
IκB-1	Cell-Signalling	DQ250326	GAAAAAGTGGCAAGAGTGTC	GAAGAGTCATCGAAAGCAAC	[17]
IκB-2	Cell-Signalling	HQ650768	GCTCGGAAGTAAATGAAGTG	CTGGAGTTCTTGAGGTCTGC	[42]
Rel	Cell-Signalling	AAK72690	GCTGAACCAGAACCTCATGA	CGAAGGACATGTTCTGATCC	[43]
MDA5	Pathogen Recognition Receptor	EKC38304	CAACAACATGGGAAGTATGGTG	TCGGTCTGTTAACTGCGGAC	
STING	Cell-Signalling	EKC29965	CTGCTATTGTCCGCCATC	GAATGGGCGTGGCATACTC	
IRF	Cell-Signalling	EKC43155	CGAAACGCAGAAACTGTTC	ATTTGCCTTCCATCTTTTGG	[17]
IK Cytokine	Cell-Signalling	EF627976	GGAGCGCGAGGAAGAGGAGATAATGG	ATCCGTCCCGGCAGAAACAGCTC	[22]
Cytokine R1	Cell-Signalling		GTCGCACAGTCCGATACAAAT	AAGGCAACAGACTCGGGTATT	
Cytokine R2	Cell-Signalling	EKC24772	AGCGCCTTTGTATGTGAGCTG	TGCTGGTCGCAGAGTTGAATG	
SOC-1	Cell-Signalling	EKC24772	CAAGAGAGAATCTGTGGGAAC	GCATCTTAGCACTAATTCTCTC	
IL-17D	Cell-Signalling	ABO93467	ACTGAGGCTCGATGCAAGTG	AGCCTTCTTGCTTCATGTGG	[44]
IL-17D Receptor	Cell-Signalling	EKC22301	TGATTGTGGACCAGCCTGAC	CACGATGATGAGACCCAGC	
PKR	IFN Stimulated Gene	EKC34807	GAGCATCAGCAAAGTGTTGAG	GTAGCACCAGGAGATGGTTC	[17]
Viperin	IFN Stimulated Gene	EKC28205	GCTTTGACCCGGAAACCAAC	TGACACCAATCCCGAACTCG	
Mpeg1	Effector Molecule	EF672979	GCCACCGAAAGCCGGAGAAGATGTC	ACCGAGACCGAGTTTCAGGGGGTAG	[22]
NOS	Effector Molecule	EKC33784	GATGGGAAAAGCTCTAGCAAG	GTTTCCAAAGGTACTGGTCAC	
MPO	Effector Molecule	EKC40014	CGGGACGTTAGCAACATTC	TGCTCTCCGCAACATGATAG	
Multicopper oxidase	Effector Molecule	EU678320	TGGTTCCTGCATTGTCACAT	AAGAGTATCAGCCGCGAAAA	[22]

A

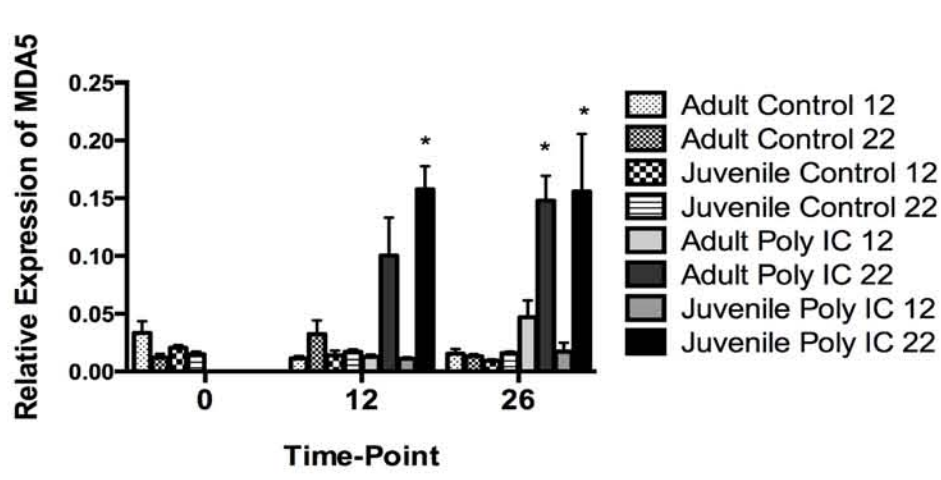
Treament	Control												Poly I:C								
Development	Adult						Juvenile						Adult				Juvenile				
Temperature	12	12	12	22	22	22	12	12	12	22	22	22	12	12	22	22	12	12	22	22	
Time-Point (hrs)	0	12	26	0	12	26	0	12	26	0	12	26	12	26	12	26	12	26	12	26	
TLR3																					
MyD88																					
IkB-1																					
Rel																					
IRF																					
MDA5																					
STING																					
SOC-1																					
PKR																					
Viperin																					
Mpeg1																					
IK																					
CytokR1																					
CytokR2																					
IkB-2																					
IL-17D																					
IL-17Dr																					
NOS																					
MPO																					
Laccase																					

Low Medium High

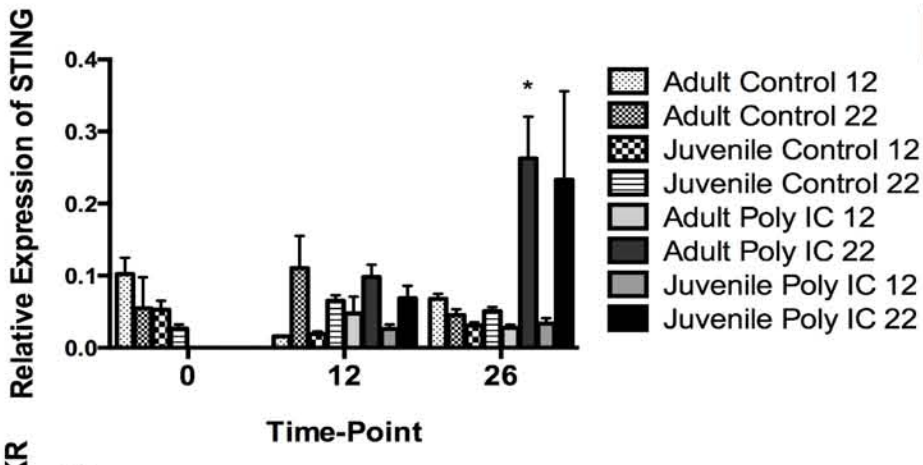
B



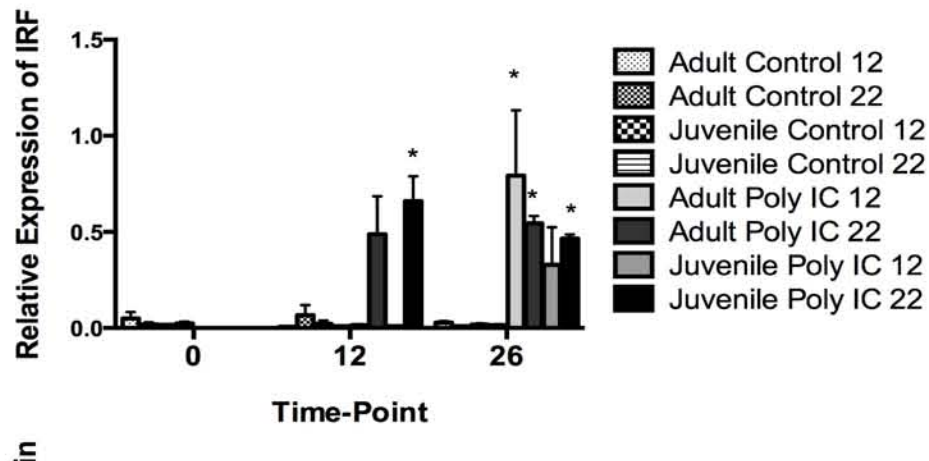
C



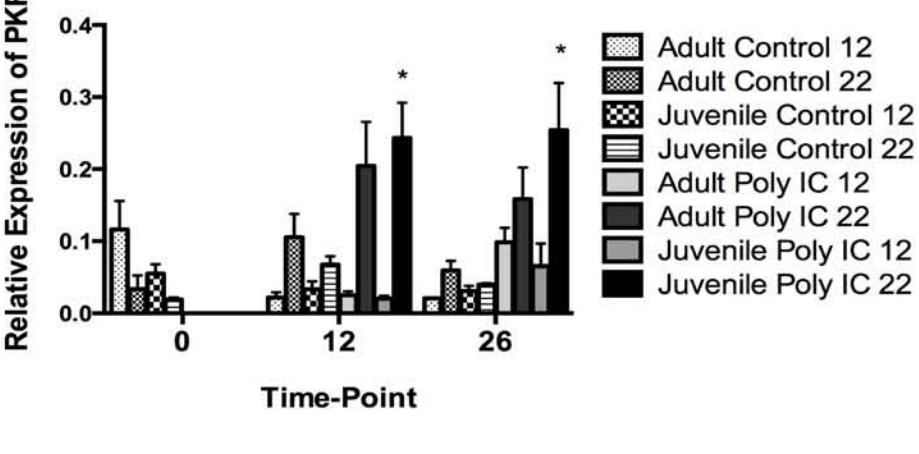
D



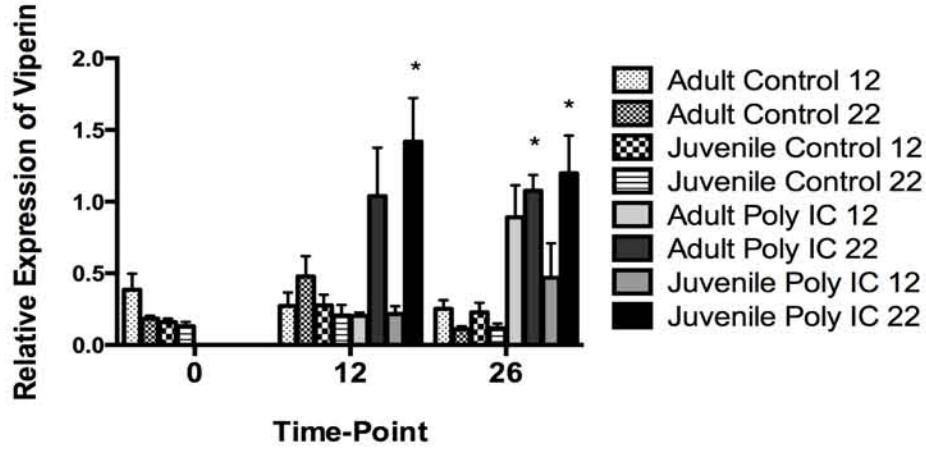
E

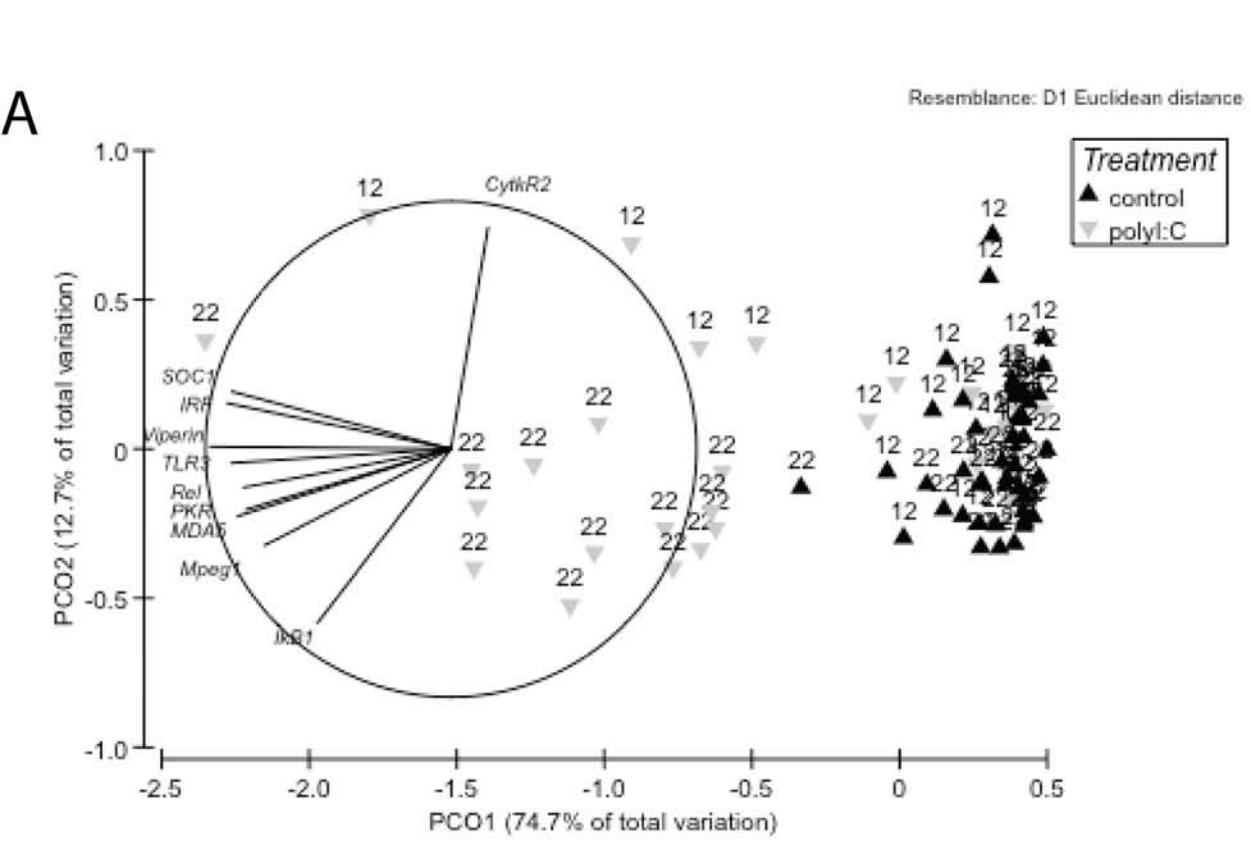


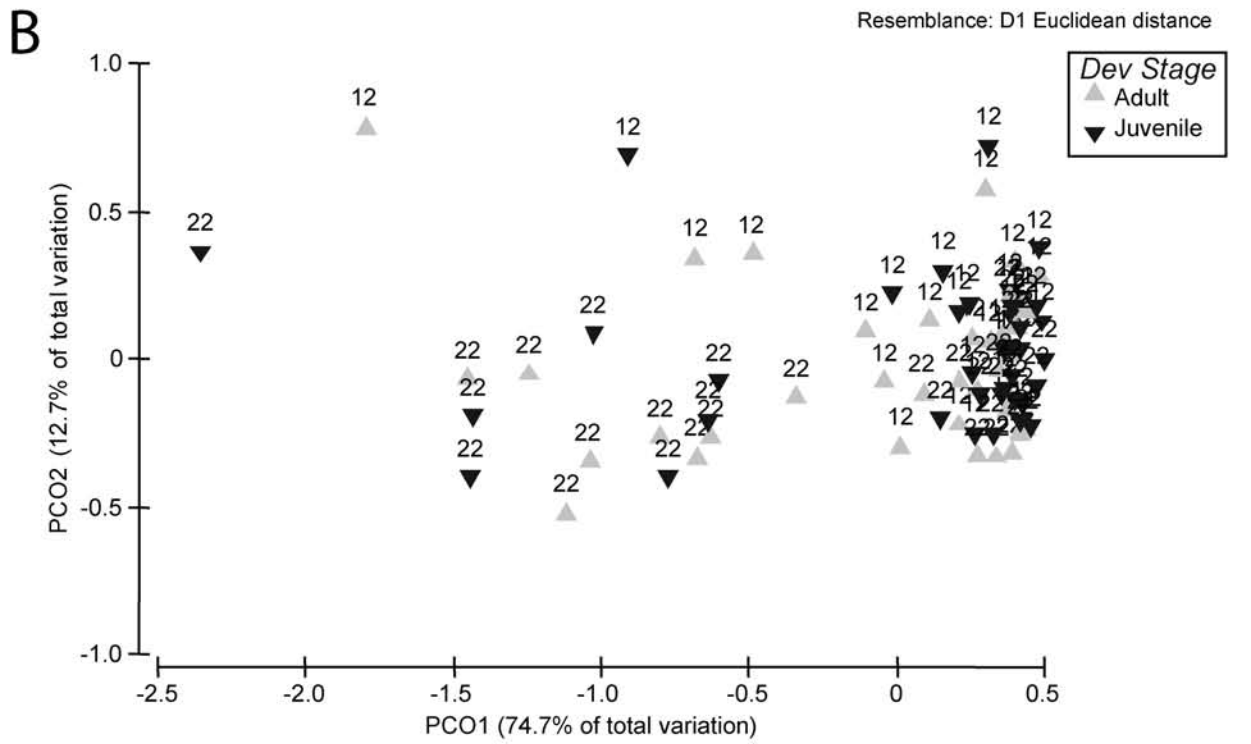
F

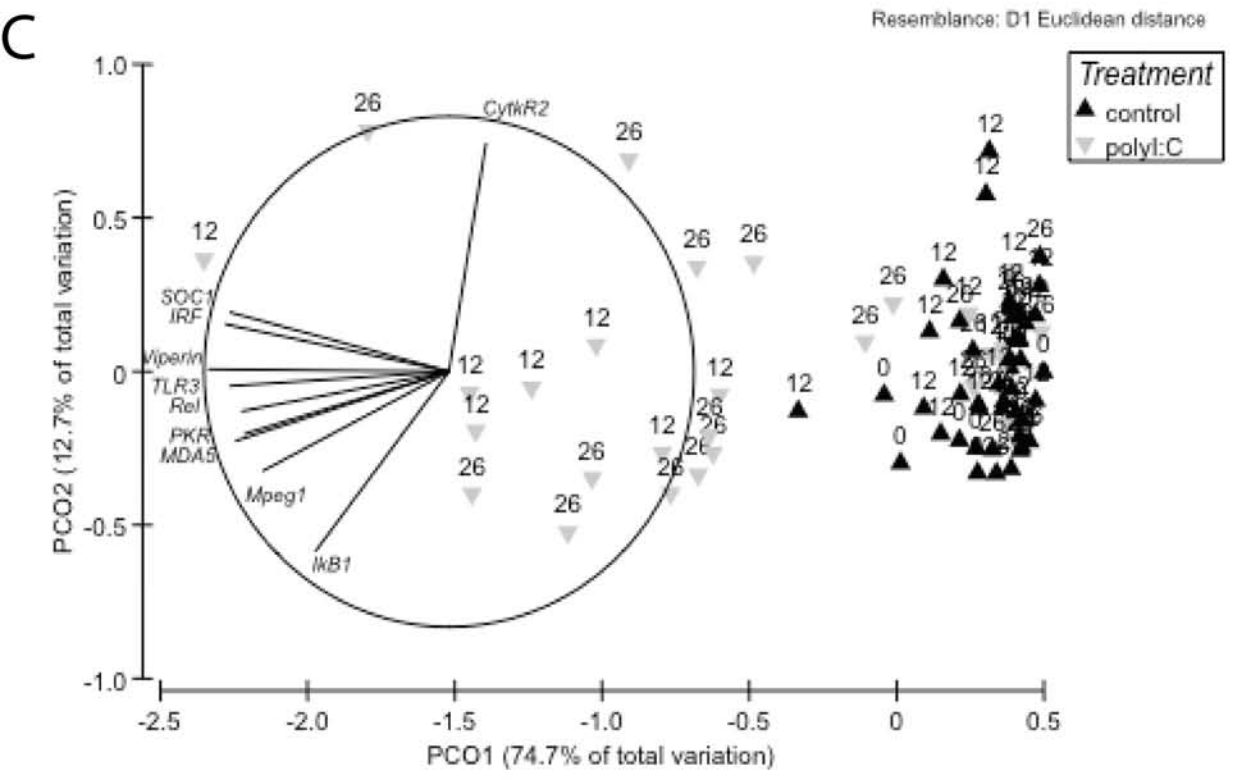


G




A
Resemblance: D1 Euclidean distance
Treatment
control
polyl:C
CytkR2
SOC1
IRF
Viperin
TLR3
Rel
PKR
MDA5
Mpeg1
IkB1
PCO2 (12.7% of total variation)
PCO1 (74.7% of total variation)


B
Resemblance: D1 Euclidean distance
Dev Stage
Adult
Juvenile
PCO2 (12.7% of total variation)
PCO1 (74.7% of total variation)


C
Resemblance: D1 Euclidean distance
Treatment
control
polyl:C
CytkR2
SOC1
IRF
Viperin
TLR3
Rel
PKR
MDA5
Mpeg1
IkB1
PCO2 (12.7% of total variation)
PCO1 (74.7% of total variation)

OsHV-1

dsRNA
ssRNA
CpG DNA

TLR

Cytokines

Cytokine Receptor

Membrane

Cytoplasm

JAK

MyD88

IRAK1

IRAK1

TRAF6

dsDNA

Short dsRNA

Long dsRNA

DDX41

RIG-1

MDA-5

STING

ISP-1

SOC

IKK

IkB

NF-kB

IRF

STAT

Cytoplasm

Nucleus

Transcriptional Activation → Immune Response

(Cytokines, Antimicrobial Peptides, *etc*)

Transcriptional Activation → ISRE genes

(*i.e.* PKR & viperin)