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

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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.

1. 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 °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 an 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 °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

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exposed to Koi herpesvirus (KHV) at 11 °C do not succumb to the disease until water temperature is elevated to 23 °C, and these fish do not shed the virus and infect naïve fish during co-habitation experiments unless temperatures are above 20 °C, suggesting the virus is in an altered transcriptional state below 20 °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 °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 *Vibrio splendidus* are well documented in the literature [reviewed by Ref. [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* haemolymph 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 °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 and 25 °C [2,8,11], whereas OsHV-1 μvar is unable to be transmitted from positive to naïve oysters at temperatures below 13 °C [12]. Our results support the hypothesis that the physiology of juvenile oysters contributes to the aetiology of Pacific oyster mortality syndrome.

2. 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 g, 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 °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 h. Next, oysters were transferred to aerated aquariums (40 L) maintained at either 12 or 22 °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 h (prior to injection), juvenile and adult oysters maintained in aquaria at either 12 or 22 °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 h post-injection. Hemolymph was immediately centrifuged, and hemocyte cell pellets and cell-free hemolymph was snap frozen in liquid nitrogen and stored at −80 °C.

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]