

THE EFFECTS OF 4-NONYLPHENOL ON THE IMMUNE RESPONSE OF THE
PACIFIC OYSTER, *CRASSOSTREA GIGAS*, FOLLOWING
BACTERIAL INFECTION (*VIBRIO CAMPBELLII*)

A Thesis
presented to
the Faculty of California Polytechnic State University,
San Luis Obispo

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Biology

by
Courtney Elizabeth Hart
June 2016

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TITLE: The Effects of 4-nonylphenol on the Immune Response of the Pacific Oyster, *Crassostrea gigas*, Following Bacterial Infection (*Vibrio campbellii*)

AUTHOR: Courtney Elizabeth Hart

DATE SUBMITTED: June 2016

COMMITTEE CHAIR: Dr. Kristin Hardy, Ph.D.
Assistant Professor of Biological Sciences

COMMITTEE MEMBER: Dr. Sean Lema, Ph.D.
Associate Professor of Biological Sciences

COMMITTEE MEMBER: Dr. Lars Tomanek, Ph.D.
Associate Professor of Biological Sciences

ABSTRACT

The Effects of 4-nonylphenol on the Immune Response of the Pacific oyster, *Crassostrea gigas*, Following Bacterial Infection (*Vibrio campbellii*)

Courtney Elizabeth Hart

Endocrine disrupting chemicals (EDCs) are compounds that can interfere with hormone signaling pathways and are now recognized as pervasive in estuarine and marine waters. One prevalent EDC in California's coastal waters is the xenoestrogen 4-nonylphenol (4-NP), which has been shown to impair reproduction, development, growth, and in some cases immune function of marine invertebrates. To further investigate effects of 4-NP on marine invertebrate immune function we measured total hemocyte counts (THC), relative transcript abundance of immune-relevant genes, and lysozyme activity in Pacific oysters (*Crassostrea gigas*) following bacterial infection. To quantify these effects we exposed oysters to dissolved phase 4-NP at high ($100 \mu\text{g l}^{-1}$), low ($2 \mu\text{g l}^{-1}$), or control (100 μl ethanol) concentrations for 7 days, and then experimentally infected (via injection into the adductor muscle) the oysters with the marine bacterium *Vibrio campbellii*. 4-NP significantly altered the effects of bacterial infection had on THC. Oysters exposed to both high and low 4-NP did not experience a bacteria-induced increase in THC, as seen in control oysters. We also determined that *V. campbellii* infection induced differential expression of a subset of immune-related genes tested (*Cg-bigdef2*, *Cg-bpi1*, *Cg-lys1*, *Cg-timp*) in some, but not all, tissues; 4-NP exposure altered expression patterns in two of these genes (*Cg-bpi1* and *Cg-tgase*). Exposure to 4-NP alone also caused differential expression in some genes (*Cg-bpi1*, *Cg-galectin1*, *Cg-clec2*). Lastly, low levels of 4-NP significantly increased lysozyme activity 24 h post-infection. These results suggest that

exposure to 4-NP can alter both cellular and humoral immune responses to bacterial infection in *C. gigas*.

Keywords

Crassostrea gigas, endocrine disrupting chemicals, 4-nonylphenol, immunotoxicology, hemocyte, gene expression, lysozyme, transcriptomics

ACKNOWLEDGMENTS

Funding

Cal Poly State University, San Luis Obispo
California Sea Grant
Herbst Foundation
Bridges to Baccalaureate

People

Cal Poly State University Biology Department
Marine Invertebrate Physiology Lab
Environmental Endocrinology Lab
Environmental Proteomics Lab
Center for Coastal Marine Science

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1. RESEARCH INTRODUCTION

1.1 Endocrine disrupting chemicals

Near-shore marine ecosystems are subject to a host of natural and anthropogenic stressors that impact resident organisms. Of particular concern in populated areas are pollutants that are endocrine disrupting chemicals (EDCs) and can enter coastal environments through wastewater treatment, industrial effluent, storm-water drainage, and dumping. EDCs include organic chemicals used heavily in the agricultural and industrial sectors; more specifically these chemicals are incorporated into plasticizers, surfactants, and pesticides (Porte et al., 2006). Despite a lack of tests and reporting on EDCs prior to the 1950s, we now know that they exist in elevated concentrations in many natural environments. EDCs are lipophilic (tending to dissolve in lipids) and continue to persist in the environment due to production abroad, long half-lives, and continued dispersal into the natural environment (Colborn et al., 1993). In particular, estrogenic EDCs and their metabolites bioaccumulate and biomagnify in environments (Colborn, 1998; Langston et al., 2005) and have the potential to interact deleteriously with resident biota.

The endocrine and reproductive impacts of EDC's can be due to their ability to mimic or inhibit the effects of hormones, alter the synthesis and metabolism of hormones, and modify hormone receptor levels (Soto et al., 1995). In all phyla, hormones help control the biochemistry, physiology and behavior of organisms. Since the late 1990s, the most potent EDCs receiving attention are natural and synthetic steroid estrogens. These xenoestrogens include 17β -estradiol (E2), estrone (E1), 17α -ethinylestradiol (EE2), as well as less potent non-steroidal chemicals such as alkylphenols and bisphenol-A (BPA);

all are widely encountered in natural environments at significant concentration levels (Pojana et al., 2007). These compounds have diverse chemical structures and can disrupt multiple biochemical and physiological systems through pathways involving membrane estrogen receptors as well as receptor-independent mechanisms (Porte et al., 2006).

There is conclusive evidence that effects of EDCs on wildlife (especially invertebrate species) might have far reaching adverse consequences for biodiversity and the sustainability of natural ecosystems (Depledge & Billingham, 1999; Zhou, 2010). Until recently, ecotoxicological assessments neglected the impacts of EDCs on the invertebrate phyla despite their being ecologically present and important in all ecosystems. Invertebrates represent 95% of known species in the animal kingdom yet there is a lack of understanding of the cause-effect relationship between exposure and response to endocrine disruptors in this group (Porto et al., 2006). It is imperative to study the deleterious effects of EDCs on marine organisms in order to identify physiological systems that are most vulnerable and to understand the mechanisms of disruption. Because these substances are expected to remain in our environment over geologic timescales, regulatory agencies need to develop tools to ensure continued monitoring of direct and transgenerational effects. Establishing coherent regulatory policy will depend on continued efforts to understand widespread physiological and behavioral consequences of EDC contamination in our natural habitats.

1.2 4-Nonylphenol

One particular estrogen agonist that attracted attention starting in the early 2000s due to its apparent deleterious interactions with endocrine systems is nonylphenol.

Nonylphenol (NP) is a product of industrial processes formed during the alkylation process of phenols. Ethoxyl groups are added to parent compounds to produce nonylphenol ethoxylates (NPE), which are used ubiquitously as industrial surfactants. NPEs account for about 80% of alkyphenol ethoxylates, which in turn are the second largest group of nonionic surfactants in commercial production (Carlisle et al., 2009). They are used as detergents, emulsifiers, and wetting agents in a variety of industrial products including paints, plastics, cosmetics, lubricant oils, construction material, vulcanized rubber, and paper. Along with once being a common additive to widely used household products, NPEs are also used as commercial applications to process fuels, metals, petroleum, textiles agriculture chemicals and leather (Carlisle et al., 2009). In 2007, U.S. and Canadian consumption of NPE surfactants was estimated to be between 300 and 400 million pounds per year (EPA, 2008).

NPE-containing products eventually reach landfills, water, and sewage treatment, as well as sludge recycling facilities where they are further broken down into multiple byproducts including nonylphenol (NP). NPs are amphipathic molecules containing a hydrophilic head and a hydrophobic tail; therefore they tend to attract water at the head and less soluble substances at the tail. NP is not a single chemical structure rather a complex mixture of highly branched structures commonly referred to as 4-nonylphenol, 4-NP). 4-NP adsorbs strongly to soils and sediments in sewage treatment plants and when released to the aquatic environment, is expected to partition between both water and sediment (EPA, 2008). In water it has a half-life of 10-15 hours (Canada, 2002); however 4-NP is taken up from water and sediment by aquatic biota and can accumulate in the tissues of these organisms (Basheer et al., 2004; Pojana et al., 2007; EPA, 2008; Diehl et

al., 2012). NP can also move up the food chain and biomagnify to a small degree. High levels of 4-NP are clearly correlated with environments impacted by human activities and the highest levels of 4-NP are documented in the waters and sediments of coastal rivers and estuaries (Diehl et al., 2012).

Exposure to 4-NP can induce myriad physiological disruptions in aquatic organisms; these include reduced fecundity in gastropods (Czech et al., 2001), inhibited settlement in barnacles (Billinghurst et al., 1998), and altered development in euryhaline copepods (Forget-Leray et al., 2005). Studies conducted by Nice et al. (2000, 2003 & 2005) have also shown a decrease in sperm motility, increase in percent hermaphroditism, and an increase of developmental abnormalities in Pacific oysters exposed to high levels of 4-NP. Currently, there is some data documenting the effects of 4-NP on the immune systems of bivalves; however, there is a lack of research studying immune responses in the economically important Pacific oyster. To date, 4-NP has been shown to decrease immune functions in mussels (Canesi et al., 2004, 2007), cockles (Matozzo et al., 2008), and clams (Matozza and Marin, 2005). Exposure to other xenobiotics (e.g., heavy metals and organotoxins) has been shown to decrease phagocytosis (Auffert et al., 2002) and impair cell-to-cell aggregations (Auffret and Obella, 1997) in oyster hemocytes. The following research aims to fill in the gap regarding the consequences of toxicological exposure to 4-NP on the immunocompetence of *C. gigas*.

1.3 The Pacific oyster (*Crassostrea gigas*)

In order to enhance our understanding of the effects of 4-NP on aquatic organisms, we are focusing on the Pacific oyster (*Crassostrea gigas*), a benthic marine invertebrate that is subject to heavy microbial loads and environmental contaminants. According to the Food and Agriculture Organization (FAO), *C. gigas* is the most heavily farmed oyster species in the world with recent global production values greater than any other species of fish, mollusk, or crustacean. *C. gigas* is a dominant shellfish produced in the United States aquaculture industry and global production of this species tops 609,000 tons with a value of USD 1.3 billion (FAO, 2014). Over the last century, California and the broader west coast of North America has developed into one of the most significant regions for growth of *C. gigas* farming worldwide. As filter feeders that are permanently attached to ground substrates, oysters are constantly exposed to sediments and the contaminants therein. Accumulation of a known xenoestrogenic contaminant in a regionally valuable seafood species should warrant particular concern given the evidence that 4-NP disturbs not only reproductive and developmental processes, but also immune processes.

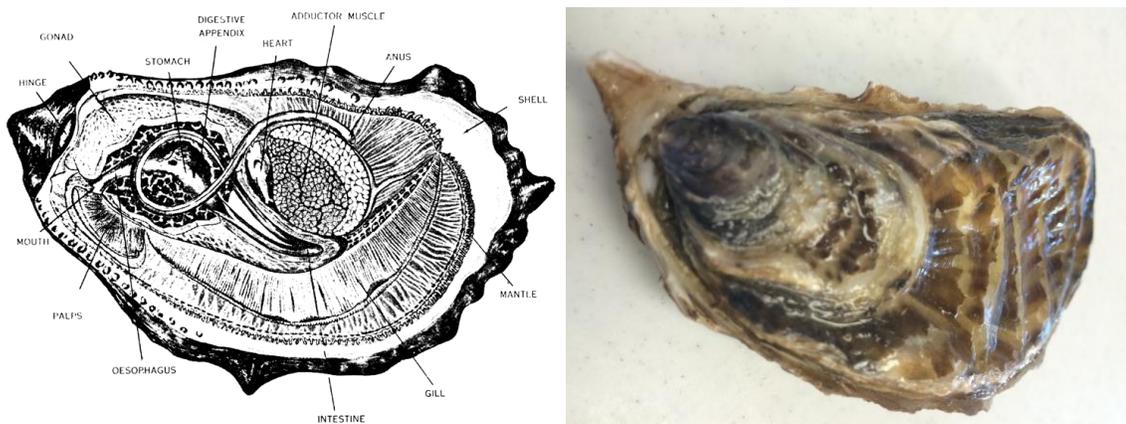


Figure 1. Anatomy of the Pacific Oyster (*C. gigas*). Diagram from Handbook for Oyster Farmers, Division of Fisheries, Australia. Photo © Courtney Hart, 2015.

1.3.1 Immune system

Oysters are bivalve mollusks and their immunity depends solely on innate mechanisms made up of physical protective barriers (Schmitt et al., 2011) as well as established internal cellular and humoral defense mechanisms. The oyster shell, the outer most layer and first line of physical defense for *C. gigas*, is secreted by the edge of the mantle. Inside the shell, the inner organs (Fig. 1) of this oyster are enclosed in the mantle, a sack-like structure that is open on all sides to allow water to flow through to the gills. Cilia beat in unison to pass water over the gills and then the water flows through the pores (ostia) into the excurrent chamber. This open water circulation system allows any particles or contaminants in the water to come into direct contact with most internal organs. All structures of the oyster have the potential to be exposed to 4-NP dissolved in filtered water; however, the mechanisms for uptake of EDCs are not yet documented. Oysters, and most filter feeders, are exposed to various microorganisms in surrounding waters and in response have dense and diversified microflora residing on the surfaces of their internal tissues, especially the mantle, gill and within the hemolymph (Thompson et al., 2004; Schmitt et al., 2012). In response to contamination, host defense mechanisms in *C. gigas* are triggered in three essential steps: detection of infecting microorganisms, activation of intracellular signaling pathways, and triggering of effector mechanisms (Montagnani et al., 2007). Subsequent to exposure, oysters mount two-fold defense mechanisms encompassed by cellular and humoral components that work in concert to recognize and eliminate pathogens. Despite lack of an acquired immunity that relies on immunological memory (i.e., antibodies), *C. gigas* has a developed system that can

discriminate advantageous microorganisms from deleterious ones (Canesi et al., 2002; Gagnaire et al., 2007).

There is a complicated relationship between bivalves, diseases, and environmental contaminants such as EDCs because of these toxins' ability to behave as estrogenic endocrine disruptors. Estrogen receptors, Cg-ER proteins, are found in the ovaries, testes, digestive, gill, mantle and labial palp tissues of the oyster at varying concentrations (Matsumoto et al., 2007). Although transcriptional activity of molluscan ERs does not appear to be activated by estrogenic compounds, studies conducted by Canesi et al. (2007) were able to mitigate deleterious effects of EDCs on hemocyte function and signaling using antiestrogens. These results suggest the presence of ER-like receptors or alternative mechanisms that can lead to EDC-related disruption. Other studies show that estrogens are not only associated with reproductive systems but also have the ability to affect the function of non-reproductive tissues including the immune system (Canesi et al., 2004). There is evidence to suggest that estrogen is an important signaling molecule (Canesi et al., 2006) and in particular E2 can affect circulating hemocytes (immunocompetent tissue) and their lysosomal activity (Moore et al., 1978, Burlando et al., 2002).

1.3.2 Cellular defense mechanisms

Oysters have an open circulatory system in which the heart pumps hemolymph through a network of arteries and veins connected to blood sinuses. Besides transporting oxygen and nutrients and removing waste products, the hemolymph is involved in immune defense against pathogens. Hemolymph is a liquid tissue that contains fluid

plasma and the main immunocompetent cells called hemocytes (Schmitt et al., 2011). Because the hemolymph is in direct contact with connective tissues throughout the body, hemocytes are able to infiltrate the tissues, particularly the mantle and gills (Galstoff, 1964). These hemocytes are the main cellular mediators of defense mechanisms (Canesi et al., 2002) in most bivalves and are important in processes including wound repair, shell recalcification, nutrient transport, digestions, and excretion (Cheng, 1996). At first contact with pathogens and other exogenous particles, hemocytes are capable of non-self recognition, chemotaxis, and active phagocytosis (Cheng, 1983; Bachère et al., 2004). Hemocytes also contain hydrolytic enzymes (Cheng et al., 1975) and eliminate pathogens by producing reactive oxygen species (Bachère et al., 1991) and antimicrobial peptides (Gonzalez et al., 2007a). All of these actions qualify the hemocytes as a tissue of concern when diagnosing immune responses in *C. gigas*.

1.3.2.1 Total hemocyte counts

With the multifaceted immune functions mediated through hemocyte activity, we expected that 4-NP or bacterial infections exposure might change the number of circulating hemocytes (total hemocyte counts, THC) in *C. gigas*. THC changes in response to bacterial infections have been observed in prawns (Sung et al., 2000), mussels (Li et al., 2008), as well as oysters (Labreuche et al., 2006). While some invertebrates show an *increase* in THC following infection, many crustaceans can have measurably lower circulating hemocyte counts immediately following pathogen exposure (e.g., Sung et al., 2000; Söderhäll et al., 2003; Yoganandhan et al., 2003; Holman et al., 2004; Johnson et al., 2011). This reaction is apparently due to hemocyte aggregations that

facilitate phagocytic/bactericidal activity and wound healing causing hemocytes to accumulate at the site of infection thereby exit the general circulation (Burnett et al., 2006; Bachère et al., 2004; Burge et al., 2007). Furthermore, some environmental contaminants have also been shown to directly influence baseline THCs in marine bivalves. For example, THCs have been shown to decline following exposure to certain heavy metals (cadmium and copper; Auffert et al., 2002; Haberkorn et al., 2014) and PCBs (Liu et al., 2009), and increase in response to certain polycyclic aromatic hydrocarbons (phenanthrene; Hannam et al., 2010), nanoparticles (nanoscale titanium dioxide; Wang et al., 2014), and other heavy metals (chromium; Ciacci et al., 2011). Understanding if and how 4-NP may alter an oyster's THC response to bacterial infection will provide insight into any deleterious effects that this EDC has on host-pathogen reactions.

1.3.3 Humoral defense mechanisms

Pathogens that succeed in overcoming external physical defenses and cellular effectors will encounter internal humoral defense systems. Secretion of these soluble defense factors by hemocytes plays an important role in the immunity of most invertebrates (Donaghy et al., 2009). The fluid hemolymph of most bivalves contains proteins including antimicrobial peptides, soluble lectins, lysins, agglutinins, and lysosomal enzymes (Pruzzo et al., 2005). These compounds act on a broad spectrum of microbial organisms and their activity has been detected in other tissue besides hemocytes and in phagocytized bacteria (Mitta et al., 2000). Subsequent microbial

elimination results from the combined action of these enzymes coordinated with cell-mediated (e.g, hemocyte) actions.

1.3.3.1 Expression of immune-related gene transcripts

In order to understand transcription level controls of the immune responses in oysters we used real-time quantitative polymerase chain reactions (RT-qPCR) to study the transcriptome. Comparison of transcription profiles of genes allowed us to identify those that are differentially expressed in response to various treatments. This research focused on a subset of genes that presumably control relevant immune responses including: antimicrobial peptides (AMPs; *Cg-bigdef1*, *Cg-bigdef2*, *Cg-defh1*, *Cg-defh2*, *Cg-bpi1*, *Cg-lys1*), lectins (*Cg-galectin*, *Cg-clec2*), a matrix metalloproteinase (*Cg-timp*), and a coagulant, (*Cg-tgase*).

AMPs are a group of immune effectors found in many bivalves and include defensins (*Cg-bigdef1-3* & *Cg-defh1-2*), bactericidal permeability-increasing proteins (*Cg-bpi1*), and lysozymes (*Cg-lys1*). AMPs are an essential part of the innate immune response of virtually all living organisms (Bulet et al., 2004). Most AMPs are characterized by a prevalence of cationic and hydrophobic amino acids, which are an integral part of their broad-spectrum antimicrobial activity against bacteria, viruses and fungi (Bulet et al., 2004; Izadpanah et al., 2005). Defensins (*Cg-defh1-2*) are a prominent group of AMPs, which are also ubiquitously distributed across unicellular and multicellular organismal groups (Schmitt et al., 2010). Representatives of a separate family of ‘big’ defensins have also been characterized in *C. gigas* (*Cg-bigdef1*, *Cg-bigdef2* and *Cg-bigdef3*; Rosa et al., 2011). These ‘big’ defensins are a group of AMPs

predominantly found in molluscs, and are genetically and structurally more similar to vertebrate defensins than to the invertebrate defensins (Rosa et al., 2011). The three big defensins are also differentially regulated; mRNA expression of both *Cg-bigdef1* and *Cg-bigdef2* are strongly induced by *Vibrio* injections, whereas the constitutively expressed *Cg-bigdef3* transcript levels are not responsive to infection (Rosa et al., 2011) so this gene was not quantified in our study. In a related transcriptomic analysis of Digital Gene Expression (DGE) data, de Lorgeril et al. (2011) found no evidence for differential expression of the *Cg-bigdefs* between oysters capable of surviving an experimental infection with a virulent *Vibrio* infection and those oysters that died following the same infection. To date, it is not entirely clear if *bigdef*-genes in oysters play a crucial role in the response to bacterial infections or EDC exposure.

The bactericidal-permeability-increasing protein (BPI) is another important peptide that plays a role in antimicrobial defenses. The toxic activities of BPI are a result of its ability to permeabilize bacterial membranes (Schmitt et al., 2011) by binding to lipid A, a component of lipopolysaccharides found on the outer membrane of gram-negative bacteria (Elsbach & Weiss, 1998; Zhang et al., 2011). The two stages of bacteria-specific attacks start with BPI's ability to arrest bacterial growth associated with the outer membrane, followed later by damage to the inner membrane (Elsbach & Weiss, 1998). Gonzalez et al. (2007b) confirmed that *Cg-bpi1* is expressed at low basal levels in oysters, but bacterial challenge induced gene transcripts up-regulation. In *C. gigas*, the expression of *Cg-bpi1* has been detected in the hemocytes, gills, mantle, adductor muscle, heart, digestive gland and gonad tissues (Zhang et al., 2011; Gonzalez et al., 2007b).

Lysozymes, the final AMP we assessed, exhibit lytic properties on peptidoglycan that comprises bacterial cell walls (Schmitt et al, 2011). They are a multigenic family detected in oyster tissues including the gills, mantle and hemocytes of *C. gigas* (Matsumoto et al., 2006). By catalyzing the cleavage of glycosidic bonds (Prager & Jolles, 1996; Bachali et al., 2002), plasma lysozyme has antibacterial activities against both Gram-positive and Gram-negative bacteria (Nilsen et al., 1999; Montenegro-Ortega and Viana, 2000). Lysozymes are important host-defense factors in that they mediate cell lysis of bacterium frequently found in tissues of filter feeding marine organisms (Olsen et al., 2003; Xue et al., 2007). Overall the defensins and BPIs are active against gram-positive bacteria (Zhang et al., 2011) while lysozymes are known to have antimicrobial activity against gram-positive and gram-negative bacteria (Song et al., 2010).

Lectins, found in species of all taxa, are sugar-binding glycoproteins that agglutinate cells or precipitate glycoconjugates (Sharon and Lis, 2004). Found in the hemolymph of many invertebrate species, bivalve lectins (*Cg-clec2* & *Cg-galectin*) are involved in non-self recognition and have an agglutination or opsonic role (promoting phagocytosis) in hemocyte phagocytosis (Vasta 2009; Vasta et al., 2012). In oysters, galectins are involved in important host-defense mechanisms because they bind to foreign particles and promote phagocytosis of macrophages (Yamaura et al., 2008). Our study examined oyster galectins (*Cg-galectin*), which are β -galactosidase-binding lectins that can act as recognition factors to pathogens by binding to glycans on the surface of microbes (Vasta et al., 2012). We also quantified the transcript abundance of *Cg-c type lectin2* (*Cg-clec2*) that encodes for C-type lectin protein believed to be a mediator of some immune responses due to their pathogen recognition ability (Schmitt et al., 2013).

Lastly, we examined the response of a matrix metalloproteinase, tissue inhibitor metalloproteinase (*Cg-timp*), and a coagulant, transglutaminase (*Cg-tgase*), which appear to be activated in invertebrate immune response (Iwanaga and Lee, 2005; Maningas et al., 2008). Tissue inhibitor metalloproteinases are multifunctional proteins that can regulate extracellular matrix homeostasis (Gomez et al., 1997). In *C. gigas*, accumulated *Cg-timp* mRNA in hemocytes were implicated in immune responses caused by bacterial infections or shell damage (Montagnani et al., 2001). TGase is an enzyme that can catalyze cross-linking of proteins and is often associated with protein modification (Whitaker, 1977); its activity in crayfish results in the formation of stable and insoluble clots in crayfish (Wang et al., 2001). Although purified multiple tissues (Kumazawa et al., 1997) of *C. gigas*, the exact function of *Cg-tgase* in immune responses to bacterial infections is unclear, as clotting mechanisms have not been reported in oysters (Gueguen et al., 2003). Quantifying the transcript abundances of these genes in oysters exposed to 4-NP and subsequently infected with a marine bacterium will help elucidate the impacts that an EDC can have on bivalve immunomodulation.

1.3.3.2 Lysozyme activity

To further explore the combined effects of bacterial infection and 4-NP on humoral immune parameters, we measured lysozyme activity in hemolymph plasma. Lysozymes are antibacterial enzymes found in many organisms and are considered to be a component of humoral defense in marine bivalves (McDade and Tripp, 1967; Cheng et al., 1975; Hardy et al., 1976). When the hemocytes of mussels were induced to perform phagocytosis there was an increased release of lysozyme from the cells into the plasma

(Cheng et al., 1975). Recently, studies hypothesize that changes in lysozyme activity as a result of exposure to 4-NP were a result of altered lysosomal membrane stability (LMS) (Matozza et al., 2008) and subsequent release of enzymes into the hemolymph. LMS is one of the most sensitive immune parameters during xenoestrogen exposure (Canesi et al., 2007) and 4-NP has been specifically shown to decrease lysosomal membrane stability in mussels (Canesi et al., 2004, 2007), cockles (Matozza et al., 2008) and clams (Matozza and Marin, 2005). 4-NP has also been shown to alter lysozyme activity in other bivalves although the findings have been contradictory (Matozza et al., 2008; Matozza and Marin, 2005). In general, the studies that examine the effects of environmental contaminants on lysozyme activity in bivalves have not generated a clear consensus on the issue (Anderson et al., 1996; Pipe and Coles, 1995); we hope that this study helps to contribute to the understanding of another component of the immune response in *C. gigas*.

1.4 Hypothesis and predictions

The overarching goal of this study is to determine how exposure to 4-NP influences immune function in the Pacific oyster (*Crassostrea gigas*) by measuring elements of the cellular (total hemocyte counts) and humoral (immune-gene expression and lysozyme activity) defense mechanisms following a bacterial infection. We seek to use various techniques in an effort to elucidate any interferences in oysters' response to bacterial challenges that may be caused by an EDC commonly found the water, sediments, and tissues of organisms in Morro Bay, California.

2. RESEARCH MANUSCRIPT

2.1 Introduction

With urban-marine interfaces concentrated near fragile coastal ecosystems, there is increasing interest in evaluating the effects of anthropogenically-derived eco-toxins on marine organism. Endocrine disrupting chemicals (EDCs) are toxic exogenous agents, synthetically or naturally derived, that alter endocrine functions by interfering with hormonal signaling pathways. EDCs are now recognized as pervasive in freshwater, estuarine, and marine waters worldwide (Ferguson et al., 2001; Pojana et al., 2004; Salgueiro-González et al., 2015). According the WHO EDC report (2012), there is a robust body of evidence demonstrating the detrimental effects of EDCs and their potential role in the worldwide decline of aquatic species diversity; lab and field studies link EDCs to developmental, reproductive, growth, and immune abnormalities (Zhou et al., 2010). One notable EDC in California, USA is the xenoestrogen 4-nonylphenol (4-NP). 4-NP is a breakdown product of the anthropogenically-derived, industrial chemical nonylphenol ethoxylate (NPE). NPEs are nonionic surfactants and are used in a wide variety of industrial, household, and agricultural applications (EPA 2014). Introduced into the environment by way of industrial and municipal wastewater discharges (Pajona et al., 2007), NPEs degrade into alkylphenol derivatives including estrogenic 4-NP. 4-NP, unlike NPEs, is hydrophobic and adheres to organic-rich sediments where it can persist for decades (Ying et al. 2002, Soares et al. 2008). 4-NP levels in bays and estuaries worldwide range from 0.001 to 2.76 $\mu\text{g l}^{-1}$ and there is evidence for moderate bioaccumulation and trophic biomagnification (Diehl et al., 2012). More troubling is that 4-NP is found at moderate levels in the seawater and benthic sediments of Morro Bay,

California, and at levels a whole order of magnitude higher in tissues of resident marine benthic invertebrates.

Exposure to 4-NP can induce myriad physiological disruptions in aquatic organisms; these include reduced fecundity in gastropods (Czech et al., 2001), inhibited settlement in barnacles (Billingham et al., 1998), and altered development in euryhaline copepods (Forget-Leray et al., 2005). Studies conducted by Nice et al. (2000, 2003 & 2005) have also shown a decrease in sperm motility, increase in percent hermaphroditism, and an increase of developmental abnormalities in Pacific oysters exposed to high levels of 4-NP (100 $\mu\text{g l}^{-1}$). The California Environmental Protection Agency (Office of Environmental Health Hazard Assessment) has recently identified a critical need for targeted studies of 4-NP toxicity on marine organisms, with particular regard to the non-reproductive and non-developmental impacts (Carlisle et al. 2009). A few recent studies show that 4-NP can induce rapid changes in certain immune parameters in marine invertebrates (Canesi et al. 2007; Matozza and Marin, 2005; Matozza et al. 2008; Sung and Ye, 2009).

This study aims to enhance our understanding of the effects of 4-NP on immune function in the Pacific oyster, *Crassostrea gigas*, a benthic filter-feeding invertebrate subject to heavy microbial loads and environmental contaminants. *C. gigas* is also a dominant shellfish produced in the United States aquaculture industry and global production of this species tops 609,000 tons with a value of USD 1.3 billion (FAO, 2014). In the last decade, summer mortality events of *C. gigas* have become more frequent, particularly in Alaska, Washington, and California (Cheney et al., 2000); these events pose a threat to an economically relevant sector of regional aquaculture

production. Environmental pressures such as warmer water temperatures and elevated nutrient levels can act as additional stressors, which may increase oyster susceptibility to adverse phenomena (Friedman et al. 1991), including concomitant exposure to EDCs and bacteria. The elaborate immune system of *C. gigas* involves only innate characteristics comprised of cellular and humoral defense mechanisms. Without adaptive traits, oysters are unable to develop long lasting protective immunity or immunological memory.

The goal of this study is to determine how exposure to 4-NP influences these immune responses of oysters during a bacterial infection. We exposed *C. gigas* to high ($100 \mu\text{g l}^{-1}$), low ($2 \mu\text{g l}^{-1}$), or control (100 μl ethanol) doses of 4-NP and then injected them with a non-pathogenic strain of marine bacteria, *Vibrio campbellii*. In order to determine the effects of this EDC, we took a three-fold approach to examine effects of acute (7 d) exposure to 4-NP on cellular (i.e., hemocytes) and humoral (e.g., inducible proteins) defense responses to bacterial challenge. We measured 1) total hemocyte counts to determine if 4-NP interfered with the cell-level immune response, 2) changes in the mRNA expression patterns of 10 immune-relevant genes (*Cg-bigdef1*, *Cg-bigdef2*, *Cg-defh1*, *Cg-defh2*, *Cg-bpil*, *Cg-lys1*, *Cg-galectin*, *Cg-clec2*, *Cg-timp* *Cg-tgase*), and 3) hemolymph plasma lysozyme activity.

2.2 Materials and Methods

2.2.1 Test organisms: animal care, maintenance, and preparation

Adult Pacific oysters, *Crassostrea gigas*, were obtained from local seafood vendors in Morro Bay, California, USA, and immediately transferred to a single large holding tank (380 l) at the California Polytechnic State University's Center for Coastal Marine Science pier facility in Avila Beach, California, USA. Here, animals were held for a minimum of two weeks in a continuous flow-through system of well-oxygenated, unfiltered seawater (~13°C; 33-34 ppt; ambient photoperiod) pumped from the ocean directly below. Animals fed off of the plankton naturally present in the raw seawater. Oysters used in this study had shell heights ranging from 69 – 130 mm and masses ranging from 40.9 – 162.1 g. Previous 4-NP testing of the seawater at this facility indicated negligible levels of 4-NP (Tomanek, unpubl. observ.).

Prior to experimentation, oysters were prepared for intramuscular injection by aseptically drilling a 1 mm hole through the shell directly above the adductor muscle. The adductor muscle is an ideal site for the injection of bacteria as it is heavily bathed with hemolymph, which rapidly enters the systemic circulation (Tripp, 1960). The hole was covered with two layers of latex dental dam and sealed with cyanoacrylate glue to simultaneously create a leak-proof bacterial injection port and hemolymph sampling site.

2.2.2 Exposure conditions and experimental design

2.2.2.1 4-Nonylphenol

Experimental tanks were dosed to a high (100 µg l⁻¹) or low (2 µg l⁻¹) concentration of 4-NP by the addition of 100 µl of stock solutions of 4-NP (99% purity,

Acros Organics), prepared in ethanol, to the 8 l of unfiltered seawater in each experimental tank. The control treatment tank received an equal volume (100 µl) of pure ethanol with no 4-NP. The low dose (2 µg l⁻¹) was chosen because it approximates ecologically relevant levels of 4-NP contamination in global coastal waters (<2.76 µg l⁻¹; Diehl et al., 2012), including those waters where *C. gigas* is ubiquitously farmed. The high dose provides an 'extreme' exposure level, which can potentially highlight the most basic immune consequences of 4-NP under severe conditions.

2.2.2.2 Bacterial preparation

The bacterial strain used in this study was *Vibrio campbellii* 90-69B3 (graciously provided by K. Burnett and L. Burnett, College of Charleston, Charleston, SC, USA), which was transfected with the stable *Vibrio*-derived plasmid pEVS146 coding for kanamycin (Kan) and chloramphenicol (Cm) antibiotic resistance (Eric Stabb, University of Georgia; as described in Burgents et al., 2005a and Macey et al., 2008). *V. campbellii* is a common opportunistic pathogen in the marine environment. It is a known crustacean pathogen (Burgents et al., 2005a; Burgents et al., 2005b; Hameed 1995; Hameed et al., 1996) that was originally isolated from diseased shrimp (D. Lightner and L. Mahone, University of Arizona, Tucson, AZ USA; Burgents et al., 2005a), and has also been isolated from *C. gigas* (Shen et al., 2005). Injections of this bacterium are non-lethal to oysters, yet still provide them an effective immune challenge (Macey et al., 2008; Williams et al., 2009). One day prior to injection, frozen aliquots of *V. campbellii* were streaked onto Tryptic soy agar plates supplemented with 2.5% NaCl and held overnight at 25°C. Following this incubation, a small amount of bacteria was suspended in 5ml of

sterile HEPES-buffered saline (10 mM HEPES, 2.5% NaCl, pH 7.5), vortexed to homogeneity, and diluted to an optical density of 0.12 at 540 nm, which is equivalent to 1×10^8 colony forming units (CFU) per milliliter (personal observation; Mikulski et al., 2000; Scholnick et al., 2006). Injection solutions were then serially diluted in HEPES saline to a final concentration of 1×10^6 CFU ml⁻¹ for injections that would take place that same day. [The bacterial concentration of the 1×10^8 CFU ml⁻¹ stock solution was confirmed following each preparation via direct CFU counts on three TSA plates (2.5% NaCl) that were streaked with 100 µl of a logarithmically diluted solution (reduced by five orders of magnitude to 1×10^3 CFU ml⁻¹) and incubated at 25°C for 24h.] In a subset of oysters, selective plating was used to confirm the presence of culturable *V. campbellii* in the hemolymph following experimental introduction. At 24 h post-injection, 100 µl of hemolymph was removed from the adductor muscle via the injection port and plated on TSA plates (2 per oyster) supplemented with 2.5% NaCl, 100 µg ml⁻¹ chloramphenicol and 5 µg ml⁻¹ kanamycin. Due to the presence of antibiotics, no other bacterial growth should be present on these plates except for the antibiotic resistant experimental strain. Plates were incubated overnight at 25°C, and the presence of culturable *V. campbellii* in the hemolymph was confirmed. We detected culturable, antibiotic resistant *V. campbellii* from the hemolymph of all bacterially challenged animals, and found no growth on plates streaked with hemolymph from our control oysters.

2.2.2.3 *Experimental protocol*

Oysters were haphazardly transferred to individual (38 l), closed-system experimental tanks (n=3-4 tank replicates per treatment, n=4 oysters per tank) where they

were held for 24 h before experimentation. Each tank contained 8 l of continuously aerated, unfiltered seawater (33-34ppt; ambient light conditions). All experimental tanks were randomly positioned within a much larger, acrylic holding tank that was continuously pumped through with seawater directly from the ocean. This arrangement served to maintain the temperature of the water in the experimental tanks at the ambient temperature conditions of the ocean below. A HOBO data-logger (HOBO Pendant® Temperature/Light Data Logger, Onset Computer Corp., Bourne, MA, USA) was maintained within a single experimental tank to record temperatures every 30 min throughout course of each experiment (mean: $15.3 \pm 0.10^\circ\text{C}$; range: $12.2 - 23.0^\circ\text{C}$).

Oysters were held in high ($100 \mu\text{g l}^{-1}$), low ($2 \mu\text{g l}^{-1}$), or control ($100 \mu\text{l ethanol}$) conditions of 4-NP for a total of 8 d. Complete water changes (using unfiltered seawater) and 4-NP re-dosing occurred on the 2nd, 4th and 6th days of exposure (every other day). Water changes were conducted primarily to maintain the appropriate concentration of 4-NP in the tank; 4-NP half-life can be as low as a few days (Mao et al., 2012). Re-dosing also served to provide a source of planktonic food, avoid tank fouling, and prevent nitrogenous waste build-up throughout the experiments. On the 7th day of exposure, oysters received an intramuscular injection of $100 \mu\text{l}$ of *V. campbellii* ($1 \times 10^6 \text{ CFU ml}^{-1}$) or sterile HEPES-buffered saline (10 mM HEPES, 2.5% NaCl, pH 7.5) using a 1 ml syringe fitted with a 25-gauge 1 ½-inch needle. The latex injection port was thoroughly cleaned with 70% isopropyl alcohol before and after injection, and oysters were monitored to confirm that there was no significant leakage at the injection site. Immediately following injection, oysters were placed back into their respective experimental tanks and held for 24 h (Macey et al. 2008; Williams et al., 2009; Rosa et

al., 2011) under their original 4-NP (or control) conditions. For the lysozyme activity measurements, we used sterile needles to collect roughly 200 μ l of hemolymph directly from the adductor muscle at 3 h and 24 h post-injection (see 2.4 below). These samples were maintained on ice for the duration of sampling, centrifuged for 10 min at 500 x g at 4°C, and the supernatant was stored at 20°C until further lysozyme activity analysis. For the THC and qPCR measurements, oysters were removed from their tank 24 h after injections, dried with a paper towel, shucked and the hemolymph, mantle, and gill tissues were collected. A needle-less 1 ml syringe was used to pull remaining hemolymph directly from inside the pericardial cavity. A 100 μ l subsample of hemolymph was placed on ice for immediate quantification of total hemocyte counts (see 2.3 below). The remainder of the hemolymph sample (~500-1000 μ l) was maintained on ice for the duration of the sampling protocol (to prevent hemocyte aggregate formation), then centrifuged for 10 min at 4000 x g at 4°C. The supernatant was discarded and the cellular pellet portion was stored at -80°C until further qPCR analysis. Gill tissue was collected from the medial portion of two superior-most gill folds, whereas a small section of mantle was collected from the posterior end of the left valve. Gill and mantle tissues were flash frozen in liquid nitrogen and stored at -80°C until further qPCR analysis. There was no oyster mortality under any treatment conditions for the entire duration of these experiments.

2.2.3 Total hemocyte counts

The 100 μ l hemolymph subsample was fixed in 100 μ l of 10% neutral buffered formalin. After mixing, 15 μ l of the fixed hemolymph suspension was loaded onto an

improved Neubauer Hemocytometer and viewed under a light microscope for direct measurement of total hemocyte counts. Two separate aliquots of the hemocyte suspension were counted for each sample, and the data was recorded as the mean of the two samples (in units of THC ml^{-1} of hemolymph). Hemolymph samples that were visibly contaminated with gametes were omitted from consideration.

2.2.4 Quantitative real-time PCR assays

Total RNA was extracted from gill and mantle tissues using Tri-Reagent (Molecular Research Center, Cincinnati, OH) with bromochloropropane as the phase separation reagent. Total RNA was extracted from hemocytes using the RNeasy Kit (Qiagen, Valencia, CA, USA). The resulting RNA was then DNase I treated (TURBO DNA-free Kit, Ambion) and quantified by spectrophotometry (NanoPhotometer P300 Implen, Inc, Westlake Village, CA, USA).

First strand cDNA was generated in 32 μl (mantle and gill) or 24 μl (hemocyte) reverse transcription reactions. Mantle (16 μl of 23.5 ng/ μl) or gill total RNA (16 μl of 29.5 ng/ μl) was combined with 4.8 μL MgCl_2 (25 mM), 1.6 μl deoxynucleotide triphosphates (dNTPs) (100 mM, Promega Corp., Madison, WI, USA), 1.6 μl random primers (500 $\mu\text{g/ml}$; random hexadeoxynucleotides; Promega), 0.125 μL recombinant RNasin® ribonuclease inhibitor (20 U/ μl , Promega), 1.2 μL GoScript™ reverse transcriptase, 0.275 μl of nuclease-free H_2O , and 6.4 μl 5X reaction buffer, as per the protocol of the GoScript™ Reverse Transcription System (Promega Corp). Hemocyte RNA was reverse transcribed as a reduced volume (12 μl of 14.4 ng/ μl), with each of the above reagents for the reverse transcription reaction scaled proportionally. All RNA was

reverse transcribed under a thermal profile of 25 °C for 5 min and 42 °C for 60 min, followed by 70 °C for 15 min to inactivate the reverse transcriptase enzyme (T100 thermal cycler, BioRad).

Quantitative real-time polymerase chain reactions (qPCR) were used to quantify changes in the relative abundance of gene transcripts. Primers for SYBR Green qPCR assays were designed to the cDNAs encoding for ten immune-related genes (Table 1): *Cg-bigdef1*, *Cg-bigdef2*, *Cg-defh1*, *Cg-defh2*, *Cg-bpi1*, *Cg-lys1*, *Cg-galectin*, *Cg-clec2*, *Cg-timp* and *Cg-tgase* from *C. gigas*. In addition, primers were also obtained from Schmidt et al. (2013) for amplification of ribosomal protein L40 (*rpl40*; AJ563473 joined with FP004478), elongation factor 1-alpha (*ef1a*; EKC33063), and ribosomal protein S6 (*rps6*; XM_011447786) as internal control genes. Nucleotide sequences for all primers are provided in Table 1. Quantitative real-time PCRs were conducted in 16 µL volumes, with each reaction containing 4.5 µL nuclease-free water (Sigma), 8.0 µL qPCR Master Mix (iTaq™ Universal SYBR® Green Supermix, BioRad), 1.0 µL of both forward and reverse primers (10 µM), and 1.5 µL of cDNA template. The PCR thermal profile for each reaction was 50 °C for 2 min, 95 °C for 3 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min, and all assays were run on a 7300 Real Time PCR System (Life Technologies, Grand Island, NY USA). For each gene, a standard curve was made from a pool of total RNA from samples representing all treatments, and each standard was assayed in triplicate. PCR efficiencies for each transcript assay are provided in Table 1 and correlation coefficients (r^2) for all transcripts were always greater than $r^2 = 0.967$. DNA contamination was assessed for each gene by analyzing RNA samples that were not reverse-transcribed, and each qPCR run also included samples without cDNA template to

further control for contamination. To confirm that one a single PCR product was amplified for each assay, dissociation curves were included for each qPCR run; select PCR products were also Sanger sequenced to confirm cDNA identity (MacrogenUSA, Rockville, MD, USA). Finally, expression of each mRNA of interest was expressed as a relative level by dividing the resulting values by the mean values of an internal control gene. The suitability of internal reference genes was found to vary with tissue type; data were therefore normalized to the internal control gene within each tissue that was unaffected by treatment exposure (gill: *rpl40*; mantle: *rps6*; hemocytes: *ef1a*). All relative mRNA values are expressed as fold increases over the mean value of the saline injection, control (i.e., no 4-NP exposure) treatment group.

2.2.5 Lysozyme activity

We measured total hemolymph plasma lysozyme activity (U ml⁻¹) using the EnzChek[®] Lysozyme Assay Kit (E-22013, Molecular Probes, Eugene, OR). This is a fluorescence-based microplate assay that measures lysozyme activity based on the change in absorbance at 450 nm resulting from the lysis of fluorescein-conjugated *Micrococcus lysodeikticus*. Lysozyme activity was calculated from the change in absorbance at 450nm for a period of 30 minutes following introduction of *M. lysodeiktocus*. Final lysozyme activity values were determined based on standard curves ($r^2 > 0.98$) generated from known concentrations (0 U mL⁻¹ to 250 U mL⁻¹) of hen egg white lysozyme (HEWL).

2.2.6 Statistical analyses

All statistical analyses were conducted using JMP Pro software (v. 11.1). Data were tested for normality using the Shapiro-Wilk test, and for homogeneity of variance using the Levene test. Data that failed to conform to these assumptions were \log_{10} or square root transformed before proceeding with parametric ANOVAs. We carried out two-way ANOVA tests to determine if there were any significant main or interaction effects of 4-NP exposure (high, low or control) and bacterial exposure (*V. campbellii* or saline) on 1) THC, 2) relative mRNA expression levels and 3) lysozyme activity levels. In the cases where data failed to conform to normality or equal variance following transformation, we performed an additional non-parametric Kruskal-Wallis test on ranks to compare the six treatments independently. In no case was there any marked difference in the statistical conclusions between the two-way ANOVA and the non-parametric Kruskal-Wallis tests. As a result, only the statistical output from the two-way ANOVA analyses will be reported. Where significant effects were detected, *post-hoc* pairwise comparisons were made using a Tukey's HSD test (THC and lysozyme activity) or a Student's t-test with a Bonferroni corrected α value (mRNA measurements). When no significant pairwise comparisons were indicated from the post-hoc analysis (following a significant effect in the two-way ANOVA), we performed a further least-squares means Tukey HSD, Dunnett's or Student's t-test on the data grouped *only* by the bacterial exposure or 4-NP exposure treatment groups. All results (excluding the Bonferroni corrected tests) were considered to be significant at the $\alpha = 0.05$ level. All values are reported as mean \pm SEM.

Table 1.
Primer sequences and PCR run-efficiencies for SYBR green quantitative real-time PCR assays

Transcript (Gen Bank No.)	Primer	Sequence (5'-3')	Amplicon size (bp)	% PCR avg. efficiency
<i>Cg-defh1</i> (DQ400101)	Forward	TTACATTGGTCGTTCTCCTGATGG	102	98.21
	Reverse	ATGGATTGGCAGTGACTGTT		
<i>Cg-defh2</i> (DQ400102)	Forward	CTTACATTAGCCGTTCTTCTGATGG	112	-
	Reverse	GCTCTACAACCGATGGACCT		
<i>Cg-bigdef1</i> (JF703138)	Forward	GCCTACGCCCTTTACAGATAC	75	98.98
	Reverse	ACTGTCATGGTCACTCCTTATTC		
<i>Cg-bigdef2</i> (JF703145)	Forward	TACGGAGTCTACGCCCTTTA	89	98.67
	Reverse	CACCACCCTCGGTTGTTAG		
<i>Cg-lys1</i> (AB179775)	Forward	CAACTGTGTCAGAGCCTACAT	87	98.48
	Reverse	CCATTGTGGATTCTGTCATAAC		
<i>Cg-bpi1</i> (AY165040)	Forward	GCTGTAGGGTGTAGCTGTAATG	125	98.39
	Reverse	TTCCGCCTCCAACCATATC		
<i>Cg-galectin</i> (AB308370)	Forward	GGGTTACTCTCCAGGCAATTC	94	97.98
	Reverse	TGGAAGGCAATATCTCCACTTT		
<i>Cg-clec2</i> (AJ579379)	Forward	GATTCCAAGACTCCTGCTACAA	108	98.28
	Reverse	CGATGGTTGTCAGGGTAACTAA		
<i>Cg-timp</i> (AY659986)	Forward	GTCAGTGATGGCGAGTTCTT	110	98.28
	Reverse	CCACAGCCTTGTCCATACAT		
<i>Cg-tgase</i> (BQ426592)	Forward	GTTCAGTGGGTGGTGAAGAA	90	98.88
	Reverse	CATCCTCTGGTGCTGATGTAG		
<i>Cg-efla</i> (EKC33063)	Forward	GAGCGTGAACGTGGTATCAC	141	98.42
	Reverse	ACAGCACAGTCAGCCTGTGA		
<i>Cg-rpl40</i> (AJ563473 & FP004478)	Forward	AATCTTGCACCGTCATGCAG	145	99.01
	Reverse	AATCAATCTCTGCTGATCTGG		
<i>Cg-rps6</i> (XM_011447786)	Forward	CAGAAGTGCCAGCTGACAGTC	129	98.73
	Reverse	AGAAGCAATCTCACACGGAC		

2.3 Results

2.3.1 Total hemocyte counts

A two-way ANOVA on log-transformed data revealed that the effect of bacterial infection on total hemocyte counts in *C. gigas* was significantly altered by exposure to 4-nonylphenol (4-NP*bacteria; $F_{2,62}=3.9048$; $p=0.0257$). A *post-hoc* Tukey's HSD test found that there was a significant increase in THC following injection with *V. campbellii* in the ethanol control group, whereas in both the low and high 4-NP exposure conditions there was no longer a significant difference in THC between naïve and challenged animals (Fig. 1).

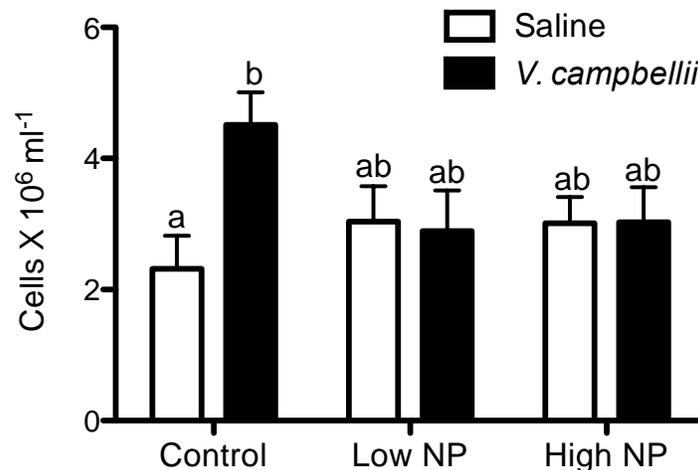


Figure 2. Effect of bacterial infection with *V. campbellii* (black bars) or injection with sterile HEPES buffered saline (open bars) on total hemocyte counts (THC) in *C. gigas* following exposure to low ($2 \mu\text{g l}^{-1}$) and high ($100 \mu\text{g l}^{-1}$) doses of 4-nonylphenol. Groups with significantly different means are indicated by different letters (Tukey's HSD test, $\alpha=0.05$). Values are means \pm SEM; $n=9-12$.

2.3.2 Expression of immune-related gene transcripts

We investigated the effects of 4-NP exposure on the transcript-level response of immune-related genes in *C. gigas* to an experimental infection with *V. campbellii*.

Relative mRNA transcript abundance was quantified in hemocyte, gill and mantle tissues for a subset of antimicrobial peptides/proteins (AMPs; *Cg-defh1*, *Cg-defh2*, *Cg-bigdef1*, *Cg-bigdef2*, *Cg-bpi1*, and *Cg-lys1*) and lectins (*Cg-galectin* and *Cg-clec2*), as well as a matrix metalloproteinase (*Cg-timp*) and a coagulant (*Cg-tgase*).

2.3.2.1 Expression of AMPs

Transcript levels of *Cg-defh1* and *Cg-defh2* mRNA were either undetectable, or very low in all of the tissues we examined. We did clearly detect *Cg-defh2* in the hemocytes, but the data was highly variable. As a result, we did not feel like the *Cg-defh* genes were reliable indicators of the influence of 4-NP exposure during an immune challenge and they were not considered in our overall analysis. *Cg-bigdef1* and, to a somewhat lesser extent, *Cg-bigdef2* displayed extremely variable expression patterns in all tissues in which they were detected. This high inter-individual variability in the expression of *Cg-defhs* and *Cg-bigdefs* has been observed before and is attributed to high rates of polymorphism and/or copy numbers in these genes (Schmitt et al., 2011; Rosa et al., 2015; see discussion). *Cg-bigdef2* was expressed in all three tissues, and *Cg-bigdef1* expression was detectable in the gill and mantle, though not the hemocytes.

Based on our data, *Cg-bigdef1* expression was not responsive to the introduction of *V. campbellii* in either the gill or mantle tissues (Fig. 2A). *Cg-bigdef2* expression, however, appeared to be strongly inducible by infection under all 4-NP conditions and in all tissues (Fig 2B.) Our two-way ANOVA model found a significant main effect of bacteria on *Cg-bigdef2* mRNA abundance in the mantle tissue ($F_{1,74}=6.6879$, $p=0.0118$). Despite a significant NP effect in the two-way ANOVA, *post-hoc* pairwise comparisons

(Bonferroni corrected student's t-test) did not reveal any significant differences between the six treatment groups in the mantle tissue. We therefore ran an additional *post hoc* least-squares means Student's t-test on the data grouped only by bacteria treatment groups (ignoring 4-NP status) and found that expression levels were significantly higher in the bacterially challenged oysters than the naïve oysters (data not shown as grouped). Unfortunately, the high variability in the expression profiles for *Cg-bigdef2* made it more difficult to statistically verify that these increases resulted from bacterial injection; however the *trend* is clearly obvious (and moderately significant) in both hemocyte ($F_{1,45}=3.0733$, $p=0.0829$) and gill ($F_{1,72}=2.9425$, $p=0.0906$) tissues (Fig. 2B). More interesting still is the fact that exposure to 4-NP – at high or low doses - did not appear to disrupt the up-regulation of *Cg-bigdef2* in any of these tissues.

There was also an evident trend of inducible *Cg-bpil* expression following *V. campbellii* injection in the hemocytes, but this response did not occur in those oysters that were exposed to 4-NP (bacteria*NP effect; $F_{2,43}=2.828$, $p=0.0702$; Fig. 2C). This was one of only two instances of an interaction effect among our entire gene expression data set and we felt it was worthy of attention despite statistical indications of only moderate significance ($p<0.1$). In the mantle tissue we also observed a significant increase in *Cg-bpil* expression following infection, but this response was *not* inhibited by 4-NP exposure (bacteria effect; $F_{1,74}=4.3587$, $p=0.0403$; Fig. 2C). *Post-hoc* comparisons on data grouped only by bacteria treatment indicated that *Cg-bigdef2* mRNA levels were significantly higher in the challenged oysters than the naïve oysters (least-squares means Student's t-test; data not shown). In the gill tissue there was a significant effect of 4-NP on *Cg-bpil* expression (NP effect; $F_{2,74}=3.558$, $p=0.0335$; Fig. 2C), whereby *Cg-bpil*

transcript abundance was significantly lower in oysters exposed to high doses of 4-NP relative to those exposed to the low dose treatment (Least squares means Tukey's HSD *post-hoc*). This pattern was exactly mirrored in the mantle tissue (NP effect; $F_{2,74}=2.8569$, $p=0.0638$), although the two-way ANOVA p-value was just slightly above the alpha value of 0.05.

The expression of *Cg-lysI* was responsive to bacteria in the gill (bacteria effect; $F_{1,74}=5.0617$, $p=0.0274$) and mantle (bacteria effect; $F_{1,71}=7.096$, $p=0.0096$) tissues, though subsequent *post-hoc* analyses revealed that the bacterium-induced *Cg-lysI* up-regulation was only significant in the low dose treatment groups (Fig. 2D; Bonferroni corrected student's t-test; $\alpha=0.0167$). Arguable, this could otherwise be interpreted as a decrease in constitutive *Cg-lysI* expression in naïve animals that have been exposed to low dose 4-NP. And in fact, this pattern was evident in all three tissues (Fig. 2D). There was no significant increase in *Cg-lysI* expression following infection in the ethanol control group in any of the three tissues.

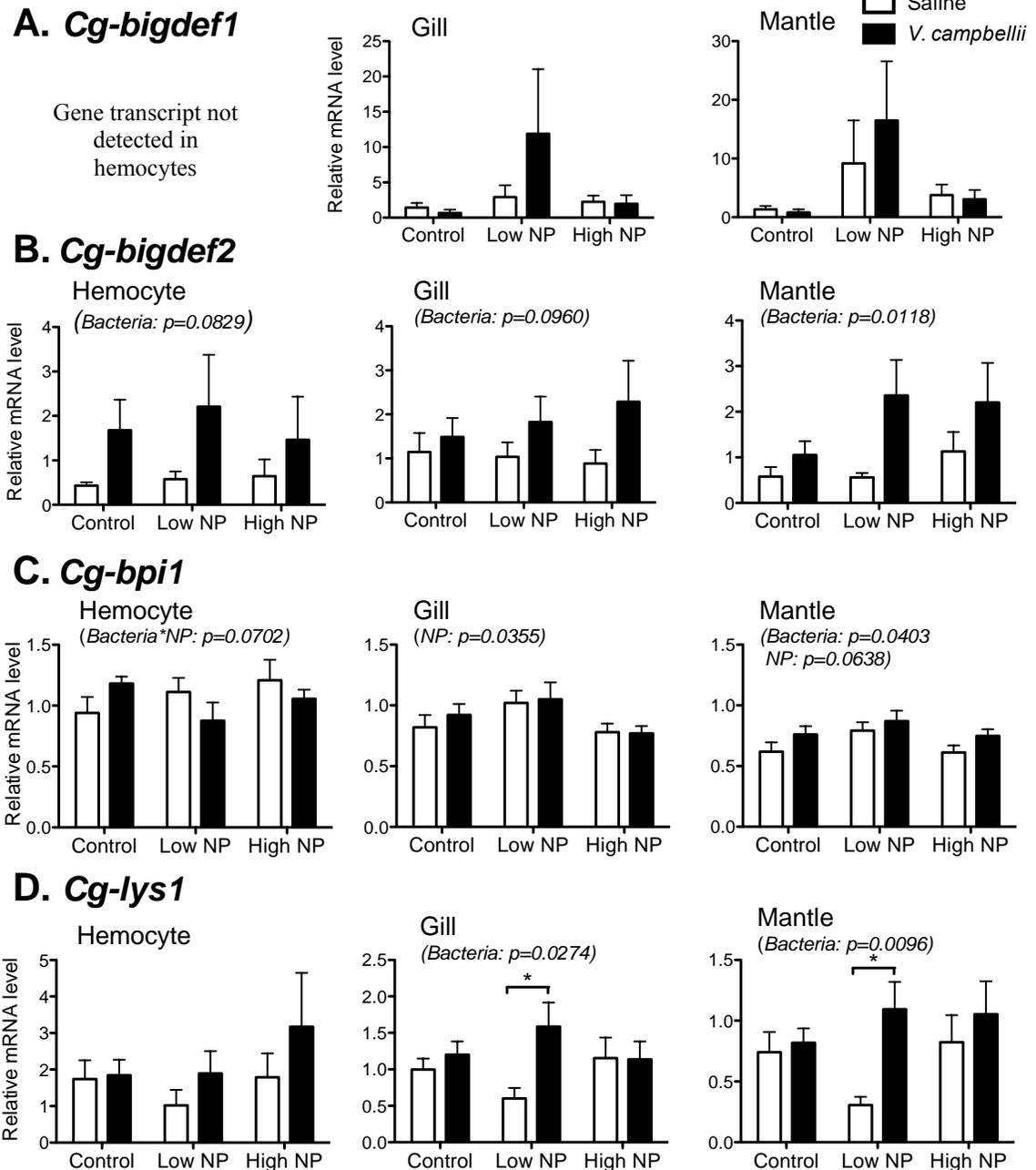


Figure 3. Effect of bacterial infection with *V. campbellii* (black bars) or injection with sterile HEPES buffered saline (open bars) on relative mRNA expression of antimicrobial peptide genes (A. *Cg-bigdef1*, B. *Cg-bigdef2*, C. *Cg-bpi1*, and D. *Cg-lys1*) in *C. gigas* following exposure to low ($2 \mu\text{g l}^{-1}$) and high ($100 \mu\text{g l}^{-1}$) doses of 4-nonylphenol. Genes in which there was a significant ($p < 0.05$) or moderately significant ($p < 0.10$) main effect (bacteria or 4-NP) or interaction effect in the corresponding two-way ANOVA have been indicated in parentheses. Asterisks indicate a significant difference between indicated treatment means (Bonferroni corrected Student's t-test; $\alpha = 0.0167$). All values are means \pm SEM; $n=5-14$ (hemocytes), $n=9-16$ (gill) and $n=10-16$ (mantle).

2.3.2.2 Expression of lectins

We found no statistical evidence that expression of either *C-type lectin2* (*Cg-clec2*) or *galectin* (*Cg-gal*) mRNA was affected by experimental infection with *V. campbellii* in any of the tissues (Fig. 3). As such, neither of these genes were useful candidates for revealing the interacting effect of 4-NP and the immune response. There was, however, evidence that 4-NP exposure independently altered gene expression in this functional group. In the mantle tissue, two-way ANOVA models revealed a significant main effect of 4-NP on the transcript abundance of both *Cg-gal* ($F_{2,71}=4.795$, $p=0.0111$) and *Cg-clec2* ($F_{2,73}=3.131$, $p=0.0496$) (Fig. 3). Exposure to the high 4-NP dose appears to significantly elevate *Cg-gal* mRNA levels relative to the control dose (Fig. 3A; Bonferroni corrected Student's t-test; $\alpha=0.0125$), whereas *Cg-clec2* mRNA levels are significantly depressed in the low 4-NP dose relative to the control treatment (Fig. 3B; least-squares means Dunnet's test).

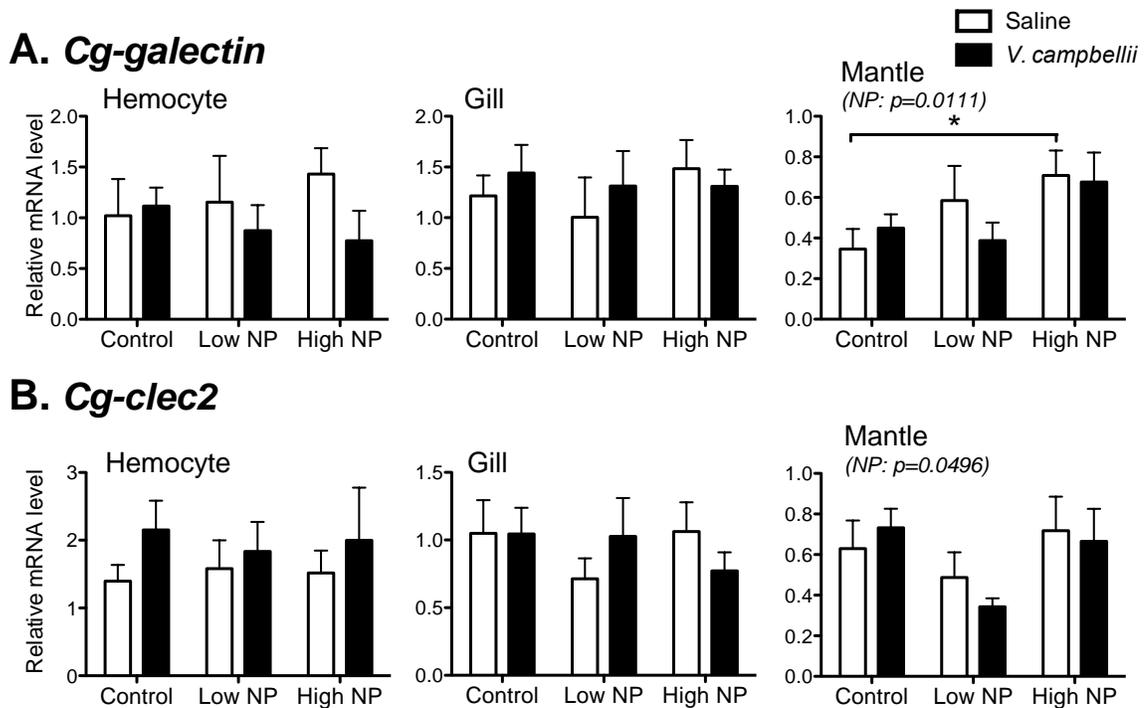


Figure 4. Effect of bacterial infection with *V. campbellii* (black bars) or injection with sterile HEPES buffered saline (open bars) on relative mRNA expression of lectin genes (A. *Cg-olec2* and B. *Cg-galectin*) in *C. gigas* following exposure to low ($2 \mu\text{g l}^{-1}$) and high ($100 \mu\text{g l}^{-1}$) doses of 4-nonylphenol. Genes in which there was a significant ($p < 0.05$) or moderately significant ($p < 0.10$) main effect (bacteria or 4-NP) or interaction effect in the corresponding two-way ANOVA have been indicated in parentheses. Asterisks indicate a significant difference between indicated treatment means (Bonferroni corrected Student's t-test; $\alpha = 0.0125$). All values are means \pm SEM; $n = 5-14$ (hemocytes), $n = 12-16$ (gill) and $n = 11-16$ (mantle).

2.3.2.3 Expression of matrix metalloproteinase and coagulants

The transcript abundance of *Cg-timp* was down-regulated by *V. campbellii* exposure in the gill tissue (bacteria effect; $F_{1,73} = 5.3795$, $p = 0.0232$; least-squares means Student's t-test *post-hoc*), and this effect appears to be independent of 4-NP exposure (Fig. 4A). In the hemocyte and mantle tissues *Cg-timp* mRNA levels were not influenced by either bacterial challenge or 4-NP exposure. Expression of *Cg-tgase* mRNA in the hemocyte and gill tissues was likewise unaffected by both bacterial challenge and 4-NP exposure (Fig. 4B). Our statistical analysis did, however, find a moderately significant

bacteria*NP interaction effect on *Cg-tgase* expression in the mantle tissue (bacteria*NP; $F_{2,74}=2.6498$, $p=0.0774$). Here bacterial challenge induced *Cg-tgase* expression in control oysters, but this response was repressed in 4-NP exposed oysters (Fig. 4B).

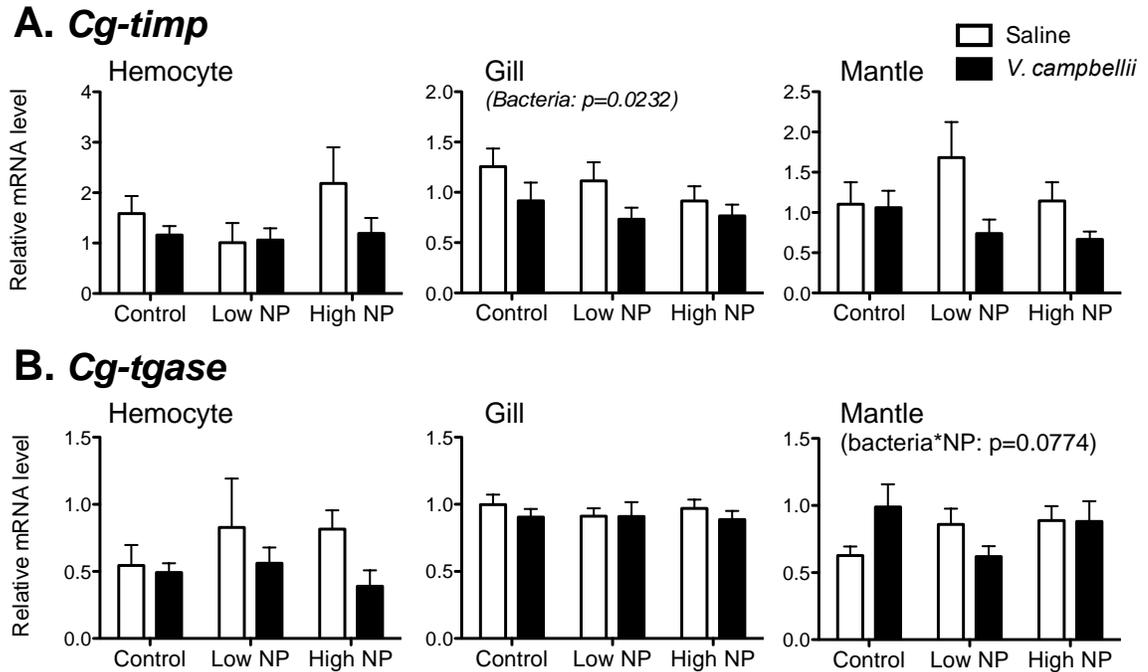


Figure 5. Effect of bacterial infection with *V. campbellii* (black bars) or injection with sterile HEPES buffered saline (open bars) on relative mRNA expression of a protease inhibitor (A. *Cg-timp*) and a coagulant (B. *Cg-tgase*) genes in *C. gigas* following exposure to low ($2 \mu\text{g l}^{-1}$) and high ($100 \mu\text{g l}^{-1}$) doses of 4-nonylphenol. Genes in which there was a significant ($p<0.05$) or moderately significant ($p<0.10$) main effect (bacteria or 4-NP) or interaction effect in the corresponding two-way ANOVA have been indicated in parentheses. Asterisks indicate a significant difference between indicated treatment means (Bonferroni corrected Student's t-test; $\alpha=0.0167$). All values are means \pm SEM; $n=7-14$ (hemocytes), $n=12-16$ (gill) and $n=12-16$ (mantle).

2.3.3 Lysozyme activity

Lysozyme activity was measured in the cell-free hemolymph plasma at 3 h and 24 h after experimental *V. campbellii* challenge in oysters exposed to low ($2 \mu\text{g l}^{-1}$), high ($100 \mu\text{g l}^{-1}$), or control ($100 \mu\text{l ethanol}$) doses of 4-NP (Table 2). The two-way ANOVA

model revealed no significant main effects of bacterial infection or 4-NP exposure on lysozyme activity at 3 h (see Table 2; graph not shown). At 24 h post-injection, we observed a significant main effect of 4-NP on lysozyme activity (4-NP; $F_{2,60}=3.7970$; $p=0.0280$). Despite a lack of significance in our *post-hoc* analysis (Bonferroni corrected Student's t-test), lysozyme activity was higher in all oysters exposed to low doses of 4-NP main effect of 4-NP (Fig. 5, main graph). Given the clear lack of bacterial infection we chose to ignore infection status and perform a least-squares means Tuckey HSP *post-hoc* comparison on the 24 h data combined (Fig. 5, inset graph).

Table 2.
Lysozyme activity ($U\ ml^{-1}$) at 3 h and 24 h post *V. campbellii* injection in oysters exposed to low ($2\ \mu g\ l^{-1}$), high ($100\ \mu g\ l^{-1}$), or control ($100\ \mu l$ ethanol) levels of 4-NP.

Treatment	Lysozyme activity ($U\ ml^{-1}$)			
	3 h		24 h	
	Saline	Bacteria	Saline	Bacteria
Control (ethanol)	58.69 ± 6.36^a	65.95 ± 7.74^a	58.85 ± 2.54^a	60.42 ± 4.53^a
Low 4-NP ($2\ \mu g\ l^{-1}$)	60.83 ± 6.69^a	67.55 ± 3.52^a	72.85 ± 4.42^a	73.87 ± 6.23^a
High 4-NP ($100\ \mu g\ l^{-1}$)	58.66 ± 7.04^a	58.02 ± 12.88^a	69.59 ± 6.48^a	59.48 ± 5.04^a

Note: sample sizes for each treatment group ranged from 10-11. Means with different letters indicate significant differences in Bonferroni corrected Student's t-test *post-hoc* comparisons

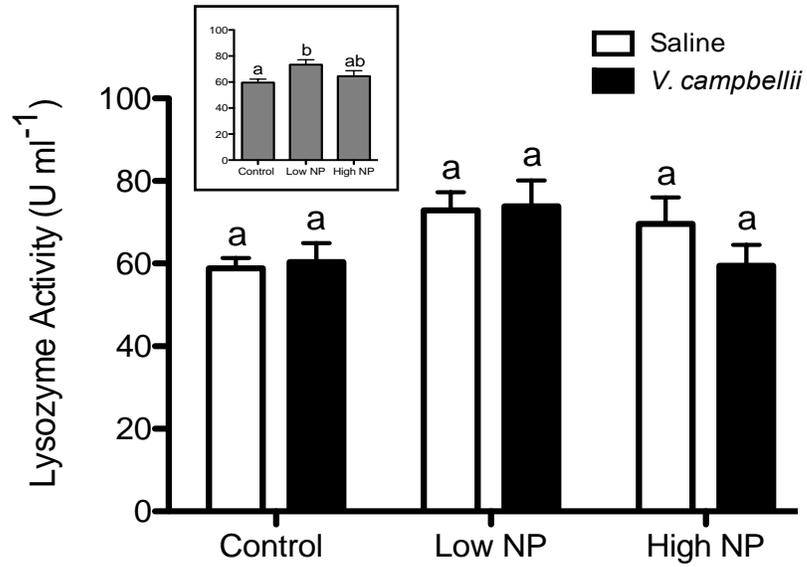


Figure 6. Effect of bacterial infection with *V. campbellii* (black bars) or injection with sterile HEPES buffered saline (open bars) on of lysozyme activity (U ml^{-1}) in *C. gigas*. We observed a main effect of 4-NP (two-way ANOVA; $p = 0.0280$), in exposure to low levels of 4-NP (2 ug l^{-1}) significantly increased lysozyme relative to controls. Inset graph shows combined data and groups with significantly different means are indicated by different letters (main graph: Bonferroni corrected student's t-test, $\alpha = 0.0125$; inset graph: least-squares means Tukey's HSD test, $\alpha = 0.05$). Values are means \pm SEM; $n = 10-11$.

2.4 Discussion

In the current study, we examined how 4-nonylphenol, a known xenoestrogenic contaminant, may be discernibly altering immunocompetence in the Pacific oyster, *Crassostrea gigas*, an aquaculture species of global economic importance. Invertebrate defenses are mediated solely by innate immunity, which is facilitated by both cellular (i.e., hemocytes) and humoral elements (e.g., inducible proteins such as AMPs, lectins, agglutinins, and lysins) (Allam and Paillard, 1998; Canesi et al., 2002; Iwanaga and Lee, 2005; Pruzzo et al., 2008). Our results point to a distinct deleterious effect of 4-NP exposure at the cellular defense level in *C. gigas* oysters that have been infected with a non-pathogenic *Vibrio campbellii* bacterium as seen by altered hemocyte counts. The results also suggest that 4-NP is moderately altering immune-gene transcript abundances and lysozyme activity during the *C. gigas*' immune response.

2.4.1 Total hemocyte counts

One of the initial cellular defense responses of an invertebrate following pathogen introduction is migration of circulating hemocytes to the site of infection for the purposes of microbial phagocytosis and encapsulation (Söderhäll and Cerenius, 1992; Cheng, 1996; Lee and Söderhäll, 2002; Smith, 2010). In bivalves, circulating hemocyte counts also tend to increase following the introduction of microorganisms (Oubella et al., 1993, 1996; Allam et al., 2006; Labreuche et al. 2006). Such hemocyte recruitment events are generally attributed to mobilization of existing hemocytes from other tissues, a process called hemocytosis, (Oubella et al., 1993, 1996; Allam et al., 2006; Labreuche et al., 2006), though cell proliferation in hematopoietic tissues is another potential cause for an

increase in total hemocytes (Söderhäll et al., 2003; Labreuche et al., 2006). In accordance, we saw a significant increase in total hemocyte counts (THC) 24 h following experimental infection with *V. campbellii* in our 4-NP control oysters (Fig. 1). These data are mirrored by several other studies that reported similar pathogen-induced THC increases in oysters specifically (Anderson et al., 1992; LaBreuche et al., 2006, Li et al., 2016).

What was more interesting than the pathogen-induced THC increase we observed in our control oysters, is that we did *not* observe any differences in the hemocyte counts between naïve and bacterially-challenged oysters that were exposed to low or high doses of 4-NP (Fig. 1). It appears that 4-NP markedly inhibited normal hemocyte recruitment and/or proliferation in *C. gigas* during pathogen introduction, and these effects are likely to have immunosuppressive consequences. Our observation that 4-NP disrupts the cell-mediated immune response in oysters is corroborated by a number of other studies that have revealed rapid, functional effects of 4-NP on hemocyte parameters in bivalves. Matozzo and Marin (2005) found that exposure to 4-NP in clams (for similar duration and lengths as in the present study) led to a substantial increase in apoptotic activity in hemocytes. 4-NP has also been shown to decrease hemocyte lysosomal membrane stability in mussels (Canesi et al., 2004, 2007) and cockles (Matozzo et al., 2008), alter hemocytic phagocytosis in mussels (Canesi et al., 2007), decrease hemocyte membrane stability and, as mentioned above, increased hemocyte apoptosis in clams (Matozza and Marin, 2005). Perhaps an increase in hemocyte cell death could explain why 4-NP exposed *C. gigas* failed to elicit an increase in cell counts following infection.

Determining the mechanisms of this repression are outside the scope of this paper and more targeted investigations into specific hemocyte activity will be necessary.

While exposure to 4-NP did repress the pathogen-induced increase in THC in our oysters, it had no significant effect on baseline THC in naïve oysters. This finding differs from previous studies, which reported an increase in THC in both clams (Matozzo and Marin, 2005) and cockles (Matozzo et al., 2008) exposed to nonylphenol (100 - 200 µg/L for 7d). Perhaps the slight (although insignificant) increases we see in our saline-injected, 4-NP exposed oysters relative to the control oysters (Fig. 1) are indicative of this pattern.

2.4.2 Expression of immune-related gene transcripts

Having established a cellular-level (i.e., THC) impediment of 4-NP to the *C. gigas* immune response, we turned our attention to the gene expression response of various humoral defense elements. After completing a comprehensive qPCR analysis of a suite of 10 immune associated genes (*Cg-defh1*, *Cg-defh2*, *Cg-bigdef1*, *Cg-bigdef2*, *Cg-bpil*, *Cg-lys1*, *Cg-galectin*, *Cg-clec2*, *Cg-timp*, and *Cg-tgase*), we did not observe any overwhelmingly clear effect of 4-NP on relative mRNA transcript abundance in bacterially-challenged oysters. There was a small subset of genes that exhibited moderate 4-NP disruptions during the bacterial response; these included *Cg-bpil* in the hemocytes, and perhaps to a lesser extent *Cg-tgase* in the mantle. Several other genes (*Cg-bigdef2*, *Cg-lys1*, and *Cg-timp*) demonstrated a clear response to *V. campbellii* infection, but this response was not modulated by 4-NP. We also found evidence that 4-NP alone can influence mRNA levels of certain genes in naïve oysters; these included *Cg-bpil* in the

gill, and *Cg-galectin* and *Cg-clec2* in the mantle. Below we have grouped our genes of interest into functional categories, and we will discuss our findings in this context.

2.4.2.1 Expression of AMPs

A selection of the genes we measured (*Cg-defh1*, *Cg-defh2*, *Cg-bigdef1*, *Cg-bigdef2*, *Cg-bpi1*, *Cg-lys1*) encode for proteins functionally grouped together as antimicrobial peptides (AMPs). AMPs are an integral part of the innate immune response of virtually all living organisms (Bulet et al., 2004). Defensins are a prominent group of AMPs in the Pacific oyster and were first identified in the mantle tissue (*Cg-defm*; Gueguen et al., 2006) and later in hemocytes (*Cg-defh1* and *Cg-defh2*; Gonzalez et al., 2007a), where they are each constitutively expressed. Representatives of a separate family of ‘big’ defensins (first identified in the horseshoe crab; Kawabata, 1997) have also been characterized in *C. gigas* (*Cg-bigdef1*, *Cg-bigdef2* and *Cg-bigdef3*) and are genetically and structurally more similar to vertebrate defensins than to the invertebrate defensins (Rosa et al., 2011).

Using RT-qPCR, we quantified gene expression patterns of *Cg-defh1*, *Cg-defh2*, *Cg-bigdef1* and *Cg-bigdef2* in *C. gigas*. Transcript levels of *Cg-defh1* and *Cg-defh2* were either undetectable, or very low and highly variable in all of the tissues we examined (mantle, gill, and hemocytes). As a result, we did not feel like these genes could be reliable indicators of the influence of 4-NP exposure during an immune challenge and they were not considered in our overall analysis. Gonzalez et al. (2007a) investigated the effects of bacterial challenge on gene expression of *Cg-defh1* and *Cg-defh2* in the same tissues, and also reported very low expression of both products. Ultimately, they too

considered the amount of *Cg-defh1* transcript measured to be too low to accurately assess pathogen-induced expression profiles. They did, however, report a significant decrease in *Cg-defh2* transcripts in circulating hemocytes 24h following a *Vibrio* bacterial challenge, whereas transcript abundance increased in the gill and mantle (presumably due to migrating hemocytes). Schmitt et al., (2012) also reported a *Vibrio*-induced decrease in *Cg-defh* (based on a universal primer for *Cg-defh1* and *Cg-defh2*) in circulating hemocytes, although they observed no significant changes in gill or mantle. At the same time though, they saw an *increase* in transcript abundance of *Cg-defh* at the injection site. Based on this data, they surmised that *Cg-defhs* might play a more important role in wound repair than defense against microbial infection.

The other group of defensins, the big defensins (*Cg-bigdef1* and *Cg-bigdef2*), had unusually variable expression patterns in all tissues in which they were detected. (*Cg-bigdef1* expression was detectable in the gill and mantle, though not the hemocytes, whereas *Cg-bigdef2* was expressed in all three tissues; Fig. 2A, B). Previous work has shown that *Cg-bigdef1-3* expression is localized exclusively to circulating hemocytes, though transcripts can also be detected in gill, gonad and digestive tissues owing to heavy hemocyte infiltration of these tissues (Rosa et al., 2011). Had we pooled our hemolymph samples, the hemocyte cDNA concentrations may have been sufficient to reliably quantify *Cg-bigdef1* in our own hemocyte samples. That being said, Rosa et al. (2011) also reported extreme variability in transcript abundances for *Cg-bigdef1*, *Cg-bigdef2* and *Cg-bigdef3* in hemocytes despite analyzing RNA from pooled (n=10 oysters) hemolymph samples. This variability has since been attributed to a high level of gene sequence polymorphisms in oysters (Hedgecock et al., 2004; Sauvage et al., 2007), which is

particularly high for the *Cg-defhs* and *Cg-bigdef*s (Schmitt et al., 2013; Rosa et al., 2015). In addition to the high polymorphism in *Cg-bigdef* mRNA expression (and hence high inter-individual expression), not all oysters express all the three *Cg-bigdef*s at the same time (Rosa et al., 2015). In *C. gigas* the *bigdef* genes demonstrate an extreme form of copy number variation called presence-absence variation, in which a large DNA segment present in some individuals can be entirely missing in others (Rosa et al., 2015).

In our own study, oysters injected with *V. campbellii* had a clear increase in *Cg-bigdef2* expression in all tissues, irrespective of 4-NP exposure (Fig. 2B). The unusual, polymorphism-driven variability, however, made it difficult to statistically assign this increase to the effect of infection. We feel confident that this is a true pathogen-stimulated increase, despite a lack of statistical support at the $p < 0.05$ level (our p -values < 0.1). And in fact, of all the genes we examined the bacterial induction of mRNA expression is the most conspicuous for *Cg-bigdef2*. It is all the more interesting then that acute exposure to low or high dose 4-NP does not appear to alter the expression of *Cg-bigdef2* following experimental infection, nor does it appear to have any independent effect on expression in naïve oysters. Thus, 4-NP does not appear to disrupt expression of the most bacteria sensitive immune gene we examined.

Expression of *Cg-bigdef1*, on the other hand, was not responsive to bacterial infection in any tissue (Fig. 2A). This result was surprising given the strong *Vibrio* induced increase in *Cg-bigdef1* reported by Rosa et al. (2011) in *C. gigas* hemocytes. We suspect that the high sequence polymorphism characteristic of this gene (mentioned just above) may have resulted in such great variation in expression that we failed to observe a significant increase in *Cg-bigdef1*. There is also the fact that different bacterial strains

have been shown to have variable effects on *Cg-bigdef* gene expression. Rosa et al. (2011) found the mRNA expression of *Cg-bigdef1* and *Cg-bigdef2* in *C. gigas* hemocytes to be significantly different 24 h following experimental infection with a virulent *Vibrio* bacterium (*V. splendidus*) and an avirulent strain (*V. tasmaniensis*). It is also possible that *V. campbellii*, which is non-pathogenic in oysters, does not itself induce strong *Cg-bigdef1* expression.

We also quantified *Cg-bpil*, which encodes for the AMP bactericidal-permeability-increasing protein. BPI's activities are a result of its ability to permeabilize bacterial membranes (Gonzalez et al., 2007b) due to their affinity for binding to lipopolysaccharides found on the outer membrane of gram-negative bacteria (Elsbach & Weiss, 1998; Levy, 2000). In this study, we detected the expression of *Cg-bpil* in all three tissues tested (gill, mantle and hemocytes), and unlike *Cg-defs* and *Cg-bigdefs* we saw lower variation among treatments, which we attribute to absence of sequence polymorphisms in the *Cg-bpil* gene (Schmitt et al., 2013). Most notable in this dataset was a moderately significant interaction ($p < 0.10$, 4-NP*bacteria) effect between exposure to 4-NP and a bacterial challenge in the hemocyte tissues. We observed that exposure to 4-NP repressed the up-regulation of *Cg-bpil* transcripts in circulating hemocytes that resulted from bacterial introduction (Fig. 2C). Interestingly, the same bacterial induction of *Cg-bpil* seen in the mantle tissues was not inhibited by 4-NP. We also found evidence that 4-NP had independent effects on *Cg-bpil* expression, whereby exposure to high and low levels of 4-NP resulted in differential *Cg-bpil* profiles in the gill tissue and to a slightly milder extent in the mantle tissue. In both cases, transcript abundance was higher in oysters that were exposed to a low dose of 4-NP relative to

those in the high dose treatment (Fig. 2C). This pattern of non-monotonic dose response is common in other estrogenic compounds (e.g. E₂, Welshons et al., 2003; BPA, Vandenberg, 2014; atrazine, Hayes et al., 2002). The decreased expression of *Cg-bpi1* transcripts following 4-NP exposure may be the result of increased hemocyte apoptosis, degranulation, and impaired hemocyte activation, which are characteristic of estrogenic EDC exposure in other marine invertebrates (Sung and Su, 2005; Canesi et al., 2004, 2006). These results make a case that 4-NP may be altering oyster immune responses mediated by *Cg-bpi1*; although changes of *Cg-bpi1* transcripts in the gill and mantle tissues do not mirror those seen in the hemocytes, 4-NP is clearly modulating expression of this gene, especially in oysters exposed to high levels of 4-NP.

Lysozymes, the final AMP we assessed, are another phylogenetically ubiquitous group of enzymes that exhibit lytic properties on the peptidoglycans that comprise bacterial cell walls (Schmitt et al, 2011; Bachère et al., 2015). They are a multigenic family detected in oyster tissues including the gills, mantle and hemocytes of *C. gigas* (Matsumoto et al., 2006). *C. gigas* is known to have at least three lysozyme forms: *Cg-lys1* (Matsumoto et al., 2006), *Cg-lys2* (Itoh and Takahashi, 2007) and *Cg-lys3* (Itoh et al., 2010); *Cg-lys3* is largely involved in digestion, whereas *Cg-lys1* and *Cg-lys3* are both involved in host defense and are expressed mainly in the mantle and hemocytes (Itoh et al., 2007; Itoh et al. 2010; Takahashi and Itoh, 2011). To date, it appears that no one has completed a targeted RT-qPCR analysis of *Cg-lys1-3* expression in oysters following experimental bacterial infection. Although we revealed no effect of *V. campbellii* infection on *Cg-lys1* expression in the mantle and gill tissues of our 4-NP control oysters, there was significant up-regulation of *Cg-lys1* in oysters exposed to low levels of 4-NP

(Fig. 2D). In other studies, *Vibrio* injections induced significant up-regulation of the lysozyme gene expression in mussels (Li et al., 2008) and clams (Zhao et al., 2010; Liu et al., 2014). It appears that this particular *Vibrio* does not induce differential expression of *Cg-lysI* in the hemocyte tissues of *C. gigas*. Based on findings that 4-NP reduces lysozyme activity in clams (Matozza and Marin, 2005) and cockles (Matozza et al., 2008), we expected to see lower overall *Cg-lysI* transcript abundance in oysters exposed to 4-NP relative to the control. We also observed that naïve oysters in the low 4-NP group had significantly lower expression of *Cg-lysI* relative to infected oysters exposed to the same of 4-NP conditions (Fig. 2D). These results suggest that regulation of *Cg-lysI* expression is sensitive to low dose of 4-NP exposure, but the resulting nonylphenol-induced inhibition of *Cg-lysI* may be overcome by bacterial infection. A similar, though non-significant, expression trend was also seen in the hemocyte tissue. Other studies on marine bivalves confirm higher relative *Cg-lysI* expression in gill and mantle tissues compared to hemocytes (Itoh and Takahashi, 2007, Matsumoto et al., 2006), which may help to explain the lack of significant changes in *Cg-lysI* expression in our hemocyte tissue.

It appears not all of these AMP genes respond in the event of a non-pathogenic bacterial infection, but our results indicate that some antimicrobials are partially involved in a response to a non-pathogenic infection of *V. campbellii*. In a similar study, oysters subjected to various virulent *Vibrio* infections showed no change in expression of antimicrobial *Cg-bigdefs*, however *Cg-lysI* transcripts were significantly increased in hemocytes from oyster surviving virulent *Vibrio* infection (de Lorgeril et al., 2011). Nevertheless, our data suggests that exposure to 4-NP may play a minor role in activating

defense mechanisms governed by antimicrobial peptides. Although there was high variability in expressions patterns of these AMP genes within each treatment, it is important to note that the polymorphism of immune genes including antimicrobials may result in variable expression level, potentially causative of variability in pathogen or disease susceptibility (Lazzaro et al., 2004; Tennessen et al., 2009).

2.4.2.2 Expression of lectins

Lectins, found in species of all taxa, are carbohydrate-binding glycoproteins involved in non-self recognition. Found in the hemolymph of many invertebrate species, they are important constituents of the innate defense system due to their ability to recognize non-self cells and aid with phagocytosis (Olafsen, 1995). Lectins reversibly bind sugar molecules on cell membranes to facilitate pathogen elimination via agglutination and encapsulation (Sharon and Lis, 2005; Vasta et al., 2012). In this study we examined oyster galectin (*Cg-galectin*), a β -galactosidase binding lectin (Vasta, 2009; Yamaura et al., 2008) that can act as a recognition factor to pathogens by binding to glycans on the surface of microbes (Vasta et al., 2012). We also quantified the transcript abundance of *Cg-C type lectin2* (*Cg-clec2*) that encodes for a membrane-associate protein also believed to be a mediator of immune responses due to its role in pathogen recognition (Schmitt et al., 2013; Vasta et al., 1982).

In oysters, galectins are involved in important host-defense mechanisms because they bind to foreign particles and promote phagocytosis of macrophages (Tasumi and Vasta, 2008; Yamaura et al., 2008). Their involvement in oyster immune function is attributed to increases in gene transcript abundance in hemocytes induced by bacterial

infection (Gueguen et al., 2003). We did not, however, see a significant change in expression of *Cg-galectin* following a *V. campbellii* challenge. These results are corroborated by other studies in which a virulent bacterial challenge (*Vibrio tubiashii*) did not up-regulate *Cg-galectin* expression in hemocytes (Yamaura et al., 2008). In fact, they found that inoculation had the effect of down-regulating *Cg-galectin* expression, a non-significant trend we also saw in our hemocyte expression profiles for this gene. The lack of this gene's change in expression may also be due to differential responses to various bacterial strains, low mRNA copy numbers found in these tissues (Yamaura et al., 2008) or differential activities of homologous galectins found throughout oyster tissues.

Despite an apparent lack of response to bacterial infection, we observed a 4-NP dose-dependent increase of *Cg-galectin* in mantle tissue, whereby expression levels were highest in the high 4-NP treatment (Fig. 3A). These results suggest that exposure to nylphenol at high enough levels has the ability to modulate *Cg-galectin* gene expression. In their study examining effects of pesticides on oyster immunity, Gagnaire et al. (2007) suggested that pesticides modified the oyster immune response by inducing an *up-regulation* of genes involved in hemocyte functions and defense mechanisms as we see here. Despite this up-regulation, they saw higher mortality in bacterially treated oysters. Increased mortality was attributed to possible tissue damage from pathogenesis of the disease and subsequent decreased regulation capacity. Our results indicate that 4-NP may be affecting baseline *Cg-galectin* gene expression in oysters, but the downstream mechanistic effects of this altered regulation remain to be determined.

Similar to *Cg-galectin* responses, we detected a significant main effect of 4-NP in mRNA transcripts of *Cg-clec2* in the mantle tissue. This gene was markedly depressed in

the low 4-NP treatment group, but was unaltered in the high dose. Considering that this C-type lectin may play an important role in activation of systems that compliment oyster pathogen recognition and elimination (Li et al., 2016), exposure to low levels of 4-NP may inhibit *C. gigas* ability to effectively respond to an immune challenge. That being said, the bacterial strain used in our study (*V. campbellii*) did not activate pathways associated with up-regulation of *Cg-lect2* (nonself-recognition proteins) in hemocytes, gills or mantle tissue. It appears that expression of lectin genes is bacteria-strain specific; recent studies have shown that mRNA expression of *Cg-lect2* in hemocytes was up-regulated significantly after bacterial challenges presented by virulent bacteria, *V. splendidus* (Li et al., 2015; de Lorgeril et al., 2011), but avirulent bacterial challenges did not induce the same increase in mRNA transcripts (de Lorgeril et al., 2011). Overall, our RT-qPCR analysis did not reveal any effects of *V. campbellii* challenge on mRNA expression of these lectin genes, however both genes were influenced by 4-NP exposure.

2.4.2.3 Expression of matrix metalloproteinase and coagulants

Tissue inhibitor metalloproteinases (TIMPs) are endogenous proteins that play important roles in the regulation of extracellular matrix (ECM) by inhibiting matrix metalloproteinases (MMPs) and affecting turnover and remodeling processes (Brew et al., 2000; Gomez et al., 1997). In *C. gigas*, *Cg-timp* is thought to be involved in host immune reactions and tissue repair (Montagnani et al., 2007). In all three tissues tested, we saw a general decrease in *Cg-timp* transcript abundance in response to bacterial exposure, but only expression changes in gill tissue exhibited a significant decrease (Fig. 4A). This trend presented itself independent of 4-NP exposure. Although our

expectations for changes in *Cg-timp* were neutral, others found that *Cg-timp* was over-expressed after injuries and bacterial challenges in *C. gigas* (Montagnani et al., 2001). However, subsequent work suggested that *Cg-timp* activation in response to infection is independent of a non-self recognition step and its precise role in responding to bacterial infection is unknown (Montagnani et al., 2007).

One possible explanation for the decrease expression we saw in the gill tissue as a result of bacterial injection is that hemocytes aggregate near tissue damage caused by injection (muscle herein) and the out-migration of these hemocytes makes it appear that there is depressed *Cg-timp* expression in the gill and mantle tissues. Furthermore, the strain of bacterial infection appears to play a role in regulating *Cg-timp*. When Labreuche et al. (2006) infected oysters with a single strand of *V. aestuarianus*, they also saw no increase of *Cg-timp* and suggested that different bacteria infect through different mechanisms, thereby activating various pathways. Regardless of the effects resulting from bacterial infection, 4-NP does not appear to interfere with baseline *Cg-timp* expression.

Transglutaminase (*Cg-tgase*) is a known coagulation factor in crustaceans (Maningas et al., 2008; Wang et al., 2001), but it's role in bivalve immunity is less clear given the lack of a true hemolymph clotting cascade (Awaji and Suzuki, 1995). Most *Cg-tgase* activity is detected in the gill and mantle of the oyster (Kumazawa et al., 1997) and pathogenic bacterial infections have been shown induce *Cg-tgase* expression (Gueguen et al., 2003). We found that expression of *Cg-tgase* was up-regulated in the mantle tissue following infection, but this response was inhibited in oysters exposed to both low and high levels of 4-NP (Fig. 4B). The interaction seen here, where 4-NP inhibits or

depresses the potential for oyster mantle tissue to express *Cg-tgase* is substantial if in fact this gene is involved in important host defenses such as pathogen recognition and clearance (Gueguen et al., 2003). We detected the same pattern in the hemocyte tissues, but expression differences were not as pronounced as the mantle tissue. These results provides more evidence that there are deleterious effects of 4-NP on the ability of Pacific oysters to effectively fight off an immune challenge from the antibiotic resistant *V. campbellii*.

2.4.3 Lysozyme activity

To further explore the combined effects of bacterial infection and 4-NP on immune parameters, we measured hemolymph plasma lysozyme activity. Lysozymes are bactericidal enzymes that can cleave the glycosidic bonds that form bacterial cell walls and therefore are important humoral defense mediators in invertebrates (McDade and Tripp, 1967; Cheng et al., 1975; Hardy et al., 1976; Bachali et al., 2002). We were able to detect lysozyme activity in the gill, mantle, and hemolymph of *C. gigas* with the highest level of activity found in the hemolymph plasma. Activity here is hypothesized to be a result of the release of lysosomal enzymes by the hemocytes, especially from granulocytes (Cheng and Roderick, 1974; Roderick and Cheng, 1974); this enzymatic release is mediated through various cytotoxic reactions in bivalve hemolymph (Canesi et al., 2002, 2006).

The results from our study indicate that lysozymal activity in cell-free hemolymph plasma was not activated by infection with *V. campbellii*. Despite the lack of response to bacterial infection, our results showed that environmentally relevant levels of

4-NP (low) stimulated an increase in lysozyme activity in cell-free hemolymph plasma of *C. gigas*. This result was interesting because we saw that low levels of 4-NP in bacterially infected oysters also caused an increase in the relative mRNA transcripts of *Cg-lysozyme1* relative to uninfected oysters. (It is important to note that the lysozyme activity does not distinguish between the 3 different lysozyme homologs.)

It was somewhat surprising that bacterial infection alone did not induce an increase of lysozyme activity but it is possible that some foreign agents were resistant to these enzymatic defense responses due to an absence of surface substrates that are susceptible to lysozyme molecules or to a lack of a triggering mechanism responsible for lysozyme release (Canesi et al., 2002). Lack of response may also be attributed to the strain of bacteria because both up- and down-regulation of lysozyme activity has been observed depending on the strain of *Vibrio* (Li et al., 2008). Seasonal changes were also known to be a factor in lysozyme activity in oysters (Chu and Peyre, 1989).

Clear changes in lysozyme activity, an important humoral defense mechanism, in response to low levels of 4-NP are notable in our attempts to understand the effects of this EDC on the oyster immune systems. Similar increases in lysozyme activity as a result of exposure to 4-NP were seen in cockles (*Cerastoderma glaucum*) and were hypothesized to be the result of altered lysosomal membrane stability and subsequent release of enzymes into the hemolymph (Matozzo et al., 2008). It has also been suggested that clams (*Mercenaria mercenaria*) experience immunosuppression when exposed to 4-NP due to decreased lysozyme activity in the hemocytes (Matozzo and Marin, 2005). Furthermore, high concentrations of a mixture of estrogenic EDCs (including 4-NP) were shown to induce lysosomal membrane destabilization in mussels (*Mytilus*

galloprovincialis) as seen by an increase in lysozyme activity in treated animals (Canesi et al., 2007). Although there are very few studies documenting effects of 4-NP on the immune system of *C. gigas*, we feel confident that low levels of 4-NP are interfering in immunomodulation. It is possible that 4-NP is affecting the stability of lysozyme membranes in hemocytes through either transcriptional pathways or alternative pathways (i.e., lysozymal stability based pathways that interact with the protein directly). A more specific analysis of all lysozyme homologs as well as longer exposure times will be needed to further elucidate the mechanisms of this immune response.

2.5 Conclusion

There are multifold immunomodulatory effects of endocrine disruptors and other environmental contaminants on both cell-mediated and humoral-mediated immunity in bivalves (see reviews: Ellis et al., 2011; Girón-Perez, 2010; Renault, 2015). To our knowledge, this is the first time that 4-nonylphenol has been shown to directly impact hemocyte counts in oysters; this is a considerable finding given that changes in THC are a definitive element of the innate immune response of *most* marine invertebrates (Bachere et al., 2004; Söderhäll and Cerenuus, 1992; Wang et al., 2014). Further, we determined that 4-NP appeared to interact with humoral defense mechanisms mediated by some genes (*Cg-bpi1* & *Cg-tgase*) that were induced by a *V. campbellii* infection. This strain of *Vibrio* induced differential expression profiles of a subset of immune-related genes tested (*Cg-bigdef2*, *Cg-bpi1*, *Cg-lys1*, *Cg-timp*) and exposure to 4-NP alone caused differential expression profiles in some genes (*Cg-bpi1*, *Cg-galectin*, *Cg-clec2*). Lastly, low levels of 4-NP significantly changed lysozyme activity 24 h post-infection. Based on these

findings we are suggesting that 4-NP can inhibit the immune response of adult Pacific oysters, which could potentially increase their disease susceptibility following microbial exposure. These deleterious effects are more prominent for cell-mediated immune responses than for humoral-mediated events. Recent laboratory studies show that there is a complex relationship between pollution exposure and increase of susceptibility to infectious diseases in marine invertebrates, and mechanisms by which this particular pollutant is interfering are not fully understood. It is clear that our work contributes to a growing body of evidence that EDC's have the potential to interfere with the continued health of commercially relevant oysters grown worldwide.

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