In-vitro model of interaction between *Vibrio splendidus* and hemocytes of soft-shell clams, *Mya arenaria*

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ABSTRACT

Mollusc aquaculture is an established industry in Canada, specifically in Prince Edward Island where the mussel production represents 80% of the total production in Canada. However, the viability of this industry is threatened by the introduction and/or emergence of pathogens with propagation of fatal diseases. In molluscs innate immunity is the sole defence against pathogens and comprises the cellular and humoral component. The cellular component, composed of different types of hemocytes, is involved in phagocytosis, encapsulation, respiratory burst activity and production of antimicrobial peptides (AMPs). The humoral component is represented by molecules, such as opsonins, antimicrobial agents and agglutinins. Some of the humoral molecules mediate pathogen recognition and facilitate the clearance of pathogens by phagocytosis, while others are involved in direct killing of pathogens. Although several studies conducted during the last few decades have advanced our knowledge about mollusc immune system, the mechanisms involved in the host defence at the early stage of infection remain unknown. Therefore, the main goal of this thesis is to develop an *in-vitro* model of interaction between hemocytes of soft-shell clams and *Vibrio splendidus* LGP32, and investigate the early immune response of soft-shell clams both at cellular and molecular levels.

Hemocytes of soft-shell clams are actively involved in immune defence. However, they lost their pseudopodia and became rounded after *V. splendidus* challenge. This phenotypic response of hemocytes (measured in percentage of rounded cells) shows a positive correlation between the ratio of hemocytes:bacteria (1:20, 1:10, 1:5 and 1:1) and the time of exposure. At a high number of bacteria per hemocyte (1:20, 1:10 and 1:5), the percentage of rounded (without pseudopodia) hemocytes increased dramatically from less than 20% in one hour to 80-90% in two. At 1:1 hemocyte to *Vibrio* ratio however phenotypic response was gradual (4.3% at 1 hour, 44.2% at 2 hours and 92% at 3 hours) and consequently, the ratio 1:1 was used in all the following experiments. Hemocyte viability in challenged (1:1 hemocyte to *Vibrio*) and control groups was very high (>91%) suggesting morphologically modified cells were alive.

In hemocytes challenged for two hours, phagocytosis and respiratory burst activities were investigated to understand the early response of hemocytes against *V. splendidus*. Hemocytes of soft-shell clams showed the ability to phagocytose and produce reactive oxygen/nitrogen species. This phagocytosis and respiratory burst activity of hemocytes was significantly reduced ($p<0.001$) post-*Vibrio* exposure. The reduction in phagocytosis with an increasing percentage of rounded cells suggests that hemocytes pseudopodia (cytoplasmic extensions) could play a key role in internalizing microbes. Consequently, *V. splendidus* seems to affect the function of hemocytes by modifying their morphology.

Besides the morphological modifications, this research also focused on the expression of genes in hemocytes post *Vibrio* challenge. However, in gene expression assays it is important to have validated housekeeping genes for accurate normalization of data. Hence, the expression stability of a set of genes was evaluated using GeNorm software.

The selected candidate genes included ribosomal proteins (rpS-15, rpS-18 and L-37), ubiquitin, receptor for activated C kinase (RACK), elongation factor 1 (EF-1) and elongation factor 2 (EF-2) as well as 18S-rRNA and actin. Our data analysis showed that actin and 18S-rRNA, usually used as housekeeping genes, were among the least stable transcripts, whereas EF-1, rpS-18 and ubiquitin were the most stable transcripts and were used as housekeeping genes in this *in-vitro* interaction model. Genes associated with cytoskeleton structural stability (actin and EF-2) were among least stable genes. Consequently, the expression of both genes was quantified and showed significant up-regulation (actin $p<0.001$; EF-2 $p<0.05$) in hemocytes challenged for two hours at a ratio 1:1.

To explore the effect of *V. splendidus* at the molecular level, suppression subtractive hybridization (SSH) technique was used to identify the up- and down-regulated genes in hemocytes of soft-shell clams. Both forward and reverse subtracted cDNA were constructed and a total of 16,000 reads were obtained and analyzed. Identity searches in protein databases were performed using BlastX program and the transcripts were clustered to different physiological processes including structural proteins, immunity, stress proteins, apoptosis, cell process, general metabolism and signal transduction. The expression level of transcripts associated with immunity such as ficolin, killer cell lectin-like receptor, natural resistance-associated macrophage protein 1, MAPK, ferritin, HSP90 and cathepsin were quantified using RT-qPCR. Some of these genes play a key role in pathogen recognition, phagocytosis, respiratory burst activity and activating innate immune response in general. The expression pattern in most transcripts showed an up-regulation at one hour followed by a down-regulation at two and three hours. For instance, ferritin and natural resistance-associated macrophage protein 1 (*Nramp* 1), active in intraphagosomal killing of pathogens in higher animals, were down-regulated at two hours, which corresponds to the decrease of respiratory burst activity observed in the previous experiment. Phagocytosis was also reduced at two hours, which could be related to the inactivation of ficolin and killer cell lectin-like receptor, which are important in pathogen recognition and phagocytosis.

This study showed that hemocytes of soft-shell clams are actively involved in immune defence. However, exposure to *V. splendidus* impaired their cellular functions suggesting the virulent nature of the bacterium. Moreover, several novel immune associated genes such as *Nramp* 1, ficolin, T cell receptor and Killer cell lectin-like receptor were identified in this study. Further investigations are needed to characterize and understand the role of those transcripts in the immune defence of soft-shell clams.

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DEDICATION

Teberh, Limeat and Minet

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List of Abbreviations

AMP	antimicrobial peptide
ANOVA	analysis of variance
ASW	artificial seawater
AVC	Atlantic Veterinary College
Bp	base pair
BRD	Brown ring disease
°C	degree Celsius
CA	California
cDNA	complementary deoxyribonucleic acid
CT	Connecticut
Ct	cycle threshold
DCFH-DA	2',7'-dichloroflourescin diacetate
DEPC	Diethylpyrocarbonate
DFO	Department of Fisheries and Oceans Canada
DHFR	dihydrofolate reductase
DHR	dihydrorhodamine 123
DNA	deoxyribonucleic acid
DMR	Department of Marine Resources
ERK	extracellular signal-regulated kinases
ECP	extracellular product
EF	elongation factor
EST	expressed sequence tag
g	Gram
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GFP	green florescent protein
GNBP	Gram negative bacteria-binding protein
HE	Hydroethidine
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSP	heat shock protein
IFREMER	Institut Français de Recherche pour l'Exploitation de la Mer
IHHNV	Infectious Hypodermal and Hematopoietic Necrosis Virus
IL	Interleukin
IRAP	Industrial Research Assistance Program
JNK	c-Jun N-terminal kinases
KLR	killer cell lectin-like receptor
LPS	lipopolysaccharide

M	Molar
MAPK	mitogen-activated protein kinase
MBL	mannan-binding lectins
ml	Milliliter
M-MLV	moloney murine leukemia virus
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate oxidase
NCBI	National Center for Biotechnology Information
NF	normalization factor
NF-kB	nuclear factor kappa B
NK	natural killer cell
NO	nitric oxide
NSERC	National Sciences and Engineering Research Council of Canada
ON	Ontario
ORF	open reading frame
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PEI	Prince Edward Island
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PKC	protein kinase C
PL	post larvae
PMA	phorbol 12-myristate 13 acetate
PRR	pathogen recognition receptors
qRT-PCR	quantitative reverse transcription PCR
RACK	receptor activated C kinase
RG	Rotor Gene
RNA	ribonucleic acid
RNS/I	reactive nitrogen species/intermediates
ROS/I	reactive oxygen species/intermediates
rRNA	ribosomal RNA
scDNA	single strand complementary DNA
SEM	standard error of the means
SSH	suppression subtractive hybridization
TCR	T cell receptor
THC	total hemocyte count
TIMP	tissue inhibitor metalloproteinases
TLR	toll-like receptors
TNFα	tumor necrosis factor alpha

tRNA	transfer RNA
TSA	trichostatin A
TSV	Taura Syndrome Virus
µl	Microlitre
µg	Microgram
µm	Micrometer
UK	United Kingdom
UPEI	University of Prince Edward Island
USA	United States of America
Vsm	*Vibrio splendidus* metalloprotease
WSSV	White Spot Syndrome Virus
xg	g-force

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Chapter 1

General Introduction

1.1 Soft-shell clams

Soft shell clams, *Mya arenaria*, are aquatic animals which belong to the Myacidae family. They are often called "steamers" or "squirt" clams. They are found buried in sediments of estuaries, intertidal and marine waters with varying salinities (5-32 ppt) and temperatures (-2-32 °C) (Abraham and Dillon, 1986). Soft-shell clams are widely distributed along the East coast of North America, Europe and West Pacific coast of Asia (Brawn, 2002).

Soft shell clams were used as food source by aboriginal people for centuries and European settlers first commercialized it not for food but for bait. In the early to mid 20th century, clams became a delicacy and since then the landing of soft shell clams has increased (Brawn, 2002). The market size of soft shell clams varies from place to place. In Prince Edward Island, the market size is 50 mm (shell length), which can be reached in approximately 3-8 years depending on the site (Robert, 1981).

Clams are filter feeders and feed primarily upon microalgae but also bacteria, zooplankton and other suspended debris. Depending on the size of the animal, soft-shell clams can filter as much as 60 liters of water a day by generating a water current using cilia in the gills (Ellis, 1998). When the water is passing through the gills, suspended microscopic food particles are trapped and transferred to the mouth. This mechanism of feeding exposes molluscs continuously to substances in the water such as toxic heavy metals and microalgae products and pathogenic microbes (Potasman et al., 2002). Hence, molluscs harvested from polluted areas can accumulate those toxic substances or

microbes, which are harmful to humans when consumed. For this reason, molluscs are widely used as environmental bio-indicators to monitor water quality (Sanders, 1993).

Robinson (1998) reported a decrease in the soft shell clam production in the Bay of Fundy due to the closure of harvesting areas contaminated with coliforms. Consequently, clam diggers were concentrated in the small open (non contaminated) areas leading to depletion of clam stocks. The soft-shell clam fishery is also a very important sector in Maine (USA), worth $5-10 million annually. It was ranked the second largest economically important industry after lobster (Beal, 2002). However, in the past few years, the soft-shell clam fishery in Maine has declined steadily. For example, in 2000 approximately 1,000 metric tons of soft shell clams were landed, making the clam fishery the third largest in value of all commercial marine species behind lobsters and sea urchins (DMR, 2000). Despite the drop and continuous fluctuation of soft-shell clam landing, it has remained one of the most commercially important species in this region. In Maine, 10 million soft shell clam seeds produced in hatcheries are restocked annually to help recover soft-shell clam fishery in the coastal communities (Beal et al., 1995).

In Canada, mollusc production is growing and generated a revenue of over $72 million in 2006 (DFO, 2009). Prince Edward Island and British Colombia are the major mussel (*Mytilus edulis*) and oyster (mainly pacific oyster, *Crassostrea gigas*) producing provinces in Canada, respectively. According to Aquaculture Canada's report, mussel and oyster production in 2006 in the respective provinces constituted 72% and 60% of Canada's production. Although, the production and revenue from soft-shell clams (*M. arenaria*) in Canada is not comparable with mussel and oysters, soft shell clams, *M.*

arenaria, is a commercially very important mollusc species in the coastal communities of Quebec and the Maritimes.

Soft-shell clams live buried in the sediment and have a close association with microorganisms in the sediment. Hence, due to the nature of the habitat and filter feeding behavior of soft-shell clam, this bivalve mollusc species is continuously exposed to microbial agents, which makes it an ideal candidate for host-pathogen interaction models. In addition, the soft-shell clam has other features which make it attractive for challenge models. They are easy to maintain under laboratory setting and large quantity of hemolymph (for *in-vitro* studies) can be obtained easily without damaging the shell or sacrificing the organism. A study conducted by Tubiash (1972) also suggested that soft-shell clams can be a good candidate to screen potential mollusc pathogens.

1.2 *Vibrio splendidus*

Vibrio species have a widespread geographical distribution in marine environments. Most *Vibrio* species favor warm waters, with temperatures exceeding 17 °C (Wright et al., 1996). The genus *Vibrio* consists of more than 30 species. Some *Vibrio* species have been linked to food borne disease and many of them are human pathogens as well (Chakraborty et al., 1997). *Vibrios* are also pathogenic to bivalve molluscs particularly to larvae and juvenile and caused high mortalities in juvenile American oyster, *Crassostrea virginica* (Le Roux et al., 2002), juvenile Pacific oyster, *Crassostrea gigas* (Costil et al., 2005) and Carpet-shell clam larvae, *Ruditapes decussatus* (Gomez-Leon et al., 2005).

Moreover, some *Vibrios* are disease causative agents in adult molluscs such as clam, *Ruditapes philippinarum* (Paillard and Maes, 1994).

Vibrio splendidus is abundant in temperate waters. It is highly diverse and consists of more than a thousand distinct genotypes (Thompson et al., 2005). Like other *Vibrio* species, *V. splendidus* has also close association with various animals. Depending on the strain, host and the environment, the nature of association ranges from commensal to pathogenic (Le Roux and Austin, 2006). *Vibrio splendidus*-related strains were first isolated from diseased juvenile *C. gigas,* which was responsible for the severe economic loss documented in the early 1990s in Britanny, France (Lacoste et al., 2001). It is also pathogenic to Great scallop larvae, *Pecten maximus* (Lambert et al., 1998; 1999). In addition, the pathogenic effect of *V. splendidus* has been demonstrated in *in-vivo* and *in-vitro* experimental infections in Pacific oysters, *C. gigas* (Allam and Ford, 2006) and Japanese clams, *R. philippinarum* (Choquet et al., 2003), respectively.

Recently, *V. splendidus* LGP32 genome has been sequenced (Le Roux et al., 2009). It contains two circular chromosomes of 3299 and 1675 kb and 4498 and 1500 predicted open reading frames (ORF) in chromosome 1 and 2, respectively. Although both chromosomes have a comparable percentage of unknown genes, chromosome 1 has by far the highest percentage of genes, which codes to various biological functions. Moreover, chromosome 1 contains a large number of rRNA and tRNA genes compared to chromosome 2. Depending on the chromosome of comparison, *V. splendidus* LGP32 appears to be closely related to *Vibrio vulnificus* and *Vibrio harveyi* based on chromosome 1 and 2 similarities, respectively. Most genes that are conserved among

Vibrio species are located in chromosome 1, suggesting that chromosome 2 is subjected to regular genomic rearrangement (Dryselius et al., 2007).

Metalloprotease (Vsm) is the only confirmed virulence factor found in *V. splendidus* LGP32 (Le Roux et al., 2007). Nevertheless, genome sequence of *V. splendidus* revealed the presence of genes homologous to potential virulence factors or toxins. These virulence factors include proteases, adhesins, hemolysins and others (Miyoshi and Shinoda, 2000; Kachlany et al., 2001; Zhang and Austin, 2005). Moreover, genes homologous to antibiotics or anti-microbial peptide resistance were found in *V. splendidus* LGP32 genome. Among them are qnrVS gene resistant to quinolone, dihydrofolate reductase (DHFR) resistant to trimethoprim and ompU gene resistant against anti-microbial peptides (Mathur and Waldor 2004; Cattoir et al., 2007; Le Roux et al., 2009).

1.3 *In vitro* model: Hemocytes-Pathogens interaction

Aquatic bacteria share habitat with molluscs and constitute a source of food for molluscs. Due to the filter feeding behavior of bivalve molluscs, they are continuously exposed to bacteria and can accumulate them in their system in large numbers (Wright et al., 1996). Some bacteria, mainly from the *Vibrio* genera are able to persist within bivalve tissues and fluids (Murphree and Tamplin, 1995). There are *Vibrio*-associated mortalities reported in bivalve molluscs such as Pacific oysters, *C. gigas* (Ottaviani and Franceschi, 1997; Le Roux et al., 2002), clams, *R. philippinarum* (Palliard et al., 1994). *Vibrio*

aestuarianus strain 01/32 was isolated during the mortality outbreak in an experimental hatchery. *In-vitro* tests showed that extracellular products (ECPs) from *V. aestuarianus* strongly inhibited hemocyte phagocytosis and adhesive abilities and it is lethal to *C. gigas* larvae (Labreuche et al., 2006a). These results suggest that the production of ECPs by the pathogen plays an important role in the pathological processes. Others like *V. tapetis,* a brown ring disease causative agent in clams (*R. philippinarum*), avoid host defence by damaging the cytoskeleton of hemocytes which led to reduced adhesion (Choquet et al., 2003).

Before a decade or so, most bivalve mollusc diseases reported were caused by protozoan species such as *Bonamia, Marteilia, Perkinsus* and *Haplosporidium* (Paillard et al., 2004). However, in recent years, bacterial diseases have become common (Borrego et al., 1996; Friedman et al., 1998; Ford and Borrero, 2001) and have become one of the limiting factors in the growth of the mollusc aquaculture industry (Kesarcodi-Watson et al., 2008; Bondad-Reantaso et al., 2005; Muroga, 2001). Bacterial diseases in general and *Vibrio*-associated diseases in particular, were initially associated with high larval mortalities in hatcheries (Sinderman 1990). However, *Vibrio*-associated adult mollusc mortalities were also reported (Paillard and Maes, 1994). As a result, several studies have been performed to understand the interaction between bacteria and hemocytes of different bivalve species, such as: mussels, *Mytilus edulis* (Mayrand et al., 2005; Hernroth, 2003), scallops, *P. maximus* (Mortensen and Glette, 1996), oysters, *C. gigas* (Labreuche et al., 2006b) and other clam species, *R. philippinarum* (Allam and Ford, 2006) both *in vitro* and *in vivo*.

In many mollusc species, a phenotypic change of hemocytes during bacterial challenge is documented. Bivalve hemocytes develop cytoplasmic extensions when in contact with flat surfaces and adhere to them (Labreuche et al., 2006b). However, loss of pseudopodia has been reported in hemocytes of various mollusc species subjected to pathogenic bacteria, leading to the reduction in hemocytes ability to adhere. For example, Labreuche et al. (2006a) demonstrated that extracellular products from *V. aestuarianus* 01/32 cause loss of pseudopodia, reduced adhesion and phagocytosis (hemocyte rounding) in the Pacific oyster, *C. gigas*. *Vibrio alginolyticus* and *V. anguillarum* also induced a loss of pseudopodia in *M. edulis* hemocytes (Lane and Birkbeck, 1999).

Allam et al. (2001) demonstrated that experimental infection of *R. philippinarum* and *R. decussatus* by *Vibrio tapetis* have different degrees of brown ring disease (BRD) signs and recovery, where 100% of the *R. philippinarum* developed BRD sign compared to the 50% *R. decussatus*. Allam and Ford (2006) exposed hemocytes of *R. philippinarum, Mercernaria mercenaria* and *C. virginica* with three different bacteria (*V. tapetis, V. splendidus* and *V. anguillarum*). Hemocytes from *R. philippinarum* and *M. mercenaria* lost pseudopodia and mortality was high when challenged with *V. tapetis* and *V. splendidus*, respectively. Mortality within *C. virginica* hemocytes resulting from the three bacteria species was very low and of no significance compared to control. In contrast, *R. philippinarum* hemocytes exposed to different doses of *V. splendidus* (ATCC 25914) maintained their pseudopodia and their adhesion capabilities were unaffected (Choquet et al., 2003). The results in these independent studies confirm that different isolates present different degrees of pathogenicity within the same host (Allam and Ford, 2006).

1.4 Immune components of molluscs

Generally, the immune system is classified into two main branches: innate and adaptive (Janeway et al., 2005). Innate immunity is the first line of defence and comprises several components such as epithelia, cellular and humoral. The epithelia serve as a physical barrier and prevent pathogens from entering. Once pathogens make their way inside the host, macrophages and neutrophils control their spread and destroy them by phagocytosis. Unlike innate immunity, adaptive immunity is only present in vertebrates and possesses antigen-specific effectors cells (B and T cells), which produce antibodies and target specific pathogens. Moreover, adaptive immunity is known for its specific response and memory cells, which prevent re-infection with the same pathogen.

Although there is a general belief that adaptive immunity is a signature for vertebrates, recent reviews by Flegel (2007) and Rowley and Powell (2007) suggest that invertebrates possess an immune mechanism, which is somehow similar to the adaptive immunity of vertebrates. For instance, antimicrobial activity in bumblebees (*Bombus terrestris*) and mealworm beetles (*Tenebrio molitor*) was found to be significantly higher in progeny descended from challenged groups than in those descendants from unchallenged groups (Sadd et al., 2005; Moret, 2006). Strong evidence of high specificity in invertebrate immunity was also demonstrated in bumblebees (Sadd et al., 2006). In this study, bumblebees were subjected to three different bacteria species and when re-infected (8 and 22 days later) with the same bacteria, the survival rate was significantly higher than those re-infected with different bacteria. Graft rejection model is another widely used approach to test if organisms have a high degree of self and nonself recognition (Janeway et al.,

2005). Cooper and Roch (1986) demonstrated that earthworms (*Lumbricus terrestris*) were able to reject grafts from other earthworms and also showed faster rejection in the second graft. Recently, the first allorecognition receptor gene was identified in golden star tunicates, *Botryllus schlosseri,* (Nyholm et al., 2006).

Another piece of evidence, which suggests the presence of adaptive-like immune response in invertebrates, is described in shrimp (Flegel and Pasharawipas, 1998). Shrimp farms around the world have suffered from a wave of viral disease outbreaks leading to severe economic losses. It was observed that two years or so after the first outbreak, the severity of disease seemed to decline in those virus-struck farms (Flegel, 2007). For instance, when White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV) and Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) were introduced for the first time, they caused high mortalities in *Penaeus vannamei* farms (Bonami and Lightner, 1991). However, 1-2 years later, good farm management in those farms gave successful harvest using infected shrimp stocks (with no sign of disease) suggesting the development of viral resistance in shrimps (Tsai et al., 1999). Immunologists call it tolerance rather than resistance because the crustacean species remains carrier of the pathogen and can transmit the disease to naïve individuals. Unlike vertebrates, shrimp do not completely clear the pathogens from their system. This gives rise to what is called the viral accommodation concept (Flegel and Pasharawipas, 1998), which suggested that "larval shrimp exposed to virus X prior to post larvae 10 (PL10) were capable of acquiring some type of memory that would specifically protect them from apoptosis triggered by virus X." Like resistance in vertebrates, shrimp tolerance was specific for a

particular viral pathogen but there was no protection from viruses to which shrimp had not been previously exposed. Indeed, high mortality was reported when TSV-tolerant *P. vannamei* stocks were exposed to white spot syndrome virus (Moss, 2002; Moss et al., 2005).

Innate immunity is conserved in both vertebrates and invertebrates. The immune system has the capability to recognize evolutionarily conserved structures, known as pathogen-associated molecular patterns (PAMPs), present in almost all micro-organisms but absent in higher organisms (Janeway, 1989). These structures of micro-organisms include lipopolysaccharides (Gram-negative bacteria), peptidoglycan (Gram-positive bacteria), beta glucan (fungi and yeast) and double strand RNA (viruses). Host cells responsible for defence use receptors known as pathogen recognition receptors (PRR), which recognize PAMP (Janeway and Medzhitov, 2002). The innate immunity has cellular and humoral components and it is the coordinated work of both components that protects the host from foreign intruders. The innate immune response includes phagocytosis, encapsulation, nodulation, antimicrobial peptides production, blood coagulation and melanization (Jiravanichpaisal et al., 2006).

1.4.1 Cellular

The cellular immune system relies on immuno-competent cells, collectively referred as hemocytes. Hemocytes are the main defence line in molluscs and are capable of chemotaxis and microbial recognition (Hoffmann, 1999). Phagocytosis is one of the main biological processes of hemocytes used to defend the organism against foreign particles

including pathogens. This mechanism involves recognition followed by adhesion, engulfing, and elimination of invaders by respiratory burst or exocytosis of antimicrobial factors (Adema et al., 1991). Hemocytes also produce extracellular products such as antimicrobial peptides, which have bactericidal or bacteriostatic effect. They also produce lectins, which are used as opsonising molecules to bridge hemocytes-pathogens contact. Other functions of mollusc hemocytes include nutrient transport and digestion, shell repair and excretion (Sunila 1991).

Although the classification of hemocytes is still in debate, mollusc hemocytes can be broadly divided into two main groups known as granulocytes and agranulocytes/hyalinocytes, based on the presence or absence of granules (Cheng 1981). Both cell types have been reported in many molluscs including *M. edulis, C. virginica, R. philippinarum, M. mercenaria, M. arenaria* and *C. gigas* (Chang et al., 2005; Allam et al., 2002a; Pipe et al., 1997, Synard, 2008). Other studies also classified hemocytes into three groups which included intermediate hemocytes (Giulianini et al., 2007) or hemoblast-like cells (Aladaileh et al., 2007). Furthermore, molluscs with only one type of hemocytes have been reported (Mortensen and Glette, 1996; Travers et al., 2008). This review focused on the two main groups of hemocytes, granulocytes and agranulocytes/hyalinocytes.

Mollusc hemocytes classification seems to vary depending on the classification approach. Some of the approaches used in hemocytes classification are flow cytometry (Hégaret et al., 2003; Allam et al., 2002a), density gradient centrifugation (Xue et al., 2000), monoclonal antibodies (Jing and Wenbin, 2006), electron microscopy (Travers et al.,

2008) and chemical staining (Cima et al., 2000). For instance, Pipe et al. (1997) separated three mussel hemocytes using Percoll density gradient and identified the three distinct hemocyte types using electron microscopy. However, based on hemocytes functional assay (phagocytosis, respiratory burst activity), lectin binding and proliferative ability, their study suggested two distinct groups of hemocytes. Hence, the conflicting reports of hemocyte classification by different authors could be due to the lack of standard approaches.

Most bivalve molluscs have the two main groups of hemocytes (granulocytes and agranulocytes) but the proportion varies between species (Cima et al., 2000; Hégaret and Wikfors, 2005, Zhang et al., 2006; Parisi et al., 2008, Allam et al., 2001, Synard, 2008). Moreover, the composition of the different types of hemocytes can fluctuate within one species depending on environmental conditions. Hégaret and Wikfors (2005) reported that hemocyte composition varies depending on season as a result of temperature, food availability and salinity variations (Hauton et al., 2000; Hégaret et al., 2003; Gagnaire et al., 2006). Gastropods, on the other hand, are reported to have either granulocytes or hyalinocytes. For instance, *Biomphalaria glabrata* possesses only granulocytes (Matricon-Gondran and Letocart 1999; Sminia, 1974), whereas *Haliotis tuberculata* and *Littorina littorea* only have hyalinocytes (Travers et al., 2008; Gorbushin and Iakovleva, 2006). In *H. tuberculata*, hyalinocytes are grouped into large and small hyalinocytes. The small hyalinocytes are referred as blast-like cells because they display morphological characteristics of undifferentiated and immature cells (high nucleus to cytoplasm ratio, acidic cytoplasm and low phagocytosis) (Aladaileh et al., 2007). Unlike *H. tuberculata*,

Haliotis asinina have granulocytes as well as hyalinocytes (Sahaphong et al., 2001), suggesting even closely related species could have different hemocyte composition. However, granulocytes of gastropods have granules of completely different shapes and sizes compared to those granulocytes observed in other molluscs, which is why many authors are reluctant to classify them as granulocytes (Matricon-Gondran and Letocart 1999).

Cytochemical analysis on hemocytes of soft-shell clam identified two hemocyte groups (granulocytes and agranulocytes) where granulocytes constitute a large portion (76.5%) of the cells (Huffman and Tripp, 1982). Similarly, Fournier et al. (2001) and Brousseau et al. (2000) found two distinct types of hemocytes in soft-shell clam using flow cytometry and found granulocytes were dominant over agranulocytes. A recent study by Mateo et al. (2009) also reported these two hemocyte populations in soft-shell clam. This study confirmed the fact that majority (69%) of hemocytes in soft-shell clam are granulocytes. Likewise, Synard (2008) found granulocytes and agranulocytes in soft-shell clam; however the author reported that agranulocytes (63%) were dominant. Based on hemocytes size Synard (2008) classified granulocytes and agranulocytes into two (type 1 and 2) and three (type 1, 2 and 3) subclasses respectively using light microscope and transmission electron microscope.

1.4.1.1 Granular hemocytes

The presence of cytoplasmic granules is the main distinguishing feature used to classify hemocytes into major groups. Those hemocytes which possess a large number of granules are known as granulocytes. The granules in those cells can be of different types

and are also heterogeneous in size. Granulocytes have small nucleus to cytoplasm ratio because they have condensed and mostly one nucleus. Other features which distinguish granulocytes from other types of hemocytes are: they are large in size and have a round to ovoid nucleus (Carballal et al., 1997b).

Some researchers classify granulocytes further into two or three groups based on cytochemistry, size and morphology. According to Cheng and Downs (1988), bivalve granulocytes are grouped into acidophiles, basophiles and neutrophiles. Zhang et al. (2006) subdivided scallops, *Argopecten irradians,* granulocytes into basophiles and acidophiles. Based on size of hemocytes and the types of granules in their cytoplasm, granulocytes were classified into five and three sub-populations in Sydney rock oysters, *Saccostrea glomerata*, (Aladaileh et al., 2007) and snails, *B. glabrata*, (Matricon-Gondran and Letocart, 1999); respectively.

Most molluscs possess granulocytes and these cells constitute the majority of the hemocytes (Hine, 1999). Based on cytochemical staining, granulocytes constitute 48% of hemocytes in *Tapes philippinarum* (Cima et al., 2000). However, granulocyte composition can fluctuate depending on season, pathogen exposure, food availability and other environmental factors (McCormick-Ray and Howard, 1991; Mayrand et al., 2005; Hégaret and Wikfors, 2005). In most aquatic molluscs, granulocytes are actively involved in phagocytosis. Several research studies have demonstrated the capability of granulocytes to phagocytose bacteria (Choquet et al., 2003; Allam and Ford, 2006; Parisi et al., 2008), yeast (Aladaileh et al., 2007) and latex particles (Labreuche et al., 2006a; Allam et al., 2001).

1.4.1.2 Agranular hemocytes

Agranulocytes (sometimes known as hyalinocytes) are hemocytes without or with few granules. Usually, they are homogeneous in size. Agranulocytes have bigger nucleus (large nucleus to cytoplasm ratio) than granulocytes, which takes most of the cell's intracellular space. Mortensen and Glette (1996) documented that *P. maximus* (scallops) only have agranulocytes or hyalinocytes. In contrast, other scallop species such as bay scallops (*A. irradians*) have both granulocytes and hyalinocytes (Hégaret and Wikfors, 2005). However, agranulocytes are the most dominant hemocyte population in bay scallops and constitute a large percentage compared to granulocytes (Zhang et al., 2006).

Agranulocytes can show little or no phagocytic activity and produce less superoxide anion compared to granulocytes, especially in molluscs where granulocytes are present in large proportions (Zhang et al., 2006; Pipe et al., 1997). Hine (1999) hypothesized that the difference in phagocytosis between granulocytes and agranulocytes may be related to the type of phagocytosed particles rather than the phagocytic capability. For instance, agranulocytes appear to be more active in phagocytosis of parasites whereas granulocytes are involved in bacterial and zymosan phagocytosis. In *Pecten maximus* where agranulocytes or hyalinocytes are the only type of hemocytes, they play a key role in phagocytosis (Mortensen and Glette, 1996). As in granulocytes, agranulocytes classification is controversial. Some researchers group them into subtypes I, II, and III (Cheng and Downs, 1988), while others classify them into small and large based on light microscope observation (Zhang et al., 2006).

1.4.2 Humoral

The hemolymph of molluscs contains humoral immune components, including antimicrobial peptides (see section 1.4.4), lysosomal enzymes (see section 1.4.5), lectins and agglutinins (Yamaura et al., 2008; Xue et al., 2004; Roch et al., 2008). Humoral components facilitate bacterial aggregation, immobilization and have bacteriostatic and bactericidal properties. Some molecules also act as a bridge between the surfaces of bacteria and hemocytes, favoring the phagocytic process by opsonization. There are two types of hemocyte receptors in bivalves which are likely involved, (1) receptors that can detect pathogens using opsonins after a complex is formed between pathogens and humoral molecules (opsonins) and (2) receptors which are capable of directly attaching to pathogens without the contribution of humoral factors (Pruzzo et al, 2005).

Hemocyte-pathogen interaction is determined by various factors (Canesi et al., 2002b). Hemolymph or serum is one important component of innate immunity which plays a crucial role by enhancing adhesion, phagocytosis and agglutination. Like higher vertebrates where phagocytosis of pathogens is mediated by serum ligands (opsonin molecules), hemolymph in molluscs and other invertebrates may have a similar function. Invertebrate hemolymph lacks immunoglobulins however; it contains soluble molecules which are necessary for self and non-self cells recognition (Yamaura et al., 2008). Lectins, glycoproteins and glycolipids are among the candidates, which have an opsonizing effect (Olafsen, 1995). The function of lectins in lower organisms could be considered similar to the role of antibodies in vertebrates. This means it would require numerous lectin types in the hemolymph with high carbohydrate-binding specificity. This

was illustrated in cockroaches, *Blaberus discoidalis*, which contain multiple lectins capable of differentiating a wide range of bacteria species (Wilson et al., 1999).

Lectins are found in the hemolymph of various molluscs such as scallops, mussels, clams and oysters (Wang et al., 2007; Kim et al., 2008; Yamaura et al., 2008; Olafsen, 1995). Lectins are sugar-binding proteins which are involved in host-pathogen adhesion during bacterial and viral infection. Canesi et al., (2002b) suggested some of the possible roles of lectin in molluscs, including acting as a bridge between bacteria and hemocytes and facilitate phagocytosis (opsonins), immobilization of invading microbes by agglutination and confining infection. Lectins isolated from oyster (*C. gigas*) hemolymph and other tissues are involved in bacteria agglutination (Yamaura et al., 2008; Olafsen et al., 1992). Recently, Wang et al. (2007) cloned a C-type lectin (CFLec-1) from the Zhikong scallop, *Chlamys farreri*, which is capable of aggregating bacteria and also inhibits bacterial growth. Although *C. virginica* lectins were also implicated in bacteria agglutination, lectins agglutinated *Cholerae* 01 but not other *Cholerae* strains, suggesting lectins are highly specific and can differentiate closely related strains of bacteria (Fisher and DiNuzzo, 1991).

1.5 Immune response of molluscs

1.5.1 Hemocyte migration

The lymph gland, the hemocyte-producing organ in insects (fruit flies), is well documented (Holz et al., 2003) however; the origin of hemocytes in molluscs still

remains unclear in spite of Smolowitz et al.'s (1989) hypothesis that proliferative cells come from connective tissues. In the pulmonate gastropod, *B. glabrata,* the hemocyte-producing organ has been identified and it is located between the pericardium and the posterior epithelium of the mantle cavity and in the blood sinuses of the ventricle (Adema et al., 1991). Although the number of circulating hemocytes varies between different mollusc species, the range is between 10^5-10^7 cells ml^{-1} of hemolymph (Zhang et al., 2006; Matozzo et al., 2008; Parisi et al., 2008, Allam et al., 2001; Labreuche et al., 2006a). It is known that total hemocyte count (THC) in the hemolymph fluctuates due to the migration of hemocytes from hemolymph to tissues and vice versa (Matozzo et al., 2008; Parry and Pipe, 2004). Recently, studies in blue mussels (Mayrand et al., 2005) and manila clams (Matozzo et al., 2008) reported that hemocytes have the ability to undergo mitotic division beside the migration of cells to reinforce the immune defence mechanism during microbial invasion. In *L. truncatula* and *L. stagnalis*, it was shown that hemocytes divide in the hemolymph, in the connective tissue as well as at the site of wound repair (Van der Knapp et al., 1993). Total hemocyte count in the hemolymph can also vary depending on season and the stage of infection. Allam et al. (2001) found a high THC in symptomatic clam (*R. philippinarum and R. decussates*) individuals compared to asymptomatic ones. Moreover, mussels (*M. edulis*) subjected to three different bacteria showed a decrease in THC in the hemolymph between 3-12h post-challenge (Parisi et al., 2008). THC was also decreased in hemolymph of mussels; *Mytilus galloprovincialis,* injected with bacteria (*V. anguillarum*, *V. splendidus*, *Micrococcus lysodeikticus*) and the hemocyte count returned to normal 48h post challenge (Ciacci et al., 2009).

1.5.2 Phagocytosis

Phagocytosis refers to the internalization of entities by single cells (Jiravanichpaisal et al., 2006). It is a conserved cellular response that occurs in different animals, ranging from mammals to simple protozoans. This primary reaction of immune cells is designed to scavenge undesirable particles, such as bacteria and yeast by engulfing them. The phagocytosis process is not only restricted to foreign invaders; it also takes care of apoptotic cells and debris during development and homeostasis of tissues (Adrem and Underhill, 1999). Hemocytes have functional resemblance to macrophages of vertebrates in that both are involved in phagocytosis and their immune response is regulated by protein kinase C (Walker and Plows, 2003). Molluscs have different types of hemocytes and the cells actively involved in phagocytosis can vary even between closely related species. In mussels and oysters, granulocytes are more actively involved in phagocytosis (Aladaileh et al., 2007; Pipe et al., 1997), while agranulocytes are in scallops (Mortensen and Glette, 1996) and abalones (Travers et al., 2008).

The phagocytosis process has well-defined stages, including recognition, chemotaxis, attachment/adhesion, ingestion and destruction of particles (Bayne et al., 2001). The targets which need to be phagocytized can be recognized either directly or indirectly. In the direct way, the cells have receptors which enable them to recognize a target by directly binding to its surfaces. However, in the indirect way of recognition, the cells cannot recognize the target by themselves. Hence, opsonin molecules such as lectins are required to bind and label the target prior to detection and phagocytosis. Internalized particles are enclosed in a primary phagosome, which then fuses with lysosomes to form

a phagolysosome. Finally, the engulfed agents are destroyed or degraded in two main systems: oxygen-dependent systems (respiratory burst activity) and oxygen-independent systems which involve reactive oxygen/nitrogen species and powerful digestive enzymes, respectively. After phagocytosis, the removal of indigestible particles to the external environment across epithelial surfaces (diapedesis) was demonstrated in oysters (Tripp, 1960) and clams (Reade and Reade, 1972) but this has not been reported in other bivalves. Particles too big to be phagocytized are encapsulated by multiple layers of hemocytes (Hooper et al., 2007).

Phagocytosis has been reported in hemocytes of different mollusc species (Labreuche et al., 2006a; Allam et al., 2001; Cima et al., 2000; Zhang et al., 2006). One of the main tasks of mollusc hemocytes is to clear foreign particles by phagocytosis. Hemocytes use their cytoplasmic extensions to seize and engulf intruders. However, pathogens have developed a means to destabilize cytoskeleton structure causing hemocytes to lose their pseudopodia. Bacterial toxins identified in *Vibrio cholerae* (Kudryashov et al., 2008) and *V. splendidus* (Le Roux et al., 2007) have been shown to interact with actin, a gene involved in cytoskeleton filament reorganization, and causing actin depolymerisation. This leads to hemocyte rounding and subsequently reduces the adhesion and phagocytosis capability of the cells (Labreuche et al., 2006a; Allam and Ford, 2006).

Several studies have demonstrated that environmental stressors could affect soft-shell clam's immune parameters such as number and viability of hemocytes, phagocytosis and respiratory burst activity. Brousseau et al. (2000) exposed hemocytes of soft-shell clam to different concentrations of various heavy metals for 18 h and showed a decreased in cell

viability and phagocytosis with increased concentration. Fournier et al. (2001) also exposed soft-shell clams to mercuric chloride and methylmercury separately for 28 days and found that at 10^{-6} M, both mercury compounds significantly reduced viability and phagocytosis activity of hemocytes. At high concentration (10^{-5} M) mercury chloride was fatal for clams. Moreover, neoplastic hemocytes of soft-shell clams showed low adhesion and phagocytosis capability (Beckmann et al., 1992).

1.5.3 Respiratory burst activity

In many bivalve species, the production of oxidative chemicals by phagocytic cells can be activated by foreign particles. This type of defence mechanism is often referred as "respiratory or oxidative burst." An oxidative burst leads to production of reactive oxygen species (ROS) also known as reactive oxygen intermediates (ROIs), catalyzed by the membrane-associated enzyme NADPH oxidase (Roch, 1999). The initial metabolite, superoxide anion (O_2^-) is transformed into hydrogen peroxide (H_2O_2), which may then be converted to other toxic ROS, such as hydroxyl radical (OH^-) and singlet oxygen (O_2^-). Hydrogen peroxide also interacts with myeloperoxidase (MPO) and halide to produce hypochlorous acid (HOCl). Nitric oxide (NO) is also an important oxidative molecule produced by bivalve hemocytes to destroy pathogens during the phagocytosis process (Franchini et al., 1995). NO is not toxic by itself, but in combination with superoxide anions, it generates the peroxynitrite anion ($ONOO^-$), a labile and highly toxic compound (Beckman and Koppenol, 1996). These metabolites play an important role in phagocytosis-mediated microbial killing.

The two main pathways in respiratory burst (Fig. 1.1) are the NADPH-oxidase and NO-synthase pathways, which lead to the production of reactive oxygen species (H_2O_2, O_2^-) and reactive nitrogen species (NO, $ONOO^-$), respectively (Dröge, 2002). Respiratory burst activity has been documented in several bivalve species including scallops (*P. maximus)*, oysters (*C. virginica and C. gigas)* and mussels (*M. edulis)* (Le Gall et al., 1991; Pipe, 1992; Hégaret et al., 2003; Goedken and DeGuise, 2004; Lambert et al., 2007). Some bivalve molluscs such as *C. gigas* have both pathways (Lambert et al., 2007), whereas reactive oxygen/nitrogen species was not detected in clams (*M. mercenaria* and *R. decussatus*) suggesting the lack of NADPH-oxidase and NO-synthase pathways (Lopez et al., 1994; Anderson et al., 2003). In contrary, Arumugan et al. (2000) and Buggé et al. (2006) have detected respiratory burst activity in *M. mercenaria.* Anderson (1994) also reported no respiratory burst activity in hemocytes of soft-shell clam (*M. arenaria*) exposed to phorbol myristate acetate (PMA). A variety of probes can be used to detect respiratory burst activity and these probes appear to be specific to different reactive oxygen intermediates (Walrand et al., 2003).

Like the respiratory burst of mammalian phagocytes, mollusc hemocyte generate various reactive oxygen intermediates (ROS) (Arumugan et al., 2000; Lambert et al., 2007) which are involved intracellular killing of the phagocytosed microbial agents. The level of ROS production can vary between species, level of infection, types of pathogens and other factors (Travers et al., 2008; Donaghy et al., 2009). For example, hemocytes from Eastern oyster, *C. virginica*, which were heavily infected with protozoan parasites *Perkinsus marinus*, produced significantly higher levels of ROS than hemocytes withdrawn from lightly infected oysters (Anderson et al., 1992).

Phagocytic cells use ROS as microbicidal agents, either alone or in combination with lysosomal enzymes, and are important in the elimination of viruses, bacteria, yeast, fungi, and protozoa. Reactive oxygen species are toxic to the host cells as well. However, the cells are protected by anti-oxidants which neutralize the toxic effect of radicals. For example, hemocytes of clams and mussels have been shown to contain anti oxidants such as superoxide dismutase, catalase and glutathione peroxidase (Wenning and DiGiulio, 1988). Thus, cells which produce toxic radicals are protected from being damaged by oxidative effect of the ROS. Nonetheless, the cells have to maintain a balance between the anti-oxidant produced and ROS. By doing so, the cells are protected from the toxic effect of ROS, but at the same time, the produced ROS should be powerful enough to kill the pathogens. Moreover, some pathogens have been shown to produce these anti-oxidants (Brown et al., 1995), a strategy to neutralize the toxic effect of oxidative radicals released by the host cells (Bramble and Anderson, 1997).

1.5.4 Antimicrobial peptides

Antimicrobial peptides (AMPs) are cationic host defence peptides which are diverse in their sequence and structures. In most cases, antimicrobial peptides are short amino acid sequences (12-50), amphipathic (have both hydrophobic/lipophilic and hydrophilic properties) and have at least two positive charges (arginine or lysine residues). They can be broadly classified into four or five groups based on their structure namely α helical AMPs, cysteine rich AMPs, β sheet AMPs, AMPs rich in regular amino acids and AMPs with rare modified amino acids (Mookherjee and Hancock, 2007; Reddy et al., 2004). AMPs possess a broad spectrum of antimicrobial activities, affecting the growth of

bacteria, fungi and yeasts (Mitta et al., 2000a; Zasloff, 2002). Most active antimicrobial peptides are able to interact with bacterial membranes and cause substantial local perturbation of the cytoplasmic membrane bilayer, which leads to increased cytoplasmic membrane permeability and bacterial cell death (Hancock and Rozek, 2002). Several mechanisms have been proposed to explain the AMP mode of action, such as barrel stave pore, carpet-like mechanism and self-promoted uptake (Shai, 1999).

Interest in the AMP research has grown rapidly and has lead to the discovery of more than 700 cationic peptides from numerous species. More than 20 AMPs, grouped in 7 classes, have been identified in fruit flies alone (Lemaitre and Hoffmann, 2007). Some of these peptides target specific groups of microbes, while others have a wide range of targets. In *Drosophila melanogaster*, AMPs are produced by the fat body and released to the hemolymph within 2-4 hours post infection (Meister et al., 1997). Toll and Imd (immune deficiency) are the two signalling pathways that induce the production of AMP (Lemaitre and Hoffmann, 2007). Toll is mostly activated by Gram-positive bacteria and fungi whereas Imd is mostly stimulated by Gram-negative bacteria.

Antimicrobial activities are also reported in various commercially important bivalve species, among which are mussels *M. edulis* and *M. galloprovincialis* (Haug et al., 2004; Mitta et al., 1999a; Mitta et al., 1999b; Mitta et al., 2000b), oysters *C. virginica* and *C. gigas* (Anderson and Beaven, 2001; Gueguen et al., 2006) and in some gastropods (Iijima et al., 2003). In mussels AMPs are produced and stored in the granules of hemocytes (granulocytes) and then released into the hemolymph at the time of microbial infection (Mitta et al., 2000a). Hemolymph from several mollusc species such as sea hares, sea

slugs, oysters, and mussels have also shown antibacterial activities (Gueguen et al., 2006; Roch et al., 2008).

In mussels (*M. edulis and M. galloprovincialis*), four types of antimicrobial peptides (AMP) are found, namely defensin, mytilin, myticin and mytimycin. Some of them target specific groups of pathogens (Mitta et al., 2000a), while others such as mytilin have a wide spectrum of activities (Roch et al., 2008). Most granulocytes express only one type of AMP and their allocation in different organs of the host seems to be dictated by the type of AMP they produce. For example, defensin-expressing granulocytes are concentrated in the intestinal epithelia, whereas mytilin and myticin expressing granulocytes are well represented in the gills. Defensin is involved in extracellular defence whereas mytilin is released both at the intracellular (post phagocytosis) and extracellular levels (Mitta et al., 2000a). Gonzalez et al. (2007) have cloned and sequenced two isoforms of defensin genes from the hemocytes of oysters, *C. gigas*. Both defensins were also identified in gills and mantle. However, only one of them (*Cg-defh2*) appeared to be involved during bacterial challenge and showed high expression in gills and mantle but down-regulated in hemocytes.

1.5.5 Lysozymes

Lysozymes are small ubiquitous antibacterial enzymes, which are found in various organisms such as bacteria, bacteriophages, fungi, plants and animals. They are characterized by their ability to catalyze the hydrolysis of the β-1, 4-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of the sugar residues in the

bacterial peptidoglycan (forming Gram-positive bacteria cell wall) leading to bacterial cell lysis (Jollès and Jollès, 1984; Prager and Jollès, 1996). Traditionally, lysozymes are characterized by: (1) reduction in the turbidity of bacterial cell walls, (2) reducing sugars, and (3) releasing a complex containing glucosamine and muramic acid (Jolles, 1964). Lysozymes isolated from some bivalves (Xue et al., 2004; Nilsen et al., 1999) and crustaceans (De-la-Re-Vega et al., 2006; Hikima et al., 2003) have bactericidal activity. Moreover, studies by Bierman et al. (1979) and Samaranayake et al. (1997) documented that lysozymes have anti-protozoan and anti-fungal activities.

Lysozymes can be classified into six types based on their catalytic and immunological characters, origin and structural differences (Xue et al., 2004). Namely, the chicken-type (c-type) lysozyme, goose-type (g-type) lysozyme, invertebrate-type (i-type) lysozyme, phage lysozyme, bacterial lysozyme and plant lysozyme (Jollès, 1996; Bachali et al., 2002; Liu et al., 2006). The i-type lysozyme has been identified in various bivalve molluscs, among them are blue mussels, *M. edulis* (Olsen et al., 2003), oysters, *C. gigas* and *Ostrea edulis* (Matsumoto et al., 2006) and *C. Virginica* (Xue et al., 2004), icelandic scallops, *Chlamys islandica* (Nilson et al., 1999), mediterranean mussels, *M. galloprovincialis*, hydrothermal-vent mussels, *Bathymodiolus azoricus* and cold-seep clams, *Calyptogena* sp. (Bachali et al., 2002).

Lysozyme activity in molluscs was first noticed in cell-free hemolymph (plasma) of the eastern oyster (*C. virginica*) (McDade and Tripp, 1967). Apart from the body fluids, lysozyme activity is also detected in tissues (Allam et al., 2000 and Cronin et al., 2001). Lysozymes, purified from several bivalve molluscs, are mostly obtained from parts of the

digestive system, such as the crystalline style and visceral mass (Nilsen et al., 1999, Miyauchi et al., 2000, and Olsen et al., 2003). However, Xue et al. (2004) purified lysozyme from hemolymph of eastern oyster, *C. virginica*, which inhibits growth of Gram-positive bacteria (*Lactococcus garvieae*, *Enterococcus* sp.) as well as Gram-negative bacteria (*Escherichia coli*, *Vibrio vulnificus*). Lysozyme is localized within granular hemocytes (Pipe, 1990) and its level of activity is high in hemocytes compared with plasma both in the mussels, *M. edulis* (Carballal et al., 1997a) and the carpet shell clams, *R. decussatus* (Lopez et al., 1997). The gene expression level of lysozyme in various mollusc tissues and cells varies between species. For instance, in Zhikong scallops, *Chlamys farreri*, mRNA transcripts of g-type lysozyme (CFLysG) were most abundantly expressed in gills, hepatopancreas and gonads, but only weak expression was measured in hemocytes and mantle (Zhao et al., 2007) whereas in Pacific oysters, *C. gigas*, i-type lysozyme was isolated and it was expressed in all tissues except in adductor muscle (Matsumoto et al., 2006). On the other hand, Itoh et al. (2007) found significantly higher lysozyme mRNA transcript in labial palps and mantles than in gills, digestive glands and hemocytes of the eastern oyster, *C. virginica*. In a recent study g-type lysozyme domain was identified in peptidoglycan recognition protein (PGRP) of *C. gigas* suggesting PGRP may be involved in binding and lysing bacteria (Itoh and Takahashi, 2009).

1.6 Immune related genes identified in molluscs

Innate immunity, the sole defence mechanism of invertebrates, is equipped with pattern recognition receptors (PRR) (Janeway and Medzhitov, 2002). These receptors play a crucial role in distinguishing between self and non-self cells. Lectins and Toll-like receptors are among several pattern recognition molecules utilized in innate immunity. Recently the presence of homologue Toll-like receptors (TLRs) in mollusc, *Euprymna scolopes* (Goodson et al., 2005), *Chlamys farreri* (Qiu et al., 2007) and *M. mercenaria* (Perrigault et al., 2009) has been reported. In the Zhikong scallop, *C. farreri,* new Toll homologues (CfToll-1) were expressed more in hemocytes challenged with lipopolysaccharide (Qiu et al., 2007). Kang et al. (2006) identified several lectin homologs in Manila clam, *R. philippinarum*. Kang and his group demonstrated that *Perkinsus olseni*-infected hemocytes of Manila clam expressed different sets of lectins from those exposed to *Vibrio tapetis* suggesting that lectins are involved in immune defence and appeared to be pathogen-specific. In vertebrates, tissue inhibitor metalloproteinases (TIMPs), inhibitor of matrix metalloproteinases (MMPs), regulate a wide range of physiological processes and its expression is also associated with wound healing and various diseases (Cawston, 1996). *Crassostrea gigas*-TIMP was expressed only in hemocytes of oysters with damaged shells or infected with pathogenic bacteria (Montagnani et al., 2001).

As soon as foreign intruders are detected, PRRs trigger a series of immune-related pathways to fight the pathogen. In *Drosophila*, there are two main immune signaling pathways, which regulate the expression of anti-microbial peptide (AMP), known as Toll

and Imd pathways (Lemaitre and Hoffmann, 2007). Gram-negative and Gram-positive bacteria are discriminated based on the specific content of peptidoglycan (PGN). Gram-positive bacteria contain lys-type PGN, whereas Gram-negative have DAP-type PGN and activate Toll and Imd pathways, respectively (Leulier et al., 2003). So far, two families of PRR have been identified in *Drosophila*. In Imd, Gram-negative bacteria-binding protein (GNBP) is used as a receptor and recognizes Gram-negative bacteria (Kim et al., 2000), whereas peptidoglycan recognition protein (PGRP) is a receptor for Toll and recognizes Gram-positive bacteria (Werner et al., 2000).

Recently, PGRPs have been identified in mollusc species including Zhikong scallops, *C. farreri*, (Su et al., 2007), bay scallops, *A. irradians*, (Ni et al., 2007) and pacific oysters, *C. gigas*, (Itoh and Takahashi, 2008). Furthermore, Zhang et al. (2007) reported the presence of GNBP gene in snails, *B. glabrata*, for which the complete gene is sequenced. Like in *Drosophila*, PGRP is up-regulated in molluscs challenged with bacteria, parasites and purified PGN (Zhang et al., 2007; Ni et al., 2007), thus suggesting that mollusc PGRP may have a similar role as in insects. PGRP is highly expressed in circulating hemocytes of molluscs compared to cells from other tissues (Su et al., 2007). GNBP is also described in shrimp species and it was up-regulated in *Penaeus stylirostris* infected with white spot syndrome virus (Roux et al. 2002). However, heat-killed *V. harveyi* and β-1, 3-glucan did not stimulate GNBP in *P. monodon* (Sritunyalucksana et al. 2002).

Several signaling pathways associated with immunity have been documented both in vertebrates and invertebrates. These signaling cascades include protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and tumor necrosis factor alpha (TNFα). In

molluscs homolog genes have been reported (Cao et al., 2004; Walker and Plows, 2003; Barcia et al., 1999; Ottaviani et al., 1995). Those signaling cascades are activated by pathogens but they are also the target of pathogens to evade or overcome the host immune defence. Roach and Schorey (2002) showed that non-pathogenic *Mycobacterium* activates MAPK and TNFα in macrophages. When subjected to pathogenic *Mycobacterium avium,* the activation was diminished, suggesting the interference of the pathogenic *Mycobacterium* with immune signaling of macrophages.

Protein kinases C (PKCs) are serine/threonine kinases with multiple cellular functions. They are also associated with innate immunity and have been shown to regulate mammalian macrophage's ability to adhere and spread, produce reactive oxygen/nitrogen species and phagocytosis (Allen and Aderem, 1995; Castrillo et al., 2001). A PKC homolog was identified in hemocytes of gastropods, *Lymnaea stagnalis*, and activated upon lipopolysaccharide (LPS) challenge (Walker and Plows, 2003). A Ca^{++}-independent PKC was purified from the mantle of *M. galloprovincialis* Lmk which is also detected in its hemocytes (Mercado et al., 2002). Cao et al. (2004) reported over expression of Ca^{++}-independent PKC in hemocytes of mussels, *M. galloprovincialis,* subjected to LPS and IL-2 confirming the involvement of PKC in mollusc immunity. These studies also suggest that the role of PKC in innate immunity may be conserved during the process of evolution.

Canesi et al. (2005) suggested that inefficient response of *M. galloprovincialis* hemocytes against mutant *V. cholerae* could be due to *V. cholerae*'s capability to down-regulate hemocytes signaling pathways. In the same study, it has been found that mutant *V.*

cholerae suppressed PKC expression and inhibited MAPK pathway leading to low anti-microbial activity of hemocytes. On the other hand, the wild type *V. cholerae* had little effect on MAPK and stimulated PKC phosphorylation in mussel hemocytes. Similar to PKC, bacteria challenge also activated MAPK pathway in *M. galloprovincialis* hemocytes (Canesi et al., 2002a). A study conducted in a gastropod (*B. glabrata*) embryonic cell line also showed MAPK activation upon PMA (PKC activator) and inhibition by PKC inhibitors (Humphries et al., 2001) suggesting PKC might be upstream of MAPK as in macrophages. So far no study has been conducted to investigate the immune response of soft-shell clams *Mya arenaria* at gene level.

1.7 Immune system in *Mya arenaria*

Like most bivalve molluscs, the immune system of soft-shell clam (*Mya arenaria*) is not well studied. In the early 80s, a study conducted by Huffman and Tripp (1982) identified two types hemocytes (granulocytes and agranulocytes) in soft-shell clams with majority of the hemocytes being granulocytes. This was later confirmed by Brousseau et al. (2000), Fournier et al. (2001) and Mateo et al. (2009). In addition, Synard (2008) found these two hemocyte groups in soft-shell clams. However, unlike the aforementioned studies Synard (2008) reported that agranulocytes are more dominant than granulocytes. Brousseau et al. (2000) and Fournier et al. (2001) also assessed the effect of heavy metals on the immune function of hemocytes and found decreased hemocyte viability and phagocytosis suggesting environmental pollutants could negatively affect the immune

function of hemocytes. In soft-shell clams, no study has been carried out to investigate the cellular and molecular immune response of hemocytes against microbial agents. Hence, to our knowledge, this is the first study investigating the effects of bacteria on the innate immunity of soft-shell clams, *Mya arenaria*.

1.8 Transcriptomics: emerging technologies for bacterial infection

For many years, researchers have used candidate gene approach to study immune response of organisms by focusing on a single gene. However, this approach has drawbacks because host response is the product of multiple transcript expression and its interactions. To capture such molecular events new technologies such as suppression subtractive hybridization (SSH) and cDNA microarray, which can screen large number of expressed genes at a time, have emerged in the last two decades. These tools open new venues in molecular biology research and gene expression studies. Immune defence in molluscs is poorly understood and these molecular methods can play a very important role in uncovering the transcriptome of hemocyte responses.

SSH and microarray have been used to investigate the global changes of gene expression level (transcriptome) in biological samples. Despite the similarities between them, both techniques have fundamental differences. The cDNA microarray is a closed system for gene discovery because every gene represented in an array is known whereas SSH can uncover novel genes. Moreover, microarrays are inefficient in detecting the expression of rare transcripts. Suppression subtractive hybridization (SSH) overcomes this limitation by

generating expressed genes, all of which are equally represented regardless of their relative abundance (Diatchenko et al., 1996). Microarray studies require prior genome knowledge of the organism under investigation whereas SSH does not. In molluscs where the genome knowledge is poor SSH is preferable to cDNA microarrays (Munir et al., 2004). Consequently, SSH is a widely used molecular technique in mollusc transcriptome studies (Gestal et al., 2007; Wang et al., 2008; Huvet et al., 2004; Tanguy et al., 2004; Perrigault et al., 2009) although recently the use of cDNA arrays, constructed from SSH libraries of pacific oyster (*C. gigas*) and zebra mussel (*Dreissena polymorpha*), have been reported (Fleury et al., 2009; Xu and Faisal, 2009; Lang et al., 2009).

The SSH allows researchers to compare two populations of messenger RNA (mRNA) that represent two experimental conditions and obtain genes regulated in one population but not in the other. It selectively amplifies differentially expressed genes and simultaneously suppresses unregulated transcripts (Diatchenko et al., 1996). The cDNA from control and exposed samples are hybridized and the hybrid sequences (transcripts common to both samples) are suppressed by PCR. Those transcripts that form hybrids are genes in which their expression is unaffected by the experimental condition. The remaining un-hybridized cDNA corresponds to the genes that are regulated. In other words the un-hybridized cDNA fragments found in challenged and control group represent genes that are up (forward subtracted cDNA library) and down-regulated (reverse subtracted cDNA library), respectively. The molecular events that occur during subtractive hybridization and selective amplification of differentially expressed genes are illustrated in Figure 1.2.

1.9 Transcript quantification and normalization strategies

The cDNA microarray and SSH are used to screen transcripts, which are regulated in experimental conditions. Neither of the approaches is quantitative, consequently transcripts reported as regulated in microarray and SSH studies must be validated using quantitative techniques such as real-time quantitative PCR (qRT-PCR). This technique is the most sensitive and accurate tool to quantify gene expression levels in biological samples. Despite the aforementioned advantages and wide application in different research areas, precautions should be taken because qRT-PCR does not take into account the variability in sample size, quantity and quality of starting material and reverse transcription-PCR efficiency in cDNA synthesis. These are some of the problems associated with the use of qRT-PCR (Dheda et al., 2004). Hence, to accurately quantify the expression level of transcripts, it is very important to normalize the qRT-PCR data. Among the normalization strategies used in gene expression studies include the use of equal sample size and total RNA and reference genes (Huggett et al., 2005).

1.9.1 Sample size

Starting with an equal number of cells/tissue weight or volume is the first step that should be taken to reduce experimental variations. The notion behind using similar tissue volume/weight is that equal tissue or sample volume have equal number of cells. However, it is not always the case. In molluscs for instance, it is well documented that hemocytes migrate between the tissue and the hemolymph during bacterial challenges. Labreuche et al. (2006a) showed that when oysters (*C. gigas*) were injected with *Vibrio*

aestruarianus strain 01/32, the number of hemocytes in the hemolymph significantly increased. In contrast, the total hemocyte number decreased substantially in mussels (*M. galloprovincialis*) injected with *V. splendidus* LGP32 and *V. anguillarum* three hours post-challenge (Parisi et al., 2008). In those cases, an equal number of hemocytes cannot be obtained from bacteria-injected and non-injected bivalves even if the same volume of hemolymph is collected. Moreover, mollusc hemocytes usually clump up together as soon as they are withdrawn from the animal therefore equal volume of hemolymph may not contain equal number of hemocytes. Hence, normalizing with equal tissue weight or hemolymph volume alone may not be enough to obtain accurate results that reflect the actual expression levels.

1.9.2 Total RNA

Before measuring the expression of genes, it is very crucial to measure concentration and assess the quality (DNA or protein contamination) and integrity of RNA. RNA is very difficult to work with because it is easily degradable by RNAse under room temperatures. Moreover, large sequences of RNA can easily be sheared during sample processing. Hence reverse transcribing RNA into cDNA is widely used as a means to maintain highly intact transcripts. The use of total RNA for normalization is more preferable than the use of sample size because it takes into consideration the variations in the amount of starting material and RNA extraction efficiency between experimental samples. The drawback of total RNA as a normalization strategy is that it does not take into account variation in reverse transcription and PCR efficiency.

The use of total RNA in normalization qRT-PCR data is driven by the fact that ribosomal RNA (rRNA) predominantly constitutes more than 80% of transcript (Huggett et al., 2005; Vandesompele et al., 2002) and the assumption that the ratio between ribosomal RNA (rRNA) to messenger RNA (mRNA) does not change (Huggett et al., 2005). This assumption may be incorrect, as studies have demonstrated that some drugs and biological factors can affect the transcription level of rRNA, which could eventually affect the ratio between rRNA and mRNA. For instance, a study by Solanas et al. (2001) showed variation in rRNA:mRNA ratios in 7.5% of mammary adenocarcinoma. Moreover, normalization with total RNA becomes impossible when cDNA is synthesized by oligo(dT) primers because these primers specifically target mRNA with polyA tail, which the rRNA (composed of 18S and 28S) do not have (Vandesompele et al., 2002).

1.9.3 Reference genes

Housekeeping or reference genes are internal controls in which their mRNA expression level is stable at different experimental conditions (Bohle et al., 2007) or period of exposure time (Arenz et al., 2007). Reference genes are also known as internal controls because they are present together with transcripts of the target genes. The advantage of reference genes over the use of total RNA normalization strategies in gene expression data is that unlike the total RNA, which contains mainly untranscribed RNA (rRNA and tRNA), reference and target genes are transcripts. Hence, reference genes become the best candidates for gene expression normalization because they reflect what the target genes are experiencing during the whole process until real-time PCR quantification. Thus, these internal control genes can minimize the systemic errors that could arise from

factors such as RNA extraction protocols, the inherent RNA variability and efficiencies of reverse transcription and PCR between samples (Huggett et al., 2005).

Housekeeping genes (HKGs) are expressed at constant level because they are associated with maintaining the homeostasis (García-Vallejo et al., 2004) and cell viability (Arenz et al., 2007). Some genes are considered as classical housekeeping genes and they are utilized without validating their suitability for a particular experimental condition. Among the widely accepted typical reference genes which are used in various studies are β-actin (He et al., 2005; Pan et al., 2005), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S-ribosomal RNA (Gunsch et al., 2006). However, recent works showed that this is not always the case and the expression of those genes involved in homeostasis or/and viability can vary in different tissues (Barber et al., 2005; Rubie et al., 2005) and cells (Bas et al., 2004) and different environmental conditions (Arenz et al., 2007; Nicot et al., 2005). For instance a study carried out by Mogal and Abdulkadir (2006) reported that 18S-rRNA regulation level varied when mouse prostate tissue explants and human prostate cancer (LNCaP) cells were treated with TSA suggesting that 18S-rRNA is not suitable internal control for this particular study. Variations in the expression level of β-actin and GAPDH was also reported in growing collateral arteries of rabbit model (Deindl et al., (2002). Moreover, β-actin expression in TO cells from Atlantic salmon (*Salmo salar*) subjected to lipopolysaccharide was unstable to be used as reference gene (Ingerslev et al., 2006). The same study showed that β-actin was a suitable reference gene in Atlantic salmon head kidney leucocytes. These contrasting findings of β-actin suggests that there exist no ideal

or classical housekeeping genes for different cells/tissues or experimental conditions (Vandesompele et al., 2002).

The importance of evaluating expression stability of classical housekeeping genes prior to utilizing them as internal control for a particular experiment was suggested long before real-time PCR was made available. Studies in the 70s and 80s showed the regulation of the so called classical housekeeping genes. For example a 1975 study demonstrated that 18S-rRNA expression in human fibroblast cells increased after cytomegalovirus infection (Tanaka et al., 1975). In 1987, another study showed differentially expression of beta-actin in different leukemia patient samples (Blomberg et al., 1987). Despite these studies, many recent published works have used these reference genes for normalization without proper evaluation of the gene's expression stability for that particular experimental condition. Therefore, it is very important to use suitable housekeeping genes which are validated for particular experimental setting (Bohle et al., 2007). Some studies also suggested the use of more than one housekeeping gene to accurately measure the expression of genes (Pfaffl et al., 2004; Arenz et al., 2007). This minimizes variation in gene expression level among samples when normalization factor of multiple HKGs genes is pooled rather than the use of normalization factor from one HKG (Jin et al., 2004).

1.10 Rationale and objectives of the study

In 2006 mollusc production accounted 27% of the world aquaculture production, with 14.1 million tonnes worth of $11.9 billion (FAO, 2008). It is the second largest sector in terms of quantity after freshwater fish. In the last decade, the growth rate of mollusc aquaculture declined from more than 11% in 1990-2000 to less than 5% in 2000-2006 due to disease and other environmental factors.

Shellfish aquaculture is one of the economic backbones of Prince Edward Island (PEI). According to a 2007 Department of Fisheries and Oceans Canada report, 50% of total shellfish production in Canada came from PEI and generated $28.5 million revenue (DFO, 2009). Traditionally, this industry is centralized on mussel production, which is now threatened by invasive species. As an alternative, soft shell clams (*M. arenaria*) are considered as an important naturally occurring species for diversification purpose. Clam fishing and harvesting represent an important economic contribution for several communities along the eastern coast of the US and Canada (Evans et al., 2002). While the clam industry has experienced a tremendous growth in Quebec and New Brunswick, in PEI it has been hindered by high mortality occurrences (McGladdery et al., 2001).

Bivalve molluscs have been used in many studies of *in-vitro* and *in-vivo* models of interaction to investigate immune defence mechanisms of a host and pathogenesis of microbial infection. As in other invertebrates, the sole defence mechanism of molluscs against pathogens and parasites is innate immunity. Despite the lack of adaptive immunity, molluscs seem to adapt to their environment and establish a sustainable

immune defence mechanism against the micro-organisms around them. Very little is known about clam immunity, which has been seen as primitive. Hence, the aim of this study is to improve our knowledge about the soft-shell clam immune system by investigating hemocyte-*Vibrio* interactions. Here, we hypothesized that when hemocytes of soft-shell clams are challenged with *V. splendidus* hemocytes' morphology is modified, their immune functions are impaired and the expression of genes related to innate immunity is disrupted. Our strategy is to develop an *in-vitro* model of hemocyte-*Vibrio* interaction in order to achieve the following goals:

1. Establish a standard working procedure for an *in-vitro* model of hemocyte-*Vibrio* interaction;
2. Investigate the phenotypical and functional responses of hemocytes exposed to *Vibrio* spp.;
3. Select suitable housekeeping genes to measure gene expression of transcripts regulated in challenged hemocytes;
4. Identify transcripts associated with immune defence of soft-shell clams response to *Vibrio* challenge; and
5. Examine the gene expression pattern of those transcripts during the challenge.

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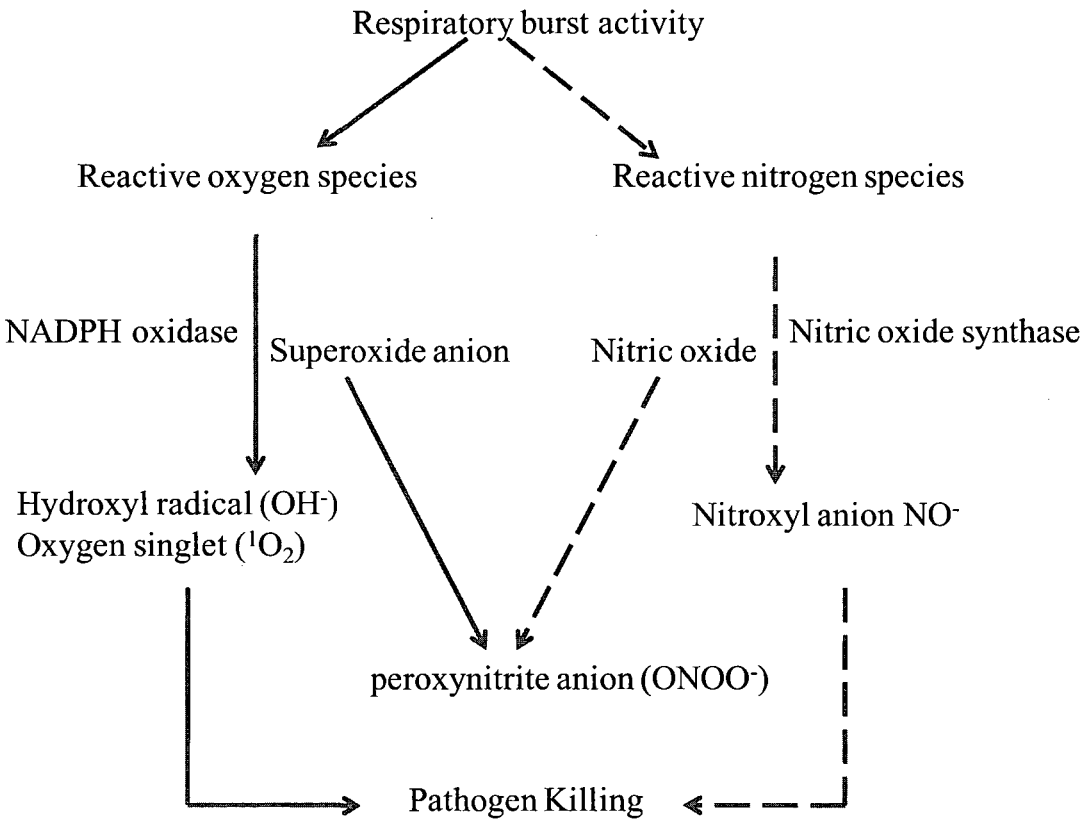
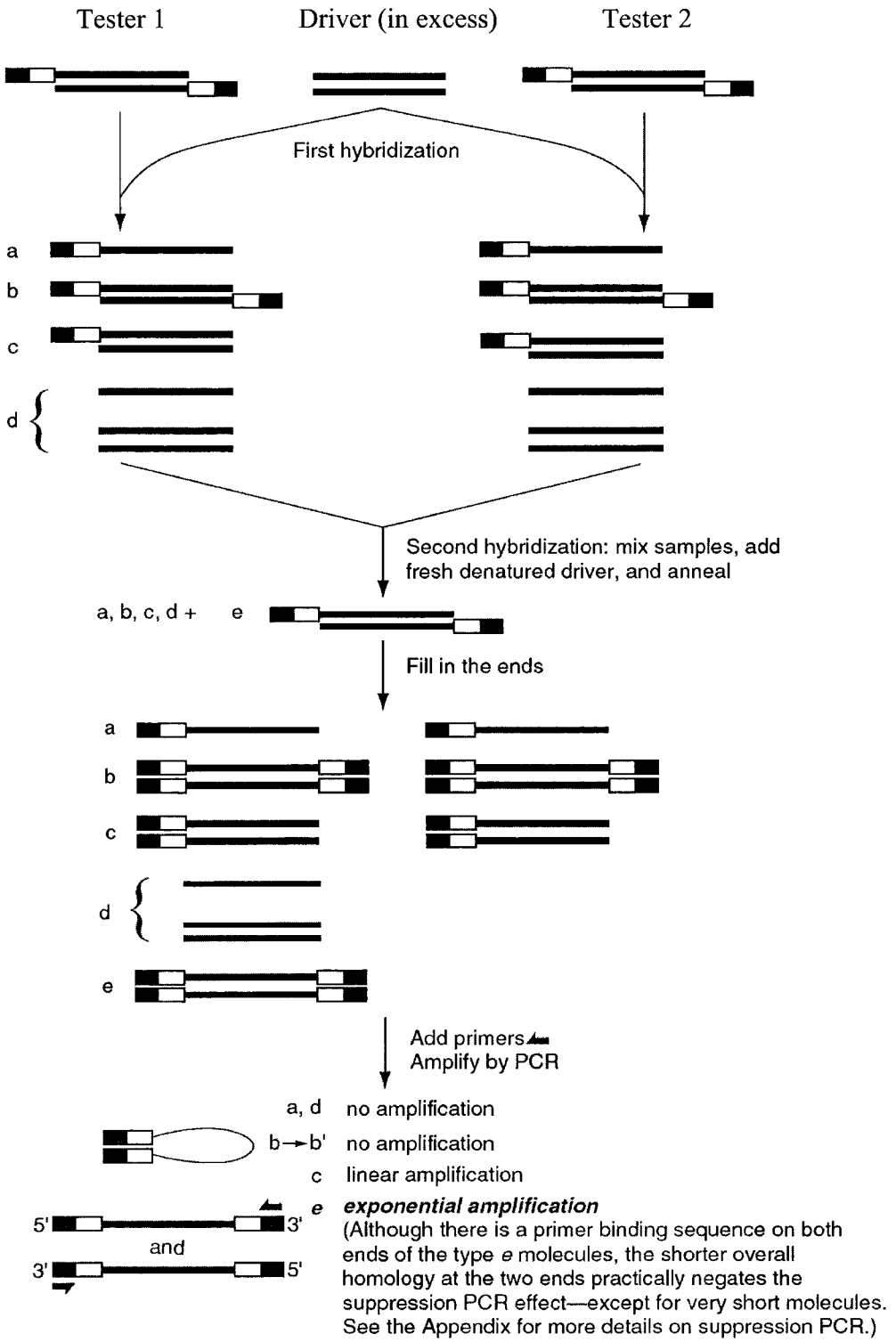


Figure1.1 Pathogens trigger respiratory burst activity either through NADPH-oxidase or NO-synthase or both pathways. These pathways generate toxic radicals which are involved in intracellular killing of pathogens. NADPH-oxidase mediates production of toxic reactive oxygen species (hydroxyl radical (OH^-) and singlet oxygen (O_2^-)) whereas NO-synthase mediates production of reactive nitrogen species (Nitroxyl anion (NO^-)). Moreover, an interaction between the intermediates of both pathways can also generate highly toxic compound such as peroxynitrite anion ($ONOO^-$).



Source: Lukyanov et al. (2007)

Figure 1.2 Suppression subtractive hybridization (SSH) is a molecular technique, which amplifies differentially expressed genes. It consists of a subtractive hybridization step and suppressive PCR step. First, tester is subdivided into two portions and each ligated to adaptator 1 and 2R separately. No adaptators are ligated to the driver. In a first hybridization, an excess driver is added to each tester. During the second hybridization, the two primary hybridization reactions are mixed together without denaturing and produces different molecules (a, b, c, d and e). During the PCR step only type (e) molecules (differentially regulated transcripts), which have different adapter sequences (primer annealing sites) at their ends can be amplified exponentially. Type (b) and (c) molecules cannot be amplified because it lacks primer annealing sites. Type (d) molecules form stem-loop pan handle-like structures that suppress amplification whereas type (a) molecules have only one primer annealing site and amplification is linear.

Chapter 2

Selection and evaluation of housekeeping genes for hemocytes of soft-shell clams (*Mya arenaria*) challenged with *Vibrio splendidus* LGP32

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2.1 Abstract

Gene expression studies have opened a tremendous field of investigation in biological research over the last decades. Expression of genes is most frequently quantified by real-time PCR (qRT-PCR), as this method has proven to be highly sensitive. One of the critical steps, however, in comparing transcription profiles is the availability of selected housekeeping genes. Expression of these genes should be steadily stable across the conditions under study so that they provide a baseline for gene expression comparison. Such a baseline is best established using a set of few housekeeping genes. Usually, those genes are involved in maintaining homeostasis and cell viability. In our study, nine candidate genes were used, including some commonly used housekeeping genes, such as ribosomal RNA (18S), ribosomal proteins (S-15, S-18 and L-37), beta actin, ubiquitin, receptor of activated C kinase (RACK) and elongation factor 1 and 2, in order to determine the most stable housekeeping genes, after hemocytes of *Mya arenaria* were exposed to *Vibrio splendidus* for two hours. Our results showed that EF-1, rpS-18 and ubiquitin appear to be the most stable genes for this experimental condition. On the other hand, both 18S-rRNA and actin, the most widely used housekeeping genes, turned out to be the least stable. This demonstrates the absolute need for preliminary assessment of housekeeping genes in gene expression studies.

2.2 Introduction

Quantitative polymerase chain reaction (qRT-PCR) is a widely used technique to quantify the expression of target genes involved in biological processes. For accurate quantification of gene expression however, it is crucial to normalize data with those genes having stable expression in the course of conditions under study. Factors, such as sample size, quantity and quality of RNA and reverse transcription PCR efficiency in cDNA synthesis, may influence qRT-PCR and need to be taken into account for normalization (Dheda et al., 2004). The use of internal controls, known as housekeeping or reference genes, usually overcomes this issue. The advantage of using housekeeping genes is that they are present together with the genes of interest in one sample. Hence the systemic errors that could arise from factors such as RNA extraction protocols, the inherent RNA variability and efficiencies of reverse transcription and PCR, are therefore eliminated (Huggett et al., 2005).

Housekeeping genes are used as internal controls because their mRNA transcription level is fairly stable during the course (intensity or duration) of the specific conditions under study (Bohle et al., 2007; Arenz et al., 2007). Hence, the differential expression of target genes is normalized and quantified in relation to those housekeeping genes. Therefore, determining the expression stability under a particular experimental situation is an essential requirement prior to the utilization of a given gene for internal standardization. It is usually accepted that housekeeping genes are expressed at constant level because they are associated with maintaining homeostasis (García-Vallejo et al., 2004) and cell

viability (Arenz et al., 2007); but their expression can fluctuate in different tissues (Barber et al., 2005; Rubie et al., 2004) and cell types (Bas et al., 2004) and following different environmental stimuli (Arenz et al., 2007; Nicot et al., 2005).

Among others, β-actin (He et al., 2005; Huang et al., 2007; He et al., 2004; Pan et al., 2005; Lockyer et al., 2007), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S-ribosomal RNA (Gunsch et al., 2006) are the most commonly used housekeeping genes. These genes were subsequently selected and included as *bona fide* housekeeping genes without preliminary evaluation of their stability for the particular experimental setting. However, Deindl et al. (2002) have demonstrated the differential expression of β-actin and GAPDH in growing collateral arteries in a rabbit model. In the case of 18S-rRNA, which is a frequently used housekeeping gene as it constitutes the largest portion (~80%) of total RNA, the assumption that the rRNA:mRNA ratio is stable is wrong (Hansen et al., 2001). Mogal and Abdulkadir (2006) have shown that 18S-rRNA levels in mouse prostate tissue explants and human prostate cancer (LNCaP) cells following TSA treatment did vary; consequently, 18S-rRNA cannot be used as an internal control in this experiment. Another drawback of normalization using 18S-ribosomal RNA is that it no longer exists in cDNA synthesized by oligo(dT) primers (Vandesompele et al., 2002). Ingerslev et al. (2006) demonstrated that β-actin expression varies when TO cells of Atlantic salmon (*Salmo salar*) were subjected to lipopolysaccharide. In this study, the group suggested β-actin as housekeeping in head kidney leucocytes but not for the TO cells. The inconsistent results obtained for these genes in different experimental conditions and

tissues clearly indicate that there is no universal housekeeping gene (Vandesompele et al., 2002). Therefore, it is of central importance to select and validate appropriate housekeeping genes for a particular experimental setting. Selection should ensure that the reference genes expression for the given cell types and organism does not vary depending on experimental conditions. Several authors have suggested the use of more than one single housekeeping gene to accurately measure the expression of genes under study (Bohle et al., 2007; Pfaffl et al., 2004; Arenz et al., 2007). The goal of our study was to select and validate a pool of genes to be used as housekeeping genes in a bacterial challenge model in the soft-shell clam, *Mya arenaria*. In this study, hemocytes of soft-shell clams were challenged with *Vibrio splendidus* and the expression profile of nine candidate genes to be used as housekeeping genes were analysed for their stability.

2.3 Materials and methods

2.3.1 Bacteria

Vibrio splendidus LGP32 was grown in 50 mL 2% NaCl Tryptic Soy Broth (TSB) for 12 hours at 16°C to a final concentration of 10^8 cells ml^{-1}. *Vibrio splendidus* was collected from the culture medium by centrifugation (5000g, 4 °C, 10 min) and cells were washed with filtered sterile saline water. Bacteria concentration was estimated using FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA) and spectrophotometer (UNICO Spectrophotometer, Montreal Biotech Inc). Before *V. splendidus* was used in the

experiments, bacteria suspension was injected to the clams to boast its virulence (Waechter et al., 2002).

2.3.2 Experimental animals

Soft-shell clams, *M. arenaria*, were obtained from the Magdalen Islands (Quebec, Canada). The clams were conditioned for two weeks in a 300L tank containing artificial seawater (Instant Ocean®, Aquarium Systems) in the recirculation system at the Atlantic Veterinary College (Prince Edward Island, Canada). Clams were fed Spat Formula (Innovative Aquaculture Products) every other day. The water was maintained at a temperature of 16-17 °C and salinity of 30 ppt (3%) with light regime of 12 h light and 12 h dark. Clams (n=60) were randomly sampled and checked for tetraploidy (hemic neoplasia) status using flow-cytometer (Delaporte et al., 2008) as this condition may interfere with the functional integrity of hemocytes (Beckmann et al., 1992). A total of 24 healthy clams (<5% tetraploidy) with shell length of 4.9-5.6 cm were included in this study. The gender of the organisms was not determined.

2.3.3 *In vitro* challenge of *M. arenaria* hemocytes with *V. splendidus*

Soft-shell clams were bled (0.5-1.5 ml from each animal) from the posterior adductor muscle using 3 ml syringe fitted to 25 gauge needle. Hemolymph quality of individual clams was checked using phase contrast microscopy (Axio Imager, ZEISS, Germany) at 400X magnification and hemolymph with lots of debris or very low number of cells was discarded. Hemolymph with high number of cells and low debris was passed through an 80 μm mesh to remove aggregated hemocytes and debris. The hemolymph was

immediately pooled into 30 ml test tube placed on ice to minimize clumping and aggregation of hemocytes. One ml from the pooled hemolymph (~10^6 hemocytes/ml) was allocated to each test tube (6 control and 6 challenged). *Vibrio splendidus* suspension (10^6 cells ml^{-1}) was added to the test tubes of experimental group to make a 1:1 hemocyte to bacteria ratio and 1 ml of filtered and sterilized artificial seawater (ASW) was added to the control. Two hours post-challenge, hemocytes were separated by centrifugation (500xg, 4 °C for 15 minutes) and washed twice with filtered sterile seawater to remove bacteria.

2.3.4 Total RNA extraction and cDNA synthesis

Total RNA was extracted from the hemocytes challenged for two hours, using the TRIzol® reagent (Invitrogen, USA) according to the manufacturer's recommendation. Total RNA was treated with DNAse-1 (Sigma, USA) to remove genomic DNA and quality and quantity of RNA was determined using Experion®, automated electrophoresis station, (Bio-Rad, CA, USA) (Fig. 2.1). First strand cDNA was synthesized using the SuperScript™ III Platinium® Two-Step qRT-PCR kit according to the manufacturer protocol (Invitrogen, USA). Briefly, 20 µl final volume of a mix containing total RNA (1-µg), 10 µl 2X RT reaction mix, 2 µl of M-MLV reverse transcriptase enzyme mix and DEPC-treated water was mixed to perform the first strand cDNA synthesis. To remove any remaining RNA, the newly synthesized cDNA was treated with 2 µl of *E. coli* RNase H.

2.3.5 Real time quantitative PCR

The expressed sequence tags (EST) of the nine candidate genes were obtained from a subtracted cDNA library of *M. arenaria* (Siah et al., 2007). Real time quantitative PCR was carried out in Rotor Gene RG-3000 (Corbett Research, Australia) using SYBR® Green I technology (Invitrogen, USA). PCR amplifications were performed on 1 µl of cDNA template (total reaction volume 25 µl). The forward and reverse primers (Table 2.1) of the nine candidate genes were cautiously designed using the Primer3 software and synthesized by Invitrogen corp. (Burlington, ON, Canada). The concentration for both reverse and forward primer to perform real time PCR was 0.4 µM. Real time PCR cycling conditions were as follows: 10 min. at 95 °C, followed by 40 cycles (95 °C for 20 sec, 60 °C for 20 sec, 72 °C for 20 sec). In all cases negative controls (without cDNA) were included to rule out DNA contamination. Melting curve and gel picture of each candidate gene was analysed in order to verify that a single PCR product was amplified for each set of primers. Standards of 10-fold dilution (10^0 to 10^{-8}) and samples of each gene were run together to calculate PCR efficiency (Table 2.2).

2.3.6 Data analysis

Real-time PCR data (calculated concentration in copies/µl) of the nine candidate genes was analysed using the geNorm software (Vandesompele et al., 2002). The program calculates the gene expression normalization factor based on geometric mean of the number of candidate genes and determines the most stable internal controls, which can be used as housekeeping genes for specific experimental condition in unchallenged and challenged cells. The gene stability measurement M was assessed by determining the

average pair-wise variation of a particular gene with all other genes. Moreover, the optimal number of housekeeping genes required for normalization was determined using geNorm.

2.4 Results

2.4.1 Quantitative Real time PCR (qRT-PCR) analysis of selected genes

The specificity of the primers was confirmed by the presence of a single peak in the melting curve and a single band of the expected size when PCR products were run in 2% agarose gel electrophoresis (Fig. 2.2b). The Ct value in the negative controls (PCR without cDNA) of two genes appeared after 35 cycles whereas in the remaining seven genes, the negative controls were null. In cases where the negative control appeared in late cycles the threshold in the melting curve was set above the control's peak in order to avoid the background noise.

To verify the absence of any genomic DNA contamination, a negative RT control was performed with specific primers (Fig. 2.2a). Ct values of the samples ranged between 20 and 30 and the Ct value difference between replicates was less than one. The PCR products were cloned and sequenced, and identity of the amplicons was confirmed. The standard curves gave correlation coefficient (R^2) and PCR efficiencies greater than 0.98 and 90% respectively. PCR efficiencies were used to calculate raw data from their corresponding Ct values. Melting curve analysis confirmed gene-specific amplification.

2.4.2 Gene expression stability analysis

The gene expression stability of the nine candidate genes was evaluated. In order to determine the internal gene stability (M), a pairwise variation of each gene was compared with all the other genes. The M value for the genes from the least stable to the most stable are as follows: 18S-rRNA (M=1.81), EF-2 (M=1.31), actin (M=1.20), rpL-37 (M=0.98), rpS-15 (M=0.50), RACK (M=0.45), Ubiquitin (M=0.39), rpS-18 (M=0.33) and EF-1 (M=0.33). The most stable genes (with the lowest *M* value) are elongation factor 1 (EF-1) and rpS-18 followed by ubiquitin, RACK and rpS-15 (Fig. 2.3). The expression stability value (*M*) of the five most stable genes ranged from 0.33 to 0.50.

The normalization factor (NF) was calculated based on the geometric mean of the expression levels of *n* genes and the optimal number of housekeeping genes used were determined by calculating the pairwise variation (V_n/V_{n+1}) by stepwise additional less stable gene to the most stable internal controls (Vandesompele et al., 2002). The data in figure 2.4 shows that there were five stable genes which can be used as internal controls in this study but only the first three (EF-1, rpS-18 and Ubiquitin) genes were selected.

2.5 Discussion

Because of the high sensitivity and robustness of the technique, quantitative real time PCR is widely used for quantification of gene expression. However, to quantify gene expression stable transcripts that can be used as internal controls are an absolute need and prerequisite (Arenz et al., 2007). Studies have shown that use of a single gene as a

housekeeping gene is not accurate for normalizing quantification of gene expression and that a set of several reference genes is needed for this purpose (Vandesompele et al., 2002). Thus, before undertaking the quantification of the expression levels of a selected target gene, the suitability and appropriateness of housekeeping gene candidates must be validated in the particular experimental conditions that are foreseen (Tang et al., 2007; Siah et al., 2008).

In our study, nine candidate housekeeping genes were selected from EST sequences of the *M. arenaria* cDNA library constructed by Siah et al. (2007). The candidate genes include ribosomal proteins (S-18, S-15 and L-37), rRNA (18S), genes involved in cytoskeleton structure (actin), gene expression pathway (RACK), protein metabolism (ubiquitin) and protein synthesis (elongation factors 1 and 2). The 18S-rRNA and actin genes were chosen because they are among the most widely used housekeeping genes and other genes which represent different pathways were selected in order to reduce the effect of co-regulation (Vandesompele et al., 2002). Among the nine candidate genes examined by geNorm software, only five transcripts could be used as potential housekeeping genes for an accurate normalization. This set of genes included elongation fctor-1 (EF1) and rpS-18 (M=0.33), ubiquitin (M=0.39), receptor activated C kinase (RACK, M=0.45) and rpS-15 (M=0.50). Surprisingly, 18S-rRNA and actin appeared as the least stable genes when hemocytes of soft-shell clams, *M. arenaria*, were challenged by *V. splendidus*. However, those genes are frequently used as housekeeping genes in molluscs (Dondero et al., 2006; Cellura et al., 2006) and crustaceans (He et al., 2004 and 2005). Tang et al. (2007) also demonstrated that 18S-rRNA was unstable in different

developmental stages of zebrafish, *Danio rerio*. The 18S-rRNA cannot be used as internal control in cDNA synthesized by oligo(dT) primers and its high abundance compared to mRNA makes it unsuitable for normalization (Vandesompele et al., 2002). Actin plays an important role in phagocytosis, encapsulation and nodule formation (Takai et al., 2001). Furthermore, He et al. (2004) suggested that up regulation of actin in hemocytes may facilitate the clearance of microbes from the host. Hence, the role of actin in the above mentioned cellular functions might lead to its regulation in hemocytes of *M. arenaria* after *Vibrio* challenge.

In order to determine the optimum number of housekeeping genes that could be used for accurate normalization, a pairwise variation V_n/V_{n+1} was calculated by adding less stable genes to the most suitable genes. The pairwise variation between the two sequential NFn and NFn+1 showed that EF-1, rpS-18 and ubiquitin could be used as housekeeping genes for accurate normalization of gene expression levels related to hemocytes from soft-shell clams challenged by *V. splendidus*. EF-1 and rpS-18 were also among the best housekeeping genes in tetraploidy analysis of soft-shell clams, *M. arenaria* (Siah et al., 2008). Moreover, Tang et al. (2007) demonstrated that EF-1 is a suitable internal control in developmental stages of zebrafish, *Danio rerio*. On the other hand, EF-1 expression in B-cells from patients with IgA nephropathy and human umbilical vein endothelial cells treated with collagenase type 2 (García-Vallejo et al., 2004) and in different life stages of *Phytophthora parasitica,* a plant pathogen, (Yan and Liou, 2006) was highly variable.

Ubiquitin was the third most stable candidate housekeeping gene in the present work. It is highly conserved in eukaryotes. It mediates the removal of short lived and damaged

proteins via non-lysosomal degradation (Finley, 2001; Marques et al., 2004) and can also be used as early marker of oxidative stress in mollusc (McDonagh and Sheehan, 2006). Ubiquitin expression remained unaffected after hemocytes were exposed to *V. splendidus* for two hours. And this could be an indication that either this cascade does not have a role in microbe degradation or two hours period was too short for the cascade to start. The fact that this pathway takes place after the microbes are phagocytosed or engulfed could mean that the activation of ubiquitin cascade may require more than two hours. In several mollusc species, the involvement of respiratory burst activity (Roch, 1999; Bugge et al., 2006), antimicrobial peptides (Mitta et al., 2000) and lysosomes (Labreuche et al., 2006) in intraphagosomal microbial degradation have been suggested. Perhaps these mechanisms of microbial degradation could be more active in soft-shell clams.

2.6 Conclusions

In our experimental conditions, 18S-rRNA and actin commonly used housekeeping genes, were found to be the least stable genes. Actin and elongation factor 2 are involved in the stability of the cytoskeleton filament and the high expression of these two genes in challenged hemocytes might suggest hemocyte's effort to maintain their cytoskeleton structural integrity. On the other hand, ubiquitin, which is mainly involved in degradation pathways, was among the most suitable genes together with elongation factor-1 and rpS-18. This study clearly shows the importance of assessing and evaluating the stability of genes for a particular condition or cells prior to their use as housekeeping genes. The use

of the EF-1, rpS-18 and ubiquitin is strongly recommended as reference genes to accurately measure gene expression in *M. arenaria* pathological studies.

2.7 Acknowledgements

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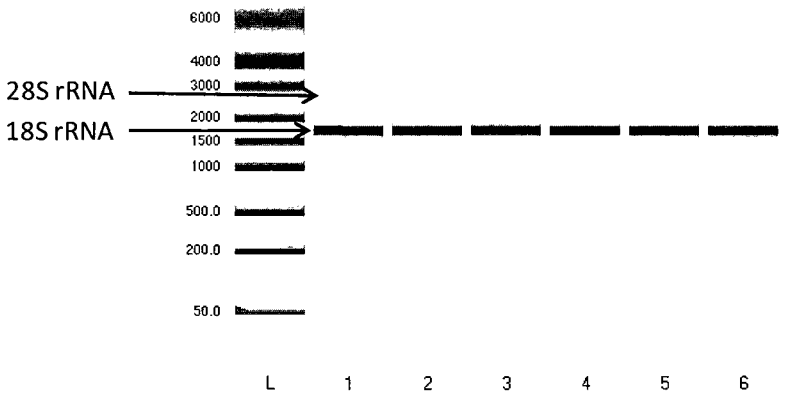
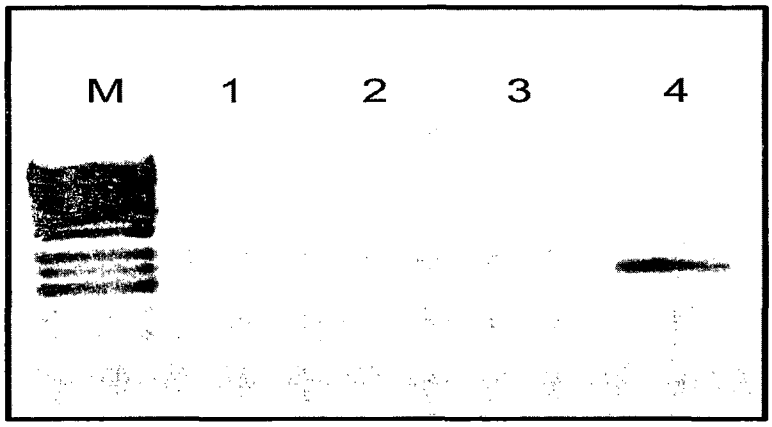


Figure 2.1 Determination of RNA integrity from *Vibrio*-challenged (lanes 1-3) and unchallenged (lanes 4-6) hemocytes of soft-shell clam samples using an automated electrophoresis station. In all cases the RNA quality indicator (RQI) is 10 indicating good quality of RNA. (L = RNA ladder).

A.



B.

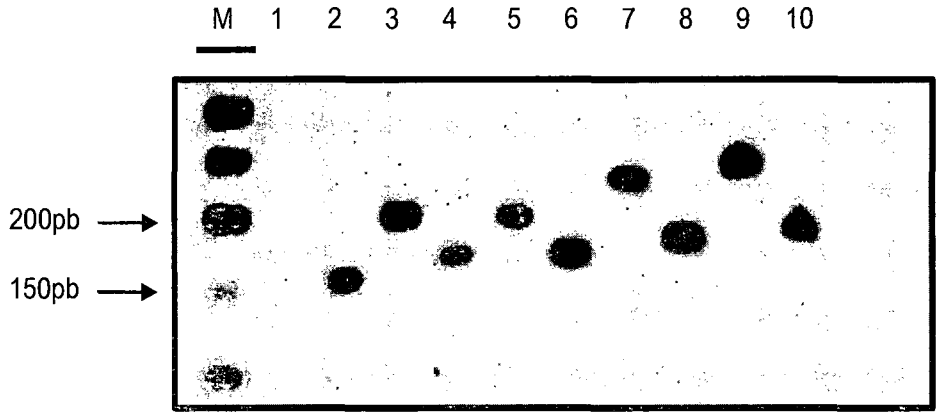


Figure 2.2 Agarose gel electrophoresis (2%) for verification of (A) genomic DNA contamination in total RNA sample using reverse transcription PCR (M, 50bp ladder; 1, without RNA; 2, with RNA; 3, RNA without reverse transcriptase and 4, RNA with reverse transcriptase) (B) primer specificity of the nine candidate genes using PCR (M, 50bp ladder; 1, negative control; 2, RACK; 3, rpS-18; 4, rpL-37; 5, Ubiquitin; 6, EF-1; 7, EF-2; 8, rpS-15; 9, 18S-rRNA and 10, actin) run in 2% agarose gel.

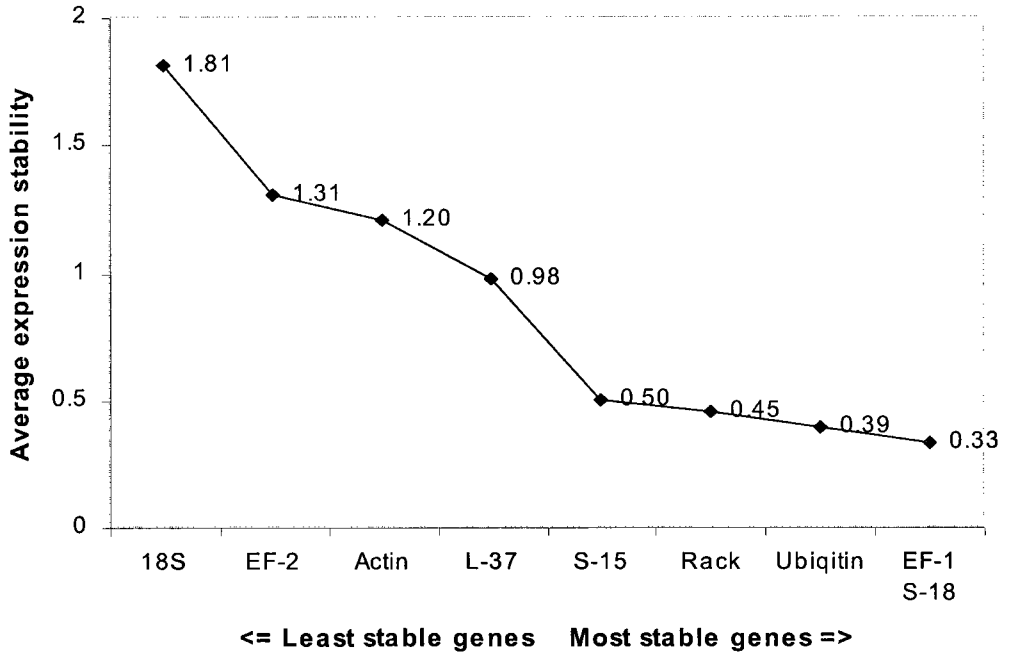


Figure 2.3 Average expression stability (*M*) value of nine candidate genes from the least stable to the most stable (18S-rRNA, EF-2, actin, rpL-37, rpS-15, RACK, Ubiquitin, rpS-18 and EF-1). Suitable housekeeping genes have small *M* value. Small *M* value signifies fairly stable regulation of a gene in the different experimental conditions under comparison. For a gene to be considered as a housekeeping gene it should have *M* value of <1.5.

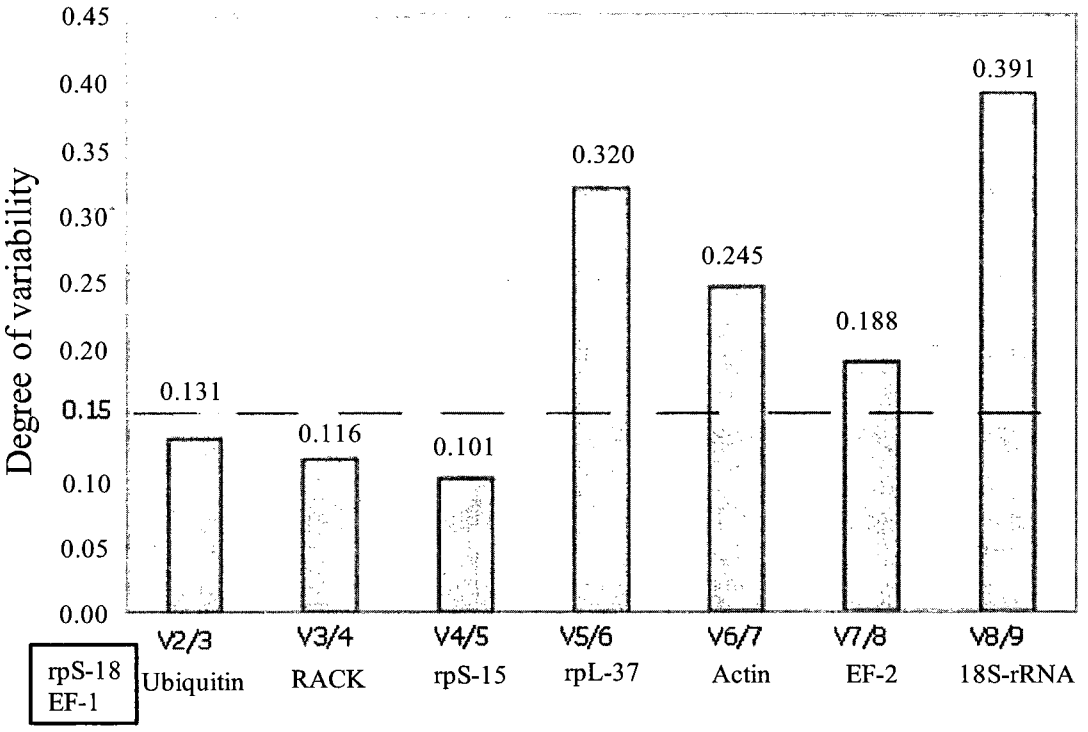


Figure 2.4 Determination of optimal number of housekeeping genes required for accurate normalization based on pair-wise variation (V_n/V_{n+1}) between candidate genes. When the first 3 (V2/3) or 4 (V3/4) or 5 (V4/5) housekeeping genes were used the degree of variation in normalization factor was less than 0.15. This means the five genes (EF-1, rpS-18, Ubiquitin, RACK and rpS-15) could be used as housekeeping genes in this study however only the first three most stable genes (EF-1, rpS-18 and Ubiquitin) were utilized for practical reasons.

Table 2.1 Sequence of primers used to quantify target gene expression using real-time PCR.

Gene name	Primers	Sequence (5'-3')	Tm	Amplicon (pb)	Efficiency (%)
Actin Gamma	Forward	GCGAAAATACTCCGTCTGGA	60	179	100
	Reverse	GCAGGTACGATCACAAGCAA			
Elongation Factor-1	Forward	GGTGGCTGTTGGTGTCATC	60	158	102
	Reverse	GGCCTAGGTGTTTTCCATGA			
Elongation Factor-2	Forward	CTACAAGCCTGGCTCAAAGG	60	218	100
	Reverse	TGACAACTGGGCTGACAGAG			
Ribosomal protein S-18	Forward	AAGATTCCCGACTGGTTCCT	60	189	90
	Reverse	GCCGGTTGTCTTTGTATGCT			
Ribosomal protein L-37	Forward	CCTAAACCTCCTGCTGGACA	60	156	99
	Reverse	GCGTGCATATCACATTCAG			
Ribosomal protein 18S	Forward	AGACTCCGGGAAACCAAAGT	60	232	93
	Reverse	AGACAAATCGCTCCACCAAC			
Ribosomal protein S-15	Forward	GTTGCGAAAGGCAAAGAAAG	60	170	97
	Reverse	GGTAATGGCCAATCATCTCC			
Ubiquitin	Forward	TCGCTAAGGAGCTGGACATT	60	194	95
	Reverse	ACCGTCGCTCCTTGTACATC			
Receptor Activated C Kinase 1	Forward	GACGGTGGCGGCATCATC	60	147	97
	Reverse	GCTTCCTGTTCCAATCACTTCC			

Table 2.2 Mean Ct values and coefficient of variance (CV) from replicates (n=10) and their quantitative PCR efficiency (E) of the candidate genes.

Genes	Actin	EF-1	EF-2	rpL-37	rpS-15	rpS-18	Ubiquitin	18S-rRNA	RACK
Ct	24.4	23.7	22.9	24.5	22.8	22.3	26.6	23.3	23.0
CV	1.1	2.0	2.0	1.5	1.6	1.2	1.1	1.8	2.1
E	100	100	100	99	97	90	95	93	97

Chapter 3

Morphological and molecular effects of *Vibrio splendidus* on hemocytes of soft-shell clams, *Mya arenaria*

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3.1 Abstract

Hemocytes constitute the cellular part of mollusc immune system and are involved in phagocytosis, production of toxic oxygen radicals, antimicrobial peptides, opsonising molecules, lysozymes, digestion, excretion and nutrient transport. In this study, we investigated the phenotypic response, phagocytosis and respiratory burst activity in hemocytes of *Mya arenaria* exposed to the bacterium *Vibrio splendidus*. Exposure to *V. splendidus* led to a loss of pseudopodia and rounding of hemocytes. The phagocytic ability of hemocytes was significantly reduced in challenged hemocytes as was the respiratory burst activity of hemocytes. The expression of actin and elongation factor 2 genes was measured to investigate a possible relation between phenotypic response of hemocytes exposed to *V. splendidus* and genes associated with cytoskeleton. Both actin and EF-2 genes were up regulated in challenged hemocytes. Further studies are under way to identify other genes in hemocytes whose expression is affected by exposure to *V. splendidus*.

3.2 Introduction

The soft-shell clam, *M. arenaria*, is a commercially important species of bivalve in the Maritime Provinces of Canada (DFO, 2001) and Maine USA (Beal, 2002) for its crucial economic contribution to livelihoods of the coastal communities. Clams live buried in the sediment, and their filter feeding behavior and close association with the sediment expose them to various aquatic micro-organisms, among which *Vibrio* species. Tubiash (1971) suggested that soft-shell clams are convenient laboratory animals, which can be used to screen potential mollusc pathogens. Although there is no report that *Vibrio splendidus* infects soft-shell clams, it is known to cause disease in other bivalve species such as oysters, *Crassostrea gigas* (Lacoste et al., 2001; Waechter et al., 2002) and scallops, *Pecten maximus* (Lambert et al., 1998; 1999). Recently, *V. splendidus* has been increasingly used in various mollusc-bacteria challenge models (Gonzalez et al., 2007; Allam and Ford, 2006; Lacoste et al., 2001; Choquet et al., 2003; Waechter et al., 2002 and Parisi et al., 2008), providing valuable information on *in-vitro* and *in-vivo* interaction between bivalve hemocytes and *V. splendidus*. This is supported by significant efforts done to explore the *V. splendidus* genome and virulence mechanisms (Le Roux et al., 2007). The soft-shell clam offers many advantages in these host-pathogen models of interaction: it is a common species, it is easy to maintain under laboratory conditions, it offers an easy access for hemolymph collection and yields large quantities of hemolymph and hemocytes.

In-vitro and *in-vivo* interactions between bacteria and molluscs have been investigated in different bivalve species: mussels, *Mytilus edulis* (Mayrand et al., 2005; Hernroth, 2003),

scallops, *P. maximus* (Mortensen and Glette, 1996), oysters, *C. gigas* and *C. virginica* (Allam and Ford, 2006; Labreuche et al., 2006a) and clam species, *Ruditapes philippinarum* and *Mercenaria mercenaria* (Allam and Ford, 2006). Hemocytes placed on flat surfaces tend to develop cytoplasmic extensions and adhere to surfaces (Labreuche et al., 2006b). However, when hemocytes associate with pathogenic bacteria (Labreuche et al., 2006b) or their extracellular products (Lane and Birkbeck, 1999), they lose their pseudopodia and become round. Consequently their adhesion and phagocytic capability are reduced (Labreuche et al., 2006a). *In-vitro* challenge of hemocytes from *R. philippinarum, M. mercenaria* and *C. virginica* with three different bacteria (*Vibrio tapetis, V. splendidus* and *Vibrio anguillarum*) suggested that pathogenicity is host-specific (Allam and Ford, 2006).

Molluscs rely on innate immunity, which constitutes both cellular (hemocytes) and humoral components (see review by Canesi et al., 2002). Among other functions, hemocytes are involved in phagocytosis (Brousseau et al., 2000; Goedken and De Guise, 2004; Allam and Ford, 2006), production of reactive oxygen/nitrogen species (Lambert et al., 2007b; Bugge et al., 2007) and antimicrobial agents (Mitta et al., 2000; Cellura et al., 2007; Gonzalez et al., 2007). *In-vitro* studies showed that mollusc hemocytes subjected to pathogens (Goedken and De Guise, 2004; Labreuche et al., 2006a; Allam and Ford, 2006) and high concentrations of heavy metals (Brousseau et al., 2000) have reduced phagocytosis. Lambert et al. (2007a) also reported inhibition of ROS production in *C. gigas* subjected to *Vibrio* sp. (strain S322).

The immune system of molluscs is very complex and involves gene networks of different pathways (Huvet et al., 2004 and Gestal et al., 2007). Some immune-related genes, which play an important role in pathogen recognition and destruction, have been identified in mussels (Mitta et al., 2000), oysters (Montagnani et al., 2001; Huvet et al., 2004 and Gonzalez et al., 2007), scallops Qiu et al., 2007), clams (Gestal et al., 2007) and abalone (Wang et al., 2008) subjected to bacteria and their components (such as lipopolysaccharide). In turn, bacteria have developed strategies to escape host defence mechanisms (Canesi et al., 2002). For instance, *Vibrio aestuarianus* 01/32 and *Vibrio anguillarum* A7 disturb the cell cytoskeleton stability and cause hemocyte rounding in *C. gigas* and *M. edulis* respectively, which hampers hemocyte adhesion (Lane and Birkbeck, 1999) and phagocytosis (Labreuche et al., 2006a).

Unlike other mollusc species, the soft-shell clam is not known to be particularly affected by any major bacterial disease, both in the wild and under controlled conditions. Hence, understanding how immune mechanisms in the soft-shell clam afford protection from disease could be useful in unraveling bacterial disease processes in other mollusc species. The overall goal of our research is to describe the hemocyte response to bacterial challenge. In this study, we examine the morphological, functional (phagocytosis and respiratory burst) and molecular responses of soft-shell clam hemocytes exposed to *V. splendidus* LGP32-GFP.

3.3 Methodology

3.3.1 Experimental animals and bacteria

Bacteria were cultured, harvested and their concentration estimated as illustrated in section 2.3.1. Clams were conditioned and screened for neoplasia as described in section 2.3.2. A total of 65 soft-shell clams (30 individuals in gene expression, 10 in challenge test for phenotypic observation, 10 in phagocytosis and 15 in respiratory burst assays) were used in this study.

3.3.2 Hemolymph collection

For hemolymph collection and quality check refer to section 2.3.3.

3.3.3 Hemocyte-*Vibrio splendidus* challenge

Hemocytes-*Vibrio* challenge was performed according to the procedure described in section 2.3.3. Hemocytes were challenged with four different ratios of hemocyte to bacteria (1:20, 1:10, 1:5 and 1:1). The morphological appearance of hemocytes was observed under phase contrast microscope (400X) at 1, 2 and 3 hours post challenge. The percentage of rounded hemocytes (without pseudopodia) was estimated by counting six different fields in each slide (each field containing between 10 to 35 hemocytes). Hemocytes subjected to *V. splendidus* for two hours at 1:1 ratio were used in the subsequent functional and gene expression assays. Hemocytes challenged in a 1:1 hemocyte to bacteria ratio for three hours were also included to follow in gene expression.

3.3.4 Hemocyte Viability

In this study, hemocytes viability was assessed using a trypan blue exclusion assay (Terahara et al., 2003). Briefly, 1 ml of *Vibrio splendidus* suspension (10^6 bacteria) was added to an equal volume of hemolymph (10^6 hemocytes). The same volume of filtered and sterilized artificial seawater was used in control groups. Both challenge and control groups were incubated for 1, 2 and 3 hours at room temperature. Then, 200 µl of 0.4% trypan blue (Sigma, USA) was added and incubated for 10 min. The percentage of viable hemocytes (dead cells stain blue whereas live cells remain unstained) was estimated by counting dead and live cells (in each field an average of 35 and 62 hemocytes were counted in control and challenged groups, respectively) in four randomly selected fields observed under phase contrast microscope.

3.3.5 Phagocytosis assay

The phagocytosis assay was adopted from Allam and Ford (2006) with some modifications. Briefly, 170 µL of *V. splendidus* suspension (~10^6 cells ml^{-1}) was placed into 5 ml flow cytometry tubes and equal volume of hemolymph (to create 1:1 *Vibrio* to hemocyte) was added. The same volume of filtered seawater was used instead of *V. splendidus* suspension for the unchallenged (control) group and each experimental condition had six replicates. Two hours post *Vibrio*-challenge, 60 µL of diluted suspension of phycoerythrin labelled (red) beads, (2 µm in diameter, Polysciences Inc., USA) was added to hemocytes in both control and challenged samples to create a 40:1 bead hemocyte ratio and incubated in the dark for two more hours at room temperature

on a gentle shaker. The internalization of the beads was terminated by placing the test tubes on ice. A FACSCalibur flow cytometer was used to estimate the percentage of hemocytes internalizing two or more beads by measuring red fluorescence (FL-2), which corresponds to the number of beads associated with hemocytes. Moreover, one group of hemocytes were treated with cytochalasin B (10 μg ml^{-1} final concentration, Sigma), an inhibitor of phagocytosis. Fluorescence (FL-2) in this group is a result of beads adhered to the hemocyte surface and not internalized. Fluorescence obtained in cytochalasin B treated hemocytes was subtracted from that of *Vibrio* challenged and control groups to exclude beads on hemocytes surfaces. Calcein AM (2.5 μM final concentration, Invitrogen, USA) was added to hemocytes and incubated for 30 min at room temperature. Stained hemocytes were washed using filtered seawater and observed under laser scanning confocal microscope (LSM 510 META, ZEISS, Germany) to verify the internalization of beads. In this study we defined phagocytosis as internalization of two or more beads because laser scanning confocal microscope observation showed that hemocytes treated with cytochalasin-B were able to internalize one bead.

3.3.6 Respiratory burst

The respiratory burst assay was adapted from Goedken and De Guise (2004) and each experimental condition had 12 replicates. Briefly, equal volumes (197 μl each) of hemolymph and *V. splendidus* suspension were placed into 5 ml flow cytometry tubes (Falcon®, BD Biosciences, San Jose, CA, USA) in the experimental group and FSSW was used in controls. Hemocytes treated with phorbol 12-myristate 13 acetate (PMA, 10 μg ml^{-1}; Sigma) served as a positive control. PMA stimulates the production of toxic

radicals by respiratory burst activity through protein kinase C (PKC) pathway. Another group of hemocytes was exposed to *V. splendidus* and PMA simultaneously. Non fluorescent 2', 7'-dichloroflourescin diacetate (DCFH-DA, 10 μM; Sigma) was added to the samples. DCFH-DA diffuses into the cells and is oxidized by hydrogen peroxide or nitric oxide to DCF which gives green fluorescence. The samples were incubated in the dark for 2 hours at room temperature and green fluorescence (FL-1) was measured using flow cytometry. *V. splendidus* LGP32-GFP is tagged with green fluorescent protein which could contribute to the fluorescence measured in *Vibrio*-challenged hemocytes. Hence, respiratory burst activity of challenged hemocytes was measured as a difference in fluorescence between *Vibrio*-challenged hemocytes treated with DCFH-DA and *Vibrio*-challenged hemocytes without DCFH-DA.

3.3.7 Gene expression

RNA was extracted and its quality and integrity was checked (see section 2.3.4) and qRT-PCR was performed as described in section 2.3.5. The primers used to amplify the target (actin and elongation factor-2) and housekeeping genes (EF-1, S-18, and ubiquitin) are listed in Table 2.1. qRT-PCR reactions with efficiencies >90% only were considered for analysis.

The relative expression of genes (actin and elongation factor-2), which are associated with cytoskeleton filament stability were quantified. In Chapter two, reference genes which are suitable for this *in-vitro* challenge model were selected (Araya et al., 2008). The housekeeping genes were utilized to normalize the expression of the target genes at

two and three hours post challenge following the instructions in geNorm manual (Vandesompele et al., 2002).

3.3.8 Data analysis

Phagocytosis, respiratory burst and relative gene expression data was normally distributed and was analyzed using student t-test and one-way ANOVA (SigmaStat, Statistical software, version 2) to determine the significance between the different experimental conditions in each assay.

3.4 Results

3.4.1 Morphological response

The morphological appearance of both challenged and unchallenged hemocytes was observed microscopically at 1, 2 and 3 hours post challenge (Figure 3.1). This morphological data was taken as a marker to select the optimal hemocyte-*Vibrio* ratio in the subsequent studies. We have observed that hemocytes subjected to *V. splendidus* lose their pseudopodia and become rounded. Especially at high *V. splendidus* to hemocyte ratios, the percentage of round hemocytes increased rapidly. For instance at 1:20, 1:10 and 1:5 hemocyte to *Vibrio* ratios, the percentage of round hemocytes rose rapidly from less than 20% in 1h to more than 80% and 100% in 2h and 3h post challenge, respectively (Table 3.1). The percentages of round hemocytes in 1:1 were 4.2, 44.3 and 92 at 1, 2 and 3 hours, respectively. Consequently, the gradual change of morphology at

1:1 ratio is more suitable for our study compared to the other ratios. The hemocytes in the control group maintained their pseudopodia and appeared to be attached to the glass slides. The percentage of round hemocytes in this (control) group was significantly low (less than 10% after three hours).

In the present report, a 1:1 hemocyte to *Vibrio* ratio was chosen as an optimal ratio for the *in-vitro* challenge model because unlike the higher ratios, at 1:1 the percentage of round hemocytes increased gradually. Within the 1:1 ratio, hemocytes subjected to *V. splendidus* for 2h (44.3% round cells) and 3h were chosen mainly for gene expression purposes. However, phagocytosis and respiratory burst activity were carried out in hemocytes exposed to *V. Splendidus* for 2 hours to link gene expression with immune functional assays.

3.4.2 Hemocyte viability

Despite the high percentage of morphologically modified hemocytes in *Vibrio*-challenged groups, the percentage of live hemocytes was very high (>91%) in both challenge and control groups (Table 3.2).

3.4.3 Phagocytosis assay

The effect of *V. splendidus* on hemocytes ability to phagocytose was investigated by providing fluorescent beads to hemocytes challenged with *V. splendidus* for 2h at 1:1 ratio. Multiple beads were observed internalized within unchallenged and challenged (exposed to *V. splendidus*) hemocytes. Although determining whether *M. arenaria*

hemocytes could phagocytose *V. splendidus* was not the aim of this study, *Vibrio* were also observed inside hemocytes (Mateo et al., 2009) and appeared to be motile.

The percentage of hemocytes phagocytosing beads in the unchallenged groups was significantly higher than in hemocytes subjected to *V. splendidus* and Cytochalasin-B (Fig. 3.2). Moreover, the percentage of phagocytosing hemocytes in *V. splendidus* treated groups was also significantly high compared to hemocytes subjected to cytochalasin B. After data was normalized to percentage of phagocytosing hemocytes exposed to cytochalasin-B, the percentage of hemocytes internalizing two or more beads in challenged and unchallenged hemocytes was 8.5% and 11%, respectively. As shown in figure 3.3 the ability of hemocytes to internalize beads was confirmed using laser scanning confocal microscope.

3.4.4 Respiratory burst assay

Hemocytes treated with PMA, a chemical which stimulates respiratory burst activity and which was used as a positive control, showed significantly higher respiratory burst activity compared to other treatment groups (Fig. 3.4) demonstrating that soft-shell clam hemocytes are able to produce toxic radicals. However, when hemocytes were exposed to *V. splendidus* alone, they generated the same level of response seen in negative controls (unchallenged hemocytes). When hemocytes were subjected to *V. splendidus* and PMA simultaneously, respiratory burst activity remained at the level of negative control and *Vibrio* only challenged hemocytes.

3.4.5 Gene expression

The quality/integrity of total RNA used to synthesize cDNA for gene expression was determined using a Bioanalyzer. Quantitative real-time PCR was performed for actin and elongation factor-2 (EF-2) genes, which are actively involved in cytoskeleton filament stability. Their relative expression was calculated by normalizing the data to three stable housekeeping genes (EF-1, rpS-18 and Ubiquitin). Actin and EF-2 genes were expressed significantly higher in *V. splendidus* challenged hemocytes than in unchallenged groups (Fig. 3.5) being up-regulated 3.5 and 2 fold, respectively. The relative expression of actin was also significantly up regulated in hemocytes exposed to *Vibrio* for three hours (Fig. 3.6). However, hemocytes challenged for three hours had significantly reduced actin expression compared to hemocytes challenged for two hours.

3.5 Discussion

Aquatic bacteria, including the genus *Vibrio* are able to persist within bivalve tissues and fluids (Murphree and Tamplin, 1995). *Vibrio* species can become pathogenic whenever the host weakens and its immune system is depressed. The high mortality rates recently reported in juvenile Pacific oysters, *C. gigas* (Lacoste et al., 2001; Waechter et al., 2002) and adult scallops, *P. maximus* (Lambert et al., 1999) could be related to such a process. Hence, in the last decade several studies focusing on the interaction between mollusc hemocytes and *V. splendidus* have been performed to elucidate mollusc immune defence system against bacteria (Allam and Ford, 2006; Choquet et al., 2003 and Parisi et al.,

2008). Here we report our results on interaction between hemocytes and *V. splendidus* strain LGP32-GFP. *V. splendidus* does not occur as a natural pathogen to soft-shell clams. Nevertheless, our study has shown that this strain has the ability to impair some of the normal functions of hemocytes: including reduced phagocytosis and respiratory burst activity.

Hemocytes from soft-shell clams were subjected to *V. splendidus* LGP32-GFP at different ratios for 1, 2 and 3 hours. When compared to a ratio of 1:1, hemocytes challenged at high ratios (1:20, 1:10, and 1:5 hemocyte to *Vibrio*) rapidly lost their pseudopodia and had a high percentage of rounded cells. However, exposing hemocytes at a 1:1 ratio for three hours also produced a high percentage (92%) of round hemocytes. Despite the morphological changes in challenged hemocytes, the percentage of viable hemocytes in both challenged (1:1 ratio) and unchallenged groups was high (>91%). Similar to our findings, a high percentage of rounded hemocytes were also reported in bivalve hemocytes exposed to *V. anguillarum* A7 (Lane and Birkbeck, 1999). In human cells *Vibrio cholerae* toxin ($MARTX_{vc}$) covalently binds to G-actin and F-actin and causes actin depolymerisation which leads to cell rounding (Kudryashov et al., 2008). Recently, a metalloprotease gene, *vsm*, an important cytotoxicity factor from the extracellular product of *V. splendidus* LGP32 was identified (Le Roux et al., 2007). This toxin was shown to cause rounding of mollusc cell lines (Le Roux et al., 2007) and may be responsible for the loss of pseudopodia (rounding) in *M. arenaria*. Although the hemocyte rounding is well documented as a cellular response to bacterial challenge, the factors responsible for this response are still unknown.

Phagocytosis is a major hemocyte function in molluscs (Labreuche et al., 2006a; Allam et al., 2001) including soft-shell clams (Brousseau et al., 2000). In the present study, we showed that hemocytes are able to internalize beads supporting results by Brousseau et al. (2000). However, *Vibrio* challenged hemocytes showed lower capacity to phagocytose beads. The reduced phagocytosis in challenged hemocytes could be due to loss of pseudopodia resulting from the toxic effect of extracellular product (vsm) produced by *V. splendidus*. This cytotoxic extracellular product causes loss of pseudopodia (Le Roux et al., 2007) which is very important during phagocytosis of pathogens and other non-self cells (Labreuche et al., 2006a). *V. tapetis* also caused hemocytes rounding in the Japanese carpet clam, *R. philippinarum* which leads to decreased phagocytosis (Allam and Ford, 2006). Loss of phagocytosis was also reported in rounded hemocytes induced by disseminated neoplasia in *M. arenaria* (Beckmann et al., 1992) and *M. edulis* (Kent et al., 1989).

The respiratory burst activity of soft-shell clam hemocytes was also investigated. We observed that, respiratory burst activity of hemocytes treated with PMA was significantly higher than in control. However, *V. splendidus* challenged group showed no significant respiratory burst activity compared to control. This suggests that either *V. splendidus* lacks a receptor which triggers respiratory burst activity or it actively suppresses the hemocyte response. When one group of hemocytes was treated with PMA and *V. splendidus* simultaneously, the respiratory burst in this group remained as low as in control and *Vibrio* challenged groups. This suggests that the low respiratory burst response of hemocytes challenged with *V. splendidus* is not due to the lack of molecules

on the surface of *V. splendidus* (receptors) which might activate hemocyte response. Instead, it appears that *V. splendidus* could have the capability to inhibit respiratory burst activity in hemocytes of soft-shell clams. Bramble and Anderson (1998) reported the inhibition of toxic radicals in *Crassostrea virginica* by the anti-oxidase activity of another *Vibrionaceae*, *Vibrio* (*Listonella*) *anguillarum*. Canesi et al. (2002) reviewed the different mechanisms involved in pathogens to escape from host immune system. These include destabilizing the structural integrity of cytoskeleton by producing toxins, capsule formation to prevent opsonisation and resist degradation, lacking receptors which triggers host immune system, lacking substrate susceptible to lysozymes and producing antioxidant compounds which inhibit respiratory burst activity of the host. Beside phagocytosis and respiratory burst activity, mollusc hemocytes are also involved in production of antimicrobial peptides (Mitta et al., 2000), lysozyme (Labreuche et al., 2006a), inflammation, wound repair, and encapsulation (Pipe, 1990).

There are two main pathways in respiratory burst (Dröge, 2002). The NADPH-oxidase and NO-synthase pathways which lead to the production of reactive oxygen species (H_2O_2, O_2^-) and nitrogen reactive species (NO, ONO_2^-), respectively. In the present study, we have not determined whether the response was from one pathway or a combination of both because the probe (DCFH-DA) can detect both NO and H_2O_2. Hence, further investigation is needed to determine the responsible pathway in hemocytes of soft-shell clams. In *Crassostrea gigas* both pathways have been reported but NADPH-oxidase and NO-synthase pathway were dominant in granulocytes and hyalinocytes, respectively (Lambert et al., 2007b). Molluscs without respiratory burst activity have also been

reported (Lopez et al., 1994 and Anderson et al., 2003). However, the low respiratory burst activity reported in those molluscs may have resulted from not using the right probes. In most molluscs respiratory burst activity assays, DCFH probe is used to detect reactive oxygen/nitrogen species (ROS/RNS). Walrand et al. (2003) demonstrated that ROS/RNS measurement in polymorphonuclear neutrophils depends on the selected probe and recommended the use of dihydrorhodamine 123 (DHR), 2',7'-dichloroflourescein diacetate (DCFH) and hydroethidine (HE) to detect H_2O_2, NO and O_2^- production; respectively.

In order to link the morphological modifications of hemocytes to gene expression, relative expression levels of actin and EF-2 transcripts were quantified in hemocytes exposed to *V. splendidus* (1:1 ratio) for 2 and 3 hours. Both genes are associated with cytoskeleton filament stability (Bektas et al., 2004; Pollard and Cooper, 1986). Actin is an abundant cellular protein, which is involved in cell division, locomotion, cytoskeleton structure formation and organelle reorganization (Walker and Garrill, 2006). In addition, actin plays an important role in phagocytosis, encapsulation and nodule formation (Takai et al., 2001) and may facilitate the clearance of microbes from the host system (He et al., 2004). On the other hand, elongation factor 2 (EF-2) is mainly involved in the elongation phase of protein synthesis in eukaryotes (Riis et al., 1990). Elongation factor two (EF-2) is also an actin binding protein which plays a key role in the regulation of intracellular actin level (Bektas et al., 1994). Bektas et al. (2004) reported that cytoskeleton disruption leads to reduced protein synthesis suggesting a link between actin and EF-2. Our results showed up-regulation of actin and EF-2 2h post challenge. Two hours post *Vibrio-*

challenge, nearly half (44.3%) of the hemocytes were round however the rest maintained pseudopodia. Hence, to determine which of the hemocytes (the ones which still maintain pseudopodia or the ones which lost it) contributed to the increased transcript level, the expression of actin was measured in hemocytes exposed for three hours, where 92% of the cells became round. Actin expression at three hours post challenge was significantly up regulated. However, it was significantly reduced compared to the hemocytes challenged for two hours. This suggests hemocytes maintaining their pseudopodia during the challenge might contribute to the expression of actin. Actively phagocytosing hemocytes need to maintain their pseudopodia to adhere and internalize particles (Labreuche et al., 2006a; Lane and Birkbeck, 1999). Similarly, an up-regulation of actin and EF-2 genes was observed in carpet-shell clams, *R. decussatus*, challenged by a mixture of dead bacteria including *V. splendidus* (Gestal et al., 2007). The up-regulation of these two genes in challenged cells could suggest that hemocytes were trying to maintain the cytoskeleton structural integrity, which plays a key role in phagocytosis. In eukaryotes, actin is involved in T-cell activation and plays a very crucial role in immune signaling cascades such as mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-kB) (see review by Kustermans et al. 2008). Since those pathways are conserved between vertebrates and molluscs, the up-regulation of actin in soft-shell clam hemocytes could also have a similar role, which is to trigger the immune signaling cascades to fight *V. splendidus* infection. MAPK and NF-kB pathways are also reported in Mediterranean mussel, *Mytilus galloprovincialis* (Betti et al., 2006) and hydrothermal vent mussel, *Bathymodiolus azoricus* (Bettencourt et al., 2007), respectively.

3.6 Conclusions

When hemocytes were challenged with *V. splendidus* strain LGP32-GFP, the percentage of round cells (without pseudopodia) increased significantly with time of exposure and concentration of bacteria. Hemocytes exposed to *V. splendidus* at a ratio of 1:1 for two hours lost their pseudopodia and showed reduced phagocytosis and respiratory burst activity. During this challenge, actin and EF-2 (two cytoskeleton associated genes) were up-regulated, which could be related to the morphological changes observed. Further studies are needed to explore this mechanism and unravel molecular pathways possibly involved in soft-shell clam hemocyte response to bacterial challenge.

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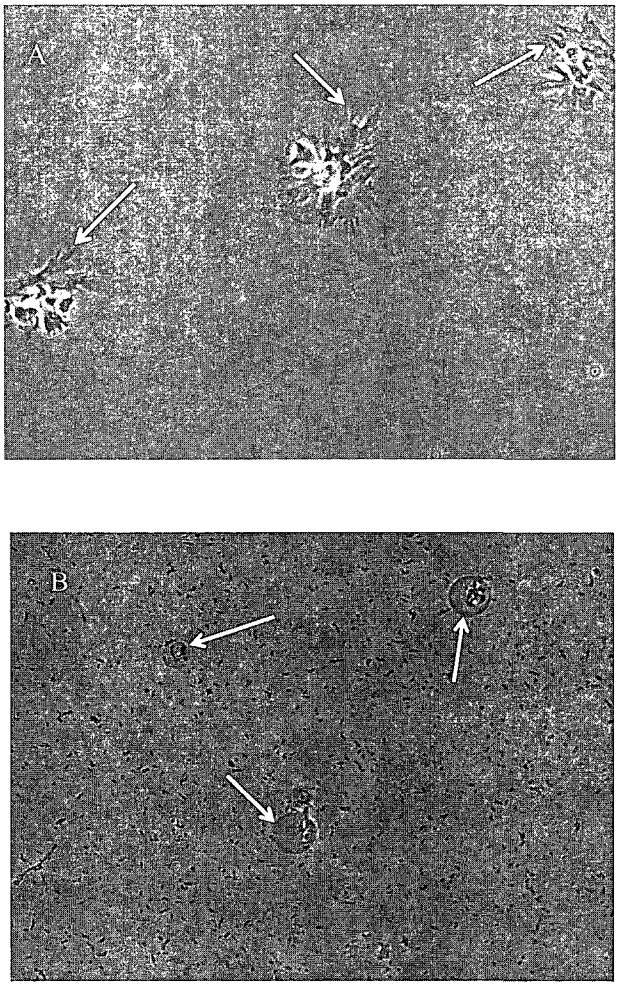

Figure 3.1 The phenotypic appearance of (A) unchallenged hemocytes of soft-shell clam (arrows indicate cytoplasmic extensions of hemocytes) and (B) *Vibrio* challenged hemocytes without cytoplasmic extensions (arrows indicate rounded hemocytes) observed under phase contrast microscope (400X).

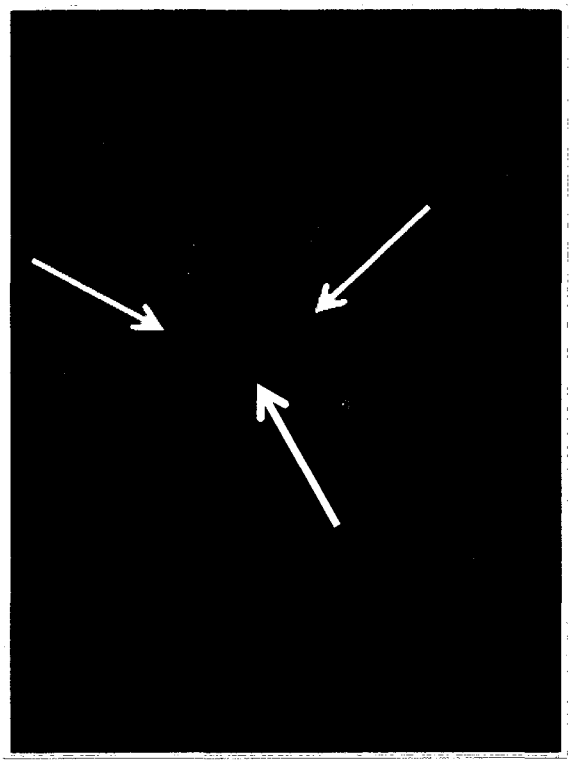

Figure 3.2 A leaser scanning confocal microscopy of hemocyte of soft-shell clam (1,000X) stained with calcein AM, phagocytosing more than two beads (orange). The arrows indicate phagocytosed beads.

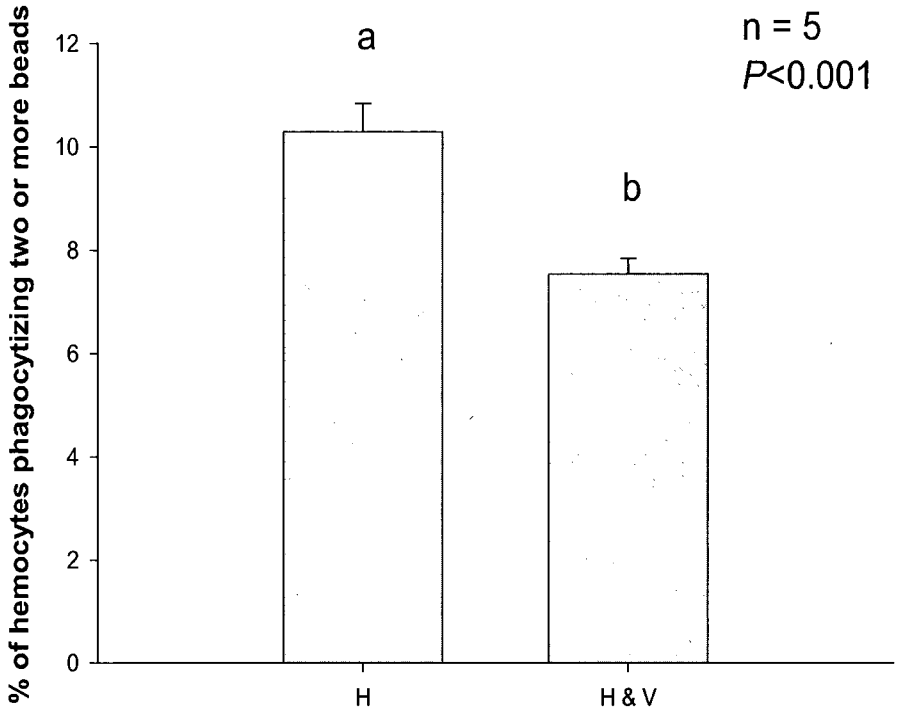

Figure 3.3 The effect of *V. splendidus* on the ability of hemocytes from soft-shell clam, *Mya arenaria*, to phagocytose phycoerythrin labelled (red) beads. Phagocytosis was significantly reduced in hemocytes subjected to *Vibrio* compared to unchallenged group. Cytochalasin-B (phagocytosis inhibitor) treated hemocytes were also included and fluorescence (phagocytosis) measured in this group was due to beads adhesion to hemocytes surface. Hence, phagocytosis in cytochalasin-B treated hemocytes was considered as a background and subtracted from challenged and unchallenged hemocytes to estimate phagocytosis in both groups (mean±SEM). (H=hemocyte; V=*V. splendidus*; letters indicate significant difference between treatments, ANOVA $p<0.001$; n = 5 replicates)

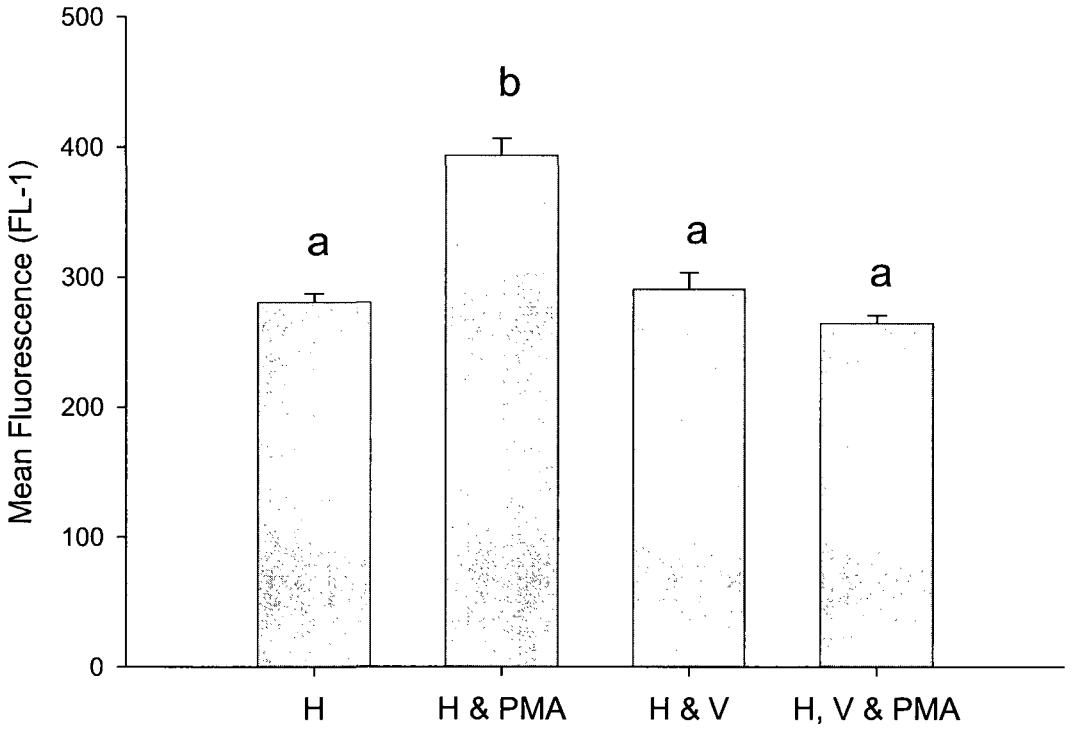

Figure 3.4 Respiratory burst activity in hemocytes of soft-shell clams (*Mya arenaria*), incubated with filtered sterile seawater (control), *V. splendidus*, PMA and *V. splendidus* and PMA simultaneously for 2 h (mean±SEM). PMA induced production of ROS/RNS suggesting a respiratory burst activity in hemocytes. However, PMA did not activate respiratory burst even in the presence of *Vibrio* suggesting the interference of *Vibrio* in ROS/RNS production induced by PMA. (H=hemocyte; V=*V. splendidus*; PMA=phorbol 12-myristate 13 acetate; letters indicate significant difference between treatments, ANOVA $p<0.001$; n = 12 replicates)

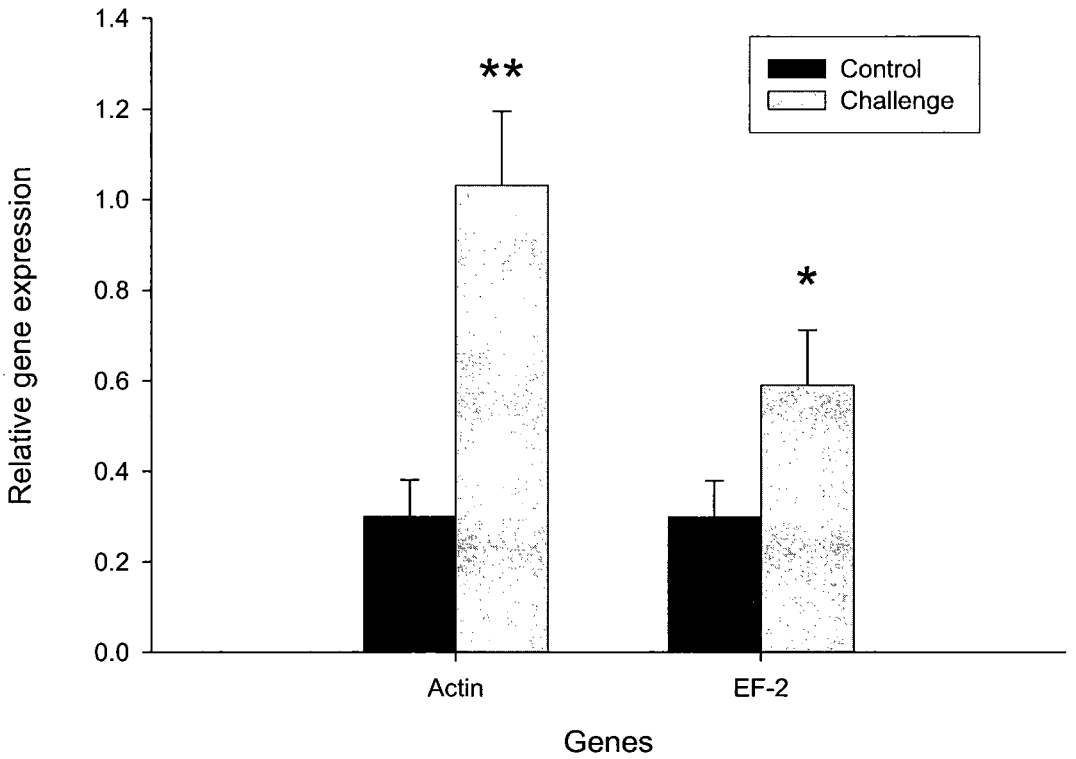

Figure 3.5 The relative expression of actin and elongation factor two (EF-2), genes associated with cytoskeleton stability, in hemocytes of soft-shell clams exposed to *V. splendidus* (1:1 ratio) for 2 h (mean±SEM). The expression of both genes is measured in reference to the three selected housekeeping genes. (*t-test*, * $p<0.05$; ** $p<0.001$ and n = 6 replicates)

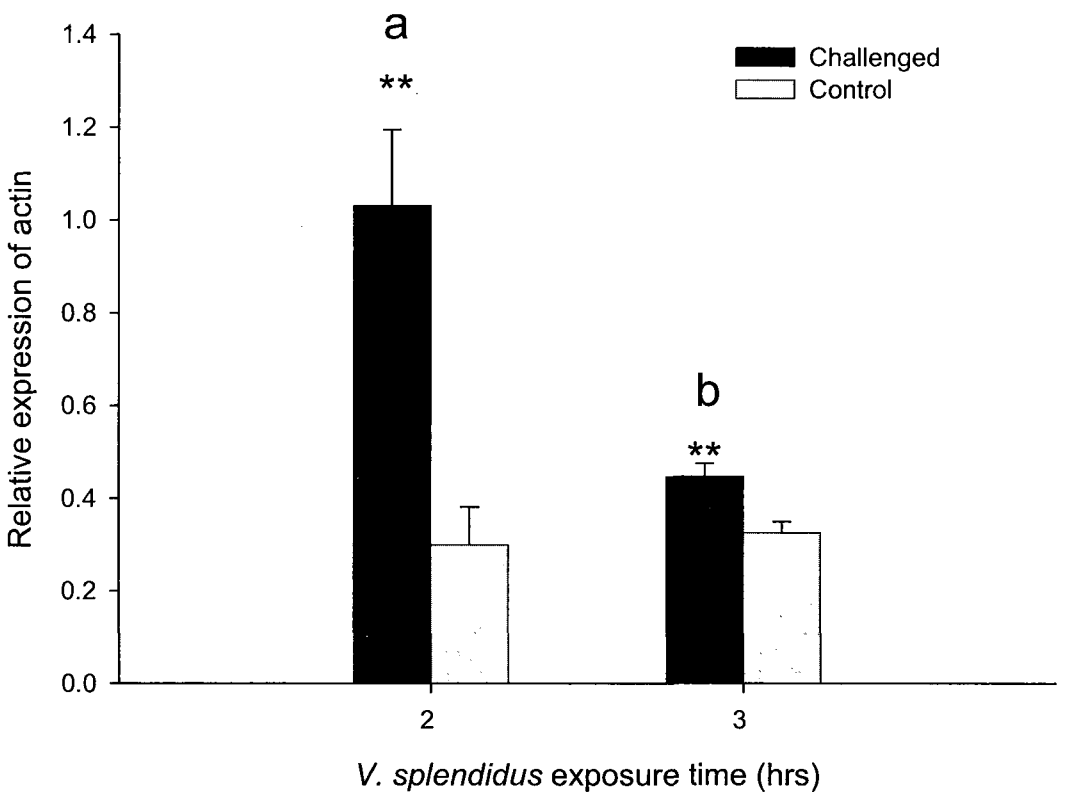

Figure 3.6 Comparison of actin expression in hemocytes of *M. arenaria* challenged with *V. splendidus* (1:1 hemocyte to *Vibrio* ratio) for 2 h and 3 h (mean±SEM). The expression of actin is measured in reference to the three selected housekeeping genes. The letters indicate significant difference ($p<0.05$) of actin expression between the challenged group at 2 and 3 hours whereas the ** indicates significance between control and challenged group at each time (*t-test*, $p<0.001$ and n = 6 replicates).

Table 3.1 Percentage of rounded hemocytes of soft-shell clams (*Mya arenaria*) challenged at different hemocyte-*V. splendidus* ratios for 1, 2 and 3 hours (Mean±SEM).

	Hemocyte to *V. splendidus* ratio				
	1:20	**1:10**	**1:5**	**1:1**	**Control**
1hr	20.76 ± 2.8	8.47 ± 2.1	8.89 ± 2.6	4.17 ± 1.3	0 ± 0
2hr	94.41 ± 4.9	86.11 ± 12.7	80.37 ± 2.8	44.29 ± 5.2	2.38 ± 4.1
3hr	100 ± 0	100 ± 0	100 ± 0	92 ± 1.2	9.09 ± 2.4

Table 3.2 Percentage of viable hemocytes in *Vibrio*-challenged and unchallenged groups after 1, 2 and 3 hours of incubation (Mean±SEM).

	% of live hemocytes	
	Control	**Challenge**
1 hr	95.4±1.6	99.0±0.4
2 hrs	91.9±1.0	96.9±0.6
3 hrs	94.8±1.6	98.4±0.5

Chapter 4

Identification and expression of immune-related genes in hemocytes of soft-shell clams, *Mya arenaria*, challenged with *Vibrio splendidus*

4.1 Abstract

Although the mollusc immune system has been studied at the cellular level, the response to pathogens at the gene level has not been thoroughly investigated. In this study, we explored the early molecular response of hemocytes of soft-shell clams, *Mya arenaria*, to *Vibrio splendidus* LGP32 GFP. Subtractive suppressive hybridization was used to selectively identify differentially expressed genes in hemocytes exposed to *V. splendidus* for two hours. Genes involved in different physiological processes were identified in forward- and reverse-subtracted cDNA pools. They include genes engaged in immunity, metabolism, cell structure, signaling and apoptosis. The relative expression of the seven genes, (ficolin, killer cell lectin-like receptor, natural resistance-associated macrophage protein 1, MAPK, ferritin, HSP90 and cathepsin) associated with immune defence, was measured by RT-qPCR at one, two and three hours post-*Vibrio* challenge. Most of these genes showed similar expression patterns, in which they become up-regulated at one hour, then began to be down-regulated at two hours and expression was further suppressed at three hours. *V. splendidus* appears to influence gene expression of hemocytes in soft-shell clams, supporting the hypothesis from our previous studies that *V. splendidus* may be pathogenic to this host as evidenced by observations of cell rounding, reduced phagocytosis and loss of respiratory burst activity. However, the down-regulation of these genes at two and three hours could be an indication that these genes are only involved in the early response against pathogens. Hence, additional studies should be conducted to investigate the molecular response of hemocytes after three hours.

This study is the first to report killer cell lectin-like receptor and natural resistance-associated macrophage protein 1 in mollusc.

4.2 Introduction

Because of the volume of water processed through filter feeding, bivalve molluscs are exposed to significant levels of pathogens naturally present in their habitat. Innate immunity is a mollusc's sole means of protection against pathogens and consists of cellular and the humoral components (see review of Canesi et al., 2002b). Hemocytes are an important component of the cellular response and engage in processes such as phagocytosis and encapsulation of non-self components (Canesi et al., 2002b). The humoral component is comprised of molecules, such as antimicrobial peptides (AMPs), lectins, agglutinins and lysozymes (Roch et al., 2008; Xue et al., 2004; Yamaura et al., 2008). The success of the host defence against pathogens depends on the coordinated effort of the cellular and humoral components of immune system.

In vertebrates, innate immunity recognizes conserved molecules found at the surface of pathogens, known as pathogen-associated molecular patterns (PAMP) (Janeway, 1989). PAMP are the signatures of microbes found in most pathogens but absent in host cells. These structures of microorganisms include lipopolysaccharide (Gram-negative bacteria), peptidoglycan and lipoteichioc acid (Gram-positive bacteria), beta glucan (fungi and yeast) and double strand RNA (viruses). The host uses opsonins (lectins) and membrane bound receptors (Toll-like receptors), referred to as pathogen recognition receptors

(PRR), to recognize PAMPs (Janeway and Medzhitov, 2002). Innate immunity in molluscs is not well understood; nevertheless PRRs have been identified in some species. Among the family of PRR proteins, lectins have been found in the Manila clam, *Ruditapes philippinarum,* infected with *Perkinsus olseni* (Kang et al., 2006). Toll-like receptors (TLRs) were also identified in the Zhikong scallop, *Chlamys farreri,* and showed differential regulation in hemocytes challenged with lipopolysaccharide (Qiu et al., 2007). These findings demonstrate the involvement of lectin and TLRs in molluscs challenged with pathogens and may suggest their role as PRR.

Once the presence of pathogenic microbes is detected in a host system, these microorganisms are phagocytosed and destroyed by toxic radicals, lysozymes and antimicrobial peptides (Beutler, 2004). Antimicrobial peptides (AMPs) are cysteine-rich peptides, which are involved in bacterial killing by destabilizing their membrane permeability (Hancock and Rozek, 2002). AMPs has been reported in several mollusc species (Anderson and Beaven, 2001; Mitta et al., 2000). Recently, Gonzalez et al. (2007) have cloned and sequenced two isoforms of defensin (*Cg-defh1* and *Cg-defh2*) genes from the hemocytes of the oyster, *Crassostrea gigas*. Hemocytes expressed *Cg-defh2* continuously however, post-bacterial challenge its expression was significantly decreased in circulating hemocytes but increased in mantle and gills indicating the possible migration of hemocytes to the tissues.

Vibrio splendidus causes high mortalities in juvenile pacific oysters and adult scallops (Lacoste et al., 2001; Lambert et al., 1999). Recently, Le Roux et al. (2007) have identified a virulence factor gene, metalloproteinase (vsm), in *V. splendidus* strain

LGP32. Our previous studies have showed that an interaction between hemocytes of *M. arenaria* and *V. splendidus* causes phenotypic and functional modifications in hemocytes (Araya et al., 2009). Soft shell clam hemocytes which came in contact with *V. splendidus* LGP32 become round and their phagocytic capability and respiratory burst activity were reduced. While post-challenge phenotypic and functional modifications in hemocytes of soft-shell clams have been observed, the molecular mechanisms involved in this interaction remain largely unknown. Hence, the aim of this work is to explore hemocyte molecular response against *V. splendidus* LGP32. In this study, we used a global scale gene expression based approach, known as suppression subtractive hybridization (SSH), to selectively identify genes (directly or indirectly involved in immunity) which are differentially expressed in response to *Vibrio* challenge and the expression of immune-related genes was measured using quantitative real-time PCR.

4.3 Materials and Methods

4.3.1 Soft-shell clam maintenance and hemolymph collection

Clams were conditioned (refer to section 2.3.2) and hemolymph was withdrawn from the adductor muscle of 20 soft-shell clams and its quality was accessed as described in section 2.3.3.

4.3.2 *In-vitro* challenge of hemocytes

Vibrio splendidus LGP32 was cultured, harvested and bacterial cell concentration estimated as in section 2.3.1. Hemocyte-*Vibrio* challenge was conducted as in section 2.3.3. In this study hemocytes exposed for one, two and three hours were used (n=6 replicates).

4.3.3 RNA extraction and cDNA synthesis

Total RNA was extracted from challenged and control hemocyte groups using RNeasy Micro Kit (Qiagen, UK) according to the manufacturer protocol and treated with 1 µl (1unit/µl) DNAse-1 (Sigma, USA). Total RNA concentration and integrity were analyzed using Experion®, automated electrophoresis station, (Bio-Rad, CA, USA). First cDNA was synthesized as described in section 2.3.4.

4.3.4 Construction of subtracted cDNA pool

Suppression subtractive hybridization (SSH) was performed on hemocytes challenged for two hours using PCR-select cDNA subtraction kit protocol (Clontech, USA). A total of two cDNA libraries were constructed corresponding to the forward (containing up regulated genes) and the reverse (containing down-regulated genes). The forward cDNA library was constructed by subtracting cDNA of control hemocytes (driver) from cDNA of challenged hemocytes (tester). In the reverse cDNA library, cDNA of control and challenged hemocytes were used as a tester and driver, respectively. Briefly, cDNA from control and challenged hemocyte groups was digested with *Rsa*I and the tester group was

equally divided into two and ligated to one of the adaptors separately (adaptor 1and adaptor 2R). In the first hybridization, excess driver was added to the adaptor-ligated testers separately and allowed to hybridize at 68 °C for 8 h. Immediately after first hybridization the two testers were mixed for the second hybridization by adding excess freshly denatured driver and incubated at 68 °C for overnight. Then, two rounds of PCR were performed to amplify only differentially expressed genes in subtracted cDNA libraries. The expressed sequence tags (ESTs) were generated using a 454 GS sequencer (Roche, USA) at Genome Quebec Platform (Mcgill University, Quebec, Canada). In this study the term subtracted cDNA pool is used instead of subtracted cDNA library because ESTs were directly sequenced from the second round PCR product without being cloned.

4.3.5 Sequence analysis

The expressed sequence tags (ESTs) were first trimmed to remove primers and GS De Novo assembler software (Roche, CT, USA) was used to construct contigs sequences. Homology searches of ESTs and contigs were performed with BlastX program using the default parameters. A threshold with E-value (<1.0E-5) and high percentage of identity (>50%) was set and the first annotated hit was retained from the output. Genes were classified into different functional groups based on the literature and their predicted function by gene ontology (Carbon et al., 2009).

4.3.6 Validating differential expression by qRT-PCR

To confirm the hemocyte response to *Vibrio*, seven genes associated with immune defence were selected from the subtracted cDNA pools and their expression level was

measured at 1 h, 2 h and 3 h post *Vibrio* challenge. Four genes (MAPK, cathepsin, natural resistance-associated macrophage protein 1 (*Nramp1*) and Killer cell lectin-like receptor) from reverse subtracted pool and three genes (HSP90, Ficolin-1 and ferritin) from forward subtracted pool were measured. Three previously validated housekeeping genes were used for normalization (Araya et al., 2008). Primers were cautiously designed using Primer3 software and synthesized by Sigma (Oakville, ON, Canada). The designed primers were analyzed for primer-dimer and secondary structure formation using M-fold (Zuker, 2003). The list of primers for the studied genes is in Table 4.1.

A standard curve was constructed for each run using a 10-fold serial dilution (10^{0}-10^{-6}) of the cDNA samples spiked with 50 ng of purified PCR product. In each run, standard curves and negative control were included. For each reaction 1 µl of scDNA template from the samples and standards are added to a total reaction volume 25 µl and SYBR Green I technology (Invitrogen, USA) based real-time qRT-PCR was performed. The PCR cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s except for ferritin and killer cell lectin-like receptor where annealing temperature was 63 °C. Both samples and standards were run in duplicate. Melting curves were analyzed to make sure that a single PCR product was amplified for each pair of primers. All reactions were performed on Rotor Gene RG-3000 (Corbett Research, Australia).

4.3.7 Data Analysis

Data was normally distributed and analyzed by parametric test. A Student t-test (SigmaStat, Statistical software, version 2) was used to compare the relative expression of each gene between control and challenged hemocytes at each sampling time. Moreover, fold-expression of each gene in challenged hemocytes at one, two and three hours was calculated by dividing the relative expression in an individual challenged sample to the mean expression in unchallenged samples (expression >1 means up-regulated whereas <1 means down-regulated). Data was analyzed by one-way ANOVA (SigmaStat, Statistical software, version 2) to determine the significance of expression of each gene between the different periods (1 h, 2 h and 3 h) of exposure time. If the expression of a gene at a particular time is greater than one, it is up-regulated however if the expression is less than one it is down-regulated.

4.4 Results

4.4.1 Suppression subtractive hybridization

The suppression subtractive approach was used in order to identify the differentially expressed transcripts in challenged hemocytes of soft shell clams, *M. arenaria*. From each subtracted cDNA pool, approximately 8000 ESTs were sequenced. More than 95% of the ESTs in each cDNA pool showed no significant homology (E value >1.0E-5) to the amino acid sequences deposited in the protein databases (NCBI and Swiss Prot). Of

the EST sequences with significant similarity, ribosomal genes constituted the largest part. Based on the gene ontology (Carbon et al., 2009) and literature, genes with significant homology were classified into different functional groups. In both subtracted cDNA pools, genes involved in different physiological processes such as immunity (T-cell receptor, ficolin, cysteine rich protein, killer cell lectin-like receptor, *Nramp1*, ferritin, cathepsin and HSP90), metabolism (26S protease, NADH dehydrogenase), cell structure (actin, profilin, septin), signaling (MAPK, calmodulin) and apoptosis (caspase 3 and 7, beta-cathenin) were identified. Selected ESTs and contigs from both subtracted cDNA pools are shown in Table 4.2.

4.4.2 Gene expression levels

Although SSH is a powerful molecular technique, which can be used to screen differentially expressed genes, it is a qualitative method. Hence, quantitative real-time PCR (qRT-PCR) was performed following SSH in order to validate the differential regulation and measure expression level of genes in the subtracted cDNA pools.

In this study, we focused on expression of genes thought to be involved in the immune response either directly or indirectly. Among those transcripts, ficolin, killer cell lectin-like receptor, natural resistance-associated macrophage protein 1, MAPK, ferritin, HSP90 and cathepsin mRNAs were quantified using qRT-PCR. In comparison to the control, the gene expression analysis at two hours post challenge revealed that all genes except cathepsin and ficolin were differentially expressed but not statistically significant. Ferritin was significantly ($p<0.01$) up-regulated after 1 hour post-challenge (Fig. 4.1b)

whereas *Nramp1* ($p<0.005$), MAPK ($p<0.05$) and cathepsin ($p<0.005$) were significantly down-regulated after 3 hours post-challenge (Fig. 4.1d, e, g).

In general, most of the transcripts quantified showed similar expression profiles, where the highest and the lowest levels were measured at one and three hours respectively. The expression of ferritin ($p<0.001$), HSP90 ($p<0.05$), *Nramp1* ($p<0.05$) and MAPK ($p<0.05$) in challenged hemocytes was significantly reduced at three hours in comparison to those challenged at one hour (Fig. 4.1b, c, d, e). Ferritin expression at two hours was also significantly ($p<0.001$) lower than those challenged for one hour (Fig. 4.1b).

4.5 Discussion

A number of studies have been conducted to understand the immune response of various mollusc species both at cellular and molecular level (Allam and Ford, 2006; Gestal et al., 2007; Labreuche et al., 2006; Parisi et al., 2008; Perrigault et al., 2009; Wang et al., 2008). In the present study, we utilized suppression subtractive hybridization to investigate differentially expressed genes in hemocytes of *M. arenaria* exposed to *V. splendidus*. Several genes related to immunity (ficolin, T-cell receptor alpha chain, killer cell lectin-like receptor, *Nramp1*, ferritin), stress (HSP90, HSP83), cell structure (actin, profilin, septin), ribosomal proteins, metabolism (26S protease, NADH dehydrogenase, cytochrome oxidase, cathepsin), cell cycle/apoptosis (Caspase 3 and 7) and signaling (calmodulin, Ras-like GTP binding protein, G-protein coupled receptor, MAPK) were identified from the subtracted cDNA pools. The expression of transcripts (*Nramp1*, Killer

cell lectin-like receptor, HSP90, Ficolin-1, MAPK, cathepsin and ferritin) associated with immune response either directly or indirectly was further investigated using qRT-PCR. The results showed that some of the genes were up-regulated one hour post-challenge. However, the expression levels of these transcripts were down-regulated at two and three hours post-challenge and this may suggest their involvement in early immune response against bacteria. It could be also an indication that *V. splendidus* has toxic effect on hemocyte of soft-shell clams.

4.5.1 Recognition receptors and proteins

The first and most important step in the immune response is to be able to detect and recognize foreign intruders. The protein groups playing this role are known as pattern recognition molecules (PRMs) or pattern recognition receptors (PRRs). They bind preferentially to particular structures on micro-organisms referred to as pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2002). In innate immunity of higher animals, several molecules involved in this process have been reported, among which are lectins (collectins, ficolins), Toll-like receptors (TLR) and various other humoral or cell membrane-associated factors (East and Isacke, 2002; Dahms and Hancock, 2002; Kilpatrick, 2002; Takeda et al., 2003). Ficolin was identified in hemocytes of soft-shell clams exposed to *V. splendidus* however its expression was not significantly affected. This protein belongs to one group of lectin family and it is composed of mainly fibrinogen and collagen-like domains (Ichijo et al., 1993). The mechanism of action of ficolin in vertebrates involves activation of mannan-binding lectin pathway. In this pathway, mannan-binding lectins (MBL) or ficolins bind to

carbohydrate moieties on bacterial surfaces and initiate a series of cascades leading to lysis of pathogens. In humans, anti-M-ficolin antibody inhibited phagocytosis of *E. coli* U937 suggesting that ficolin also serves as an opsonin and facilitates the internalization of bacteria. In mice, ficolin A serves as an opsonin and enhances the uptake of microbes (Endo et al., 2007). Jensen et al. (2006) also demonstrated that ficolin-L binds to DNA, which indicates its involvement in the clearance of apoptotic cells. Various types of ficolin have been reported in several vertebrates including in humans (L, H and M ficolins), pigs (ficolin alpha and beta) and mice (Ficolin-A and B). Ficolin homolog genes were identified from subtracted cDNA libraries of bivalve molluscs (*C. gigas, Saccostrea glomerata, R. philippinarum*) exposed to bacteria and parasites (Gueguen et al., 2003; Gagnaire et al., 2007; Green et al., 2009) however its molecular mechanism of action in mollusc is not known. Based on their origin, ficolins can be divided generally into two major groups: serum type such as human ficolin H and L, mice ficolin A and pig ficolin alpha (mostly synthesized in the liver) and cell associated type such as human ficolin M (predominantly produced by phagocytic cells). Ficolin found in the present study can be classified as cell-associated ficolin because it is obtained from phagocytic hemocytes. So far, no serum ficolin (ficolin isolated from hemolymph) has been reported in molluscs.

Another important immune related transcript found in the subtracted cDNA of soft shell clam hemocytes is killer cell lectin-like receptor (KLR). It is one of the structurally distinct receptor families regulating the cytotoxic activity of Natural Killer cells (NK). NK cells constitute the first line of defence in vertebrate immune system directed against

transformed (tumor) cells, virally infected cells, intracellular bacteria and parasites (Trinchieri, 1990; Biron, 1997; Scott and Trinchieri, 1995). The NK cells kill target cells either by releasing perforin which leads to cell lysis or granzyme that mediates apoptosis or a combination of both (Liu et al., 1995). To our knowledge, this is the first study to report stimulatory killer cell lectin-like receptor in mollusc species. Although no cytotoxic cells have been identified in molluscs, hemocytes with cytotoxic activity have been reported in mussels (Hannam et al., 2009) and tunicates (Peddie and Smith, 1993). Hence, the discovery of KLR in hemocytes of soft shell clams suggests that hemocytes may also have functional resemblance to NK cells, which play a key role in innate immunity. Mollusc hemocytes have been also related to macrophages based on their similarity in functional (phagocytosis and ROS) (Cima et al., 2000; Zhang et al., 2006; Bugge et al., 2006; Lambert et al., 2007; Araya et al., 2009) and molecular (Walker and Plows, 2003) response against pathogens. Despite the general belief that invertebrates lack adaptive immunity, some invertebrates have been shown to possess an immune response related to adaptive immunity (Flegel and Pasharawipas, 1998; Cooper and Roch, 1986; Flegel, 2007; Rowley and Powell, 2007; Sadd et al., 2005; Moret, 2006). In the current study, T cell receptor alpha homolog transcript has been isolated from hemocytes of soft-shell clams. T cell receptor (TCR) are found on the surface of T cells and used to recognize antigen bound to the major histocompatibility complex (MHC) molecules (Fields and Mariuzza, 1996). Although additional investigation into TCR will be needed to determine the exact role of this transcript in hemocytes, the presence of this gene in soft-shell clams could be an indication that hemocytes use receptors similar to that of T cells for pathogen detection. T cell receptor alpha was also up-regulated in

pacific oysters challenged with *Perkinsus marinus* (Tanguy et al., 2004) suggesting its role in immune response against parasites. The fact that KLR and TCR were found in hemocytes of *M. arenaria* could be also an indication that the immune system involves hemocytes similar to natural killer T (NKT) cells of vertebrates, which have characteristics of T and NK cells (Arase et al., 1992). Further investigations are needed to determine the functions of these genes in mollusc immune defence.

4.5.2 Signaling molecules

A mitogen-activated protein kinase (MAPK) was regulated in hemocytes of soft shell clams subjected to *V. splendidus*. MAPK is one of the most ancient and evolutionarily conserved signaling pathways, which is important in diverse physiological functions, including the immune response. Pathogen-associated molecular patterns (PAMPs) such as bacterial flagella, lipopolysaccharide, and peptidoglycan activate MAPK signaling cascades in animals (Rao, 2001). There are three major subfamilies of MAPK: the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and stress-activated p38 proteins (review by Dong et al., 2002). In invertebrates, stress-activated p38 is activated by pathogens and appears to induce antimicrobial peptide gene expression following exposure to lipopolysaccharide (LPS) (Han et al., 1998). The stress-activated p38 is reported in hemocytes of mussels (*M. galloprovincialis* Lam.) (Betti et al., 2006). Canesi et al. (2002a) also demonstrated that hemocytes of *M. galloprovincialis* subjected to *E. coli* activated stress-activated p38 MAPK. As shown in *Drosophila* and mussels, the differentially expressed MAPK in soft-shell clam hemocytes subjected to *Vibrio* can be stress-activated p38 MAPK. However, further investigations are needed to

verify this hypothesis. MAPK gene expression could be up- or down-regulated depending on the pathogens. For instance, MAPK was activated in macrophages exposed to non-pathogenic *Mycobacterium* however the activation was diminished when subjected to pathogenic *Mycobacterium avium* (Roach and Schorey, 2002). More recently, Travers et al. (2009) reported that p38 MAPK in hemocytes of abalone (*H. tuberculata*) was activated by non-pathogenic *Vibrio harveyi* and induced phagocytosis and ROS production whereas pathogenic *V. harveyi* deactivated p38 MAPK. Hence, the down-regulation of MAPK in hemocytes of soft-shell clam could also signify the virulent nature of *V. splendidus* which support the cellular responses of hemocytes described in our previous findings (Araya et al., 2009).

4.5.3 Metabolism

Involved in the protein metabolisms, cathepsins (L and K) are among the group of cysteine proteases, which were regulated in soft shell clam hemocytes subjected to *V. splendidus*. Cathepsins are lysosomal proteases, known for their role in food digestion. Beside their role as scavengers of undesired proteins, these proteases also play a key role in immune defence of vertebrates (Zavasnik-Bergant and Turk, 2006; Turk et al., 2000) and invertebrates (Loseva and Engstrom, 2004) suggesting a conserved function of these proteases during evolution. Our result showed an up-regulation of cathepsin L in the first hour of challenge followed by a down-regulation at 2 and 3 hours post-challenge. Cathepsin L was identified in *Biomphalaria glabrata* infected by a parasite *Echinostoma caproni* (Bouchut et al., 2007) and its expression was found highly elevated as in shrimp (*Litopenaeus vannamei*) resistant to white spot syndrome virus (Zhao et al., 2007). These

studies support the hypothesis that cathepsins are involved in the innate immunity of invertebrates during an infection.

4.5.4 Oxidative burst

Our investigation also led to the identification of a transcript homolog to ferritin, an iron chelating protein. This protein is a critical component of iron homeostasis in various organisms. Iron is crucial for white blood cells to perform phagocytosis and generate reactive oxygen species (Murakawa et al., 1987; Knovich et al., 2009). Moreover, iron is an important growth factor for bacteria and it is one of the prerequisites for successful bacterial infections (Goldoni et al., 1991). During infection, the host uses iron-sequestering proteins (ferritin) to limit access of bacteria to iron as a means to contain infection (Lipiński et al., 1991). Iron is also involved in respiratory burst activity by catalyzing the fenton reaction which led to the production of reactive oxygen species (McCord, 1996). Hence ferritin can regulate iron concentration to protect cells from oxidative stress (Harrison and Arosio (1996). Ferritin was up-regulated one hour post-challenge and the expression level came back to normal at three hours suggests that ferritin may be involved in the early stages of hemocyte-*Vibrio* interaction, a crucial time to contain and destroy bacteria before the infection is well established. Ferritin was also identified in cDNA libraries of carpet clam (*Ruditapes decussatus*), zhikong scallop (*Chlamys farreri*) and variously colored abalone (*Haliotis diversicolor*) challenged with bacteria (Gestal et al., 2007; Wang et al., 2008; Wang et al., 2009a) and white spot syndrome virus (WSSV) resistant shrimp, *Penaeus japonicus*, (Pan et al., 2005).

Some pathogens have been reported to resist the intracellular killing by respiratory burst activity and survive inside phagocytic cells or macrophages and this has been associated with the disruption of natural resistance-associated macrophage protein 1 (*Nramp1*) in the host cells (Vidal et al., 1993). *Nramp1,* a membrane protein, facilitates intraphagosomal bacterium killing by depriving bacteria of Fe^{2+} and other divalent cations (Zn^{2+}, Mn^{2+}) that are critical for bacteria to mount an effective antioxidant defence such as superoxide dismutase (see review Wyllie et al., 2002). *Nramp* identified in higher animals including different fish species (Chen et al., 2002; Burge et al., 2004; Chen et al., 2007) showed high expression during bacteria challenge. *Nramp* of striped bass, *Morone saxatilis*, (*MsNramp*) was highly expressed when the fish was injected with live *Mycobacterium marinum* and respiratory burst was suppressed. However, when injected with LPS *MsNramp* expression was dose dependent and showed increased respiratory burst activity (Burge et al., 2004). One hour post-*V. splendidus* challenge, *Nramp1* was up-regulated in hemocytes of soft-shell clams followed by its down-regulation at two and three hours post-challenge. The down-regulation of *Nramp1* and ferritin two hours post-challenge corresponds to reduced ROS production in hemocytes (Araya et al., 2009) may suggest its role in respiratory burst activity. These results may also imply *V. splendidus*'s effective strategy to weaken immune defences of soft-shell clams. To our knowledge this is the first study to report *Nramp1* in mollusc.

4.5.5 Stress proteins

Heat shock proteins (HSPs) have been implicated with stress-related responses including bacterial infection (Gestal et al., 2007; Wang et al., 2009b). In the present study, HSPs

such as HSP90 and HSP83 were differentially regulated in hemocytes exposed to bacteria suggesting HSPs may play a role in soft-shell clam immune defence as in other organisms. Heat shock proteins (HSPs) are highly conserved and are the most abundant protein families, which are grouped into several major categories based on their molecular mass. HSPs are also referred as molecular chaperones because they are involved in the folding of newly synthesized proteins, stabilization and refolding of denatured proteins, preventing protein accumulation and directing misfold and damaged proteins for degradation. Several studies have suggested the use of HSPs as general markers of non specific stress due to their involvement in various stress-related cellular conditions such as bacteria and parasite infection, high temperature, anoxia, salinity and heavy metals (Dowling et al., 2006; Farcy et al., 2007; Gao et al., 2007; Choi et al., 2008; Perrigault et al., 2009; Wang et al., 2009b). HSP90 belongs to a well studied heat shock protein family, which is involved in different regulatory pathways and controls a wide range of cellular processes such as cell cycle control, cell proliferation, cell differentiation, apoptosis, tumorigenesis and immune response (Csermely et al., 1998; Helmbrecht et al., 2000; Tsutsumi and Neckers, 2007). There are four main types of HSP90 proteins identified in vertebrates, including two major cytosolic isoforms: the HSP90-alpha (inducible form) and HSP90-beta (constitutive form); one endoplasmic reticulum isoform (Grp94 or 94-kDa); and the mitochondrial HSP90 homologue (HSP75/TRAP) (Chen et al., 2005). Although this study is the first to report HSP90 in *M. arenaria* exposed to bacteria, HSP homologs such as HSP90, HSP70 and HSP40 have been reported in other mollusc species exposed to bacteria (Gestal et al., 2007; Wang et al., 2008; Wang et al., 2009b; Perrigault et al., 2009) suggesting their involvement in

immune defence. In hard clams, pearl oysters and variously colored abalones, HSP70 seems to be more actively involved in bacteria and parasite response (Wang et al., 2008; Wang et al., 2009b; Perrigault et al., 2009). Heat-killed *V. anguillarum* triggered HSP70 expression in hemocytes of *M. galloprovincialis*, however, heat-killed *Micrococcus lysodeikticus* and *V. splendidus* did not affect its expression suggesting HSPs responses against bacteria may be pathogen specific (Cellular et al., 2006).

4.6 Conclusions

In soft-shell clams, several differentially regulated genes associated with immune response and involved in the early stages of hemocyte-*V. splendidus* interaction, were identified. Most of the genes showed a similar trend of expression patterns over time. During the first hour of challenge, *Vibrio* triggered over expression of transcripts involved in stress (HSP90), oxidative burst (ferritin and *Nramp1*) as well as the signaling molecules (MAPK). The expression of these genes decreased after 2 and 3 hours post-challenge. This research highlights the role of those genes in soft-shell clam immune defence against bacteria. Unanswered questions arise from this study: Are these genes specific to *V. splendidus* challenge? Is there a correlation between the expression of those genes with the virulence of the bacteria? Further studies will be needed to characterize those genes and investigate their specific role in hemocytes response against bacterial infection.

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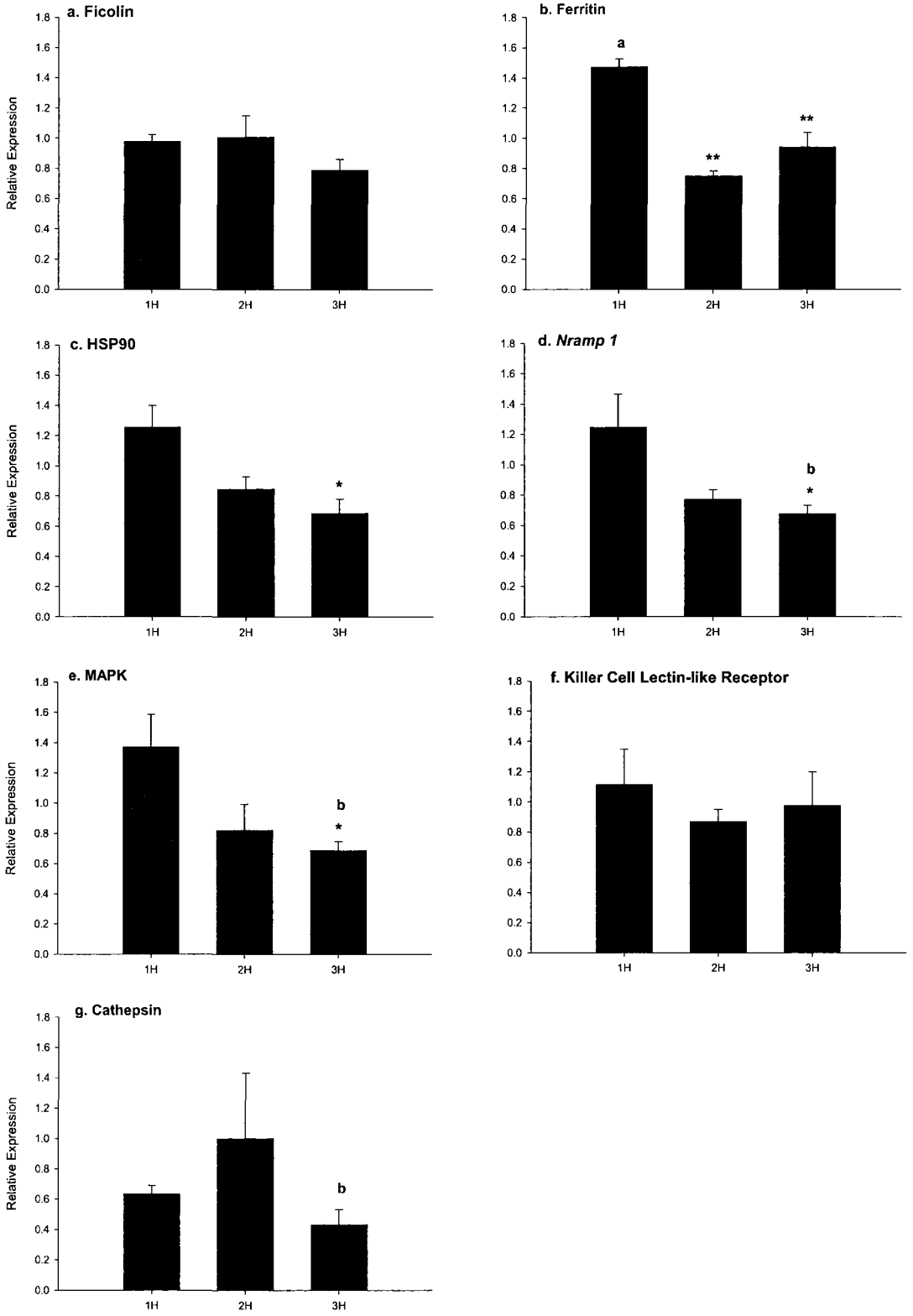
a. Ficolin
b. Ferritin
c. HSP90
d. Nramp 1
e. MAPK
f. Killer Cell Lectin-like Receptor
g. Cathepsin
Relative Expression
1H
2H
3H
a
b
*
**

Figure 4.1 The relative expression of (a) ficolin (b) ferritin (c) HSP90 (d) natural resistance-associated macrophage protein 1 (*Nramp 1*) (e) MAPK (f) killer cell lectin-like receptor and (g) cathepsin-L in hemocytes of soft-shell clams (*Mya arenaria*) subjected to *V. splendidus* for 1h, 2h and 3h. The expression of genes in challenged and unchallenged groups was normalized in reference to three housekeeping genes: elongation factor-1, rpS-18 and ubiquitin. Furthermore, the expression of the genes in challenged group was normalized to their expression in the respective control (expression >1 means up-regulated whereas <1 means down-regulated) (mean±SEM). (* indicate significant difference in comparison to hemocytes challenged for 1 hour; ANOVA * $p<0.05$; ** $p<0.001$; whereas letters indicate expression significance of a gene in challenged hemocytes in relation to unchallenged hemocytes in each exposure time; *t-test* (a) up-regulation (b) down-regulation $p<0.05$; n = 4 replicates).

Table 4.1: Primer sequences and PCR efficiencies of target and reference genes

	Gene name	Primers	Sequence (5'-3')	Tm	Amplicon length (pb)	PCR efficiency (%) 1h	2h	3h
Reference genes	Elongation Factor-1	Forward Reverse	GGTGGCTGTTGGTGTCATC GGCCTAGGTGTTTTCCATGA	60	158	104	100	104
	Ribosomal protein S-18	Forward Reverse	AAGATTCCCGACTGGTTCCT GCCGGTTGTCTTTGTATGCT	60	189	99	94	99
	Ubiquitin	Forward Reverse	TCGCTAAGGAGCTGGACATT ACCGTCGCTCCTTGTACATC	60	194	94	101	101
Target genes	Killer cell lectin-like receptor	Forward Reverse	AGGCTGACGGCTAGATCCTT GAAGGAACGCACATCCTGTT	63	135	96	93	91
	Cathepsin-L	Forward Reverse	CGGTGCTGGTCGTAGGATAC TCGGCACATGTTTCCCTTAT	60	125	100	104	93
	Nramp1	Forward Reverse	CTCAATATTACCGGGGTCCA GGCAAATACCAGTGCCAGAT	60	119	102	100	97
	Ferritin subunit	Forward Reverse	CATCTGCCTGCACACCATAC GGAGGTGTCATGGTTCGACT	63	139	97	92	97
	Ficolin-1	Forward Reverse	TAGCACGCTTTAAACCACCA TGGACCAACAGTGATGGGTA	60	158	102	95	100
	HSP90	Forward Reverse	GTGCCATGTTGTTTGTTCCA TTCAGGGATGAGTTCTTCACA	60	129	95	95	92
	MAPK	Forward Reverse	TGTCTGAGGAGGCAAAGGAT GGGTAGCGAGAGGTGTAGCA	60	129	97	91	98

Table 4.2:
A. Homolog genes identified in forward subtracted cDNA pool from hemocytes of soft-shell clam subjected to *V. splendidus*

Category and gene identity (BlastX)	cDNA length (bp)	Homolog species	NCBI/Swiss Prot gi	E-value
1. Immunity/ Stress response				
Ficolin-1	252	*Mus musculus*	O70165	4.0E-09
Cysteine rich protien 1	216	*Rattus norvegicus*	P63255	3.0E-09
T-cell receptor alpha chain	208	*Mus musculus*	P01849	1.0E-11
HSP90	261	*Brugia pahangi*	O61998	6.0E-30
HSP83	253	*Anopheles gambiae*	Q7PT10	6.0E-24
Ferritin subunit	544	*Meretrix meretrix*	DQ069277	1.0E-39
2. Antioxidants/ Metabolism				
NADH-ubiquitone oxidoreductase chain 4	273	*Rhipicephalus sanguineus*	O99825	4.0E-17
NADH dehydrogenase sub unit 4	1016	*Ruditapes philippinarum*	AB065375	4.0E-49
17-beta-hydroxysteroid dehydrogenase 14	250	*Homo sapiens*	Q9BPX1	8.0E-19
Cytochrome b	278	*Heteromys anomalus*	Q2N2J7	2.0E-13
Cytochrome c	240	*Pisaster ochraceus*	P25001	2.0E-17
Cytochrome oxidase subunit 1	542	*Venerupis philippinarum*	AB244374	3.0E-53
3. Cell process/ Cell structure				
Profilin	797	*Clypeaster japonicas*	P18321	3.0E-42
Tubulin alpha chain	259	*Lytechinus pictus*	P02553	4.0E-24
Septin-8-B	166	*Xenopus laevis*	Q6IRQ5	7.0E-07
Matrilin	645	*Biomphalaria glabrata*	DQ113393	3.0E-14
Zinc finger protein 207	234	*Homo sapiens*	O43670	4.0E-27
Actin	226	*Strongylocentrotus purpuratus*	P69005	1.0E-27

Continuation of Table 4.2 A

Category and gene identity (BlastX)	cDNA length (bp)	Homolog species	NCBI/Swiss Prot gi	E-value
4. Signaling				
Calmodulin	257	*Xenopus laevis*	P62155	2.0E-07
Guanin nucleotide-binding protien	141	*Biomphalaria glabrata*	Q93134	2.0E-11
5. Ribosomal proteins				
40S ribosomal protein S2	786	*Salmo salar*	BT044869	2.0E-98
ubiquitin/ribosomal protein S27a fusion protein	528	*Dermacentor variabilis*	EU551647.1	3.0E-60
ribosomal protein S8	623	*Haliotis diversicolor*	EU244378.1	6.0E-89
ribosomal protein L13	618	*Danio rerio*	AF385081	9.0E-61
ribosomal protein S4	572	*Argopecten irradians*	AF526210	3.0E-85

Table 4.2: **B.** Homolog genes identified in reverse subtracted cDNA pool from hemocytes of soft-shell clam subjected to *V. splendidus*

Category and gene identity (BlastX)	cDNA length (bp)	Homolog species	GenBank/Swiss Prot gi	E-value
1. Immunity/ Stress response				
Cathepsin K	242	*Bos taurus*	Q5E968	2.0E-13
Cathepsin L	256	*Brugia pahangi*	O17473	4.0E-16
Killer Cell lectin-like receptor	229	*Mus musculus*	P27814	8.0E-06
Natural resistance-assocaited macrophage protein 1	272	*Ovis aries*	P49280	3.0E-23
Nucleoside diphosphate kinase B	242	*Rattus norvegicus*	P19804	2.0E-20
2. Apoptosis / Cell cycle related genes				
Caspase 3	177	*Bos taurus*	Q08DY9	5.0E-15
Caspase 7	238	*Homo sapiens*	P55210	4.0E-09
Catenin beta	211	*Urechis caupo*	P35224	2.0E-23
Heterogeneous nuclear ribonucleoprotein A3	225	*Xenopus laevis*	P51992	1.0E-14
3. Cell process/ cell structure				
Myosin heavy chain	240	*Drosophila melanogaster*	Q99323	9.0E-21
Plastin 2	213	*Danio rerio*	Q6P698	8.0E-06
Dystrophin	249	*Mus musculus*	P11531	3.0E-06
Kinesin-like protein KIF1B	248	*Mus musculus*	Q60575	6.0E-24
Severin	227	*Dictyostelium discoideum*	P10733	5.0E-08
Radixin	254	*Gallus gallus*	Q9PU45	7.0E-26
Histone H2A	254	*Mytilus galloprovincialis*	Q6WV88	5.0E-18

Continuation of Table 4.2 B

Category and gene identity (BlastX)	cDNA length (bp)	Homolog species	GenBank/Swiss Prot gi	E-value
4. Signaling				
MAPK-interacting serine/thrionine protein	229	*Mus musculus*	O08605	2.0E-16
Flotillin 2	228	*Rattus norvegicus*	Q9Z2S9	5.0E-27
Ras-like GTP-binding protein	223	*Discopyge ommata*	P22122	4.0E-18
G-protein coupled receptor	229	*Lymnaea stagnalis*	P46023	2.0E-07
Inositol-trisphosphate 3-kinase B	222	*Homo sapiens*	P27987	5.0E-19
5. Antioxidants / metabolism				
Sulfotransferase family cytosolic 1B member 1	610	*Homo sapiens*	D89479	3.0E-09
Glutamine synthetase	229	*Squalus acanthias*	P41320	7.0E-07
collagen type XXI-like protein	677	*Haliotis diversicolor*	EU244340	6.0E-06
ATP synthase F0 subunit 6	622	*Xestospongia muta*	EU237490	3.0E-07
26S protease regulatory subunit 6A	207	*Rattus norvegicus*	Q63569	1.0E-27
6. Ribosomal Proteins				
ribosomal protein rpl7a	553	*Arenicola marina*	EU124958	6.0E-78
putative 40S ribosomal protein RPS2	547	*Novocrania anomala*	EU558290	1.0E-93
60S ribosomal protein L24	656	*Pediculus humanus corporis*	DS235306	2.0E-49
Others				
translationally controlled tumor protein-like variant I	646	*Madurella mycetomatis*	DQ218143	2.0E-08
Serine protease 29	246	*Mus musculus*	Q99MS4	4.0E-12

Chapter 5

General Discussion

The immune system of molluscs plays a key role in the organism's defence against pathogens. Details as to the functioning of the mollusc immune system are lacking although it is generally considered to be primitive and involving only innate immunity. Several studies have been conducted in soft-shell clams to understand the pathology and diagnosis of neoplasia (Brousseau and Baglivo, 1991; McGladdery et al., 2001; Barber et al., 2004) and parasitic disease (McLaughlin and Faisal, 1999; Dungan et al., 2002); however, knowledge of the immune defence of soft-shell clams against bacteria is very scarce. In this study we developed an *in-vitro* model of interaction between hemocytes of soft-shell clams and the bacterium, *V. splendidus* LGP32 to understand the early immune response both at cellular and molecular levels.

Molluscs are widely used in different *in-vivo* and *in-vitro* bacterial challenge models (Goedken and De Guise, 2004; Allam and Ford, 2006; Labreuche et al., 2006a). Soft-shell clams, *M. arenaria*, are a good candidate organism for bacterial challenge models. Firstly, soft-shell clams like other molluscs are always in close contact with marine bacteria due to their habitat (sediment) and filter feeding behavior. Secondly, they are easy to maintain under laboratory conditions and provide a direct access to large amounts of hemolymph without dissection (Tubiash, 1971). Soft-shell clams have been also suggested to screen potential mollusc pathogens (Tubiash, 1971). Hemocytes' immune function can be compromised by various factors such as exposure to heavy metals, microbial agents and other environmental factors (Fournier et al., 2001; Labreuche et al., 2006a). In soft-shell clams hemic neoplasia is one of the leading causes of mortality (McGladdery et al., 2001) and neoplastic hemocytes of soft-shell clam have displayed

reduced phagocytosis (Beckmann et al., 1992). Hence, soft-shell clams utilized in this study were collected from pristine (free of pollutants and pathogens) coastal waters of Magdalen Islands where the clam population in this Island is one of the healthiest (< 5% neoplastic hemocytes) populations in Eastern Canada (Delaporte et al., 2008).

In the present study, *V. splendidus* LGP32 was used to investigate hemocyte-bacteria interaction. There are several reasons why this *Vibrio* species has been chosen for this model of interaction. First, *V. splendidus* is a pathogen and can cause disease to closely-related bivalve species both in the natural environment and laboratory setting. *Vibrio splendidus* has been isolated from diseased *C. gigas* hatcheries and associated with high mortality (Lacoste et al., 2001). Moreover, this *Vibrio* species is also reported to have a pathogenic effect on other mollusc species such as clams (*R. philippinarum*) and scallops (*P. maximus*) (Lambert et al., 1999; Choquet et al., 2003). Secondly, *V. splendidus* LGP32 genome is sequenced (Le Roux et al., 2009), which makes it easier to manipulate the virulence genes and study their effect on the host. Last but not least, *V. splendidus* is genetically engineered to express green fluorescent protein making it easy to monitor with fluorescence microscopy (Le Roux et al., 2007).

Molluscs possess only innate immunity, composed of cellular (hemocytes) and humoral components. Hemocytes are responsible in molluscs for cell-mediated immune responses including phagocytosis, a predominant mechanism of cellular defence (Lopez-Cortes et al., 1999). Moreover, hemocytes produce lysosomal enzymes and antimicrobial peptides, which have antimicrobial activity (Cheng and Downs, 1988; Mitta et al., 2000). Mollusc hemocytes also have the capability to generate highly reactive oxygen/nitrogen

intermediates (ROI/RNIs), an important cellular immune defence to protect the host against pathogens (Arumugam et al., 2000; Lambert et al., 2007; Bugge et al., 2007; Gourdon et al., 2001). Hence, hemocytes were the focus of this thesis, which was aimed at increasing our knowledge of the soft-shell clam's immune response against bacteria.

The phenotypic response of hemocytes of the soft-shell clam subjected to different ratios of hemocyte:*V. splendidus* LGP32 (1:20, 1:10, 1:5 and 1:1) showed a positive correlation between the percentage of rounding hemocytes, the ratio of hemocytes:bacteria and the time of exposure. The percentage of rounded hemocytes in all ratios except in 1:1 hemocyte to *Vibrio* ratio increased sharply from below 20% in one hour to 80-90% and 100% rounded cells in 2 and 3 h, respectively. With a 1:1 hemocyte to *Vibrio* ratio, the percentage of rounded hemocytes increased gradually (4.3% at 1 h, 44.2% at 2 h and 92% at 3 h). Unchallenged hemocytes had less than 10% rounded cells in 3 hours indicating a significant effect of *V. splendidus* on the phenotype of soft-shell clam hemocytes. Similar to our finding, Lane and Birkbeck (1999) observed that in hemocytes of *M. edulis* exposed to *V. anguillarum* 2981 or *V. alginolyticus* 1339 at 10 or 50 bacteria per hemocyte led to 100% rounding within 120-150 minutes. The same study also reported that in a 1:1 ratio, *V. anguillarum* 2981 caused 50% hemocytes rounding at three hours. Exposure of hemocytes of *C. gigas* to the extracellular products of *V. aestuarianus* 01/32 also led to hemocyte rounding (Labreuche et al., 2006a). The loss of pseudopodia has been associated with some toxins produced by the pathogens (Kudryashov et al., 2008). A toxin or virulence factor such as metalloprotease (*vsm*) has been identified in the genome of *V. splendidus* LGP32 (Le Roux et al., 2007). Such toxins may have interfered

with actin polymerization and disrupt the cytoskeleton filament stability in challenged hemocytes of soft-shell clams. The low percentage (<10%) of rounded hemocytes in unchallenged hemocytes however could be related to the stress caused during hemolymph agitation (by gently pippeting up and down) to resuspend hemocytes adhered to the sides and separate clumped hemocytes.

This study also showed that hemocytes of soft-shell clams are actively involved in phagocytosis and production of reactive oxygen/nitrogen intermediates. However, when exposed to *V. splendidus,* these hemocytes showed reduced phagocytosis, which could be associated with the loss of pseudopodia. Cytoplasmic extensions (pseudopodia) of hemocytes are very important in adhesion and internalization of microbes and parasites. Several studies also reported that mollusc hemocytes subjected to pathogenic bacteria or their extracellular product significantly affected hemocytes' normal function (Labreuche et al., 2006b; Lane and Birkbeck, 1999). *In-vivo* and *in-vitro* studies conducted in Pacific oysters (*C. gigas*) challenged with *V. aestuarianus* strain 01/32 showed that hemocytes in both cases lost their ability to adhere and consequently phagocytosis was significantly inhibited (Labreuche et al., 2006a; 2006b). Likewise, Allam and Ford (2006) demonstrated that hemocytes of *R. philippinarum* exposed to extracellular products of *V. tapetis* and *V. anguillarum* showed a marked decrease in phagocytosis. Soft-shell clam studies conducted by Brousseau et al. (2000) and Fournier et al. (2001) also reported low phagocytosis in clams exposed to heavy metals.

In the present study, hemocytes of soft-shell clam incubated with PMA (phorbol 12-myristate 13 acetate) showed an increased respiratory burst activity compared to controls

suggesting the ability of these hemocytes to produce reactive oxygen/nitrogen species. However, the respiratory burst activity in hemocytes exposed to *Vibrio* was diminished suggesting the capacity of *V. splendidus* LGP32 to deliberately inhibit respiratory burst activity. Lambert and his coworkers (2003) demonstrated that zymosan activates respiratory burst activity in oysters, whereas *Vibrio sp.* known to be pathogenic to molluscs has shown direct negative effect on the ROS production. Such pathogens are known to produce anti-oxidants (superoxide dismutase) to neutralize the toxic effect of ROS produced by host cells (Brown et al., 1995). Hence, it is possible that *V. splendidus* may have such molecules to interfere with respiratory burst activity of soft-shell clams. The current study demonstrated that *V. splendidus* has the ability to impair the normal function of hemocytes.

In gene expression assays, qRT-PCR is the method of choice for measuring regulation of genes mainly due to its high sensitivity and accuracy. Despite this, qRT-PCR has a shortcoming when it comes to comparing gene expression between samples because it does not take into account variations in volume/mass of starting material, RNA extraction methods and reverse transcription efficiency. To overcome the problem, the use of housekeeping (sometimes referred as reference or internal control) genes becomes the gold standard approach for standardizing qRT-PCR data. The expression of housekeeping genes should be reasonably stable in experimental and control groups as well as along the course of the experiment. In the present study, suitable housekeeping genes were first selected before measuring the expression of target genes in Chapters three and four. Nine candidate genes, which are involved in different physiological processes, were selected.

Among them, some commonly used housekeeping genes, such as 18S-rRNA and actin were included. Interestingly, these classical housekeeping genes were not suitable reference genes in this hemocyte-*Vibrio* interaction model confirming the need for prior validation of housekeeping genes stability before used in gene expression analysis.

Until now, many studies are utilizing commonly used housekeeping genes such as actin and 18S-rRNA without validating their expression suitability in a particular experiment. Although these genes are good candidate genes to be considered, several researchers have demonstrated the regulation of these genes in various conditions or cells/tissue types (Bas et al., 2004; Rubie et al., 2005; Nicot et al., 2005). In the current study, 18S-rRNA and actin were among the least stable genes supporting those reports arguing there are no classical or ideal reference genes. For instance, Vandesompele et al. (2002) examined the stability of ten candidate genes in different human tissues/cells and found that actin and 18S-rRNA were suitable housekeeping genes in some tissues/cells but not in others even though they were subjected to the same conditions. Tang et al. (2007) also showed that housekeeping genes for zebrafish developmental stages and its various tissues are different. They found that actin was a suitable housekeeping gene in developmental stages but not in tissue panels and the reverse is true for 18S-rRNA. All these studies emphasize the importance of validating the expression stability of reference genes prior to using them. Several studies have suggested the use of more than one reference gene (Vandesompele et al., 2002; Pfaffl et al., 2004). When multiple reference genes are used for normalization, the selected reference genes should preferably be from different physiological processes in order to avoid potential co-regulation. The most stable

housekeeping genes shown to be suitable for the current *in-vitro* challenge model were EF-1, rpS-18 and ubiquitin.

The molecular response of soft-shell clam hemocytes to bacteria is largely unknown. To understand the immune defence of soft-shell clams at the gene level, we utilized a powerful molecular technique known as "Suppressive Subtractive Hybridization" (SSH). In the present work, genes involved in the early response of hemocytes against *V. splendidus* were uncovered using this technique. SSH has the ability to selectively amplify differentially expressed genes and suppress unregulated genes. Unlike microarrays, SSH does not require prior genome knowledge of the organism under investigation. Hence, it becomes the most suitable method to explore molecular mechanisms of immune system in molluscs and other invertebrates whose genome is not well studied. This technique has been used in several mollusc studies to understand the molecular mechanism of their immune response against different pathogens and parasites (Lockyer et al., 2007; Gestal et al., 2007; Huvet et al., 2004; Prado-Alvarez et al., 2009). This is the first study, to our knowledge, which investigated immune response of soft-shell clam hemocytes against bacteria using SSH. In both subtracted cDNA pools, we identified several genes from different biological processes. The relative gene expression of seven transcripts, which are directly or indirectly associated with immunity was measured by normalizing to three housekeeping genes selected in Chapter two.

Moreover, the expression of actin and EF-2 (genes associated with cytoskeleton) was measured to investigate a possible link between loss of pseudopodia in challenged hemocytes and genes associated with it. Actin is involved in cell migration, adhesion,

cytokinesis, encapsulation, nodule formation and phagocytosis (Walker and Garrill, 2006; Takai et al., 2001) whereas EF-2 is an actin binding protein and regulates the level of intracellular actin in cells (Bektas et al., 1994). Both genes were significantly up regulated in hemocytes challenged for two hours. Three hours post-challenge actin was still up-regulated however its expression was significantly reduced compared to hemocytes exposed for two hours. This decreased actin expression three hours post-challenge corresponds with increased percent of rounded hemocytes. The inverse correlation between the percentage of hemocytes without pseudopodia (rounded) and actin expression could be an indication that hemocytes with pseudopodia expressed actin to maintain these extensions in order to perform their normal function.

Innate immunity uses a non-specific pathogen recognition system known as pattern recognition receptors (PRR). Those receptors preferentially bind to pathogens associated molecular patterns (PAMPs), which are evolutionary conserved molecules found on the surface of pathogens (Janeway, 1989). Mollusc lectins have PAMP recognition domains and may serve as "antibodies" in lower animals (Kang et al., 2006; Zhang et al., 2009). Lectins have been considered as innate immune effectors, which can induce bacterial agglutination, enhance phagocytosis by opsonization and activate the phenoloxidase and oxidative processes (Bayne, 1990; Vasta et al., 2004; Soria et al., 2006; Yu and Kanost, 2001). The molecular mechanisms by which hemocytes of soft-shell clams react to the infection remain by far understudied. In the present study, genes involved in pathogen recognition (ficolin, killer cell lectin-like receptor (KLR) and T cell receptor (TCR)) were identified in hemocytes of *M. arenaria* challenged with *V. splendidus*. KLR and TCR are

found in killer cells and T cells of higher animals and play a crucial role in pathogen recognition in innate and adaptive immunity, respectively (Fields and Mariuzza, 1996; Middleton et al., 2002). The down-regulation of ficolin (3 hrs post challenge) and KLR (2 hrs post challenge) suggest that the reduced phagocytosis of hemocytes may not only be due to the loss of pseudopodia but also the result of down-regulation of opsonin molecules and receptors, which are crucial in detection and phagocytosis. In genus *Crassostrea* and *Mytilus,* several immune related genes have been reported and were regulated in response to microbial challenge. These genes include lectins, antimicrobial peptides, toll-like receptors, lysosymes and tissue inhibitor metalloproteinases (Montagnani et al., 2001; Mitta et al., 2000; Gonzalez et al., 2007; Gueguen et al., 2006; Haug et al., 2004; Itoh et al., 2007). Similar studies exploring the expression of immune genes have been also conducted in several mollusc species such as abalones, scallops, snails and clams (*R. philippinarum* and *R. decussatus*) (Qiu et al., 2007; Kang et al., 2006; Su et al., 2007; Travers et al., 2009; Zhang et al., 2007) and revealed that some pathogens are capable of suppressing immune genes to undermine host defence systems.

Ferritin, an iron binding protein, has been associated with disease resistance in invertebrates. Iron is an important catalyst in the Fenton reaction which leads to the production of reactive oxygen species in the immune cells of higher animals (Murakawa et al., 1987). In *Biomphalaria glabrata,* a link between ferritin expression and respiratory burst activity (H_2O_2 production) has been suggested as a resistance mechanism against parasite exposure (Lockyer et al., 2007). Ferritin was also up-regulated in white spot syndrome virus (WSSV)-resistant shrimps and snails resistant to parasites in comparison

to their susceptible counterparts (Pan et al., 2005; Bayne et al., 2001). Natural resistance-associated macrophage protein 1 (*Nramp* 1) is another gene associated with disease resistance, as it regulates iron during bacterial infection to induce interphagosomal killing by producing reactive oxygen species (see review Wyllie et al., 2002). In our study, ferritin and *Nramp* 1 were up-regulated one hour post-challenge. However, one hour later, the expression was suppressed and this could be linked to the decrease of respiratory burst activity observed in hemocytes (Chapter three). Three hours post-challenge cathepsin-L, a cysteine protease, was also down-regulated in hemocytes of soft-shell clam. Cathepsin expression in response to parasites and viruses has been reported and all these findings may suggest the involvement of cathepsin in immune defence (Onishi et al., 2004; Zhao et al., 2007). Cathepsin L, which exists in all mammalian tissues, also plays an important role in degradation of both exogenous and endogenous proteins (Ishidoh et al., 1999).

Mammalian MAPKs are involved in the immune response and play a key role in the initiation of innate immunity, activation of adaptive immunity, dendritic cell maturation and apoptosis (Dong et al., 2002; Nakahara et al., 2006). MAPK signaling pathways (c-JNK, ERK, and p38 MAPK) are activated by a series of phosphorylation cascades where MAPK kinase kinases (MAPKKKs) are phosphorylated and activate MAPK kinases (MAPKKs). In turn, MAPKKs are phosphorylated and activate MAP kinases (MAPKs), which interact with substrates in the cytoplasm to form complexes. These complexes are translocated into the nucleus to initiate transcription of MAPK target genes depending on the type of stimuli (Widmann et al., 1999; Shan et al., 2007). In the current study, MAPK

was up-regulated one-hour post hemocyte-*Vibrio* interaction. However, 2 and 3 hours post-challenge, it was down-regulated. Some pathogens have targeted MAPK signaling pathway to evade the immune defence mechanism of organisms. For instance, *Vibrio* outer protein A (VopA), a virulence factor (effector) isolated from *Vibrio parahemolyticus*, inhibits MAPK signaling pathways in yeast and mammalian cells (Trosky et al., 2004). Anthrax lethal toxin was also shown to hinder MAPK (Chopra et al., 2003). Hence, it is possible that *V. splendidus* may possess some effector molecules which can interfere with MAPK molecular mechanisms of soft-shell clam hemocytes to escape immune reaction.

In the present study, the early response of soft-shell clam hemocytes challenged with *V. splendidus* LGP32 was investigated. Our data showed that *V. splendidus* impaired the hemocyte functions (phagocytosis and respiratory burst activity) and also down-regulated immune-related genes (ferritin, Nramp-1 and MAPK) analysed. The altered immune response of hemocytes both at the cellular and gene level may suggest that *V. splendidus* is virulent or has toxic effect to the host. However, we have to be very careful in interpreting *in-vitro* data into *in-vivo* because the conditions are different in many ways. *In-vitro* studies are conducted in isolated cells under very controlled environment and therefore *in-vitro* models do not take into account physiological processes such as reproductive status which could affect immune response of cells (Gauthier-Clerc et al., 2006). Consequently, *in-vitro* studies should be validated with *in-vivo* (Schilter et al., 1996).

In mollusc *in-vitro* studies, hemocytes interact with pathogens either in hemolymph free medium (mainly filtered sterile seawater) or with hemolymph but diluted with sterile seawater containing bacteria suspension. By removing or diluting hemolymph the activity of different molecules (opsonins, lysozymes and antimicrobial peptides), which play a key role in protecting the organism from pathogens could be compromised. Bear in mind that the success of the immune system depends on the coordinated work of the cellular and humoral components. Secondly, in *in-vivo* challenge models of molluscs, hemocyte migration from hemolymph or tissue to site of infection was reported (Matozzo et al., 2008; Parry and Pipe, 2004). However, in *in-vitro* settings migration of hemocytes cannot happen and this could affect the overall response of the cells against pathogens.

This study has improved our understanding about the immune defence of soft-shell clams. It answered some important questions about the hemocytes' role in the immune system both at the cellular and molecular level. Moreover, we uncovered novel immune genes and numerous unknown transcripts which need to be further characterized. Several genes obtained from the subtracted cDNA can be also used to build cDNA microarrays, which can be utilized for research and diagnosis purposes in *M. arenaria* pathological studies. Soft-shell clams, unlike other commercially important bivalve molluscs, appear to be resistant to bacterial diseases as no diseases of bacterial origin have been reported in this clam species. If any of the identified transcripts happen to be associated with resistance to bacterial disease in soft-shell clams, such genes can be used to genetically engineer susceptible mollusc species to become resistant against bacterial disease.

5.1 Future Directions

In this study, we developed an *in-vitro* model of interaction between *V. splendidus* LGP32 and hemocytes of soft-shell clams to understand the hemocyte's role in immune defence both at cellular and molecular levels. The results of our work revealed that hemocytes are actively involved in immune defence of soft-shell clams. Early immune response of hemocytes subjected to *Vibrio* suggested that *V. splendidus* LGP32 could have toxic effects to hemocytes because it damaged cytoskeletal architecture, reduced phagocytosis and respiratory burst activity and down-regulated genes associated with immunity post-challenge. Additional studies will be needed to understand more about the cellular and molecular response of hemocytes in this hemocyte-*Vibrio* interaction model. Here are 4 suggestions for future of research:

1. Assessment of hemocyte viability

The cellular responses of hemocytes were impaired by *V. splendidus* LGP32, in that the hemocytes lost their pseudopodia and showed reduced phagocytosis and respiratory burst activity. Moreover, the molecular response of hemocytes against *Vibrio* also showed down-regulation of immune related genes. Our results suggest the potential pathogenicity of *V. splendidus* to hemocytes of soft-shell clams. Despite the indication of *V. splendidus* pathogenic potential, additional studies need to be performed to address some of the questions below:

A. What happens to the rounded hemocytes after three hours (do they recover or die)?

B. In the context of whole animals, what happens to those rounded hemocytes in challenged clams? i.e. can the host make up for the hemocytes impaired by bacteria challenge so that infection can be avoided? Indeed, it is worth remembering that virulence of *V. splendidus* or resistance of soft-shell clams is measured whether or not infection is successfully established and causes disease and mortality.

2. Identification of the pathway involved in respiratory burst activity (NADPH-oxidase or NO-synthase pathways)

Hemocytes of soft-shell clams showed increased respiratory burst activity upon PMA exposure, which suggests their ability to produce toxic molecules, which are involved in interphagosomal killing. However, the chemical (DCFH-DA) used to detect these toxic radicals is not specific enough to distinguish between reactive oxygen or nitrogen species. Hence, it could be interesting to investigate which pathway is involved in hemocytes of soft-shell clams and also determine whether the type of toxic molecule/s produced is dependent on the pathogen.

3. Assessment of the hemolymph role on the function of hemocytes

In our study, the cellular and molecular response of hemocytes to *V. splendidus* was examined in the presence of diluted hemolymph (2x diluted by adding equal volume of bacteria suspension in filtered sterile seawater). It is known that hemolymph (serum) of vertebrates and invertebrates (including molluscs) contain opsonizing molecules which recognize non-self cells and also facilitate adhesion and phagocytosis. Hemolymph also

contains antibacterial agents such as lysozymes, antimicrobial peptides and prophenoloxidase activity. Hence, further investigations will be needed to determine the role of hemolymph in immune function of hemocytes. Moreover, the reproductive status and gender of soft-shell clams should be determined in future studies as these factors have shown to affect the immune system of molluscs.

4. Characterize some novel genes and determine their role in immune defence against bacteria using gene knockout

Several immune associated novel genes have been identified in hemocytes of soft-shell clams challenged with *Vibrio*. The differential expression of genes identified in this *in-vitro* challenge model could suggest their involvement in hemocytes response against *Vibrio* however, the exact role of those transcripts is not known in hemocytes of soft-shell clams. Hence, RNA interference (RNAi) technology can be used to determine the exact function of each gene of interest. In RNAi, short double strand RNAs (dsRNA) are used to trigger the degradation of matured mRNA of target gene before it is translated into protein. By doing so, the expression of a specific gene can be silenced. This way we can see how silencing of a particular gene affects the hemocytes' response to *Vibrio*. Moreover, more than 95% of the ESTs found in our subtracted cDNA pools represent genes with unknown biological functions. For this reason, it could be also interesting to look at those transcripts with abundant ESTs to determine their function in this challenge.

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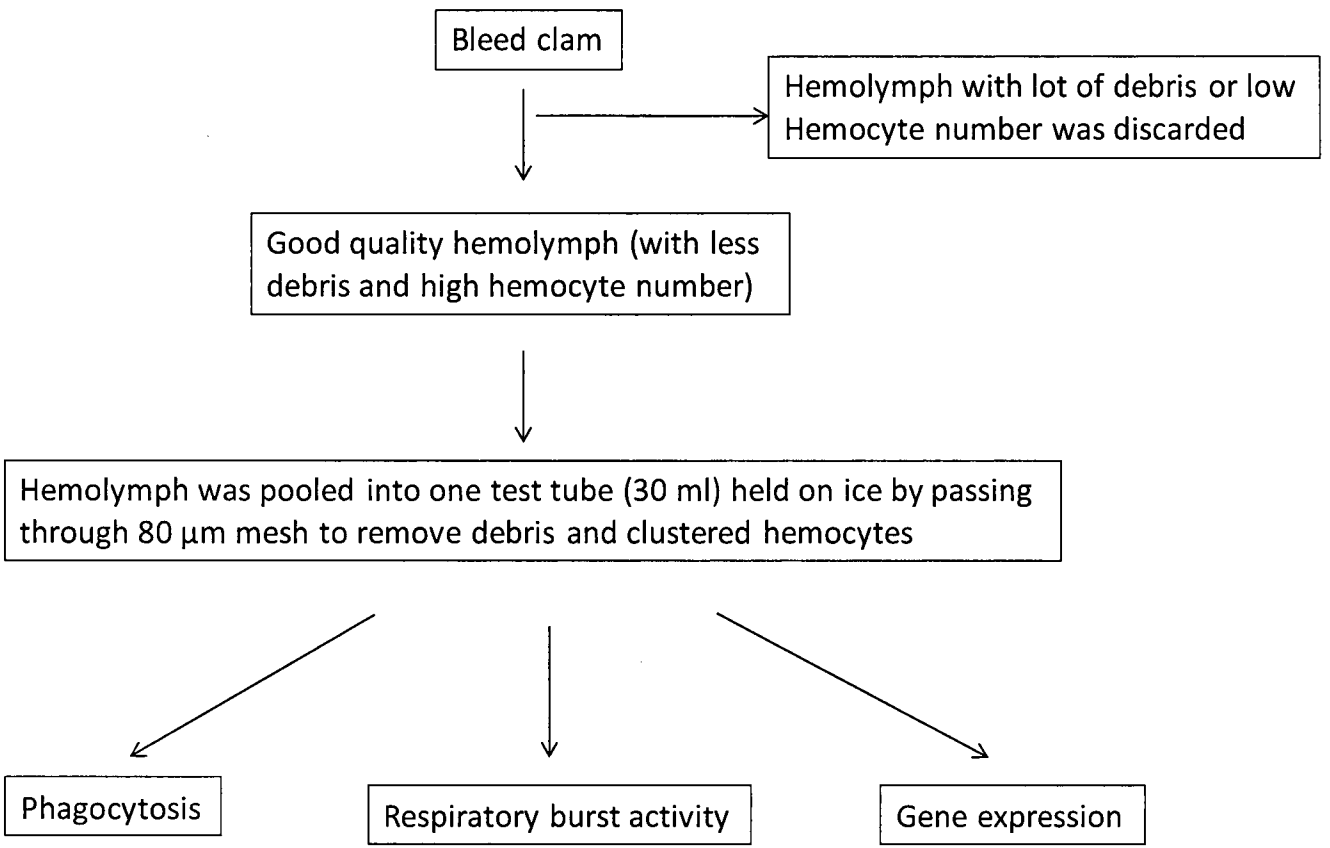
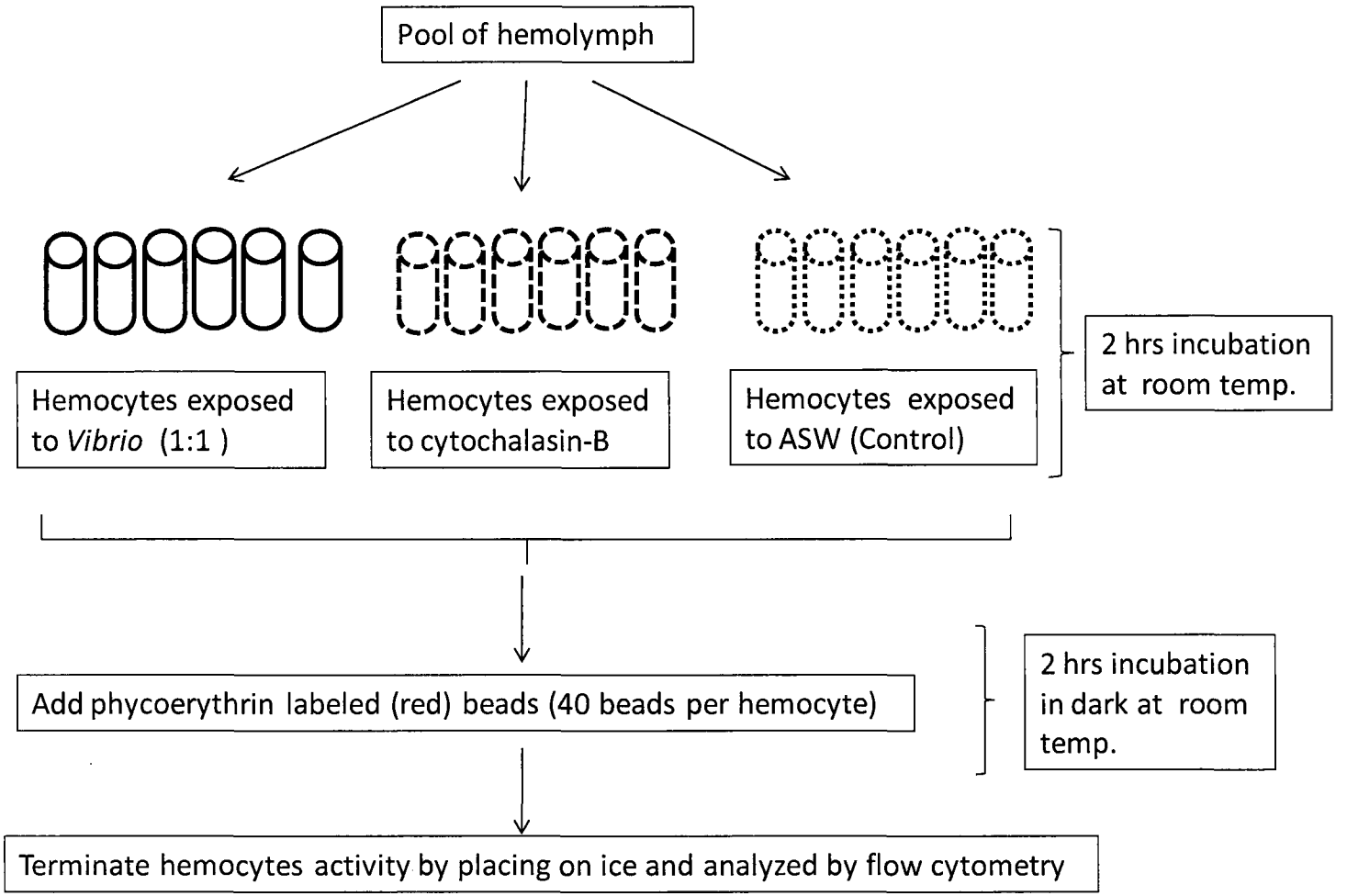
Appendix

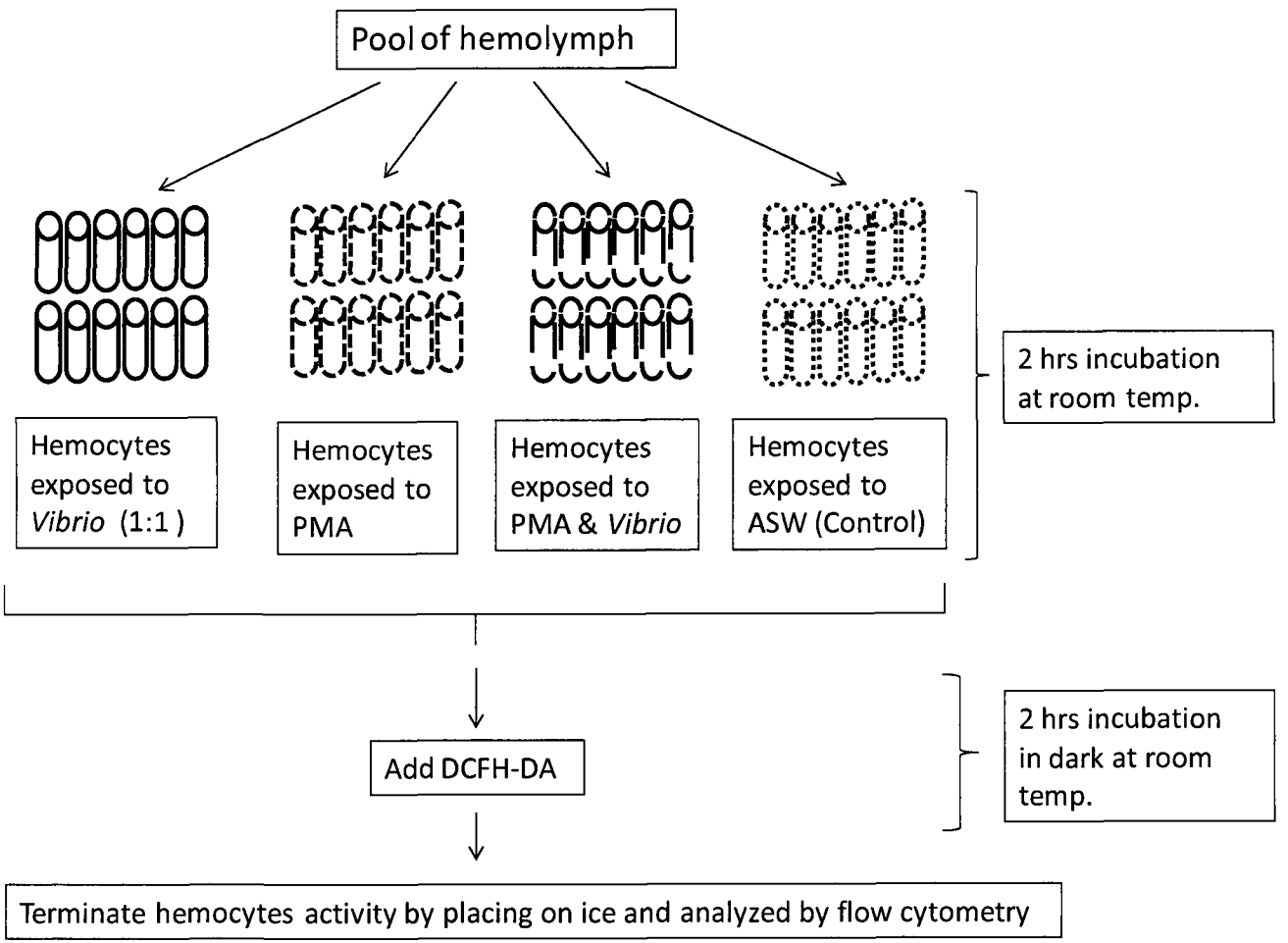
Hemolymph collection



Phagocytosis



Respiratory burst activity



Gene expression