In vivo model of the response of soft-shell clam (*Mya arenaria*) haemocytes differentially induced by two strains of *Vibrio splendidus*

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Abstract

Constantly exposed to different microorganisms, molluscs have developed means to recognize and mount defense mechanisms against potential pathogens. Conversely, pathogenic bacteria are able to subvert the host immune system by modulating their responses. Although several studies on host responses have contributed to the understanding of these interactions in various bivalves challenged with bacteria, there is no information in this respect on soft-shell clams *Mya arenaria*. In this study, an *in vivo* model of interaction between *M. arenaria* haemocytes and the marine bacteria *Vibrio splendidus* was developed by assessing a series of host responses against two bacterial strains: LGP32-GFP, derived from a pathogenic strain (LGP32) associated with oyster mortalities in France, and 7SHRW, an environmental isolate from PEI marine sediments.

Using contrast phase microscopy and flow cytometry, phenotypic responses were measured at cellular and sub-cellular levels. In addition, the expression of genes involved in physiological and immune processes was assessed with RT real time q-PCR. Also, lysozyme activity in haemolymph was studied in clams from two different origins (i.e. Ireland and Canada) challenged with the pathogenic strain. Finally, degranulation mechanisms were briefly explored *in vitro*. Responses of the *in vivo* model were assessed 24 h after injecting 4.5 x 10^6 bacteria/g of clam.

Increased percentages of rounded haemocytess and total numbers were found in response to both strains. However, values were significantly higher ($p \leq 0.01$) after infection with LGP32-GFP compared to 7SHRW. Also, LGP32-GFP induced markedly diminished cell adherence ($p < 0.001$) while no significant changes were found after infection with 7SHRW. Flow cytometry forward and side scatter profiles showed two haemocyte subpopulations: hyalinocytes and granulocytes. Granulocytes exhibited significantly higher levels of lysosomal staining ($p < 0.01$), revealed by the fluorescent dye LysoTracker. Following infection with LGP32-GFP, and not with 7SHRW, both subpopulations merged into a single continuous group. Also, the lysosomal content significantly decreased in hyalinocytes ($p < 0.001$) and granulocytes ($p = 0.001$), suggesting the possible occurrence of precursor cells and degranulation, respectively. Among the housekeeping genes evaluated, using the software geNorm, elongation factor 1α (EF-1α) and 2 (EF-2) had the most stable expression under *in vivo* challenge with LGP32-GFP, whereas EF-1α and the ribosomal protein S-18, after challenge with 7SHRW. By using these genes as internal controls, it was detected that LGP32-GFP induced significant up-regulation of γ-actin ($p < 0.001$), IRAK-4 homologue ($p < 0.001$) and down-regulation of TLR-2 ($p < 0.001$) and lysozyme-2 ($p < 0.05$) homologues. While actin regulation is likely associated with the cytoskeleton changes, IRAK-4 regulation would suggest activation of the NF-κB pathway. Only the down-regulation of lysozyme was significant ($p < 0.05$) after challenge with 7SHRW.

Lysozyme activity in haemolymph of clams from Ireland significantly increased after challenge, with a peak at 12 h ($p < 0.001$), while lysozyme levels did not change significantly in clams from Canada. Lysozyme levels were higher in haemocytes than in plasma. In addition, haemocytes could be induced to degranulate with the compound

48/80 suggesting a signaling pathway involving G proteins that could not be confirmed as inhibitors of such pathway did not prevent degranulation.

These results suggest specific responses at a cellular and molecular level induced by different *V. splendidus* strains. In contrast to 7SHRW, the strain LGP32-GFP induced marked responses at both levels that would impair the defense mechanisms of haemocytes. The differential responses assessed in this model have potential to be used as a tool to evaluate health status of clam populations.

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Dedication

"…What though thy shell protects thy fragile head

From the sharp bailiffs of the briny sea?

Thy valves are, sure, no safety-valves to thee

While rakes are free to desecrate thy bed,

And bear thee off – as foemen take their spoil -

Far from friends and family to roam;

Forced, like a Hessian, from thy native home

To meet destruction in a foreign broil!..."

(…or a lab bench!)

From "Sonnet to a clam" by John G. Saxe (1816-1887)

Hoping that the sacrifice of the beautiful creatures
used for this study was meaningful…

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List of Abbreviations

α_2-M	Alpha$_2$-macroglobulin
ANOVA	Analysis of variance
AMP	Antimicrobial peptide
ECP	Extracellular products
EF	Elongation factor
ERK	Extracellular regulated kinase
EST	Expressed sequence tag
GFP	Green fluorescence protein
FSSW	Filtered sterile seawater
HSP	Heat shock protein
IκB	Inhibitor protein κB
IKK	IκB kinases
IL	Interleukin
IL-R	Interleukin receptor
IRAK	Interleukin-1 receptor-associated kinase
JNK	c-jun N-terminal kinases
LGBP	LPS and β-1,3-glucan-binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mpeg	Macrophage expressed gene
NF-κB	Nuclear factor-κB
$NO^{\cdot}$	Nitric oxide
$ONOO^{-}$	Peroxynitirite
OmpU	Outer membrane protein U
PAMP	Pathogen-associated molecular pattern
PKA	Protein kinases A
PKC	Protein kinases C
proPO	Prophenoloxidase
PRR	Pattern recognition receptor
ROI	Reactive oxygen intermediates
S-18	Ribosomal protein S-18
SEM	Standard error of the mean
SPAK	Stress–activated protein kinases
TIMP	Tissue inhibitor of metalloprotease
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Vsm	*Vibrio splendidus* metalloprotease

Chapter 1: General Introduction

Molluscs are among the most primitive metazoans. Their successful evolutionary history suggests a highly adapted immune system despite lacking the complexity found in other metazoans. Their filter-feeding behavior and open circulatory system expose them to constant challenges from their fluid environment which carries microorganisms in both high numbers and diversity.

One of the remarkable characteristics of the innate immune system of bivalves is their capacity to distinguish among bacteria before mounting defensive mechanisms (Bachère et al., 2004). Likewise, it is interesting how different bacteria (i.e. pathogenic, non-pathogenic, indigenous and non-indigenous) can modulate the immune responses of their hosts (Canesi et al., 2005). Once potential pathogens have gained intimate access to a host, they can provoke a cascade of responses that involve molecular and cellular mechanisms to neutralize the intruders (reviwed by Roch, 1999; Canesi et al., 2002).

Conversely, some bacteria have various strategies to circumvent the defenses of their host in an effort to survive and proliferate. During the last decade, several studies have investigated the mechanisms by which mollusc haemocytes interact with pathogenic microorganisms (Pruzzo et al., 2005). However, no model of host-pathogen interaction has been developed in order to unravel the cellular and molecular mechanisms in *Mya arenaria*. Such a model of interaction would contribute to our further understanding of the pathogenesis of bacterial diseases and also facilitate the assessment of soft-shell clams health.

This thesis, explores the development of an *in vivo* model of the response of soft-shell clam (*M. arenaria*) haemocytes that have been differentially induced by two

strains of the Gram-negative bacteria, *Vibrio splendidus*. Prior to delineating the hypothesis and objectives of this research, relevant background information on the host (*Mya arenaria*), the bacterial pathogen (*Vibrio*) and the current understanding of both bivalve mollusc immunity and host-pathogen interactions are provided.

1.1. Soft-shell clam *Mya arenaria*

The soft-shell clam *Mya arenaria* (Linnaeus, 1758) is widely distributed in the Northern hemisphere as part of the intertidal and subtidal infauna of bays and estuaries. On the Atlantic coast of North America, this species is especially abundant intertidally along New England and the Canadian Maritime coasts and subtidally in Chesapeake and Delaware bays (Newell, 1990). Despite their wide distribution, there is low genetic variation among soft-shell clam populations of the Atlantic and Pacific Oceans (Strasser and Barber, 2008).

Mya arenaria lives buried in a broad spectrum of sediment substrates, with preference to fine sands mixed with clay or black mud with high organic content due to its adaptive physiology to the presence of H_2S and O_2 deficiency. This clam also tolerates wide salinity and temperature ranges; the latter seems to be related to its geographical distribution (Strasser, 1999). The shell growth rate is usually faster during the first few years and it reaches sizes of 6 to 10 cm, with a life span of about 10-12 years. The species is dioecious and spawning usually occurs once or twice a year in response to the temperature and food availability. *Mya arenaria* uses its siphon to pump water to respire and feed. It filters phytoplankton and is also capable of absorbing dissolved organic matter (Strasser, 1999). Like other bivalves, *M. arenaria* has an open

circulatory system, meaning that the haemolymph circulates through vessels and permeates through tissues (Cheng, 1981).

For centuries, soft-shell clams have been considered an important resource in coastal communities of the Canadian Maritimes and New England, either for direct consumption or as bait (Hawkins, 1985; Newell, 1990). Marketed in the shell or as fresh and frozen shucked meats (Brown et al., 1995), *M. arenaria* is still considered a significant resource in the region (Hidu and Newell, 1989; Beal, 2002). According to the Department of Fisheries and Rural Development of Prince Edward Island, 314 metric tonnes were landed in the province in 2008 which corresponded to a value of $692 thousand. Several efforts for extensive clam culture by seeding clam flats with juveniles, either from hatcheries or collected from the wild, have been tried in the region with a certain degree of success (Beal, 2002; Chevarie et al., 2005).

While bacterial outbreaks have rarely been reported (Kaneko et al., 1975), *Rickettsia*-like organisms and some parasites, including ciliated protozoans (i.e. trichodinids, *Ancistrocoma* spp. and *Spenophrya*-like) and trematode metacercariae of various species of Digenea, usually cause chronic but light infections not associated with disease (Harshbarger et al., 1976; Bower et al., 1994). However, proliferative disorders including haemic and gonadal neoplasias considerably affect wild populations in Canada (McGladdery et al., 2001; Delaporte et al., 2008) and USA (Walker et al., 2009). Disseminated neoplasia is characterized by the presence of large, anaplastic cells in the connective tissue, blood vessels and sinuses showing high nucleus to cytoplasm ratio and mitotic figures, whereas in gonadal neoplasias undifferentiated germ cells are initially focalized in gonads where they proliferate, rather than mature, eventually

invading other tissues (Barber, 2004). The etiology of these neoplasias is not clearly defined and although retroviral infection, in association with detrimental environmental factors, have been implicated (Barber, 2004), recent studies have detected endogenous reverse transcriptase activity in healthy haemocytes and absence of viral particles in neoplastic haemocytes that challenge this theory (AboElKhair et al., 2009).

Due to its easy manipulation and wide accessibility this bivalve has been considered a convenient animal for screening bacterial pathogens in experimental models as well as in ecotoxicology studies (Tubiash, 1971). Indeed, *M. arenaria* is widely used as a sentinel species in several biomonitoring programs due to its capability to bioaccumulate xenobiotics present in the environment (Fournier et al., 2001, 2002; Siah et al., 2003; Gagné et al., 2006; Yang et al., 2006; Pichaud et al., 2008). Consequently, studies have investigated the effect of contaminants on the physiology and immunity of *M. arenaria* in order to identify biological markers responding to the effect of specific and/or a mixture of contaminants (Fournier et al., 2002; Gagne et al., 2009).

1.2. Vibrios

The term "vibrios" is commonly used to refer to bacteria belonging to the genus *Vibrio* or to the Vibrionaceae family (class Gammaproteobacteria), which also includes the genera *Photobacterium* and *Salinivibrio* (Farmer and Janda, 2005). Vibrios are Gram-negative small rods (0.5 – 0.8 x 1.4 – 2.6 μm), curved in early stationary phase, with flagella. They are facultative anaerobes, ferment D-glucose, and mostly possess oxidase and reduce nitrates to nitrites. They are primarily aquatic and their species

distribution depends on environmental factors including nutrient concentration and temperature but especially on the Na^+ concentration of the water (Farmer et al., 2005).

Vibrios are ubiquitous in marine and estuarine environments including sediments and water and, in higher densities, in/on associated organisms, i.e. corals, fish, molluscs, seagrass, sponges, shrimp, zooplankton, macroalgae (Huq and Colwell, 1995; Vandenberghe et al., 2003; Thompson et al., 2004; Pruzzo et al., 2005).

Several vibrios are of human clinical importance causing serious intestinal infections (e.g. *V. cholerae*, *V. parahaemolyticus*), wound infections and primary septicaemia (*V. vulnificus*) (reviewed in Huq and Colwell, 1995; Thompson et al., 2004; Farmer et al., 2005; Farmer and Hickman-Brenner, 2006). Other vibrios have been associated with infections of aquatic organisms including fish (e.g. *V. anguillarum*, *V. ordalii*, *V. salmonicida*), crustaceans (e.g. *V. harveyi*, *V. penaeicida*), bivalve molluscs (e.g. *V. tapetis*, *V. splendidus, V. pectenicida*) and corals (*V. shiilonii*, *V. coralliilyticus*) (reviewed in Thompson et al., 2004; Paillard et al., 2004; Austin, 2006a, b; Crosa et al., 2006; Le Roux and Austin, 2006; Owens and Busico-Salcedo, 2006). Vibrios are not exclusively pathogenic to a single host species or even to a major taxonomic group. For example, *V. alginolyticus* is known as a human pathogen (Farmer et al., 2005) and has also been reported to be pathogenic to fish, crustaceans and bivalve molluscs (Austin, 2006b).

Several virulence factors have been described in pathogenic vibrios including haemolysins, siderophores, adhesive factors, proteases, lipases, and different toxins from which haemolysins are probably the most widely distributed among the pathogenic strains (Zhang and Austin, 2005). Vibrios pathogenic to humans and to aquatic

vertebrates and invertebrates are believed to acquire virulence factors by horizontal transfer of plasmids or transposons from other bacteria and bacteriophages (Cotter and DiRita, 2000; Austin and Zhang, 2006).

Although biochemical tests have previously been established to differentiate *Vibrio* species (Alsina and Blanch, 1994), the large phenotypic variability of this genus precludes making an accurate identification to the level of species by conventional methods, or even by using sophisticated automated systems such as Biolog (Vandenberghe et al., 2003; Thompson et al., 2004; Le Roux and Austin, 2006). The phenotypic variability observed is derived from the vast genotypic diversity of vibrios as a consequence of the high rates of horizontal gene transfer and recombination occurring between related species such as *V. harveyi*, *V. tubiashii*, and *V. splendidus* (Thompson et al., 2006). Hence using a combination of phenotypic and genomic analyses, described as a polyphasic approach, is crucial for species identification (Vandamme et al., 1996; Stackebrandt et al., 2002; Le Roux and Austin, 2006). The analysis of 16S ribosomal DNA sequence similarity has commonly been used to establish phylogenetic relationships among vibrios (Kita-Tsukamoto et al., 1993). Other methods including genomic fingerprinting (i.e. fluorescent amplified fragment length polymorphism, repetitive extragenic palindromes or multilocus sequence typing) represent good alternatives for *Vibrio* identification (Thompson et al., 2004).

1.2.1. *Vibrio splendidus*

Vibrio splendidus is a halophilic species commonly present as a component of the marine bacterioplankton (Thompson et al., 2005). This species seems to be

especially dominant among culturable vibrios in low temperature geographical areas (Urakawa et al., 1999). *Vibrio splendidus* is bioluminescent; a feature shared with few other marine vibrios such as *V. fischeri*, *V. harveyi* and *V. logei* (Farmer et al., 2005).

This species is classically divided into two biotypes. Biotype I is characterized by being luminescent and positive for arginine dihydrolase, while biotype II is negative for both tests (Farmer and Hickman-Brenner, 2006). A third unnamed biotype has also been considered, but is not well characterized (Farmer et al., 2005). This classification into classic biotypes has recently been challenged because it is based mainly on phenotypic features. Moreover, the low DNA-DNA homology ($\leq 61\%$) between biotype I and II suggests they belong to different species (Le Roux and Austin, 2006).

At present, seven species, *V. lentus, V. cyclitrophicus*, *V. kanaloae*, *V. pomeroyi*, *V. tasmaniensis*, *V. chagasii*, and *V. crassostreae*, are closely related to *V. splendidus* (Le Roux and Austin, 2006). Given the great limitations of phenotypic characterization to distinguish among species, a polyphasic approach has been suggested (Thompson et al., 2004; Le Roux and Austin, 2006). In fact, due to the limitations of the 16S ribosomal DNA for species delineation, the type II topoisomerase *gyrB* has been shown to be an alternative for phylogenetic analysis for *V. splendidus*-related strains given its congruency with DNA-DNA homology analysis (Le Roux et al., 2004).

It is hypothesized that there are at least one thousand different genotypes of *V. splendidus* occurring at very low concentrations in the environment. Although they display seasonal variation in abundance, it is considered that the genotype variation is neutral, meaning that numerous genotypes coexist without a dominant ribotype (Thompson et al., 2005).

Vibrio splendidus has been isolated from sediments (Sobecky et al., 1998; Mateo, 2006) and marine organisms including as part of the common flora of bivalve molluscs (Hariharan et al., 1995; Mateo, 2006). Different strains of this species however have also been reported as pathogenic to cultured bivalves (Sugumar et al., 1998; Gómez-León et al., 2005; Kesarcodi-Watson et al., 2009) and fish (Gatesoupe et al., 1999; Jensen et al., 2003; Thomson et al., 2005). A strain of *V. splendidus* biotype II causes loss of cilia and velum followed by death of *Crassostrea gigas* larvae (Sugumar et al., 1998). The strains TA2 and TA15 of the same biotype cause loss of velar cells, disorganization of muscle fibers and death of larvae and spat of clam *Ruditapes decussatus* (Gómez-León et al., 2005). In mussel *Perna canaliculus* larvae, a *V. splendidus* strain causes detachment of cilia and necrosis and vacuolation in the digestive region (Kesarcodi-Watson et al., 2009). Various *V. splendidus* strains, including TNEMF6, TNNIII7, Mel31 and LGP32 (previously called Mel 32), have also been associated with mortalities of juvenile oysters *C. gigas* (Lacoste et al., 2001a; Waetcher et al., 2002; Le Roux et al., 2002; Gay et al., 2004a, b). The presence of an extracellular protease is considered an important factor in *V. splendidus* strains pathogenicity (Farto et al., 2006; Vásquez et al., 2006), and the vulnerability of juvenile *C. gigas* to *V. splendidus* is increased by stress (Lacoste et al., 2001b).

1.2.1.1. *Vibrio splendidus* strain LGP32

Vibrio splendidus LGP32 was isolated from diseased oysters after an outbreak of "summer mortality" syndrome in France (Gay et al., 2004a, b). The complete genome of this strain has recently been sequenced and characteristic of other vibrios, it contains

two circular chromosomes. Chromosome 1 and 2 contain 3.29 Mb and 1.67 Mb with 2998 and 1500 predicted open reading frames, respectively. In contrast to other vibrios, LGP32 contains a small chromosomal integron with a limited number of gene cassettes (Le Roux et al., 2009).

The LGP32 genome contains several homologous sequences to genes associated with virulence including haemolysins, proteases, a type IV secretion system, siderophore transport and utilization genes, adhesion and genes associated with antimicrobial peptide and antibiotic resistance (Cattoir et al., 2007; Le Roux et al., 2009). The different distribution of these genes among *V. splendidus* strains may explain the virulence variability observed against aquatic animals (Le Roux et al., 2009).

Based on experimental infections using gene deletions, the metalloprotease Vsm is considered to be an important toxicity factor in extracellular products (ECPs) as determined by the cytopathic effect in mollusc cell lines and *C. gigas* mortality (Binesse et al., 2008; Hasegawa et al., 2009). Vsm share high homology with other metalloproteases of various *Vibrio* species including *V. tubiashii*, *V. cholerae*, *V. fluvialis*, and *V. vulnificus* (Hasegawa et al., 2008) which are also toxic to oyster larvae (Hasegawa et al., 2009). However, Vsm is not necessary for bacterial virulence as infection with *vsm* deleted *V. splendidus* induced similar mortality rates (Le Roux et al., 2007). Recently, the major outer membrane protein OmpU has been identified as a factor that not only confers resistance to oyster antimicrobial peptides but is required for virulence against *C. gigas* (Duperthuy et al., 2010). *V. splendidus* LGP32 OmpU has fibronectin-binding properties (Duperthuy et al., 2010) which, as in case of *V. vulnificus* OmpU, would favour adherence to host cells (Goo et al., 2006).

1.3. Immunity in bivalve molluscs

In bivalves, as in other invertebrates, the defense against pathogens relies on the innate immune system. Innate responses lack the complexity and memory capacity, given by lymphocytes and immunoglobulins, possessed by vertebrates (Roch, 1999; Iwanaga and Lee, 2005). Innate responses of invertebrates are based on the recognition of common antigens on cell surfaces of potential pathogens (Iwanaga and Lee, 2005) and yet are able to elicit discriminative responses based on the character of the microorganism (Bachère et al., 2004).

The immune response involves first the recognition of non-self followed by the activation of a defensive response in order to kill or inactivate the invader, and finally the removal of damaged host cells (Mydlarz et al., 2006). For distinguishing self from non-self, conserved molecules in bacteria, such as lipopolysaccharide (LPS) or peptidoglycan, known as pathogen-associated molecular patterns (PAMPs) are identified through pattern recognition receptors (PRRs) (Medzhitov and Janeway, 2000). The recognition and the responses generated are performed by humoral and cellular components represented by the haemocytes and various soluble factors in a complementary and highly efficient fashion.

1.3.1. Cellular component: haemocytes

Haemocytes are the circulatory cells in molluscs. They have several functions including digestion and transport of nutrients, shell formation and repair, wound repair, excretion and internal defense (reviewed in Cheng, 1981, 1984).

1.3.1.1. Types of haemocytes

By using microscopy or, more recently, flow cytometry, several types and sub-types of haemocytes have been described based on morphology, staining affinity and cell function. The consensus, however, is that two cell types, proposed by Cheng (1981), are dominant: granulocytes and hyalinocytes (agranulocytes). Granulocytes have a varied number of granules, form filopodia and are actively involved in phagocytosis, whereas hyalinocytes possess a clear cytoplasm containing few or no granules, form lobopodia instead of filopodia, and are smaller than granulocytes (Cheng, 1984). Hyalinocytes might be involved in the production of extacellular matrix during wound healing (Cajaraville and Pal, 1995). Additionally, Auffret (1988) defined "agranular" cells as those of the same hematopoietic lineage as granulocytes that have no visible granules (but are different from degranulated granulocytes), and "non granular" cells as those that have few or no granules regardless of cell lineage.

Synard (2007) classified haemocytes of *M. arenaria* into five types based on microscopic features and staining affinity. Type 1 and 2 granulocytes were basophilic and eosinophilic respectively, and type 1, 2 and 3 agranulocytes were smaller than granulocytes with higher nucleus to cytoplasm ratio, lacked granules and could be differentiated based on size and the presence of vacuoles (Type 1 agranulocyte). It is, however, recognized that these types might represent different maturation and degranulation states of haemocytes.

1.3.1.2. Ontogeny

There is no clear evidence of the origin of haemocytes, but some studies suggest that haemocytes arise from differentiation of connective tissue cells (Cheng, 2000). From microscopic observations several theories about the ontogeny of haemocytes have been proposed and summarized by Auffret (1988). Some propose a common origin in leucoblasts, while others consider separate lineages for granulocytes and hyalinocytes. Granublast or agranular prohaemocyte are considered the precursor cells of granulocytes while hyalinoblast or prohyalinocyte are considered the precursor cells of hyalinocytes. While these cells are hypothetical some researchers have reported cells that appear to be precursor haemocytes. They have been described as blast-like cells or haemoblasts, and are characterized by a high nucleus to cytoplasm ratio and lack of organelles (Hine, 1999; Cima et al., 2000; Chang et al., 2005, Aladaileh et al., 2007a; Matozzo et al., 2008).

1.3.1.3. Role of haemocytes in inflammation

Haemocytes are actively involved in the inflammatory process and can be activated either through injury or by a living or inert body (Feng, 1988). When an injury is involved, the process of healing engages the infiltration of haemocytes to the injured tissues, formation of a plug by aggregated haemocytes, replacement of the damaged tissues, deposition of collagen and removal of the necrotic tissues. When microorganisms cause the inflammation, haemocytes are attracted by chemotaxis (Feng, 1988).

Infectious agents such as bacteria are neutralized by humoral factors and by the direct intervention of haemocytes through initial adhesion and subsequent phagocytosis. Three ways in which foreign bodies such as bacteria are taken up into the ectoplasm have been described in oysters *C. virginica* (Cheng, 1984, 2000). The first involves the adherence of the particle by filopodia and gliding of the filopodia in order to take the particle into the ectoplasm. The second mechanism involves the invagination of the cell surface. The third mechanism is through funnel-like pseudopodia in which the particle glides into a phagosome. In the case of large pathogens that cannot be phagocytosed, such as parasites, haemocytes react by encapsulating the pathogen (Cheng, 1984).

1.3.2. Humoral components

The humoral components are composed of several soluble molecules in the plasma. These are mainly lectins, antimicrobial peptides, lysozyme (and other hydrolytic enzymes), prophenoloxidase (proPO), and heat shock proteins. Other constituents such as nitric oxide, protease inhibitors and cytokines play important roles in the immune system but have been reported less frequently in bivalves.

1.3.2.1. Lectins

Lectins are carbohydrate-binding proteins or glycoproteins that facilitate phagocytosis in animals of all taxa (Olafsen 1995, 1996). In chordates, mannose-binding lectins activate the complement system through the lectin pathway (Endo et al., 2006). Lectins identify non-self through the recognition of carbohydrate structures in microorganism surfaces (Vasta and Marchalonis, 1987). In invertebrates, the lectin

ligand interaction may be regulated by sialic acid (Tunkijjanukij et al., 1998). When lectins have multivalent capacity they are known as agglutinins (Vasta and Marchalonis, 1987).

There are different structural families of lectins. The most common are the C-type lectins and the S-type (or galectins) that are sulfhydryl-dependent or beta-galactosidase binding lectins, but other families include I-type, P-type, and ficolins (Kilpatrick, 2002). Most invertebrate lectins, including those from bivalves, are C-type, and they require divalent cations, in particular Ca^{2+} for agglutination, but may be inhibited by Zn^{2+} (Olafsen 1995, 1996).

In bivalves, lectins are synthesized in haemocytes and found in serum (humoral lectins) as well as on the plasma membrane of circulating phagocytic cells (cell bound lectins), and have both an opsonic and agglutinating role mediating binding between the haemocyte surface and the invader microorganism (Olafsen 1995, 1996). In other aquatic invertebrates (i.e. crustaceans) lectins seem to activate the proPO system in addition to the non-self recognition role (Marques and Barracco, 2000).

Lectins have been detected in oyster *C. gigas*, known as gigalins (Olafsen 1995), Manila clams *R. philippinarum* (Kang et al., 2006) and bay scallops *Argopecten irradians* (Zhu et al., 2008, 2009).

1.3.2.2. Antimicrobial peptides

Antimicrobial peptides (AMPs) are small peptides, usually less than 50 amino acid residues, with an overall positive charge (cationic) and a substantial portion (≥50%) of hydrophobic residues (Powers and Hancock, 2003). These peptides have the ability to

adopt an amphipathic conformation with opposing hydrophobic and positively charged faces when in contact with bacterial membranes (Papo and Shai, 2003). In this way they interact with membranes, disrupting or translocating them, which eventually kill the invading microorganisms (Powers and Hancock, 2003). In addition to their antimicrobial activity, AMPs are involved in other immune functions such as pro- or anti-inflammatory, stimulating wound healing, and anti-tumor activity of the cells (Brown and Hancock, 2006).

Bivalve AMPs are synthesized within circulating granular haemocytes, processed from precursors to active peptides and stored in granules (Mitta et al., 2000). They were considered to be constitutively expressed (Bachère et al., 2004; Mitta et al., 2000), but recent reports have indicated AMP induction by bacterial challenge (Cellura et al., 2007; Zhao et al., 2007; Gueguen et al., 2009). AMPs are mostly concentrated in tissues such as the gut and respiratory organs that are exposed to elevated concentrations of microorganisms (Tincu and Taylor, 2004).

Mussel AMPs are characterized by having high cysteine content. According to their primary structure and consensus cysteine array AMPs are part of four groups: defensins (6 cysteines), mytilins (8 cysteines), myticins (8 cysteines) and mytimicins (12 cysteines) (Mitta et al., 2000). Two defensin isoforms, A and B, have been isolated from plasma of the mussel *Mytilus edulis* (Mitta et al., 2000). A defensin-like peptide MGD-1 was isolated from plasma and haemocytes of the mussel *M. galloprovincialis*, and MGD-2 from haemocytes. Five isoforms of mytilins have been described: isoforms A and B were isolated from plasma of *M. edulis*, and isoforms B, C, D and G1 were isolated from haemocytes of *M. galloprovincialis*. Isoforms A and B of myticins have

been described; A isolated from haemocytes and B from plasma and haemocytes of *M. galloprovincialis*. Mytimicyn has been isolated from plasma of *M. edulis* (Mitta et al., 2000).

Mussel AMPs have complementary and specific antimicrobial activities. Defensins and myticins have been found to be active against Gram-positive bacteria and less active against Gram-negatives and fungi whereas mytimicin is solely antifungal. Mytilins B, C and D are active against Gram-positive and -negative bacteria, including vibrios, whereas G1 is only active against Gram-positives (Mitta et al., 2000, Tincu and Taylor, 2004). Different AMP encoding genes are expressed in different haemocyte subtypes and transported to different mussel organs. Haemocytes expressing defensin genes infiltrate the digestive tubule epithelia whereas those expressing mytilins and myticins are found in gills (Mitta et al., 2000).

In oysters, two AMPs belonging to the defensin family have recently been isolated and characterized. The defensin AOD was isolated from gills of the oyster *Crassostrea virginica*, and another from the mantle of the oyster *C. gigas* (*Cg*-Def) (Seo et al., 2005; Gueguen et al., 2006). AOD posses 6 cysteine residues and shares high sequence similarity with arthropod and mussel defensins. It has 71% sequence similarity with defensin A and 69% with defensin B from *M. edulis*, 73% with MGD-1 and 72% with MGD-2 from *M. galloprovincialis* (Seo et al., 2005). *Cg*-Def is a singular defensin that has two extra cysteine residues and shares 50% sequence similarity with MGD-1 (Gueguen et al., 2006).

Both, mussel and oyster defensins are active against Gram-positive bacteria, and *Cg*-Def has limited or no activity against Gram-negative bacteria and fungi like most

invertebrate defensins whereas AOD has significant activity against Gram-negative bacteria, including *Escherichia coli* and *V. parahaemolyticus* (Seo et al., 2005; Gueguen et al., 2006).

1.3.2.3. Hydrolytic enzymes

Eukaryotic cells have membrane bound cytoplasmic bodies called lysosomes that contain several acid-dependent hydrolytic enzymes (Dell'Angelica et al., 2000). In bivalves, lysosomes are prominent in granulocytes (Cheng, 1983). Among these enzymes, the presence of β-glucuronidase, alkaline and acid phosphatase, lysozyme, lipase and non-specific esterases has been detected in different bivalves (Chen and Rodrick, 1975; Carballal et al., 1997; Xing et al., 2002), including *M. arenaria* (Rodrick, 1979; Huffman and Tripp, 1982). During infection these enzymes are released into the serum to neutralise invader microorganisms (Cheng, 1983). The lysosomal enzymes, especially lysozyme, are among the most studied proteins involved in the invertebrate immune system.

1.3.2.3.1. Lysozyme

This enzyme has a role in digestion and host defense and these functions have been associated with its tissue location and antibacterial activity (Olsen et al., 2003; Xue et al., 2004, 2007; Itoh et al., 2007). Digestive lysozymes are found in the crystalline style and visceral mass (McHenery, et al., 1979), and are mostly active against Gram-positive bacteria (Cheng and Rodrick, 1974), whereas host defense lysozymes are found in haemolymph (haemocytes and plasma), extrapallial fluids, labial palps and mantle

(McHenery et al., 1979; Allam et al., 2000; Itoh et al., 2007) and are active against both Gram-positive and -negative bacteria (Nilsen et al., 1999; Xue et al., 2004).

Based on structural, catalytic and immunological criteria, lysozymes are classified into different types. Animal lysozymes involve c-lysozyme, initially isolated from chickens but also found in other birds, mammals, reptiles and insects, g-lysozyme, isolated from goose and other birds (Jollès and Jollès, 1984), and i-lysozyme, found in invertebrates, including bivalve molluscs, Penaeid crustaceans and some insects (Bachali et al., 2002).

Lysozymes are characterized by their ability to hydrolyse glycoside bonds of peptidoglycan wall, the major component of Gram-positive bacteria, and chitin (Jollès and Jollès, 1984). Gram-negative bacteria can also be hydrolyzed through a similar mechanism to antimicrobial peptides. A helical hairpin or C-terminal helix allow lysozyme to cross the outer membrane via self-promoted uptake causing damage to the inner membrane through channel formation (Ibrahim et al., 2001).

Increased levels of lysozyme activity have been reported during experimental challenge with pathogens such as in clams *R. philippinarum* and *R. decussatus* infected with *V. tapetis* and *V. anguillarum* (Allam et al., 2000, 2006), and mussels *M. galloprovincialis* infected with *V. splendidus*, *V. anguillarum* and *M. lysodeiktycus* (Ciacci et al., 2009). In oysters, *O. edulis,* infected with the protozoan parasite *Bonamia ostreae*, however, their levels have no clear relationship with the level of resistance (Cronin et al., 2001).

Lysozymes have been detected in the plasma and haemocytes of several bivalves in different proportions. Also, different lysozymes have been described in bivalves.

Lysozymes have been reported in higher levels in haemocytes than plasma in mussels *M. galloprovincialis* (Carballal et al., 1997) and oysters *O. edulis* (Cronin et al., 2001). In *M. edulis* three lysozymes involved with digestion (sA-, sB- and sC-lysozyme) and one involved in host defense (bm-lysozyme) have been described (Olsen et al., 2003). In oysters *C. virginica*, higher levels of lysozyme in plasma than haemocytes were found (Chen and Rodrick, 1975). Lysozyme levels in *C. virginica* haemocytes were similar to those found in the clam *Mercenaria campechiensis* and higher than in the clam *Anadara ovalis* (Rodrick and Ulrich, 1984). Two lysozymes have been described in *C. virginica*, cv-lysozyme type 1 found in the labial palps, mantle and gills and cv-lysozyme type 2 in the digestive tubules (Itoh et al., 2007; Xue et al., 2007). In *C. gigas* two types of lysozyme, CGL-1 and 2, have been identified in digestive tubules (Itoh and Takahashi, 2007). In *O. edulis,* lysozyme activity has been reported (Cronin et al., 2001) and the enzyme has been characterized as i-type lysozyme (Matsumoto et al., 2006).

Lysozyme has also been reported in scallops *C. opercularis* (McHenery et al., 1979), *Chlamys islandica*, known as chlamysin, (Nilsen et al., 1999), and *C. farreri* (Xing et al., 2002), mussels *Modiolus modiolus* (McHenery et al., 1979), and clams *M. arenaria* (Cheng and Rodrick, 1974; Rodrick, 1979; McHenery et al., 1979), *R. philippinarum* (Allam et al., 2000), *R. decussatus* (López et al., 1997), *Tellina tenuis* (McHenery et al., 1979) and *Mercenaria mercenaria* (Cheng and Rodrick, 1975). In *M. arenaria*, lysozyme is mostly associated with gill, labial palps, and digestive glands (McHenery et al., 1979), and its activity was found at higher levels in plasma than haemocytes (Rodrick, 1979).

1.3.2.4. Heat shock proteins

Heat shock proteins (HSPs) are phylogenetically conserved proteins present in all prokaryotes and eukaryotes (Feder and Hofmann, 1999; Pockley, 2003; Tsan and Gao, 2004; Multhoff, 2006). HSPs perform a variety of chaperoning functions, including folding of polypeptides and facilitation of protein transport (Multhoff, 2006). Under stress conditions HSPs are expressed to stabilize and refold partially denatured proteins or mediate degradation of irreversibly damaged proteins (Feder and Hofmann, 1999; Zügel and Kaufmann, 1999; Wallin et al., 2002; Routsias and Tzioufas, 2006).

Upon necrotic cell death (Tsan and Gao, 2004) or secreted from lysosome vesicles (Ireland et al., 2007), HSPs are released into the extracellular environment. There, they are thought to have an important role activating the immune response of vertebrates including cell signaling, through CD14, Toll-like receptor 2 (TLR-2), and TLR-4, and antigen presentation, carrying antigenic peptides or through antigen presentation cells, via the major histocompatibility complex (Routsias and Tzioufas, 2006; Wallin et al., 2002).

Stress involved in HSP induction could be due to environmental, pathological (i.e. infections), or physiological (i.e. cell differentiation) factors. Environmental stressors include not only raised temperature (Moseley, 1997) but also exposure to oxidative stress, nutritional deficiencies, ultraviolet irradiation, heavy metals, chemicals, and ethanol (Pockley, 2003; Tsan and Gao, 2004).

Bivalve HSPs have been used as biomarkers in biomonitoring programs and environmental toxicology (Feder and Hofmann, 1999). Studies on the expression of Hsp genes in oysters *C. gigas* and mussels *M. galloprovincialis* have been related to

exposure to thermal stress and heavy metals (Boulet et al., 2003; Piano et al., 2004; Franzellitti and Fabri, 2005). Pathogen infections have also been related to expression of bivalve Hsps. Infection of mussels *M. galloprovincialis* with *V. anguillarum* can induce the expression of *Hsp70* (Cellura et al., 2006; 2007). In *Argopecten irradians, Hsp70* gene expression is up-regulated after exposure to naphthalin and also infection with *V. anguillarum* (Song et al., 2006). Hsp gene homologues have also been found in an expressed sequence tag (EST) library of *M. galloprovincialis* after bacterial challenge with a mixture of bacteria including *M. lysodeiktycus*, *V. splendidus* and *V. anguillarum* (Gestal et al., 2007).

1.3.2.5. Prophenoloxidase system

The prophenoloxidase (proPO) system is a cascade of molecule activation that leads to melanisation of tissues and pathogens. During melanin formation, toxic quinolones are produced by phenol oxidation mediated by phenoloxidase (Cerenius and Söderhäll, 2004). The system is activated by serine proteases in the presence of bacterial and fungal compounds such as β-glucan, peptidoglycan and LPS and is regulated by protease inhibitors. Subsequent activation of serine proteases lead to cleavage of pro-prophenoloxidase-activating enzyme (pro-ppA) into ppA and subsequent cleavage of proPO into PO after release from the haemocyte into the plasma (Cerenius and Söderhäll, 2004).

This defensive cascade is common in crustaceans but the phenoloxidase enzyme has also been reported in haemolymph of bivalve molluscs such as mussels *M. edulis*, *Perna viridis*, oysters *Saccostrea glomerata, C. gigas*, clams *M. arenaria, R.*

philippinarum, *Chamelea gallina*, *Tapes decussatus*, scallops *Argopecten ventricosus*, *Nodipecten subnodosus*, and penshell *Atrina maura* (Smith and Söderhäll, 1991; Coles and Pipe, 1994; Cong et al., 2005; Muñoz et al., 2006; Aladaileh et al., 2007b, c; Hellio et al., 2007). Similarly to arthropods, proPO was observed to be induced by PAMPs, such as LPS, and exogenous proteases in bivalves such as mussels *Perna viridis* (Asokan et al., 1997), scallops *N. subnodosus*, oysters *C. gigas* (Luna-Gonzáles et al., 2003), and oysters *S. glomerata* (Aladaileh et al., 2007b, c). In clams *M. arenaria* and mussels *M. edulis*, however, proPO was not activated by proteases (Smith and Söderhäll, 1991).

1.3.2.6. Inhibitors of proteases

Inhibitors of proteases act against unwanted endogenous and pathogen proteases. Endogenous proteases are involved in the physiological functions of digestion (Neurath, 1984), morphogenesis, tissue remodeling, and acting in the turnover of the extracellular matrix (Sottrup-Jensen, 1989; Brew et al., 2000). In addition, endogenous proteases play a role in immune processes such as complement and proPO cascade activation in invertebrates (Cerenius and Söderhäll, 2004; Iwanaga and Lee, 2005). Conversely, pathogen proteases are virulent factors used for invasion and nutrition through tissue degradation (Miyoshi and Shinoda, 2000) and immune evasion by destruction of defense mediators (Armstrong, 2006).

With the exception of macroglobulins, protein inhibitors are effective only against one class of protease (i.e. serine, carboxyl, metallo and sulfhydryl proteases) (Laskowski and Kato, 1980). Serine protease inhibitors, also known as kazals, have been

identified in scallops *A. irradians* and *Chlamys farreri* (Zhu et al., 2006; Wang et al., 2008).

Proteases inhibitors from the humoral phase are mainly represented by the α_2-macroglobulins whereas those from tissues are mostly represented by the tissue inhibitors of the metalloproteases (TIMPs) (Brew et al., 2000).

1.3.2.6.1. Alpha$_2$-macroglobulins

Alpha$_2$-macroglobulins (α_2-Ms) are high molecular weight glycoproteins found in the plasma of vertebrates and invertebrates. Alpha$_2$-Ms bind a wide variety of proteases, including endogenous and exogenous proteases, using a bait region, which is a substrate for most proteases, triggering conformational changes that trap the enzyme (Laskowski and Kato, 1980; Sottrup-Jensen, 1989). Besides their physiological function, they modulate the immune response by trapping proteases in excess and forming complexes and, as a consequence, exposing previously concealed receptor-binding domains. These sites are recognized and bound by receptors on macrophages and other phagocytic cells leading to a rapid clearance of the protease (Sottrup-Jensen, 1989).

The α_2-Ms has been associated with immune responses of marine animals including molluscs (Armstrong and Quigley, 1992), and their inhibitory activity have been observed against bacterial metalloproteases and protozoan serine proteases (Faisal et al., 1998). Alpha$_2$-Ms has been found in the surf clam *Spisula solidissima* (Armstrong and Quigley, 1992), the oysters *C. virginica* and *C. gigas* (Faisal et al., 1998), and the soft-shell clam *M. arenaria* (Elsayed et al., 1999). In *M. arenaria* with haemic neoplasia the inhibitory activity of α_2-M was diminished (Elsayed et al., 1999).

1.3.2.6.2. Tissue inhibitors of metalloproteases

Tissue inhibitors of metalloproteases (TIMPs) are ancient eukaryotic proteins found in humans, birds, amphibians, fish, insects (Brew et al., 2000) and other invertebrates, including bivalves (Montagnani et al., 2001). Their most common function is to maintain the balance in the metabolism of the extracellular matrix, thus TIMP expression is controlled during tissue remodeling and physiological conditions (Brew et al., 2000). However, TIMPs are also involved in defense mechanisms since expression can be regulated in response to pathogen invasion (Montagnani et al., 2001).

In oysters *C. gigas*, expression of TIMP genes is induced by shell damage and bacterial challenge with a mixture of *V. anguillarum*, *V. metshnikovii*, *V. tubiashii* and *Vibrio* S322 (Montagnani et al., 2001, 2007) but not with *V. aestuarianus* (Labreuche et al., 2006b). Other *Vibrio* species, such as *V. tubiashii*, *V. splendidus* and *V. cholerae* produced metalloproteases during *C. gigas* larvae infection but the effect decreased with age (Hasegawa et al., 2009). The expression of TIMP in older stages would account for a lower susceptibility.

1.3.2.7. Nitric oxide

Nitric oxide ($NO^{\cdot}$) is an oxidant species that kills pathogens. $NO^{\cdot}$ is oxidized by $NO^{\cdot}$ synthetase and, although its formation seems to be independent from reactive oxygen intermediates (ROIs), its interaction with the supeoxide anion $O_2^{\cdot}$ leads to formation of peroxynitirite ($ONOO^{\cdot}$), a more toxic compound than its precursors (Splettstoesser and Schuff-Werner, 2002).

Nitric oxide, or nitrite/nitrate (their stable end products), has been detected in some bivalves such as mussels *M. galloprovincialis* (Ottaviani et al., 1993; Arumugam et al., 2000; Tafalla et al., 2002), *M. edulis* (Akaishi et al., 2007), clams *R. decussatus* (Tafalla et al., 2003), and oysters *C. virginica* (Villamil et al., 2007). Production of NO^- is induced by *E. coli* LPS in *M. galloprovincialis* and *R. decussatus* (Ottaviani et al., 1993; Tafalla et al., 2003) and by *Micrococcus lysodeikticus* and *V. anguillarum* challenge in *M. galloprovincialis* (Costa et al., 2009).

1.3.2.8. Cytokines

Cytokines, including interleukins (IL), interferons and chemokines, are recognized regulatory molecules in host defense and homeostatic mechanisms of vertebrates (Cohen and Cohen, 1996). There is evidence that invertebrate cytokines are involved in functions including cell motility, chemotaxis, phagocytosis, and cytotoxicity, similar to vertebrates (Ottaviani et al., 2003).

Cytokine-like molecules and gene homologues to cytokines, cytokine factors and receptors have been identified in some bivalves. The LPS-induced Tumour Necrosis Factor-α (TNF-α) factor, that mediates expression of TNF-α was ubiquitously found in haemocytes and tissues of scallops *C. farreri* (Yu et al., 2007) and oysters *C. gigas* (Park et al., 2008). TNF-α expression was up-regulated by challenge with *E. coli* LPS and a mixture of *V. anguillarum, V. metschnikovii, V. tubiashi, and V. parahaemolyticus*, respectively. Also in *C. gigas*, an IL 17 homolog has been found in haemocytes and its expression is up-regulated by a mixture of *M. luteus*, *V. splendidus* and *V. anguillarum*

(Roberts et al., 2008). The interferon-inhibiting cytokine IK was up-regulated by the anti-viral inducer poly I:C in *S. glomerata* haemocytes (Green and Barnes, 2009).

In addition, α, β, and γ subunits of the cytokine receptor IL-2R have been previously found in mussels *M. galloprovincialis* and synthesis of IL-1Rα was up-regulated by LPS (Barcia et al., 1999; Cao et al., 2003). In mussel *M. edulis* haemocytes, LPS induced the production of cytokines IL-1 and TNF (Hughes et al., 1991). In *C. gigas*, IL-17 and transforming growth factor-β (TGF-β) homologues have been reported. Interleukin 17 was found in haemocytes and its expression is up-regulated by a mixture of *M. luteus*, *V. splendidus* and *V. anguillarum* (Roberts et al., 2008), while TGF-β was found in various tissues and behaved as an immune cytokine since it was also up-regulated by challenge with *V. metschnikovii*, *V. alginolyticus* and *V. harveyi* and *E. coli* LPS (Lelong et al., 2007).

Recombinant cytokines could also activate bivalve haemocytes. In mussels *M. edulis* recombinant IL-1 induced the production of TNF (Hughes et al., 1990). In *M. galloprovincialis* haemocytes recombinant TNF-α activated signal cascades leading to increased phagocytosis (Betti et al., 2006). In oysters *Pinctada fucata* recombinant interferon-ω reduced mortality and increased the tissue repairing functions produced by a viral infection (Miyazaki et al., 2000).

Similarly to vertebrates, mollusc cytokines play also a role in stress responses (Ottaviani et al., 2003). Some interleukins and growth factors, such as IL-2 and PDGF, induced haemocyte release of catecholamine hormones in mussels *M. galloprovincialis* (Cao et al., 2004a, 2007). PDGF, in conection with catecholamines, would have an anti-inflammatory role (Cao et al., 2007).

1.4. Host–pathogen interactions (bivalve–bacteria)

Due to their anatomical and physiological characteristics, bivalve molluscs are in intimate contact with microorganisms co-inhabiting their environment. The aquatic environment carries a great variety of microorganisms, including indigenous and non-indigenous bacteria, pathogens and non-pathogens, which can reach high densities inside these filter-feeding organims (Jones et al., 1991; Pruzzo et al., 2005). This implies a constant exposure of the host tissues and fluids to bacteria. During these encounters a series of host and bacterial molecules interact with each other and activate mechanisms leading to the control of potential infections and to the bacterial survival in a hostile environment, respectively.

1.4.1. Cellular mechanisms in the host

Bivalve haemocytes display chemotatic and chemokinetic migration stimulated by bacterial components (Schneeweiss and Renwrantz, 1993). Important mediators of such behavior are lectins. Bivalve lectins are considered important pattern recognition receptors (PRR) (Kang et al., 2006). After binding to bacteria, they facilitate phagocytosis by binding haemocytes as opsonins or just immobilizing bacteria as agglutinins (Olafsen 1995, 1996). The fact that bivalve haemocytes possess receptors that bind bacteria directly or indirectly, through lectins, has been deduced by assessing phagocytosis in the presence or absence of plasma (reviewed in Robhom, 1984; Pruzzo, et al., 2005). Once the haemocyte has bound a bacterium, phagocytosis is carried out by a series of steps initiated by a localized perturbation of the plasma membrane and ending with the endocytosis of the bacterium into a phagosome (Robhom, 1984). Subsequently,

activation of the ROIs cascade leads to the release of toxic compounds, such as nitrogen and hydroxyl radicals, singlet oxygen and hydrogen peroxide, which aim to kill the phagocytized bacterium (Roch, 1999). In addition, lysosomes fuse with the phagosome and release hydrolytic enzymes such as β-glucuronidase, alkaline and acid phosphatase, lysozyme, lipase and non-specific esterases (Chen and Rodrick, 1975; Rodrick, 1979; Huffman and Tripp, 1982; Carballal et al., 1997; Xing et al., 2002). Similarly, antimicrobial peptides are secreted from their granules into the phagosome. If the infection becomes systemic, lysozymes and antimicrobial peptides are released into the plasma (Cheng, 1983; Mitta, et al., 1999, 2000; Canesi et al., 2002; Tincu and Taylor, 2004). The activation of proPO cascades seem to occur during bacterial infections in bivalves (Asokan et al., 1997). Disintegrated haemocytes would release HSP which constitute "danger signals" that promote immune responses (Cellura et al., 2006). Similarly to lysozyme and AMPs, HSP could also be secreted from the cells (Ireland et al., 2007). In addition, the host is able to counteract the insult of bacterial proteases by the action of protease inhibitors such as tissue inhibitors of metalloprotease (Montagnani et al., 2001, 2007). All these cellular components directly eliminate invading bacteria or their components and stimulate other haemocytes to activate defensive mechanism aimed to control the infection.

1.4.1.1. Bacterial virulence factors

During the infection process, bacteria release a series of virulence factors to aid survival and proliferation within the host. Exotoxins have been reported in *Vibrio* species. In *V. alginolyticus* a protease with cytotoxic effect and a ciliostatic toxin have

been identified as virulence factor against oysters *O. edulis* larvae (Nottage and Birkbeck, 1987). In *V. pectenicida* a toxin, known as *Vibrio* haemocyte-killer toxin VHKT, has been identified as lethal to scallop *Pecten maximus* haemocytes (Lambert et al., 2001). *Vibrio tapetis* possesses adherence factors, pili, haemolysins and cytotoxins that are released to gain nutrients and inhibit pseudopod formation and adherence of clam *Ruditapes philippinarum* haemocytes (Borrego 1996). In addition, siderophores have also been identified in strains of *V. alginolyticus* and *V. splendidus* characterized as the main cause of reduced viability of carpet shell clam *R. decussatus* haemocytes (Gómez-León et al., 2005).

Pathogenic strains of *V. tubiashii* and *V. splendidus* possess metalloproteases (VtpA and Vsm, respectively) considered major virulent factors for oysters *C. gigas* (Le Roux et al., 2007; Binesse et al., 2008; Hasegawa et al., 2008, 2009). In addition, in pathogenic strains of *V. splendidus* (i.e. LGP32), the outer membrane protein OmpU is an important virulence factor against *C. gigas* that confers AMP resistance and adhesive properties (Duperthuy et al., 2010).

1.4.1.2. Changes in cellular immune parameters

Changes in immune parameters have been reported in many *in vivo* and *in vitro* bivalve models of bacterial infection. While many immune parameter assessments reflect an effective defense of the host to control infection, other responses suggest bacterial manipulation of the bivalve immune system. Bacterial infection is usually followed by fluctuations in the number of circulating haemocytes (Suresh and Mohandas, 1990; Oubella et al., 1993; Allam et al., 2001; Labreuche et al., 2006b;

Allam et al., 2006; Parisi et al., 2008). These changes in numbers might be due to activation of haematopoiesis or the redistribution of circulating haemocytes towards infected tissues (Bachère et al., 2004).

Another common manifestation that occurs following bacterial infection is host cytoskeletal changes that produce rounded haemocytes with a decreased adherence capacity (Lane and Birkbeck, 2000; Choquet et al., 2003; Allam and Ford, 2006; Labreuche et al., 2006a, b). This change of shape probably corresponds to a bacterial strategy to subvert phagocytosis as the cytoskeleton appears to be permanently impaired. Indeed, bacterial challenge induces a decreased capacity of haemocytes to phagocytose (Allam et al., 2001; Allam and Ford, 2006; Labreuche et al., 2006a, b; Costa et al., 2009). In addition, the oxidative burst capacity in bivalve haemocytes is modulated after bacterial challenge. This modulation depends on the host species and the pathogenicity of the bacteria (Lambert and Nicolas, 1998). In some cases, however, this association is not totally clear. For instance, *V. tapetis*, a clam pathogen that does not cause clinical signs or mortalities in oysters, induced the highest inhibition of oxidative burst in *C. gigas* haemocytes (Lambert et al., 2003).

1.4.2. Molecular mechanisms modulated by bacteria

Genes encoding proteins involved in immune responses are normally activated by bacterial infection. Some bacteria, however, have the ability to regulate the expression of genes that are involved in the host molecular process of defense responses. Studies have been conducted to identify the transcripts involved in such mechanisms using open source strategies such as EST libraries. Up-regulation of genes encoding

antimicrobial peptides, proteases and protease inhibitors, lectins, cytokines, receptors, stress response and signal transduction proteins have been reported (Huvet et al., 2004; Gestal et al., 2007; Tanguy et al., 2008; Roberts et al., 2009; Wang et al., 2009).

The expression of many of these genes however is differentially modulated according to the bacterial species or strain and the host species. For instance, *V. anguillarum* up-regulates the expression of the defensin in the scallop *Argopecten irradians* but does not alter its expression in *M. galloprovincialis* (Zhao et al., 2007). Similarly, the expression of the mytilin and *Hsp70* in *M. galloprovincialis* haemocytes is not affected by *V. splendidus* but is up-regulated by *V. anguillarum* (Cellura et al., 2007). Lysozyme expression in *M. galloprovincialis* is also up-regulated by *V. anguillarum* (Costa et al., 2009) but down-regulated by *V. splendidus* (Li et al., 2008).

1.4.3. Cell signaling in bacterial infection

Bacteria can activate cellular signaling pathways that lead to the activation of host immune responses. This signal transduction could be initially activated through Toll-like receptors (*Drosophila* Toll homologues) upon binding to their ligands (Takeda and Akira, 2004). These signaling pathways that lead to the synthesis of pro-inflammatory cytokines could involve the nuclear factor-κB (NF-κB) and the mitogen-activated protein kinase (MAPK) pathway (Fig. 1) (Mogensen et al., 2009). The MAPK transduction pathway is constituted by a family of serine/threonine kinases implicated in various cellular processes including growth, cell cycle, stress and host defense (Travers et al., 2009). Signal transduction occurs through a three-tier regulatory cascade of phosphorylation that involves three main signaling pathways: the extracellular regulated

kinase (ERK), the c-jun N-terminal kinases or stress–activated protein kinases (JNK/SPAK), and the p38 kinase family (Cowan and Storey, 2003). MAPK p38 and JNK activate the transcription factor activator protein-1 (AP-1) for synthesis of inflammatory cytokines (Mogensen, 2009).

The activation of MAPK and other kinase cascades in response to bacteria or their products have been reported in mollusc haemocytes (Canesi et al., 2002, 2005; Cao et al., 2004b; García-García et al., 2008; Travers et al., 2009). In mussel *M. galloprovincialis* haemocytes, it was observed that *Escherichia coli* mainly activate p38 and JNK MAPKs (Canesi et al., 2002, 2005). Also, in *M. galloprovincialis*, the phosphatidylinositol-3-OH-kinase (PI-3K) cascade can be activated by *E. coli* and *V. alginolyticus* (Canesi et al., 2002, 2005; García-García et al., 2008). In addition, *E. coli* LPS can also activate the protein kinases A (PKA) and C (PKC), whereas *V. cholerae* activates mainly PKC in mussel haemocytes (Cao et al., 2004b; Canesi et al., 2005).

NF-κB is involved in the transcription of genes encoding cytokines and chemokines. NF-κB exists in an inactive form associated to the inhibitor protein IκB and is activated when its inhibitor is degraded by IκB kinases (IKK) allowing translocation to the nucleus (Li and Verma, 2002). Components of the NF-κB pathway are activated by bacteria in bivalve haemocytes. *Vibrio* species induce up-regulation of components such as IκB, in oysters *S. glomerata* haemocytes (Green and Barnes, 2009). In haemocytes of the scallop *C. farreri*, LPS induces up-regulation of a Toll receptor gene (Qiu et al., 2007).

Conversely, bacteria can evade or disrupt signaling pathways through different strategies that include modification of their PAMPs (e.g. LPS), to avoid signal

activation, and secretion of toxins that inhibit pathway activator proteins (Mogensen, 2009). Bacterial toxins such as pertussis toxin from *Bortadella pertussis* and cholera toxin, from *V. cholerae*, are known to inhibit GTPase activity of G proteins by ADP-rybosilation, disrupting MAPK cascades (Gilman, 1995; Morris and Malbon, 1999). In epithelial cells of the human intestine, the deregulation of cAMP production caused by cholera toxin results in massive ion and water efflux (Kaper et al., 1995). The *Vibrio* outer protein A of *V. parahaemolyticus* is an effector that disrupted MAPK pathways (but not NF-κB) in mammalian cells (Trosky et al., 2004). Inhibition was reported to be through MAPK kinases acetylation preventing their phosphorylation (Trosky et al., 2007). This mechanism is supposed to inhibit production of inflammatory cytokines during *V. parahaemolyticus* infection in humans and to allow survival within shellfish, its commensal hosts.

It has been also noted that *V. cholera* has a reduced capacity of activating MAPK in *M. galloprovincialis* haemocytes in compare to *E. coli* which is associated with the lower anti-bacterial activity of haemocytes towards *V. cholera* (Canesi et al., 2005). This activation of MAPK is related to the presence of fimbriae and mannose-sensitive haemagglutinin (type IV pilus) in *E. coli* and *V. cholerae*, respectively. Moreover, a pathogenic strain of *V. harveyi* induced a retarded activation of p38-MAPK pathway, in abalone *Haliotis tuberculata* haemocytes, in compare to non-pathogenic strains, and this was also associated with the inability to induce ROI production and phagocytosis (Travers et al., 2009).

1.5. Rationale of the research, hypothesis and objectives

The development of *in vivo* and *in vitro* models of host-pathogen interactions are required to gain insight into the bivalve defense mechanisms and the pathogenicity of their diseases. Moreover, the host responses induced by bacteria have the potential to be used as early warning markers of bacterial infection.

Most studies involving bivalve responses to pathogens have used species of major economic importance, such as oysters (*Crassostrea gigas*, *C. virginica*, and *Ostrea edulis*), mussels (*Mytilus galloprovincialis*, *M. edulis*), clams (*Ruditapes philippinarum*, *R. decussatus*, and *Mercenaria mercenaria*) and more recently scallops, (*Argopecten irradians*, *C. farreri*). A proportion of these studies have focused on responses induced by protozoan parasites, (*Perkinsus*, *Haplosporidium*, *Bonamia*) and by bacteria (*V. anguillarum*, *V. tapetis*, *V. alginolyticus* and more recently *V. splendidus*). Studies on immune responses of soft-shell clams *M. arenaria* have been focused mainly on toxicological aspects caused by diverse pollutants.

Soft-shell clams are a resource of considerable economic importance to the local populations of the Canadian Maritimes and the North Atlantic coast of USA, and with a significant value as a bioindicator of environmental toxicity. Except for haemic neoplasia, other serious pathological problems affecting this species are unknown. The possible resistance to infectious agents, including bacteria, suggests that this species would possess a uniquely adapted immune system or that no significant environmental stress occurs to significantly alter its immunity. In fact, bivalve disease outbreaks commonly occur in aquaculture systems where stress associated with high animal densities, suboptimal water quality and frequent handling affect the immune system or

occur as a consequence of animal and potential pathogen movements to naïve environments.

Several strains of *Vibrio* species have been associated with disease and mortality of bivalves. Various experimental models in the literature using pathogenic and non-pathogenic strains have produced differentiated effects on bivalves depending on the host species, and the experimental conditions. Cellular and molecular responses induced by *Vibrio* species have been assessed using different techniques that reflect the many ways that the host's immune system responds in order to neutralize and eliminate the pathogen or, conversely, the ability of the pathogen to manipulate the host's immune system in order to survive and proliferate. Some of these studies include *V. splendidus* LGP32, a strain associated with mortalities of juvenile oysters, *C. gigas*, in France.

Given the environmental and economic importance of soft-shell clams *M. arenaria* and their particular absence of major pathogens it was decided to use this species to develop a model of interaction with bacterial pathogens. One of the bacterial strains chosen for this study was *V. splendidus* LGP32-GFP, derived from LGP32 that has been associated with the occurrence of "summer mortality" syndrome in juvenile oysters, *C. gigas* in France (Gay et al., 2004a, b) but possesses a green fluorescent protein as a tag that facilitates its traceability. Since the clam populations of interest for this study were mostly from the Gulf of Saint Lawrence (Canada), it was decided to also study the responses that are induced by bacteria isolated from such environment. In a previous study on antimicrobial resistance assessments in Prince Edward Island, several strains of *V. splendidus* were identified (Mateo 2006). One of these strains, 7SHRW, was chosen as an environmental strain for this study.

The general hypotheses of the study are that *in vivo* challenge of *M. arenaria* with *V. splendidus* strains LGP32-GFP and 7SHRW induce differential responses at: (1) a cellular level with changes in haemocyte structure, adherence, numbers, and distribution according to their size and complexity and to the lysosome content, (2) a molecular level with regulation of gene expression changes impacting both physiological and immune functions. The specific objectives of this thesis are:

Chapter 2 - To determine the changes in the percentage of rounded haemocytes, adherence and haemocyte numbers induced by each of the *V. splendidus* strains.

Chapter 3 - To characterize the changes in the distribution of the haemocyte subpopulations (i.e. granulocytes and hyalinocytes) according to their lysosome content induced by each of the *V. splendidus* strains.

Chapter 4 - To determine the expression of various genes, some of which are involved in immune functions, induced by each of the *V. splendidus* strains.

Chapter 5 - To determine and compare the lysozyme activity after challenge with *V. splendidus* LGP32-GFP in clams from Canada and Ireland.

Chapter 6 - To assess degranulation after exposing haemocytes to degranulating stimulants and test inhibitors of pathways involved with the activation of G proteins and assess the expression of genes directly and indirectly involved with degranulation mechanisms.

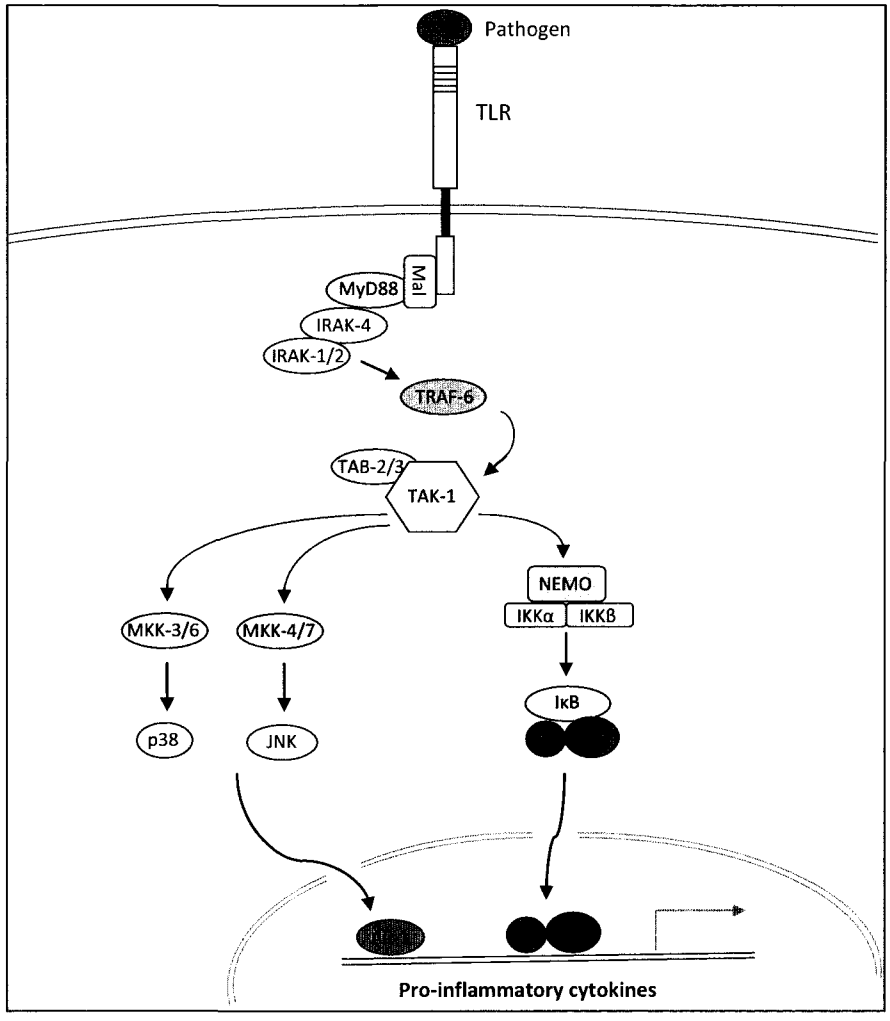

Figure 1.1. Activation of the MAPK and NF-κB signaling pathways by bacteria (after Mogensen et al., 2009). Upon PAMP stimulation, the adaptor molecules MyD88 adaptor–like protein (Mal) and MyD88 associate with the Toll-IL-1 receptor (TIR) domain of TLR inducing recruitment of interleukin-1 receptor-associate kinases (IRAK). Following phosphorylation, IRAK-4 and IRAK1/2 associates with the tumor necrosis factor receptor-associated factor-6 (TRAF-6) which in turn ubiquitinate itself, the transforming growth factor-activated protein kinase-1 (TAK-1) and IκB kinase (IKK) subunit NF-κB essential modifier (NEMO). Subsequent recruitment of the TAK-1-binding protein-2 (TAB-2) and TAB-3 to ubiquitinated TRAF-6 activate the signaling complex leading to the stimulation of separate pathways: the NF-κB (via the IKK complex) and the MAPK pathways. IKK complex activation by TAK-1 results in the phosphorylation and subsequent degradation of the inhibitory protein IκB allowing the translocation of NF-κB to the nucleus. There, it binds to κB sites of promoters and enhancers of several pro-inflammatory genes. In the MAPK pathway, TAK-1 phosphorylates the MAPK kinases MKK-3/6 and MKK-4/7 that subsequently phosphorylate and activate p38 and c-Jun N-terminal kinase (JNK), respectively. Both, p38 and JNK, translocate across the nuclear membrane and activate the transcription factor activator protein-1 (AP-1).

1.6. References

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Chapter 2. Differential *in vivo* response of soft-shell clam haemocytes against two strains of *Vibrio splendidus*: changes in cell structure, numbers and adherence

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2.1. Abstract

Host-pathogen interaction models in aquatic species are useful tools for understanding the pathogenicity of diseases in cultured and wild populations. In this study we report the differential *in vivo* response of soft-shell clam (*Mya arenaria*) haemocytes against two strains of *Vibrio splendidus*. Responses were measured 24 h after injecting into the posterior adductor muscle either an endemic environmental strain (7SHRW) or a strain associated with oyster mortalities (LGP32-GFP). Changes in haemocyte structure (percentage of rounded cells) were assessed microscopically. Changes in adherence and haemocyte numbers were analysed by flow cytometric cell counting. Increased percentages of rounded cells were found in response to both strains. However values from the group infected with LGP32-GFP were significantly higher ($p < 0.01$) than with 7SHRW. The cell adherence was markedly diminished ($p < 0.001$) by LGP32-GFP whereas 7SHRW did not change it significantly. Increased numbers of haemocytes ($p < 0.001$) were induced by LGP32-GFP, while lower increase was found after infection with 7SHRW. These results show the regulatory capacity of soft-shell clams haemocytes to perform specific responses against different strains of *V. splendidus*.

2.2. Introduction

Host-pathogen interaction models are gaining more attention in aquatic species since they are useful tools for understanding the pathogenicity of diseases in cultured and wild populations. In bivalve molluscs, haemocytes play a major role in the host defence as mediators of cellular and, indirectly, of humoral defences. Hence, host-pathogen interaction studies have focused their attention on haemocytic responses against pathogens.

Common responses attributed to bacterial challenge are changes in (total and differential) haemocyte numbers, viability, structure, adherence, phagocytosis, production of ROIs, and enzymatic activities (Nottage and Birkbeck, 1990; Allam et al., 2001, 2006; Choquet et al., 2003; Lambert et al., 2003; Allam and Ford, 2006; Allam et al., 2006; Labreuche et al., 2006a,b; Parisi et al., 2008). Although the innate immune systems of bivalves are known to be less complex and specific than the adaptive systems of vertebrates their haemocytes have the capacity to discriminate microorganisms (Bachère et al., 2004). Thus, several of these responses have been shown to be specific to certain bacterial species or strains. In soft-shell clams, however, there is no information on haemocytic responses induced by bacterial infections.

Mya arenaria is an important resource for coastal communities of North-Eastern North America with potential for aquaculture (Chevarie et al., 2005). This species has also been deemed to be a convenient bivalve for screening bacterial pathogenicity (Tubiash, 1971). Although bacterial outbreaks are rare in soft-shell clams (Kaneko et al., 1975) their vulnerability to haemic neoplasia, a common disorder that considerably affects some populations in Atlantic Canada (McGladdery et al., 2001) and the US

(Farley et al., 1991), might render them susceptible to opportunistic bacteria (Kent et al., 1989). Among the most common opportunistic bacteria are vibrios, normally found in marine and eurohaline environments (Huq and Colwell, 1995). While most *Vibrio* spp. outbreaks are associated with mortalities of bivalve larvae in hatcheries, few species are deemed to be primarily pathogenic to adult or juvenile bivalves (Paillard et al., 2004). Among them, some strains of *Vibrio splendidus* have been associated with the "summer mortality" syndrome of juvenile oysters *Crassostrea gigas* in France (Lacoste et al., 2001a; Waechter et al., 2002).

Given the importance of both, host and pathogen, we are developing a model of interaction between soft-shell clams and two strains of *V. splendidus*. In this study, we report differential changes in haemocyte structure, numbers, and adherence induced by a pathogenic and a environmental strain of *V. splendidus*.

2.3. Materials and methods

2.3.1. Experimental animals and conditioning

Wild soft-shell clams of 4 - 6 cm in size and an average weight of 21.5 ± 3.1 g, shipped from the Iles-de-la-Madeleine (Gulf of Saint Lawrence, Canada), were used in our experiments. This site was chosen due to its minimal level of pollution. The clams sampled have reduced environmental bacterial loads and undetected levels of haemic neoplasia. These factors have been shown to have detrimental effects on haemocytic parameters (Kent et al., 1989; Fournier et al., 2002; Mayrand et al., 2005; Gagnaire et al., 2007). Likewise, wild bacteria might have an agonist/antagonist effect to the experimental bacteria (Gay et al., 2004a,b).

On arrival at our facility, clams were held within 300 L tanks with re-circulating artificial sea water (Instant Ocean®). During the acclimation period (at least one week before trials were performed) the water temperature was kept at 16°C and the salinity at 30 ppt. During this period, clams were fed with an algae paste every other day.

2.3.2. Bacterial strains, growth conditions and suspension preparation

Vibrio splendidus LGP32-GFP has been associated with the occurrence of "summer mortalities" syndrome in juvenile oysters *Crassostrea gigas* in France (Gay et al., 2004a,b). To facilitate its traceability this strain has a GFP gene insertion that confers green fluorescence when excited with UV light. *V. splendidus* 7SHRW is a environmental strain isolated from sediments from Hillsborough River, Prince Edward Island (Gulf of Saint Lawrence, Canada). This strain was identified as *V. splendidus* based on conventional biochemical tests and BIOLOG automated identification. Both strains, 7SHRW and LGP32-GFP, form green colonies in Thiosulfate Citrate Bile Sucrose agar, produce catalase and oxidase, and cannot utilize citrate, neither can degrade urea and aesculin. They can produce acid from glucose but not from lactose and are able to grow at temperatures up to 28°C in blood agar. The strain 7SHRW is haemolytic and sensitive to the vibriostatic agent O/129 while LGP32-GFP is not. Although the strain 7SHRW could be identified as *V. splendidus* based on the utilization of 96 carbon sources, the strain LGP32-GFP could not.

Both strains were cultured overnight to reach exponential growth phase in Trypticase Soy Broth (TSB, BD-Bacto™) supplemented with 2% NaCl at 16°C in 250 mL Erlenmeyer flasks, shaken at 100 rpm. Bacteria were collected by centrifugation at

5000 x g and washed twice with filtered (0.22 µm) sterile seawater (FSSW). The bacterial concentration was determined by optical density measured with a spectrophotometer (2802 UV/VIS Unico) at 600nm, and adjusted to approximately 4.8 x 10^8 bacteria/mL in FSSW according to 1 OD_{600nm} = 4 x 10^8 bacteria/mL as estimated by flow cytometry cell counting.

2.3.3. Clam haemocytes pre-screening

Clams were pre-screened through microscopic observation of their haemolymph immediately before experiments. Haemolymph was extracted as described in the next section. A drop of haemolymph from each sample was placed on Snow coat X-tra™ poly-lysine-coated slides, cover slipped and placed in a humid chamber for 20 min to allow haemocytes to adhere to the glass surface. Samples were analysed with an Axio Imager A1 (Carl Zeiss) fluorescent light microscope with phase contrast (400x). The numbers of stretched (normal) and rounded haemocytes were counted in a total of five fields per sample. The percentage of rounded haemocytes per sample was estimated by calculating the average of five measurements. Samples whose percentage of rounded haemocytes was higher than 5% on average were excluded from the experiment as we previously established that higher values are related to abnormal levels of stress (data not shown). Likewise, those clams whose haemolymph showed obvious presence of bacteria were excluded to avoid interference.

2.3.4. Clam infection and haemolymph withdrawal

Approximately 4.5 x 10^6 bacteria of either strain per gram of clam, contained in 200 µL of bacterial suspension, were injected into the posterior adductor muscle. FSSW was injected into the control group clams. After injection, clams were kept out of the water for 1 h, to assure the bacterial suspension was retained prior to the clams being transferred to containers with non-circulating artificial seawater at 16°C for 24 h.

Haemolymph was withdrawn from the posterior adductor muscle of each clam with a 3 mL syringe fitted with a 25-gauge needle. For the microscopic assessment of the percentage of rounded haemocytes and the flow cytometric assessment of the number of haemocytes, haemolymph was collected 4 d before and 24 h after injection. In the former assessment one drop was extracted while in the latter assessment 300 µL were required. For the adhesion assay, 400 µL of haemolymph was withdrawn per clam 24 h after injection. In all cases, haemolymph was screened through an 80 µm mesh to avoid large particles. The haemolymph used in flow cytometric assessments was immediately placed on ice to prevent formation of aggregates of haemocytes.

2.3.5. Phase contrast and confocal microscopy

Samples were analysed as described above for the haemocyte pre-screening. Samples were also examined with a Zeiss 510 Meta laser confocal microscope to monitor internalization of phagocytosed bacteria after infection.

2.3.6. Adhesion assay

The capacity of the haemocytes to adhere after injection was estimated using a modification of the method described by Choquet et al. (2003). Briefly, a 200 µL aliquot of haemolymph was taken into a control tube and mixed with 200 µL of 6% formalin in FSSW for fixation and flow cytometric assessment. Another 200 µL sample of haemolymph was taken into a 48-well microplate and incubated at 16°C for 3 h. After incubation, 200 µL of fixative was added. The supernatant was then analysed using a FACSCalibur flow cytometer (BD Biosciences). The proportion of adhered and non-adhered cells was estimated in relation to the total number of haemocytes from the control tube.

2.3.7. Haemocyte counts

Haemocyte counts, before and after injection, were determined by using a 300 µL haemolymph sample read through a FACSCalibur flow cytometer according to gates previously established to discriminate bacteria from other particles. Readings of the number of events were obtained after 30 sec under low flow rate.

2.3.8. Statistical analysis

Statistical analysis was done using the MINITAB 15.1.0.0 statistical software. The differences of the percentages of rounded haemocytes before and after infection were tested by using the non-parametric Sign test, and the differences between groups and strains, after infection, were tested by using the Mann–Whittney non-parametric test. For the differences of the number of haemocytes a paired *t*-test and 2 sample *t*-test

was applied to the logarithmic transformed data. A paired *t*-test was used to compare values before and after infection and two sample *t*-test for comparison between groups and strains after infection. The differences of the percentages of adhered cells were tested using the one way general linear analysis of variance (ANOVA), followed by Bonferroni pairwise comparison, also after logarithmic transformation of the data. A statistical significance level of 0.05 was considered for the differences in all tests.

2.4. Results

2.4.1. Phase contrast microscopy before and after infection

Microscopic analysis revealed significant differences between the percentage of rounded haemocytes assessed 4 d before and 24 h after injection in the infected groups with both *V. splendidus* strains (Figs 2.1.a and b). When the clams were infected with the strain 7SHRW, there was a significant ($p < 0.01$), increase of the percentage of rounded haemocytes from 2.0 ± 0.7% to 24.0 ± 3.2% (Fig. 2.1.a). When the clams were infected with the strain LGP32-GFP the increase was much greater, with values from 0.6 ± 0.3% to 88.5 ± 8.0% ($p < 0.01$, Fig. 2.1.b). The percentage of rounded haemocytes induced by the strain LGP32-GFP was significantly higher ($p < 0.01$) than that induced by 7SHRW 24 h after infection. Healthy haemocytes with the ability to stretch and adhere to the substrate were dominant in control samples and most samples challenged with 7SHRW (Fig. 2.2.a), whereas there were few adhered haemocytes and a vast majority of rounded haemocytes in the samples challenged with LGP32-GFP (Fig. 2.2.b). Rounded haemocytes were diverse in size and appearance; some appeared to have their contents compacted towards one side of the cell while others looked empty.

While few bacteria could be visualized in samples challenged with the 7SHRW, all of the samples challenged with LGP32-GFP had high numbers inside and outside haemocytes 24 h after infection (Fig. 2.3).

2.4.2. Adhesion assay

There was a strong significant decrease ($p < 0.001$) in haemocyte adherence from clams infected with the LGP32-GFP in relation to the control group after 3 h of incubation (Fig. 2.4). Only 11.7 ± 4.4% of cells from the LGP32-GFP infected group could adhere to the micro-wells whereas 96 ± 0.8% of cells from the control group were adhered. The haemocyte adherence from the clams injected with 7SHRW did not change significantly (85.2 ± 4.1%, $p > 0.05$) with respect to the control group and was significantly higher ($p < 0.001$) than the group infected with LGP32-GFP (Fig. 2.4).

2.4.3. Haemocyte counts before and after infection

There were strong significant differences between the number of haemocytes assessed 4 d before and 24 h after injection in the infected group with *V. splendidus* strain LGP32-GFP ($p < 0.001$), whereas with 7SHRW there was lower, although significant, increase found ($p > 0.05$, Figs. 2.5.a and b). When clams were infected with the strain 7SHRW there was a relatively small increase in the number of haemocytes from 3.6 ± 0.7 x 10^5 to 8.0 ± 1.5 x 10^5 (Fig. 2.5.a). Clams infected with the strain LGP32-GFP had a very significant increase, with values from 2.7 ± 2.5 x 10^5 to 1.9 ± 0.1 x 10^7 (Fig. 2.5.b). The difference in the number of haemocytes between infected

groups of each strain 24 h after infection was also significant ($p < 0.001$, Figs. 2.5.a and b).

2.5. Discussion

Given the economic potential of soft-shell clams and the importance of vibrios as common inhabitants of aquatic animals, also associated with disease outbreaks, we have assessed phenotypic immune responses of *M. arenaria* haemocytes induced by *in vivo* challenges with two strains of *V. splendidus*. The magnitude of haemocytic responses against bacterial infections is usually associated with the type of bacteria, the bacteria-haemocyte ratio and the time of exposure. These factors are controllable *in vitro* but under *in vivo* conditions some parameters are obviously not manageable. An estimation of the bacteria-haemocyte ratio would be unrealistic because not all haemocytes are evenly circulating in haemolymphatic vessels. A portion of haemocytes is distributed within the tissues throughout the body (Bachère et al., 2004). Normalization of the bacterial concentration according to the clam weight was the approach that was therefore chosen to standardize the model assuming that the total number of haemocytes positively correlates to the mass/weight of the individual.

The outcome of bacterial infections in bivalves is usually dose dependant (Allam and Ford, 2006). In this study, we chose to measure the haemocytic responses using a concentration of 4.5×10^6 bacteria/g of clam after 24 h because with these conditions there is a clear difference in the responses among groups and results have high reproducibility. Based on our observations, lower concentrations of LGP32-GFP were

cleared by the host while higher concentrations killed the clams before 24 h with both outcomes being highly variable.

Soft-shell clam haemocytes demonstrated multiple phenotypic changes in response to the bacterial challenges with *V. splendidus*. Changes in haemocyte structure, numbers and adherence were all regulated in response to two different strains of *V. splendidus*. In fact, the bivalve immune system is known to have a discriminative capacity towards microorganisms (Bachère et al., 2004). This specificity against different vibrios has been previously reported in studies assessing a variety of phenotypic responses *in vitro* (i.e. viability, adherence, ROIs) (Nottage and Birkbeck, 1990; Choquet et al., 2003; Lambert et al., 2003) and *in vivo* (i.e. mortality, kinetics of bacterial clearance) (Gay et al., 2004a; Parisi et al., 2008). It is believed that the high specificity shown in bivalves is related to the bacterial ability to activate or deactivate cell signaling cascades, such as the mitogen-activated protein kinase (MAPK) signaling pathway (Pruzzo et al., 2005).

One of the initial and obvious responses in bivalve haemocytes are cytoskeletal changes that eventually produce rounded cells. Rounding of haemocytes induced by vibrios has been observed in other bivalves after *in vitro* challenges (Lane and Birkbeck, 1999, 2000; Choquet et al., 2003; Allam and Ford, 2006; Labreuche et al., 2006a). In the current study, we observed a drastic increase in the proportion of rounded haemocytes 24 h after challenge (from 0.6 ± 0.3% to 88.5 ± 8.0%, $p < 0.01$) with the strain LGP32-GFP whereas the increase with the strain 7SHRW, although significant (from 2.0 ± 0.7 to 24.0 ± 3.2%, $p < 0.01$), was less striking (Figs. 2.1.a and b) and significantly lower than with LGP32-GFP ($p < 0.01$). The specific mechanism of bacterial induced cell

rounding has been associated with the presence of surface components on the bacterium or by excreted products, i.e. toxins (Lane and Birkbeck, 1999). It has been recently demonstrated that a metalloprotease, called Vsm, is the most important factor of toxicity in extracellular products (ECPs) from *V. splendidus* LGP32 (Binesse et al., 2008). This toxin has cytopathic effects in mammal and mollusc cell lines causing cell rounding. In fact, muscular lesions in oysters *C. gigas*, including rounded fibres, have previously been associated with a possible toxic effect of the same strain of *V. splendidus* (Gay et al., 2004b). In *Vibrio tapetis*, a thermosensitive protease was suggested to be associated with changes in structure, phagocytic activity and viability of *Ruditapes philippinarum* haemocytes (Allam and Ford, 2006).

The effect of cell rounding has been previously seen in human pathogenic vibrios. Toxins such as $MARTX_{Vc}$ from *V. cholerae,* which are known to covalently cross-link G-actin and depolymerise F-actin, have been causally implicated (Fullner and Mekalanos, 2000; Kudryashov et al., 2008). It is probable that the differential effect of the two strains of *V. splendidus* for provoking haemocyte rounding in *M. arenaria* is due to the secretion of the toxin Vsm, produced by LGP32-GFP, which would be absent in 7SHRW. These differences in virulence, and other phenotypic features, among *V. splendidus* strains are not uncommon and are attributed to the extremely high genetic diversity of this species (Le Roux et al., 2009). Further studies are needed to investigate the effects of ECPs on haemocytes.

The adherence of haemocytes was shown to vary between groups. It slightly decreased from 96 ± 0.8%, in the control group, to 85.2 ± 4.1%, in the group infected with 7SHRW, to a significant decrease in the group infected with LGP32-GFP (11.7 ±

4.4%, $p < 0.001$). Significant cytotoxic effects, expressed as non-adherent ratios, against haemocytes of *R. philippinarum* and, to a lesser extent, of *C. gigas* were caused by several strains of *V. tapetis* (Choquet et al., 2003). It is interesting to mention that Choquet et al. (2003) also tested a strain of *V. splendidus* (ATCC 25914) associated with oyster "summer mortalities" syndrome but surprisingly it induced the lowest non-adherent ratios in *C. gigas* haemocytes. Labreuche et al. (2006a,b) observed a progressive decrease in haemocyte adherence in oysters *C. gigas* after infection with *Vibrio aestuarianus* 01/32 and their ECPs. The results of our adhesion assay are consistent with the rounded cell phenotype. This suggests that the rounded cells indeed lose their ability to adhere, and thus impairs the defense capacity against pathogens. It was previously suggested that rounded haemocytes, induced by haemic neoplasia, have a diminished capacity to phagocytise pathogens (Kent et al., 1989). However, it is noteworthy that several rounded cells observed after infection contained *V. splendidus* LGP32-GFP (tracked by their green fluorescence) within their cytoplasm (Fig. 2.2). While the implication of this observation is currently unclear, further characterization of this phenomenon would be required to determine whether rounding occurred after bacteria were phagocytised.

Usually, the strongest haemocytic responses *in vitro* are against bacteria isolated from diseased hosts of the same species with the source animals (Lane and Birkbeck, 2000). Since primary bacterial infections are unknown in soft-shell clams, we used *V. splendidus* LGP32-GFP which was originally isolated from a case of "summer mortality" syndrome in juvenile oysters *C. gigas* (Waechter et al., 2002; Gay et al., 2004a,b). Nevertheless, this strain produced a cytotoxic effect on soft-shell clam

haemocytes, at a concentration that a environmental strain (7SHRW) did not cause significant damage. It is notable that Manila clams, *R. philippinarum*, are also highly susceptible to *V. splendidus*-related strains isolated from oysters (Le Roux et al., 2002; Gay et al., 2004a). It is possible that haemocytes of Pacific oysters and some clams share receptors for the same toxin produced by *V. splendidus* LGP32-GFP. It is also believed that the particular genetic diversity of *V. splendidus*, which is reflected by their wide distribution, may be associated with a wide host range susceptibility (Gay et al., 2004a). Other vibrios known to be pathogenic for certain bivalves but also able to affect haemocytes from bivalves belonging to a different genus or species have been reported in the literature (Lane and Birkbeck, 1999, 2000; Lambert et al., 2003).

Haemocyte cell counts in bivalves may increase on exposure to stress (Malagoli et al., 2007), toxins (Jones et al., 1995), pollutants (Fournier et al., 2002), pathogenic insults (reviewed by Ford and Tripp, 1996; Bachère et al., 2004), among other factors (reviewed by Oliver and Fisher, 1999). In mammals, the increase in the number of circulating leukocytes, known as leukocytosis, is a consequence of the movement of cells from the bone marrow to the circulatory pool, usually accompanied by increased haematopoiesis (Opdenakker et al., 1998). This occurs in response to infection, inflammation and some diseases and disorders, and it is mediated by cytokines and proteases (Opdenakker et al., 1998). In bivalves, due to the nature of their haemolymph open circulation, there is a constant flux of haemocytes from the circulatory vessels to the sinuses within tissues (Cheng, 1981). In response to infections, chemotactic molecules induce temporal unidirectional migration of haemocytes from one compartment to another resulting in increased numbers of haemocytes, known as

haemocytosis (Feng, 1988). However, this is not necessarily equivalent to the mammalian leukocytosis since the involvement of concurrent haematopoiesis in bivalves has not been demonstrated (Cheng, 1981). Increased numbers of haemocytes seem to be an attempt of the host to return to homeostasis following perturbations or to respond to infections more effectively (with or without success). During bacterial infections these variations may be acute or delayed depending upon the host (Suresh and Mohandas, 1990) and/or upon the antigenic character of the bacteria. In our experiments we found that 24 h after challenge *V. splendidus* LGP32-GFP induced a very significant increase ($p < 0.001$) in haemocyte counts of *M. arenaria* while the environmental strain 7SHRW induced a lower increase, although also significant ($p < 0.05$), when compared to the clams before infection (Figs. 2.5.a and b). It is tempting to think that such marked increase in total haemocytes is the result of an active process of haematopoiesis and proliferation induced by LGP32-GFP infection. Although sham injections with saline have also been reported to induce increased haemocytic numbers (Suresh and Mohandas, 1990), we did not find significant differences between the number of haemocytes before and after injection with FSSW in the control group.

Suresh and Mohandas (1990) found a significant increase of haemocyte counts after challenging clams *Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis* with *Vibrio alginolyticus*. The increase was noticed as early as 6 h in the former host species and only at 48 h in the latter. Oubella et al. (1993) found a significant increase of haemocyte counts when clams *R. philippinarum* and *R decussatus* where injected with *Vibrio* P1 (subsequently identified as *V. tapetis*), the causative agent of brown ring disease in clams. Although *R. decussatus* is less sensitive to this bacterial species, both

host species experienced an increase of haemocyte numbers as early as 24 h after infection and were significantly higher than controls at 72 h after infection. Allam et al. (2001) also found significant increases in total number of haemocytes in clams, *R. philippinarum*, from the USA and France, and *R. decussatus* that were symptomatic for brown ring disease, 4 weeks after infection with *V. tapetis*. Labreuche et al. (2006b) found significantly increased numbers of total haemocyte counts 3 d after infecting oysters (*C. gigas*) with *V. aestuarianus* 01/32, a strain associated with mortalities of *C. gigas* in hatcheries. Parisi et al. (2008) noticed an initial decrease of total haemocytic counts in mussels *M. galloprovincialis* a few hours after challenge with *V. splendidus* followed by an increase 24 h after infection. Similar, although milder, kinetics were found in response to *V. anguillarum*. Allam et al. (2006) also found increased numbers of total haemocytes in *R. philippinarum* and *Mercenaria mercenaria* 3 d following injection with *V. tapetis*, although changes were not found in *R. decussatus* and *C. virginica*. By studying the kinetics of the haemocyte responses in terms of total haemocytic numbers these authors compared the specificity of *R. philippinarum* haemocytes against three bacterial species. Increased cell numbers were significant at 6 h after injection with *V. tapetis* and reached their maximum number (5.3 x 10^6 cells/mL) after 72 h, whereas the maximum response to *V. anguillarum* was reached at 6 h (4.2 x 10^6 cells/mL) and at 24 h after infection for the non-Vibrionaceae R2.

Since our measurements were obtained at only one time point after challenge there is no information about the kinetics of the cell numbers during early infection. Very few bacteria were observed in the haemolymph 24 h *post*-infection with 7SHRW. It is possible that the number of haemocytes in those clams did increase at an earlier

point and were returning to normal levels following effective clearance of bacteria. Conversely, even when the number of haemocytes increased after infection with LGP32-GFP, the clams appeared moribund and most would succumb to infections after 24 h. Similarly, during some protozoan infections in bivalves increased haemocyte counts are not always accompanied by an effective immune response (Oliver and Fisher, 1999). In those cases the pathogen has the ability to inhibit the oxidative killing mechanism inside phagosomes (Ford and Tripp, 1996). This is not likely the case in our study since at lower concentrations LGP32-GFP was effectively cleared by the clams (data not shown). Possibly, at high concentrations LGP32-GFP, and/or their products, inhibits phagocytic ability and also impairs other basic haemocyte functions. Alternatively, in an equivalent manner that acute sepsis does in mammals (Bone, 1991), uncontrolled amounts of degranulation products (i.e. nitric oxide, ROIs, hydrolytic enzymes) and mediators of inflammation (i.e. cytokines and chemokines), triggered by such bacterial insult, would cause host tissue damage.

In conclusion, specific phenotypic responses of the soft-shell clam *M. arenaria* haemocytes against two strains of *V. splendidus* were characterized by changes in their structure, numbers and adherence. These changes were regulated in response to these strains. Responses were severe after infection with LGP32-GFP. Further studies are needed to confirm the production of cytotoxic products by LGP32-GFP and the molecular mechanisms underlying the observed haemocytic changes.

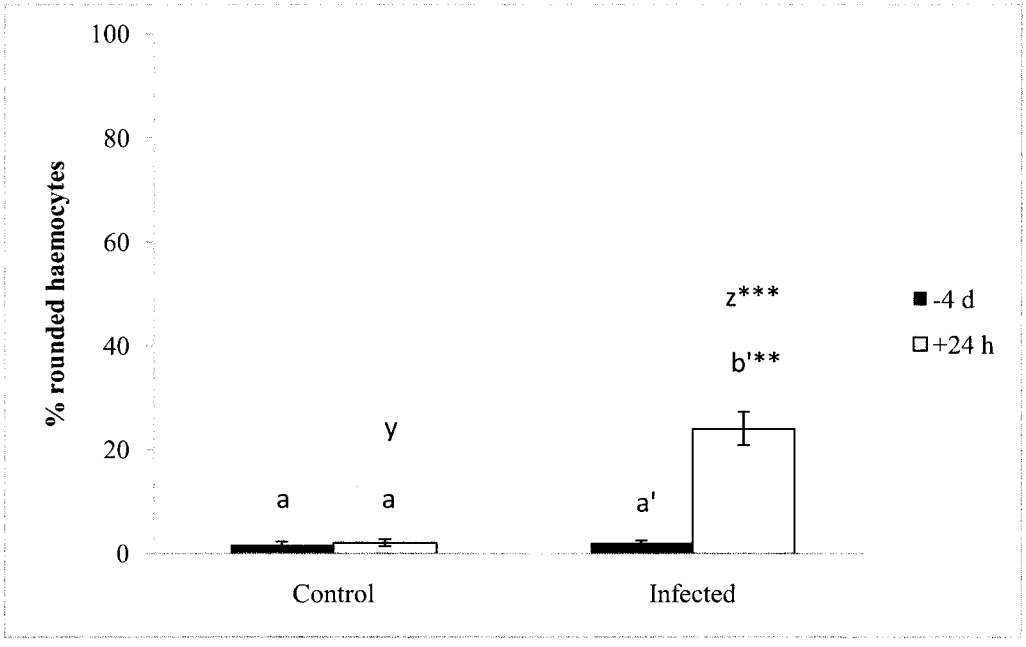

Figure 2.1.a. Percentage of rounded haemocytes of the soft-shell clam *M. arenaria* 4 d before and 24 h after injection with *V. splendidus* strain 7SHRW (n = 36). Each bar represents the mean ± standard error. Letters show statistical equivalence (same letters) or difference (different letters) between assessments obtained before and after each treatment (a, a',b') or between treatments (y, z). Asterisks indicate the levels of significant differences (**: $p < 0.01$; ***: $p < 0.001$).

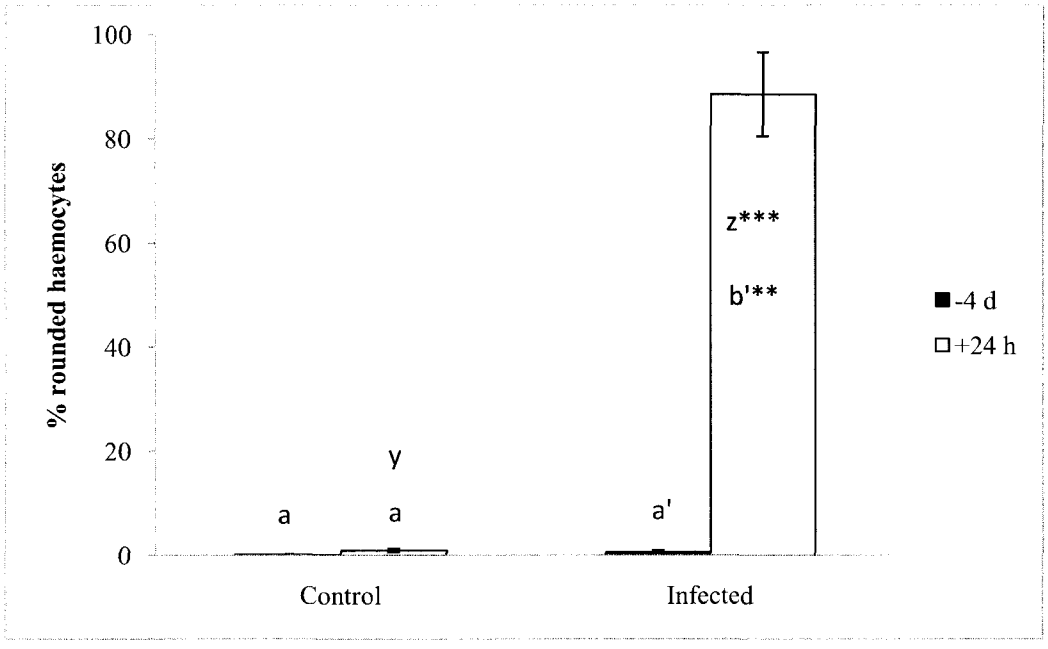

Figure 2.1.b. Percentage of rounded haemocytes of the soft-shell clam *M. arenaria* 4 d before and 24 h after injection with *V. splendidus* strain LGP32-GFP (n = 36). Each bar represents the mean ± standard error. Letters show statistical equivalence (same letters) or difference (different letters) between assessments obtained before and after each treatment (a, a',b') or between treatments (y, z). Asterisks indicate the levels of significant differences (**: $p < 0.01$; ***: $p < 0.001$).

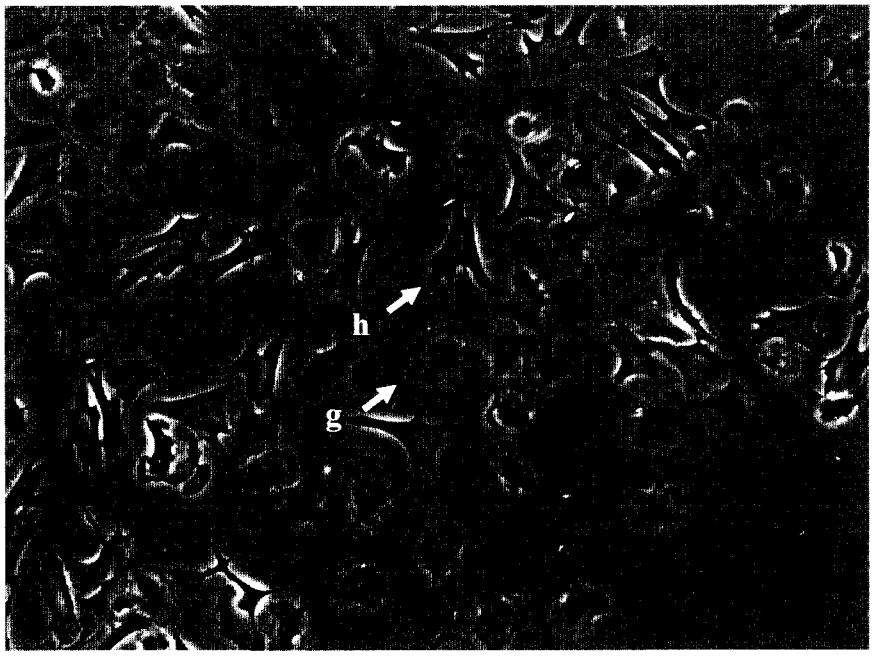

Figure 2.2.a. Healthy and stretched haemocytes from *M. arenaria* 24 h after injection with FSSW (h: hyalinocytes; g: granulocyte). Haemocytes were left for 20 min on glass slides (400x).

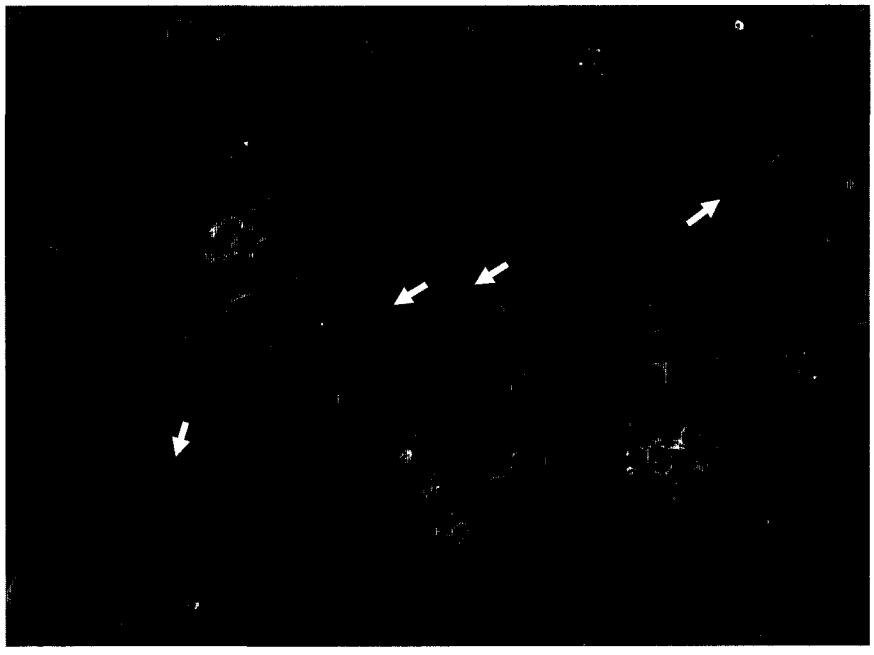

Figure 2.2.b. Rounded haemocytes of *M. arenaria* 24 h after injection with *V. splendidus* LGP32-GFP (Arrows - notice different sizes and cell content). Haemocytes were left for 20 min on glass slides (400x).

Figure 2.3. Confocal microscopy of the soft-shell clam *M. arenaria* haemocytes showing bacteria *V. splendidus* LGP32-GFP outside and inside the cells (arrow - green fluorescent bacteria) (630x).

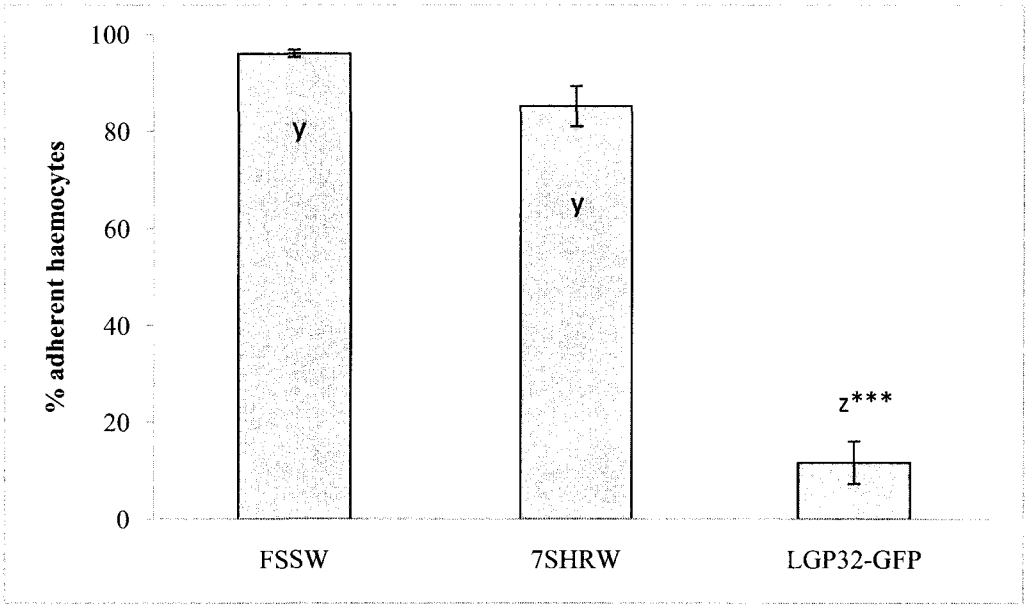

Figure 2.4. Percentage of adherent haemocytes of *M. arenaria* challenged with FSSW, *V. splendidus* 7SHRW and LGP32-GFP 3h after incubation in microplates at 16°C (n = 18). Each bar represents the mean ± standard error. Letters show statistical equivalence (same letters) or difference (different letters) between treatments (y, z). Asterisks indicate the level of significant difference (***: $p < 0.001$).

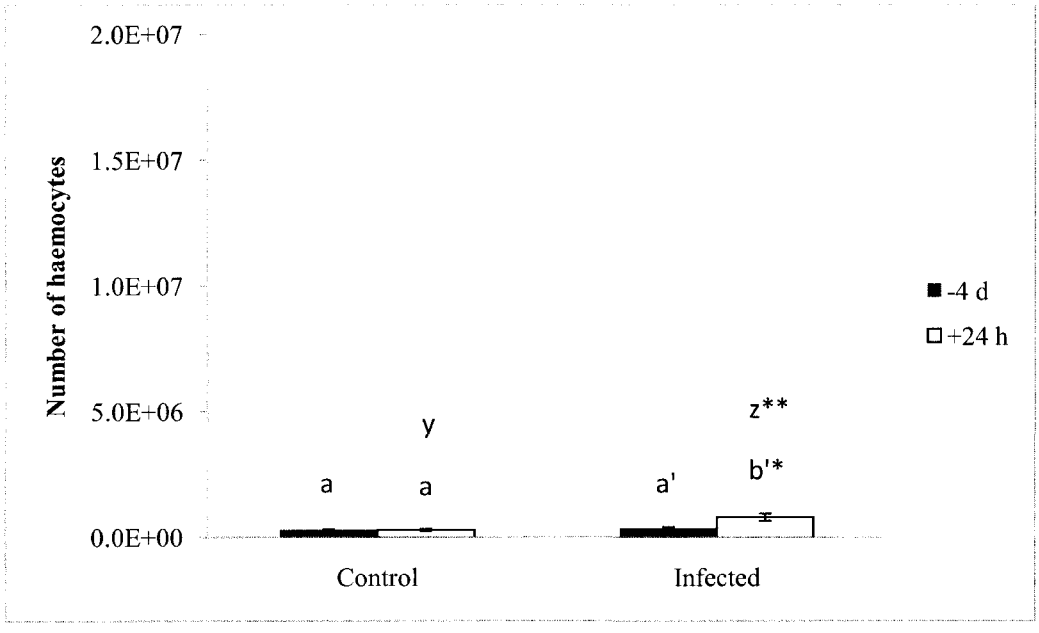

Figure 2.5.a. Number of haemocytes of the soft-shell clam *M. arenaria* 4 d before and 24 h after injection with *V. splendidus* strain 7SHRW (n = 36). Each bar represents the mean ± standard error. Letters show statistical equivalence (same letters) or difference (different letters) between assessments obtained before and after each treatment (a, a', b') or between treatments (y, z). Asterisks indicate the levels of significant differences (*: $p < 0.05$; **: $p < 0.01$).

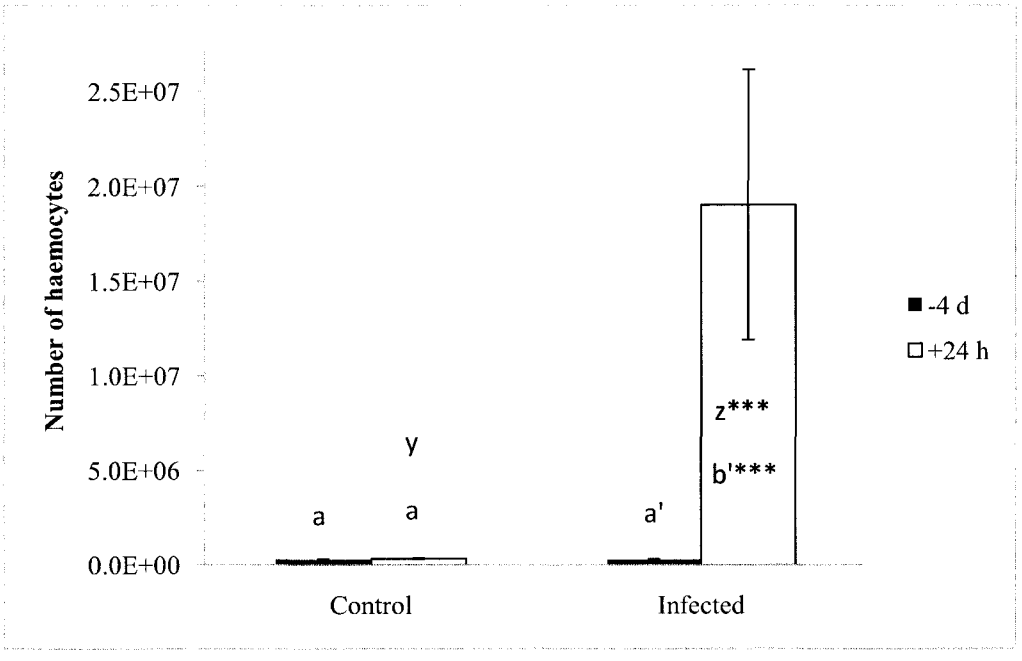

Figure 2.5.b. Number of haemocytes of the soft-shell clam *M. arenaria* 4 d before and 24 h after injection with *V. splendidus* strain LGP32-GFP (n = 36). Each bar represents the mean ± standard error. Letters show statistical equivalence (same letters) or difference (different letters) between assessments obtained before and after each treatment (a, a', b') or between treatments (y, z). Asterisks indicate the levels of significant differences (***: $p \leq 0.001$).

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Chapter 3. Changes induced by two strains of *Vibrio splendidus* in haemocyte subpopulations of *Mya arenaria*, detected by flow cytometry with LysoTracker

Mateo D.R., Spurmanis A., Siah A., Araya M.T., Kulka M., Berthe F.C.J., Johnson G.R., Greenwood S.J. (2009) Changes induced by two strains of *Vibrio splendidus* in haemocyte subpopulations of *Mya arenaria*, detected by flow cytometry with LysoTracker. Diseases of Aquatic Organisms 86: 253-262.

3.1. Abstract

Flow cytometric characterization of bivalve haemocytes is usually performed by light scatter profiles based on size and complexity of the cells. Additional means of characterization such as specific fluorescent dyes are not commonly used to discriminate cell subpopulations in challenged and unchallenged haemocytes. In this study, we characterize the changes of haemocyte subpopulations of soft-shell clam (*Mya arenaria*) induced by *in vivo* challenge with 2 strains of *Vibrio splendidus* by using a fluorescent probe. Responses were measured 24 h after infection with either an endemic environmental strain (7SHRW) or a strain associated with oyster mortalities (LGP32). Changes in haemocyte subpopulations were analysed through flow cytometry based on two-parameter scatter profiles and lysosomal content reflected by LysoTracker staining. Forward and side scatter profiles revealed 2 haemocyte subpopulations: hyalinocytes and granulocytes. Granulocytes exhibited significantly higher levels of lysosomal staining ($p < 0.01$). Following infection with LGP32-GFP, both subpopulations merged into a single continuous group and their lysosomal content significantly decreased ($p < 0.05$). Independent modifications after infection were observed in the proportions of subpopulations established by their (low or high) lysosomal content. While the subpopulations of hyalinocytes had lower levels of lysosomal content after infection, especially with LGP32-GFP ($p < 0.001$), the subpopulations of granulocytes had similar levels of lysosomes after infection with 7SHRW and significantly decreased after infection with LGP32-GFP ($p = 0.001$). Our data suggest specific modulation of bivalve responses against pathogenic bacteria that would include degranulation.

3.2. Introduction

Haemocytic ontogeny and typing are undefined aspects of haemocytic characterization in bivalve molluscs. In the absence of well-defined immunophenotypic markers comparable to those typically employed to study analogous mammalian peripheral blood leukocytes, the characterization of haemocytic subpopulations in bivalves relies upon morphological features including cell size, complexity or granularity, or functional characteristics including phagocytosis and oxidative burst (Huffman & Tripp, 1982; Cajaraville & Pal, 1995; López et al., 1997; Pipe at al., 1997; Cima et al., 2000; Chang et al., 2005; Aladaileh et al., 2007; Zhang et al., 2006; García-García et al., 2008). Interspecies variability and methodological differences in specimen collection and processing have been considered as the principal reasons for the current lack of consensus with regard to the actual number of distinct haemocytic subpopulations that can be discerned morphologically (reviewed in Cheng, 1981, 1984; Auffret, 1988; Hine, 1999). There is general agreement, however, that bivalve haemocytes can readily be classified into at least two morphologically distinct groups, namely the granulocytes, which tend to be larger and contain cytoplasmic granules, and hyalinocytes (or agranulocytes), which tend to be smaller and contain fewer or no granules.

The cytoplasmic granules that characterize granulocytes are mostly lysosomes, membrane-bound organelles containing hydrolytic enzymes at low pH (Luzio et al., 2000). In bivalves, haemocyte lysosomes are known to be involved in the intracellular degradation of digestible material and the release of hydrolytic enzymes during phagocytosis in response to infection (reviewed in Cheng, 1983). Since their

membranes are susceptible to be destabilized by different stressors, this feature has been frequently used as a biomarker to monitor pollution and animal health (reviewed in Moore et al., 2004, 2006).

Since the introduction of flow cytometry to the study of bivalve haemocytes, this tool has gained gradual acceptance due to the practical use and avoidance of subjectivity in comparison with the traditional methods of cell characterization (Fisher and Ford, 1988; Ashton-Alcox et al., 2000). The application of light scatter profiling has been used to differentiate bivalve haemocyte subpopulations (Ashton-Alcox and Ford, 1998; Allam et al., 2002; García-García et al., 2008) and to monitor changes in several immune indicators after bacterial challenges (Allam et al., 2001; Choquet et al., 2003; Lambert et al., 2003; Allam et al., 2006; Allam and Ford, 2006; Labreuche et al., 2006). In addition, haemocyte characterization by using fluorescent dyes with affinity to cellular organelles and monoclonal antibodies for specific cellular types conjugated with fluorescent dyes have also been used in combination to light scatter profiles (Renault et al., 2001; Tu et al., 2007).

In soft-shell clams, granulocytes have been successfully discriminated from agranulocytes based on both light microscopic (Huffman and Tripp, 1982) and flow cytometric analyses (Brousseau et al., 2000; Fournier et al., 2001, 2002). Recently, using flow cytometric analysis, we reported changes in cell numbers and adhesion of *M. arenaria* haemocytes infected with 2 strains of *V. splendidus* (Mateo et al., 2009). A shift in the distribution of Manila clams, *Ruditapes philippinarum*, granulocytes after *in vitro* challenge with *V. tapetis* has been previously reported using two parameter scatter

profiles (Allam and Ford, 2006). Whether bacterial challenge induces changes in the distribution of haemocyte subpopulations of *M. arenaria* is still unknown.

Given its acidic tropism, the commercially available probe LysoTracker has been used to detect lysosomes in studies of human cells (Haller et al., 1996; Via et al., 1998; Blander and Medzhitov, 2004). Here, we report the changes in the distribution of haemocyte subpopulations of *M. arenaria* induced by *in vivo* challenges with two strains of *V. splendidus* using LysoTracker Red. By using this method we supplement light scatter characterization of subpopulations of soft-shell clam haemocytes with profiles of cellular lysosomal content.

3.3. Material and methods

3.3.1. Clams

Wild soft-shell clams (approximately 4-5 cm in length and 17 g in weight), shipped from the Centre Maricole des Iles-de-la-Madeleine, CEMIM (Gulf of St Lawrence, Canada), were used as they are exposed to minimal levels of pollution. Clams were held within 300 L tanks with re-circulating synthetic sea water (Instant Ocean®, Aquarium Systems, Sarreburg, France). Throughout the acclimation period clams were fed with Spat Formula (Innovative Aquaculture Products Ltd., Skerry Bay, Canada) every other day, and the water temperature was kept at 16°C and the salinity at 30 ppt.

3.3.2. Bacteria

Vibrio splendidus LGP32 is a strain associated with mortalities in juvenile oysters *Crassostrea gigas* in France (Gay et al. 2004a, b), and we used a modified strain

(LGP32-GFP) which has a green fluorescence protein (GFP) gene insertion that confers fluorescence through UV light. *V. splendidus* 7SHRW (GenBank accession no. FJ610758) is an environmental strain isolated from sediments from Hillsborough River, Prince Edward Island (Gulf of Saint Lawrence, Canada) (Mateo, 2006). The identification of this strain as *V. splendidus* was based on the combination of conventional biochemical tests, BIOLOG automated identification and determination of its 16S rDNA sequence which possesses 98% similarity to the 16S rDNA on chromosome 1 of LGP32 (GenBank accession number FM954972).

Bacterial exponential growth was achieved overnight in Trypticase Soy Broth (TSB, BD-Bacto™) supplemented with 2% NaCl at 16°C. Bacteria were suspended in filtered (0.22 µm) sterile seawater (FSSW) after 2 steps of centrifugation (5000 x g, 16°C, 10 min) and rinse. The bacterial concentration was adjusted to approximately 3.8 x 10^8 bacteria/mL in FSSW according to 1 OD_{600nm} = 4 x 10^8 bacteria/mL as estimated by flow cytometry cell counting.

3.3.3. Clam pre-screening and inoculation

In order to avoid samples from unhealthy or stressed clams, haemolymph was pre-screened through microscopic observation immediately before experiments as previously described (Mateo et al., 2009). Briefly, a drop of haemolymph from each clam was placed on a slide for over 15 min to allow healthy haemocytes to adhere and stretch onto the glass surface. An Axio Imager A1 (Carl Zeiss) light-fluorescent microscope with phase contrast (400x) was used to detect bacteria and assess the percentage of rounded haemocytes estimated by calculating the average count from 5

different fields. A threshold of > 5% and/or obvious presence of bacteria were used to exclude unfit animals.

A total of 21 clams were injected into the posterior adductor muscle with 200 µL of either FSSW or bacterial suspension, containing approximately 4.5 x 10^6 bacteria per gram of clam. To assure the bacterial suspension was retained, clams were kept out of the water for 1 h after injection before transferring them to containers with non-circulating artificial seawater at 16°C.

Twenty four hours after infection, haemolymph was withdrawn from the posterior adductor muscle of each clam with a 3 mL syringe fitted with a 25-gauge needle containing 200 µL of anti-aggregate Alsever's solution (Sigma). From each clam an aliquot of 400 µL of haemolymph was collected and screened through an 80 µm mesh to avoid large particles, and immediately placed on ice to prevent formation of haemocyte aggregates.

3.3.4. Morphological profiling

For the analysis of haemocyte subpopulations, 400 µL haemolymph samples were treated with LysoTracker™ Red (Invitrogen-Molecular Probes), a fluorescent probe that stains lysosomes (Fig. 3.1). A working solution of LysoTracker was prepared by diluting the stock solution 1:1000 in phosphate buffered saline (PBS) supplemented with 2% NaCl and added to 400 µL haemolymph samples at a ratio of 1:20. Samples were incubated on ice and in the darkness for 2 h, to allow adequate staining prior to analysis in a FACSaria cell sorter (BD Biosciences). PE-Texas Red channel (600-620 nm) was used for LysoTracker detection. Excitation was performed with a blue laser

(488nm) and the detector PMT voltage was adjusted so that unstained cells appeared in the first decade (i.e. relative fluorescence < 300). The distribution of haemocytes was characterized according to their size and complexity (dependant on the presence of granules and organelles) using two-parameter scatter profiling: side scatter (SS) *vs.* forward scatter (FS). Arbitrary gating was drawn around distinct subpopulations of cells that were readily discernible in healthy specimens. The intensity of fluorescence emitted by the LysoTracker was simultaneously measured in a separate fluorescence channel from each gate both before and after infection.

Haemocytes from control and LGP32-GFP infected clams belonging to each of the subpopulations established by combining the light scatter and the LysoTracker staining profiles were physically sorted and immediately fixed with 6% formalin (prepared with FSSW) for observation by light-fluorescent microscopy under the rhodamine filter. Images were obtained by using an Axio Cam imaging system.

3.3.5. Statistical analysis

Statistical analysis was performed using the MINITAB 15.1.0.0 statistical software. The differences in fluorescence intensity among haemocyte subpopulations were tested using the one way general linear analysis of variance (ANOVA), followed by Bonferroni pairwise comparison, to the base 10 logarithm transformed data. The differences of the proportions of haemocytic subpopulations were also tested using ANOVA and Boferroni pairwise comparison of the arcsine of the square root transformed data. When normal distribution was not achieved, the Kruskal-Wallis non parametric model was applied followed by Mann-Whitney test for pairwise

comparisons. A statistical significance level of 0.05 was considered for the differences in all tests.

3.4. Results

Forward vs. side scatter profiles revealed that healthy *M. arenaria* haemocytes are distributed into two discernable subpopulations: one group (69.1 ± 3.1%) composed of larger and more complex cells, considered to be granulocytes, and another group (30.9 ± 3.1%) composed of smaller and less complex cells, considered to be hyalinocytes or agranulocytes (Fig. 3.2 and 3.3.a). After infection with 7SHRW, the latter subpopulation in the scatter profile became less discernible (Fig. 3.3.b), while with LGP32-GFP both subpopulations appear to coalesce into a single continuous group (Fig. 3.3.c). When the arbitrary gates established for healthy clam haemocyte subpopulations were maintained, the proportions of these two subpopulations did not change significantly after infection with either strain ($p > 0.05$, Fig. 3.2 and 3.3).

Comparison of the median of fluorescent intensity values of LysoTracker showed that in control clams the subpopulation of granulocytes significantly exhibited more lysosomal staining than hyalinocytes ($p < 0.01$) (Fig. 3.4). A similar tendency was found after infection with 7SHRW ($p < 0.001$) and with LGP32-GFP ($p < 0.001$). However, the median fluorescence intensity revealed that the lysosomal staining in both haemocyte subpopulations in LGP32-GFP infected clams was significantly lower than those from control ($p < 0.01$ for granulocytes and $p < 0.001$ for hyalinocytes) and from 7SHRW infected clams ($p < 0.001$ for granulocytes and $p = 0.001$ for hyalinocytes) (Fig. 3.4).

When each of these two haemocyte subpopulations was further subdivided according to the lysosomal content (herein defined by the relative amount of LysoTracker fluorescence observed) into low and high groups (Figs. 3.5.a and 3.5.b), the proportions among groups significantly changed after infection, in both hyalinocytes ($p < 0.001$) and granulocytes ($p = 0.001$) (Figs. 3.6.a and 3.6.b). Among the subpopulation of hyalinocytes, the proportion of cells with high lysosomal content significantly decrease from 85.3 ± 2.2%, in the control clams, to 69.4 ± 4.7%, in the 7SHRW infected clams, and to 21.1 ± 5.4% in the LGP32-GFP infected clams (Fig. 3.6.a). Among the subpopulation of granulocytes, the proportion of cells with high lysosomal content varied little between the control and the 7SHRW infected groups, both having more than 80% of cells with high lysosomal content (87.8 ± 0.4% and 86.6 ± 1.8%, respectively) and less than 15% of cells with low lysosomal content (12.2 ± 0.4% and 13.4 ± 1.8%, respectively). These proportions, however, were somewhat inversed when infected with LGP32-GFP having 60.2 ± 10.6 % of cells with low lysosomal content and 39.8 ± 10.6 % of cells with high lysosomal content (Fig. 3.6.b).

The microscopic observation of the sorted cells belonging to each of the four subpopulations established by the two-scatter and LysoTracker staining profiles revealed cells of different sizes and cytoplasm content (Fig. 3.7). Hyalinocytes have a diameter of approximately 5-7 μm (Fig. 3.7.a, b, e, f) whereas in granulocytes it was around 9-13 μm (Fig. 3.7.c, d, g, h). Haemocytes sorted from the high LysoTracker staining subpopulation (Fig. 3.7.b, d, f, h) appeared to have a higher granularity when compared against those with low staining (Fig. 3.7.a, c, e, g). To some extent, the cytoplasm content of the control haemocytes appeared to be homogeneously distributed

while in LGP32-GFP infected cells it appear localized towards one side of the cell and the nucleus was often indistinguishable.

3.5. Discussion

Flow cytometry is a useful technique for the characterization of haemocyte subpopulations in bivalves. Analyses using these methods are mostly based on 2 side scatter profiles that delineate subpopulations according to cell size and complexity. Additional fluorescent dyes can be used to reveal complementary information of cellular components for a more complete characterization. In this study, the use of LysoTracker, as an indicator of lysosomal content, in addition to the light scatter profiles, revealed different haemocyte subpopulations in *M. arenaria*. These subpopulations were shown to experience changes in proportions that suggest interesting cellular processes induced by 2 strains of *V. splendidus*, an environmental and a strain associated with oyster mortalities.

Through the flow cytometry scatter profiles (forward and side-scatter) we observed two discernible subpopulations of haemocytes in *M. arenaria*: a discrete subpopulation of smaller and less complex cells and another, more dispersed, subpopulation of larger and more complex cells. We consider that the former subpopulation is composed of hyalinocytes and/or agranulocytes (with no or few cytoplasmic granules) and the latter subpopulation of granulocytes. This latter subpopulation might include what others have classified as small and large granulocytes (reviewed in Cheng, 1981). Previously, using flow cytometric toxicological studies of

M. arenaria haemocytes, two discernible subpopulations using two-scatter profiles were also noticed (Brousseau et al., 2000; Fournier et al., 2001, 2002).

Prior reports involving flow cytometric light scatter profiling have revealed that haemocytes from a number of bivalve species can typically be classified into 2, 3 or 4 subpopulations. Haemocytes from clams *R. philippinarum*, *R. decussatus* and *Mercenaria mercenaria* have been classified in two subpopulations: hyalinocytes and granulocytes, located in the lower and higher channels of both light scatter axes, respectively (Allam et al., 2001, 2002, 2006; Allam and Ford, 2006). In the scallop, *Chlamys farreri*, two haemocyte types has been identified: granulocytes and hyalinocytes (Xing et al., 2002). The hard clam *Meretrix lusoria* has been reported to have three subpopulations: hyalinocytes, small and large granulocytes (Tu et al., 2007). In mussels *Mytilus galloprovincialis*, three subpopulations (hyalinocytes, small and large granulocytes) (Parisi et al., 2008) and four subpopulations (large granulocytes, large semigranulocytes, smaller granulocytes and small agranulocytes or hyalinocytes) (García-García et al., 2008) have been considered. Oysters, *C. virginica* and *C. gigas*, were shown to possess three haemocyte subpopulations, although regarded slightly differently by different researchers (Ashton-Alcox and Ford, 1998; Allam et al., 2002; Lambert et al., 2003; Goedken and Guise, 2004). In the Sydney rock oyster, *Saccostrea glomerata*, up to four subpopulations were distinguished, although hyalinocytes and granulocytes were the two most abundant phenotypes (Aladaileh et al., 2007).

Following infection with *V. splendidus* 7SHRW, the proportion of hyalinocytes slightly decreased (Fig. 3.2) and became less discernible in the scatter profile (Fig. 3.3.b), while with the strain LGP32-GFP changes are more striking as both

subpopulations merge into a single continuous group (Fig. 3.3.c). Drastic changes in the haemocyte distribution according to their size and granularity have been previously noticed by Allam and Ford (2006). They observed a clear shift of granular cells toward the agranular cell population resulting in unimodal distribution of haemocytes of clams *R. philippinarum* and *M. mercenaria* after *in vitro* exposure to *V. tapetis* and *V. splendidus*.

We supplemented our light scatter profiling data with LysoTracker staining in order to provide a measure of lysosome content. LysoTracker is a fluorescent probe that accumulates in acidic compartments (Freundt et al., 2007) and has been used previously in studies of human cell lysosomes (Haller et al., 1996; Via et al., 1998; Blander and Medzhitov, 2004). This approach, applied for the first time to bivalve haemocytes in our study, revealed independent responses in haemocyte subpopulations (Figs. 3.4, 3.5 and 3.6). LysoTracker showed that the subpopulation of granulocytes of the control clams contained significantly more lysosomes than the subpopulation of hyalinocytes. This is in agreement with microscopical observations, and indirectly through enzymatic studies, which have shown that granulocytes have abundant lysosomes (Cajaraville and Pal, 1995; Cima et al., 2000; Pipe, 1990; Matozzo et al., 2007).

When challenged, the subpopulation of hyalinocytes had lower levels of lysosomal content, especially after infection with the strain LGP32-GFP (Figs. 3.4, 3.5.a and 3.6.a), whereas the subpopulation of granulocytes had similar levels of lysosomes after infection with the strain 7SHRW and significantly decreased after infection with LGP32-GFP (Figs. 3.4, 3.5.b and 3.6.b). Lysosomes are cellular organelles that contain hydrolytic enzymes (Luzio et al., 2000), including lysozymes that are involved in

intracellular degradation and host defense (Olsen et al., 2003). Many lysosomes have secretory functions (Holt, 2006). As it occurs in mammalian macrophages, it has been reported in several mollusc bivalves that lysosomes release their content upon infection during degranulation of actively phagocytising cells (Cheng and Rodrick, 1974; Rodrick, 1979; reviewed in Cheng 1983; Chu 1988). Lysosomal degranulation may be one possible mechanism accounting for the decrease of lysosomal content, expressed as loss of fluorescent intensity (Figs. 3.4, 3.5) and proportions (Fig 3.6.a and 3.6.b) in haemocytes from *V. splendidus* LGP32-GFP infected clams. The decreased responsiveness to *V. splendidus* 7SHRW insult could, by extension, be attributable to a decreased capacity to activate degranulation in *M. arenaria* haemocytes.

Considering that 7SHRW is an environmental endemic strain isolated from sediments from an area relatively close to the source area of our clams where there is no history of bacterial infections this strain may be recognized by the haemocyte pattern recognition receptors as a non threat non self particle. On the other hand, LGP32 is a non native strain that has been associated with mortalities of juvenile Pacific oysters, *Crassostrea gigas* in Europe (Gay et al., 2006a, b). Moreover, it has been found that this strain possesses the pathogenic factor Vsm, a metalloprotease recently demonstrated to be the most important factor of toxicity in its extra cellular products (Binese et al., 2008). It is likely that this pathogenic factor is associated with the significant degranulation of *M. arenaria* haemocytes in this study. The specific response of *M. arenaria* haemocytes against the strain LGP32-GFP is in accordance to our previous findings that this strain induces significant changes in haemocyte structure, number and

adhesion on this host species whereas changes induced by 7SHRW are minor or nonexistent (Mateo et al., 2009).

The decrease of lysosomal content in hyalinocytes (Figs. 3.4 and 3.5.a) or the increase of hyalinocytes with low levels of lysosomal content (Figs. 3.5.a and 3.6.a) might not only be due to degranulation but possibly due to the increased presence of precursor haemocytes that have not yet developed cytoplasmic granules. They might be released prematurely (from an unknown haematopoietic tissue) to fight the infection. A similar phenomenon, known as "left shift", occurs in mammalian leukocytes upon inflammation and septic shock (Opdenakker, 2001). Undifferentiated and small stem cells have been described as blast-like cells or haemoblasts, a subtype of agranular cells with high nucleus:cytoplasm ratio and which lack organelles (Hine, 1999; Cima et al., 2000; Chang et al., 2005; Aladaileh et al., 2007; Matozzo et al., 2008). Further research is, however, required to confirm the nature of the abundant smaller and less complex cells we found after *V. splendidus* LGP32-GFP challenge.

In conclusion, we found that responses of haemocytic subpopulations are not only specific to the pathogen strain, but are modulated independently of each other and possibly through independent cellular mechanisms. These changes in haemocytic subpopulations were monitored by using LysoTracker as an indicator of the lysosomal content and more striking responses were found after infection with *V. splendidus* LGP32-GFP. Functional studies are needed to confirm the activation of degranulation and the suspected release of immature haemocytes.

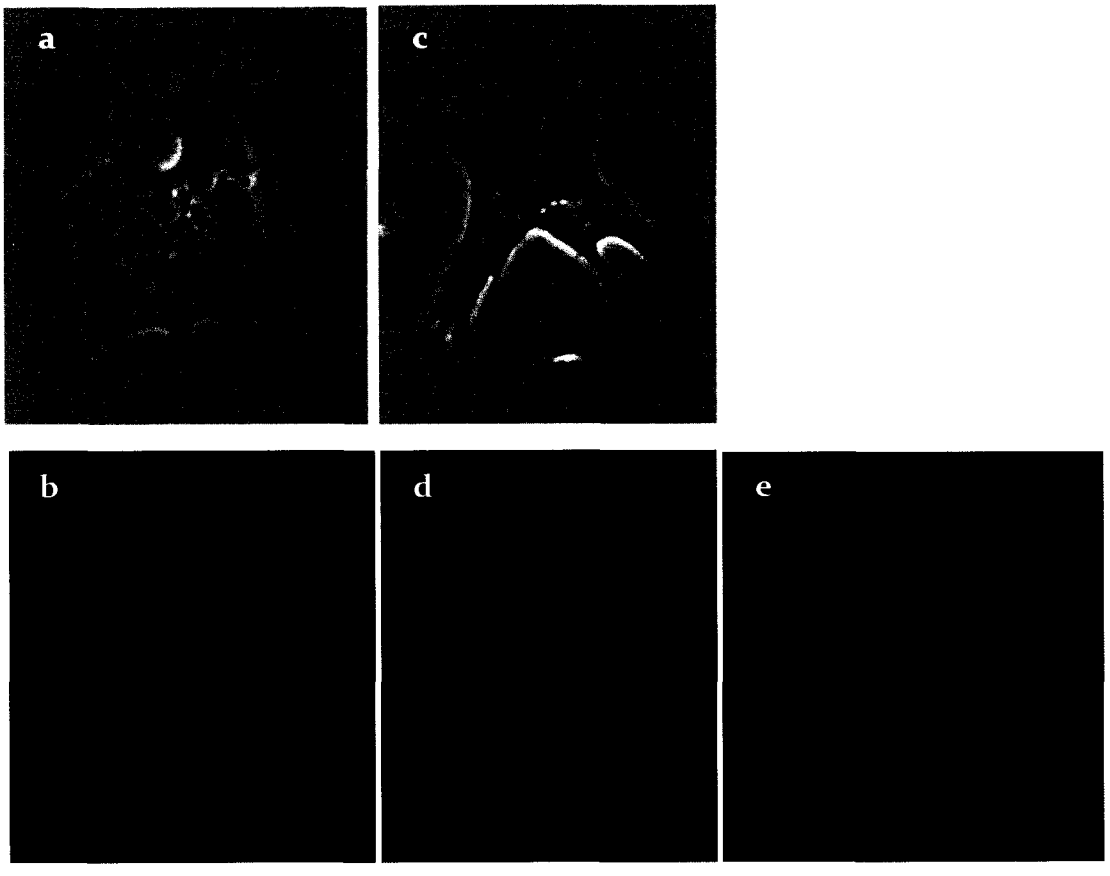

Figure 3.1. Soft-shell clam *M. arenaria* haemocytes (1000x). Granulocyte under (a) normal light, and (b) fluorescent light showing many lysosomes stained by LysoTracker. Hyalinocytes under (c) normal light, and (d) fluorescent light showing few lysosomes stained by LysoTracker. (e) Rounded cell after infection showing lysosomal content after LysoTracker staining.

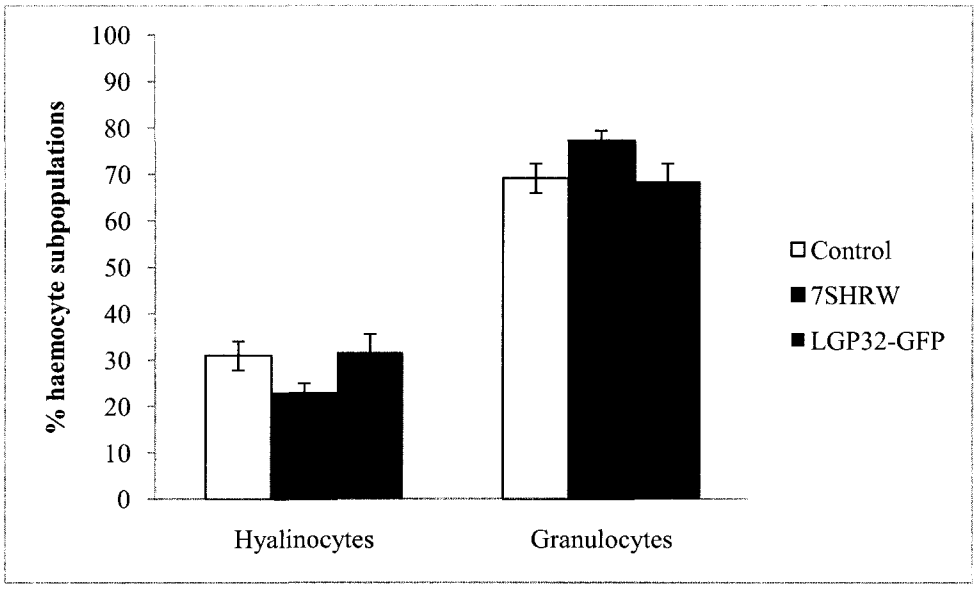

Figure 3.2. Proportions (± SEM) of *M. arenaria* hyalinocytes and granulocytes 24 h after injection with filtered sterile seawater (FSSW; control), *V. splendidus* 7SHRW or *V. splendidus* LGP32-GFP (n=21). Differences among treatments were not significant ($p > 0.05$).

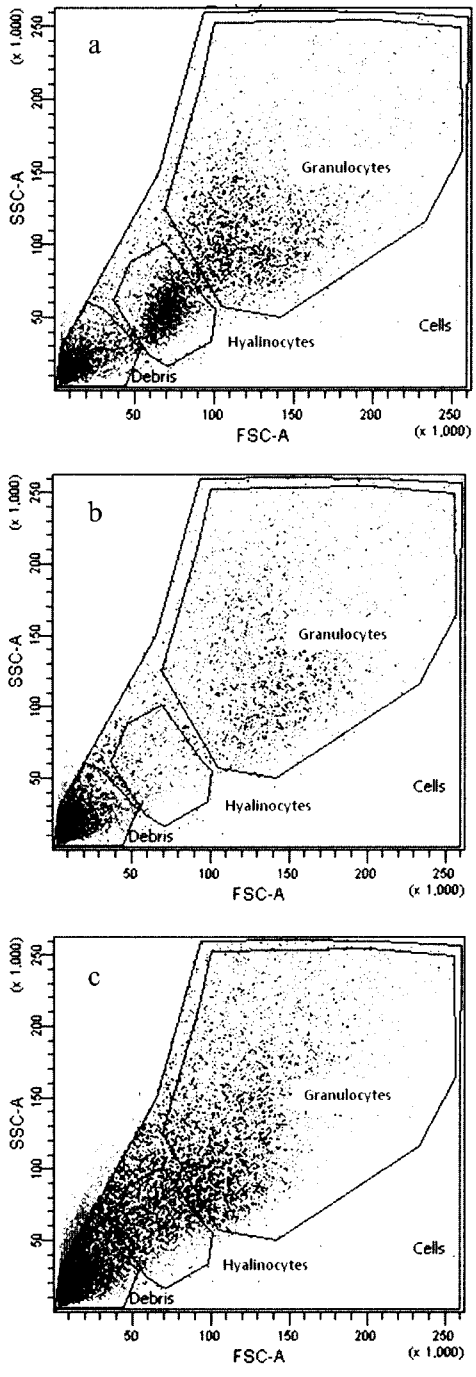

Figure 3.3. Forward-scatter (FSC) and side-scatter (SSC) plot profiles of *M. arenaria* granulocytes and hyalinocytes from representative clams 24 h after injection with (a) filtered sterile seawater (FSSW), (b) *V. splendidus* 7SHRW, or (c) *V. splendidus* LGP32-GFP.

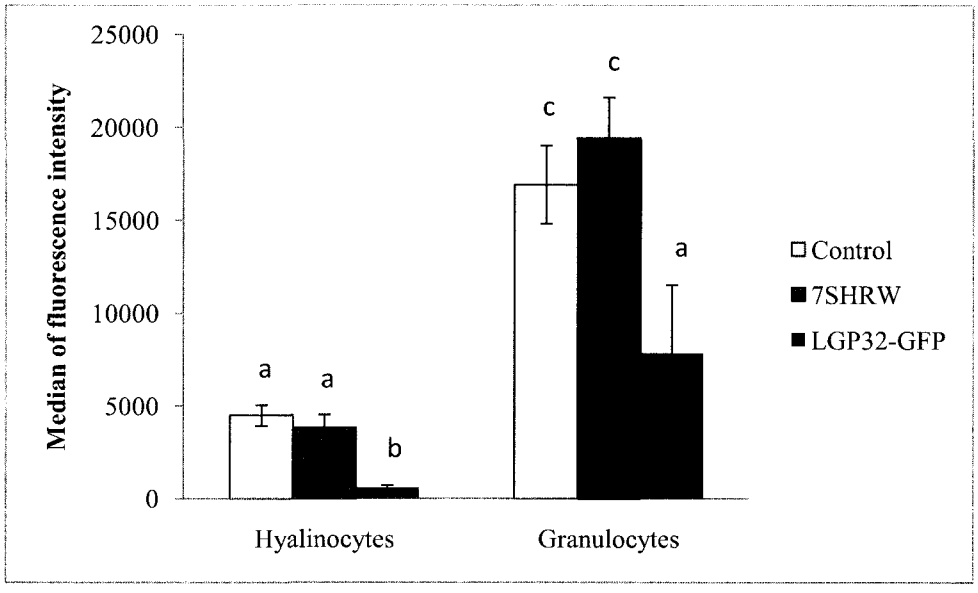

Figure 3.4. Median (± SEM) fluorescence intensity of LysoTracker retention in granulocytes and hyalinocytes 24 h after injection with filtered sterile seawater (FSSW; control), *V. splendidus* 7SHRW or *V. splendidus* LGP32-GFP (n = 21). Letters (a, b, c) show statistical equivalence (same letters) or difference at $p < 0.05$ (different letters) between pairs of haemocyte subpopulations (granulocytes and hyalinocytes) and treatments.

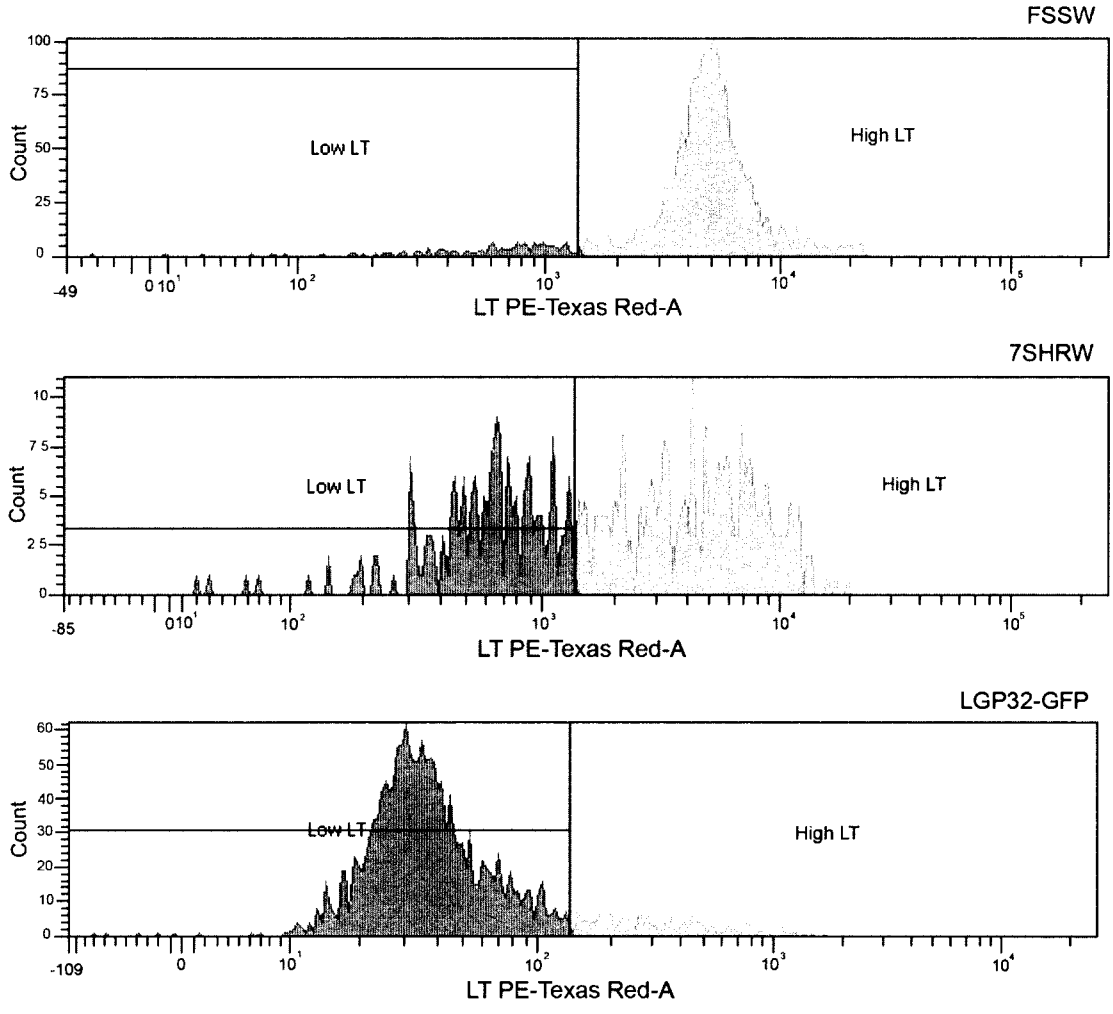

Figure 3.5.a. Median fluorescence intensity of LysoTracker (LT) retention (arbitrarily established as "high" and "low") in hyalinocytes from representative clams 24 h after injection with filtered sterile seawater (FSSW; top), *V. splendidus* 7SHRW (middle) and *V. splendidus* LGP32-GFP (bottom).

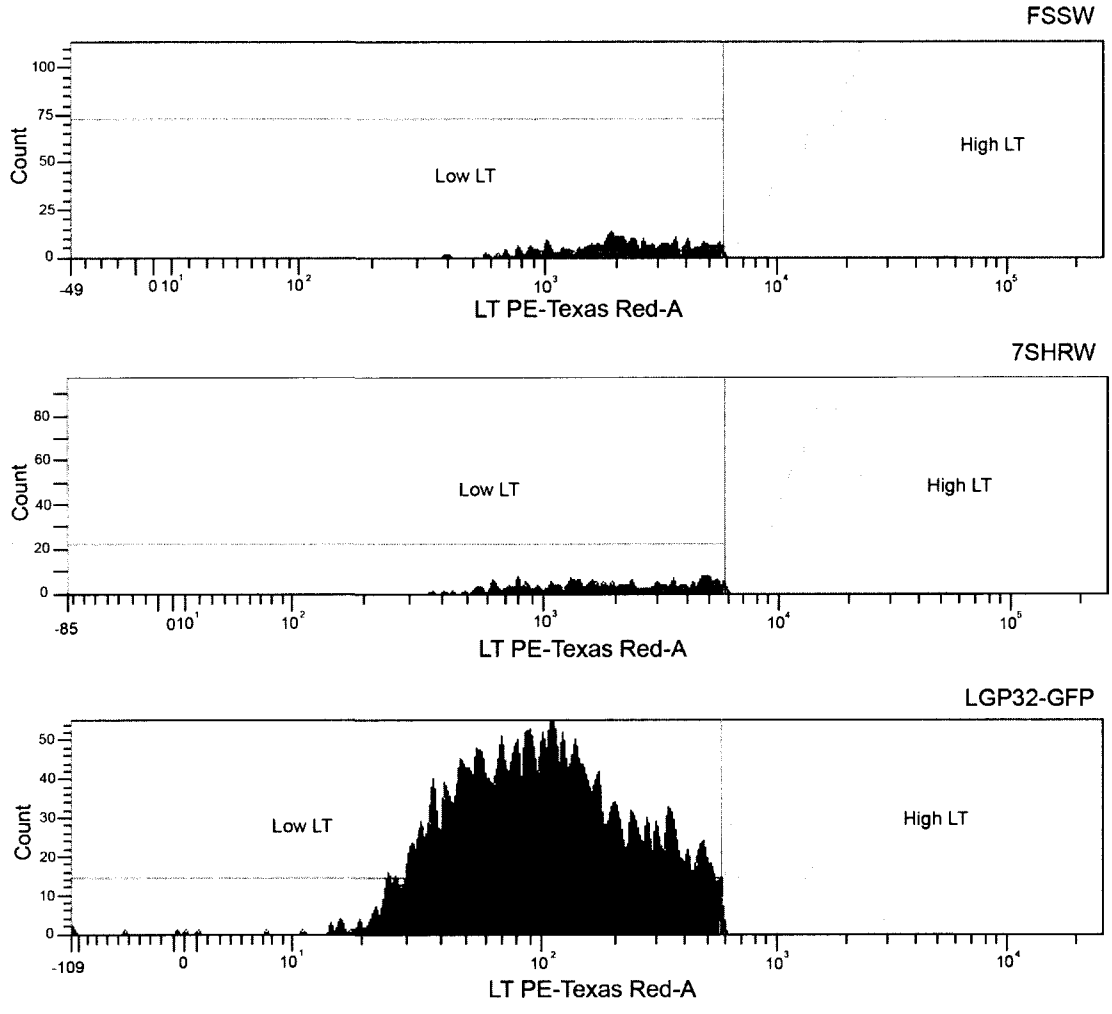

Figure 3.5.b. Median fluorescence intensity of LysoTracker (LT) retention (arbitrarily established as "high" and "low") in granulocytes from representative clams 24 h after injection with filtered sterile seawater (FSSW; top), *V. splendidus* 7SHRW (middle) and *V. splendidus* LGP32-GFP (bottom).

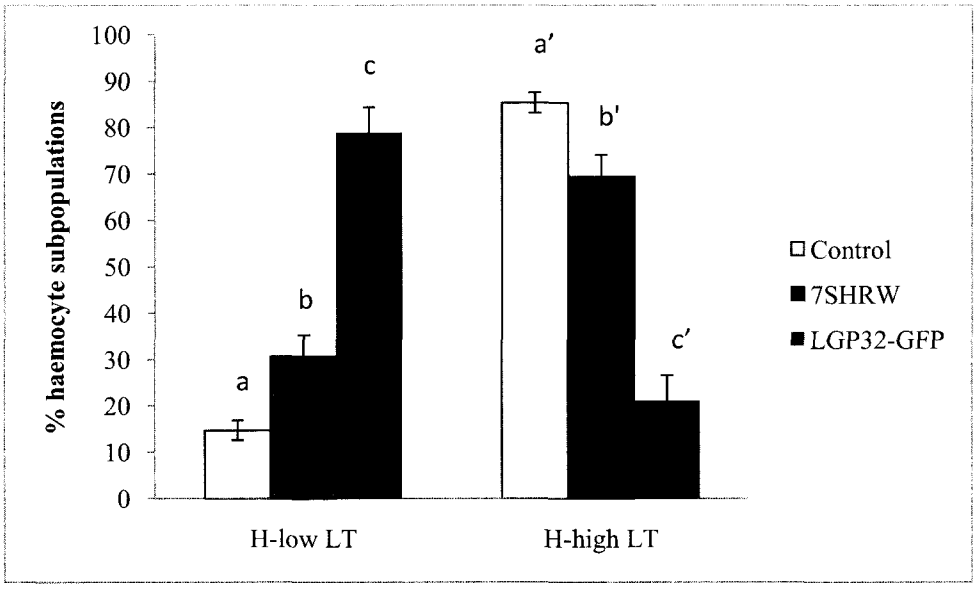

Figure 3.6.a. Proportions (± SEM) of hyalinocytes according to LysoTracker (LT: "high" or "low") staining 24 h after injection of clams with filtered sterile seawater (FSSW; control), *V. splendidus* 7SHRW or *V. splendidus* LGP32-GFP (n = 21). Letters (a, a', b, b', c, c') show statistical equivalence (same letters) or difference at $p < 0.05$ (different letters) among treatments.

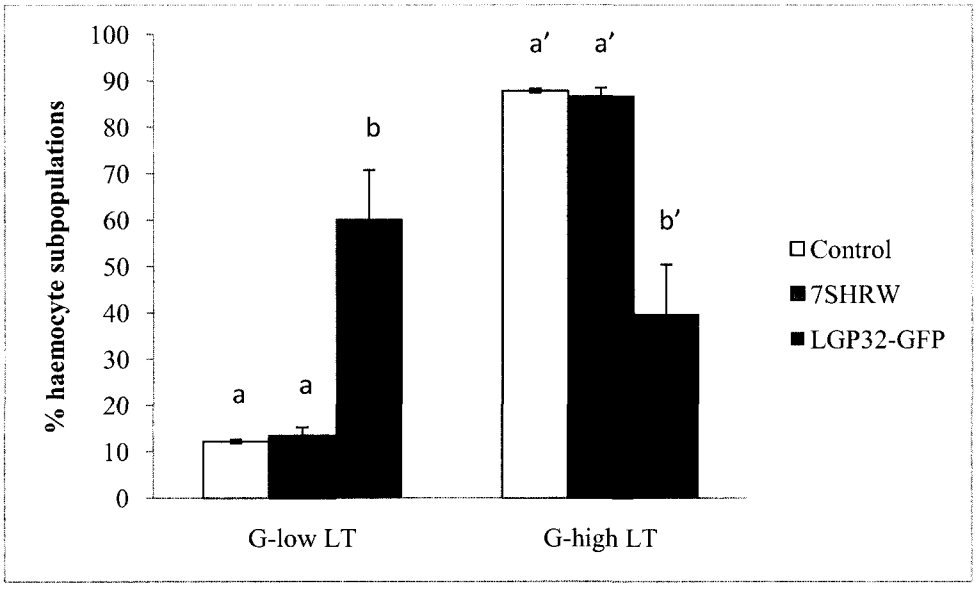

Figure 3.6.b. Proportions (± SEM) of granulocytes according to LysoTracker (LT: "high" or "low") staining 24 h after injection of clams with filtered sterile seawater (FSSW; control), *V. splendidus* 7SHRW or *V. splendidus* LGP32-GFP (n = 21). Letters (a, a', b, b', c, c') show statistical equivalence (same letters) or difference at $p < 0.05$ (different letters) among treatments.

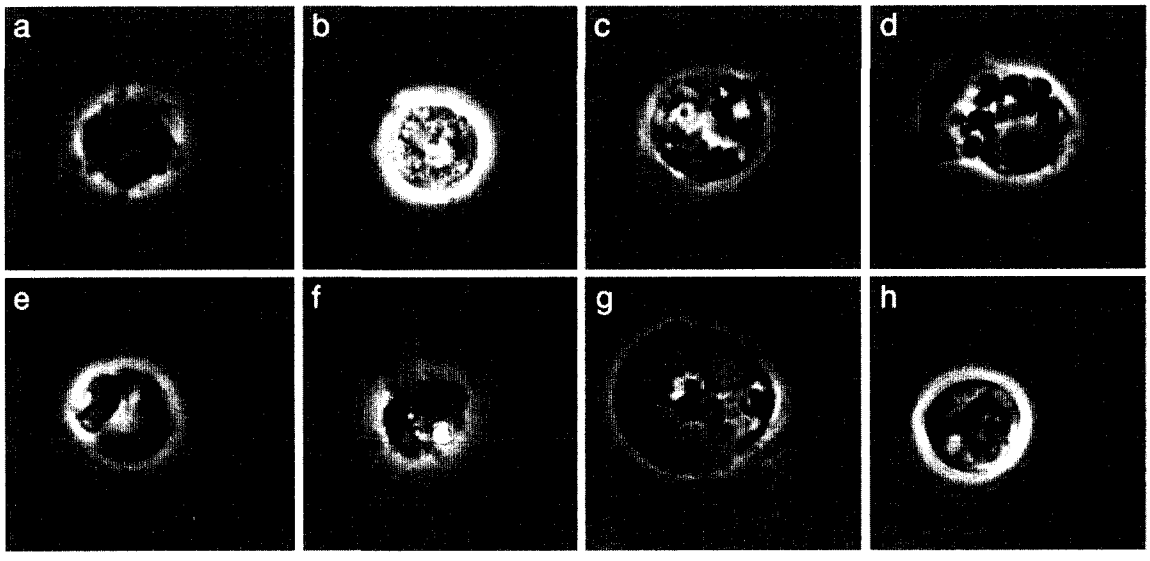

Figure 3.7. Contrast phase images (1000x) of immediately fixed haemocytes from each subpopulation. Control group: hyalinocytes with (a) "low" and (b) "high" LysoTracker staining; granulocytes with (c) "low" and (d) "high" LysoTracker staining. *Vibrio splendidus* LGP32-GFP-infected group: hyalinocytes with (e) "low" and (f) "high" LysoTracker staining; granulocytes with (g) "low" and "high" LysoTracker staining.

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Chapter 4. Differential gene expression in *Mya arenaria* haemocytes induced by *in vivo* infections with two *Vibrio splendidus* strains

Mateo D.R., Greenwood S.J., Araya M.T., Berthe F.C.J., Johnson G.R., Siah A. (2010) Differential gene expression of γ-Actin, Toll-Like Receptor 2 (TLR-2) and Interleukin 1 Receptor-Associate Kinase 4 (IRAK-4) in *Mya arenaria* haemocytes induced by in vivo infections with two *Vibrio splendidus* strains. Developmental and Comparative Immunology 34: 710-714.

4.1. Abstract

Immune function gene expression in *Mya arenaria* haemocytes was evaluated following *in vivo* infection with *Vibrio splendidus* LGP32-GFP and 7SHRW. Elongation factor 1α (EF-1α) and 2 (EF-2), after challenge with LGP32-GFP, and EF-1α with the ribosomal protein S-18, after challenge with 7SHRW, were found to be the most stable housekeeping genes. By comparison to these internal controls, LGP32-GFP induced significant up-regulation of γ-actin ($p < 0.001$), interleukin-1 receptor-associated kinase-4 (IRAK-4) ($p < 0.001$) and defensin ($p < 0.05$), and down-regulation of Toll-Lke receptor-2 (TLR-2) ($p < 0.01$) and lysozyme-2 ($p < 0.05$) homologues. The strain 7SHRW only induced significant down-regulation of lysozyme-2 ($p < 0.05$). Comparing the regulation induced by both strains, up-regulation of γ-actin, down-regulation of TLR-2 and up-regulation of IRAK-4 was significantly higher after challenge with LGP32-GFP ($p < 0.001$, $p = 0.001$ and $p < 0.05$, respectively).These results suggest specific responses at a molecular level modulated by the bacterial strains. LGP32-GFP induced marked responses which coincide with a similar trend previously found on phenotypic responses under the experimental model.

4.2. Introduction

Bivalve molluscs are constantly and intimately exposed to a wide number and variety of microorganisms in their aquatic environment. Their innate immune system is regarded as primitive but has elaborated mechanisms to differentiate pathogens from commensal bacterial flora and mount defensive mechanisms to neutralize potential infections (Bachère et al., 2006). Pathogenic bacteria, in turn, are able to differentially modulate host responses by interfering with cell signaling necessary for mollusc immune responses (Canesi et al., 2005; Travers et al., 2009).

The development of experimental host-pathogen interaction models provides an excellent system to gain insight into the immune system of marine bivalves. The genus *Vibrio* contains several marine bivalve pathogens and has been well studied (reviewed in Paillard et al., 2004). Various studies have explored a wide variety of bivalve phenotypic immune responses against different *Vibrio* species. Several studies have revealed, specific immune responses from assessing haemocyte numbers, cellular shape, adherence, phagocytosis, production of ROIs and nitric oxide, lysosome membrane stability, and viability (Lambert and Nicolas, 1998; Lane and Birkbeck, 1999, 2000; Choquet et al., 2003; Lambert et al., 2003; Canesi et al., 2005; Allam and Ford, 2006; Costa et al., 2009).

Vibrio splendidus is receiving more attention in mollusc health-related studies. This species was initially viewed as an environmental isolate and only later was associated with abnormal mortalities (Le Roux and Austin, 2006). *Vibrio splendidus* has been found to be the cause of disease in early bivalve life stages, including larvae of oysters *Crassostrea gigas* (Sugumar et al., 1998), clams *Ruditapes decussatus* (Gómez-

León et al., 2005), and mussels *Perna canaliculus* (Kesarcodi-Watson et al., 2009), and juvenile oysters *C. gigas* (Lacoste et al., 2001a; Waechter et al., 2002; Le Roux et al., 2002; Gay et al., 2004a, 2004b). The strain LGP32 was isolated from affected oysters and is believed to play an important role in the outbreaks of "summer mortalities" (Gay et al., 2004a, 2004b). In fact, major virulence factors against oysters, such as the metalloprotease Vsm and the outer membrane protein OmpU, have been identified in LGP32 (Le Roux et al., 2007; Binesse et al., 2008; Duperthuy et al., 2010).

Recently, studies on host response in the mussel, *Mytilus galloprovincialis,* challenged with *V. splendidus* LGP32 have demonstrated the pathogenic capacity against haemocytes and the host differential response at a cellular level (i.e. total cell counts, lysozyme activity and lysosome stability) as compared to other *Vibrio* species (Li et al., 2008; Parisi et al., 2008; Ciacci et al., 2009). In previous studies using soft-shell clams *Mya arenaria,* our data showed differential changes in cell structure, numbers, adherence (Mateo et al., 2009a) and lysosome content (Mateo et al., 2009b) in haemocytes induced by *in vivo* infections with two strains of *V. splendidus*: LGP32 (tagged with GFP) and 7SHRW. These studies suggested the modulatory capacity of *V. splendidus* strains to induce specific responses on *M. arenaria* haemocytes at a cellular level.

Nevertheless, studies at a molecular level are necessary to elucidate the mechanisms involved in the observed phenotypic responses. In addition, to unveil pathways of immune processes activation, gene regulation patterns can potentially be used in the selection of immune markers (Roch, 1999). Previously, differential expression in genes associated with immune responses (i.e. antimicrobial peptides, heat

shock proteins and lysozyme genes) were found in *M. galloprovincialis* haemocytes exposed to *V. splendidus* LGP32 (Cellura et al., 2006, 2007; Li et al., 2008). Recently, γ-actin and elongation factor-2 were found to be significantly up-regulated in *M. arenaria* haemocytes after *in vitro* infection with *V. splendidus* LGP32-GFP (Araya et al., 2009).

Following previous studies assessing differential *in vivo* phenotypic responses in *M. arenaria* haemocytes induced by *V. splendidus* LGP32-GFP and 7SHRW (Mateo et al., 2009a, 2009b), in this study the experimental model is complemented assessing responses at a molecular level against the same two bacterial strains. The study aims at investigating changes in expression of different genes including some with clear roles in immune function.

4.3. Materials and methods

4.3.1. Clams

A total of 34 wild soft-shell clams (approximately 5-6 cm in length and 22 g in weight) obtained from a relatively pristine area in the Magdalen Islands (Gulf of Saint Lawrence, Canada) were used in this study. Clams were acclimatized for a week in 300 L tanks with re-circulating artificial seawater (Instant Ocean) at 16°C and 30 ppt of salinity, and fed every other day with Spat formula (Innovative Aquaculture Products Ltd.).

4.3.2. Bacteria

Two strains of *V. splendidus* were used for comparison. The strain LGP32-GFP was derived from a strain (LGP32) associated with the "summer mortalities" syndrome of juvenile oysters *Crassostrea gigas* in France (Gay et al., 2004a, 2004b). The strain 7SHRW was isolated from marine sediments of Prince Edward Island, Canada.

Both bacterial strains were grown and their concentrations estimated according to methods described previously (Mateo et al., 2009a, 2009b). Briefly, bacteria were cultured overnight in 2% NaCl Tryptic Soy Broth (TSB, BD-Bacto) to achieve exponential growth, harvested by centrifugation (5000 g, 4°C, 10 min) and rinsed in filtered sterile seawater (FSSW). Bacterial concentration was estimated using a spectrophotometer (2802 UV/VIS Unico) with a FACSCalibur flow cytometer (Becton-Dickinson).

4.3.3. Clam infection and sample collection

Using 1 mL syringes fitted with 25-gauge needles, 200 µL of either FSSW or 4.5 x 10^6 bacteria/g of clam were injected into the posterior adductor muscle. After injection, clams were held out of water 1 h to monitor retention of injected material, clams were then placed in separate containers with non-circulating artificial seawater.

Twenty four hours after infection, 2-3 mL of haemolymph were collected from the posterior adductor muscle of each clam, using 3 mL syringes fitted with 25-gauge needles. Haemolymph was passed through an 80 µm mesh to screen aggregated haemocytes, gametes, or other debris, and placed in glass tubes on ice. Haemocytes were collected after centrifugation (500 x g, 4°C, 15 min).

4.3.4. Assessment of the percentage of rounded haemocytes

In order to corroborate the success of the infection, analysis of the percentage of rounded haemocytes was performed microscopically as previously described (Mateo et al., 2009a). Briefly, stretched (healthy) and rounded (distressed) cells were counted using a contrast phase microscopy Axio Imager A1 (Carl Zeiss) microscopy (400x) and the mean percentage was estimated from 5 counts per sample.

4.3.5. RNA extraction and cDNA synthesis

Total RNA from haemocytes was extracted using a Qiagen DNEasy Tissue kit according to the manufacturer's protocol. RNA was quantified using a Nanodrop spectrophotometer (ND-1000) and its integrity was analysed using a Bioanalyzer (Agilent Technologies). Complementary DNA was synthesized using the SuperScript III Platinium Two-Step qRT-PCR kit (Invitrogen) according to the manufacturer's protocol. Briefly, to synthesize the first cDNA strand, 1 μg of total RNA was mixed in a total volume of 20 μL containing 10 μL of 2x RT reaction mix, 2 μL of M-MMLV reverse transcriptase enzyme mix and DEPC-treated water, and incubated (25°C, 10 min; 42°C, 50 min; 85°C, 5 min). Treatment with *E. coli* RNase H (2 μL) was used to remove remaining RNA.

4.3.6. Real time quantitative polymerase chain reaction

Subtracted cDNA libraries of *M. arenaria* haemocytes were the source for the expressed sequence tags (EST) of housekeeping genes (Siah et al., 2007) and target candidate genes (Siah, pers. com.) (Table 4.1). Primers were designed using the program

Primer Blast at NCBI and their properties analysed using the program PCR Primer Stat from The Sequence Manipulation Suite 2 (Stothard, 2000) (Table 4.2). Quantitative PCR was performed in a Rotor Gene RG-3000 real time thermocycler (Corbett Research) using SYBR Green I chemistry (Invitrogen) on 1 μL of cDNA template (25 μL total reaction volume). The PCR conditions were 10 min at 95°C followed by 40 amplification cycles (95°C for 20 s, 60°C for 20 s, 72°C for 20 s). Analysis of gel images and melting curves confirmed amplification of the specific PCR products for each primer set.

4.3.7. Gene expression analysis

The Visual Basic Application geNorm was used to determine the most stable genes among the candidate housekeeping genes. For infections using both bacterial strains, the housekeeping genes γ-actin, S-18, ubiquitin, elongation factor 1α (EF-1α) and 2 (EF-2) were evaluated. The most stable genes were used as internal controls to normalize the relative expression of the target immune/stress genes (Table 4.1) following geNorm software (Vandesompele et al., 2002).

4.3.8. Statistical analysis

Statistical analysis was accomplished using the statistical software Minitab 15.1.0.0. Analysis of the data related to the percentage of rounded haemocytes was done using the non-parametric Kruskal-Wallis model followed by Mann-Whitney test for pairwise comparisons. For the gene expression analysis, comparisons were done between treated and control groups for challenges with each strain (values expressed as

relative expression) and between treated groups (values expressed as x-fold in relation to the controls). Data were transformed to the base 10 logarithm to achieve normality when necessary. Two-sample *t*-tests were used in all cases when data were normally distributed; otherwise the Mann-Whitney test was performed.

4.2. Results

4.4.1. Percentage of rounded haemocytes

Results of the percentage of rounded haemocytes after challenge are shown in Table 4.3. While challenge with 7SHRW did not induce significant increase of rounded haemocytes, challenge with LGP32-GFP induced a strongly significant increase ($p < 0.001$). The percentage of rounded haemocytes of the group challenged with LGP32-GFP was significantly higher than the group challenged with 7SHRW ($p = 0.001$).

4.4.2. Housekeeping genes

The application geNorm established the gene expression stability among the candidate housekeeping genes by determining the internal gene stability M. The most stable genes under experimental infection using LGP32-GFP were EF-1α and EF-2 (M = 0.685), whereas the least stable was γ-actin (Fig. 4.1). Based on the pairwise variation analysis (Fig. 4.2) the lowest variation was obtained by using 2 genes (V2/3 = 0.272), therefore it was decided to use the 2 least variable genes as internal controls (i.e. EF-1α and 2). When using 7SHRW for the experimental infection EF-1α and S-18 were the most stable (M = 0.89). The normalization factor was then calculated using these sets of

2 genes to normalize the relative expression of the target genes (Vandesompele et al., 2002).

Given that γ-actin was among the least stable among the housekeeping gene candidates, its relative expression was assessed and found to be significantly up-regulated after infection with LGP32-GFP ($p < 0.001$) but with 7SHRW there was no significant difference (Fig. 4.3).

4.4.3. Target genes

Under the experimental infection using LGP32-GFP, it was found that the gene homologues of Defensin, HSP70, HSP90, IRAK-4 and Mpeg were up-regulated (Fig. 4.4). However, this up-regulation was significant only for Defensin ($p < 0.05$) and IRAK-4 ($p < 0.001$) homologues. Three genes homologues were down-regulated using the same bacterial strain: LGBR, lysozyme-2 and TLR-2 (Fig. 4.4). Down-regulation was significant for lysozyme-2 ($p < 0.05$) and TLR-2 ($p < 0.01$).

In order to compare the gene expression induced by LGP32-GFP and 7SHRW, only those candidate target genes whose expression was significantly regulated after infection with LGP32-GFP (i.e. IRAK-4, TLR-2, Defensin, lysozyme-2) were also tested using 7SHRW. After infection with 7SHRW, only IRAK-4 was up-regulated, but not significantly, whereas TLR-2, defensin, and lysozyme-2 were down-regulated (Fig. 4.5). Only the down-regulation of lysozyme was significant ($p < 0.05$).

The changes in gene expression (fold change) for clams challenged with LGP32-GFP and 7SHRW were compared (Fig. 4.6). There was significant difference in the

expression of TLR-2 ($p = 0.001$) and IRAK-4 ($p < 0.05$) between clams challenged with both strains.

4.5. Discussion

Due to the importance of host-pathogen interaction models in revealing characteristics of the immune responses of marine bivalves (reviewed in Canesi et al., 2002; Pruzzo et al., 2005), a model of *in vivo* interaction between soft-shell clam *M. arenaria* haemocytes and *V. splendidus* has been developed. To explore the modulatory effect of bacteria in host responses, haemocyte responses at the cellular (Mateo et al., 2009a, 2009b) and molecular level against 2 strains of *V. splendidus* (LGP32-GFP, associated with mortalities of juveniles oysters *C. gigas* in Europe, and 7SHRW, an environmental strain isolated from local sediments) were compared.

In order to confirm the establishment of the infections after bacterial injection the percentage of rounded haemocytes was determined. The percentages obtained were in accordance with previous assessments under the same experimental model (Mateo et al., 2009a, 2009b) and show an association between the observed changes in gene expression and the phenotypic responses reported previously.

4.5.1. Housekeeping genes

Five candidate housekeeping genes for the infection with each bacterial strain were evaluated. These genes are involved in different cellular functions as recommended to avoid co-expression (Vandesompele et al., 2002). Under infection with LGP32-GFP, the pairwise variation increased with 3 and 4 genes (Fig. 4.2), thus it was

decided to use only the 2 most stable housekeeping genes as internal controls under both experimental conditions to keep uniformity on the quantitative analysis of the targeted transcripts.

The use of more than one housekeeping gene as internal control has recently been addressed in studies on gene expression in *M. arenaria* haemocytes under different ploidy status (Siah et al., 2008), and under *in vitro* bacterial infection (Araya et al., 2008). The importance of validating housekeeping genes under specific experimental conditions (reviewed in Vandesompele et al., 2002; Pfaffl 2004; Huggett et al., 2005; Vandesompele et al., 2009) is reflected by the different ranking of variability of housekeeping genes obtained in *M. arenaria* haemocytes. While in this *in vivo* model EF-2 was found among the most stable genes using LGP32-GFP, the same gene was among the least stable ones using 7SHRW. Similarly, Araya et al. (2008), using *V. splendidus* LGP32-GFP *in vitro*, found EF-2 to be one of the least stable genes. Conversely, the similar common finding between this study and others with *M. arenaria* haemocytes (Siah et al., 2008; Araya et al., 2008) was that EF-1α and S-18 are among the most stable genes. Future work on gene expression in *M. arenaria* haemocytes should consider these 2 genes as important candidates to be evaluated for internal controls.

4.5.2. Cytoskeleton

In the present study, significant ($p < 0.001$) up-regulation of γ-actin using *V. splendidus* LGP32-GFP was found (Fig. 4.3). Similarly, Araya et al. (2009) found up-regulation of this gene in an *in vitro* model using the same bacterial strain, and Gestal et

al. (2007) found actin to be among the up-regulated genes of a suppression subtractive hybridization library obtained after challenging *Ruditapes decussatus* with a bacterial mixture that included *V. splendidus*. Actins (β and γ) are proteins involved in constant rearrangement of the eukaryotic cell cytoskeleton (Schmitt-Ney and Habener, 2004). Genes encoding for actin have also been identified in marine bivalves (Mohsin et al., 1996; Cadoret et al., 1999; Ma et al., 2007; Wang Z. et al., 2008). Actin is believed to play an important role in bivalve haemocyte functions (Panara et al., 1996). Processes such as cell locomotion and microorganism engulfment require rapid polymerization of actin filaments (Belyi, 2002).

In addition, the up-regulation of actin in haemocytes from clams infected with LGP32-GFP observed in this study was accompanied by an increased level of rounded cells (Table 4.3). This similar trend was observed previously under *in vivo* (Mateo et al., 2009a) and *in vitro* infections (Araya et al., 2009). Conversely, the expression of actin in haemocytes from 7SHRW infected clams did not change significantly which coincides with the low increase of rounded haemocytes (Table 4.3). Changes in cell configuration (Farmer et al., 1983) and altered G-actin to F-actin ratios (Schmitt-Ney and Habener, 2004) have been observed to change the gene expression of actin. A strategy of bacteria to subvert the defensive mechanism of host cells is to induce changes in their cytoskeleton (Belyi, 2002; Gruenheid and Finlay, 2003). *Vibrio* toxins have the capacity to induce cell rounding by depolymerisation of F-actin (Fullner and Mekalanos, 2000; Kudryashov et al., 2008). Moreover, the metalloprotease Vsm, from *V. splendidus* LGP32, altered actin filaments in Bge cell line (Binesse et al., 2008). In a study assessing the expression of actin (named Pfact1) in haemocytes of the pearl oyster

Pinctada fucata at different times after challenge with *V. alginolyticus*, Wang et al., (2008) did not find significant difference compared to controls. Unfortunately, the authors did not report changes in haemocyte structure.

The expression of genes associated with diverse functions, including some directly associated with immune responses (Table 4.1), were assessed 24h after bacterial infection. In clams infected with *V. splendidus* LGP32-GFP the gene expression of HSP70, HSP90, LGBP, and Mpeg did not vary significantly compared to controls.

4.5.3. Chaperone proteins

Heat shock proteins are conserved chaperone proteins expressed under environmental stress (reviewed in Feder and Hofmann, 1999), and also believed to be associated with immune functions (reviewed in Multhoff, 2006; Routsias and Tzioufas, 2006). In scallops, *Argopecten irradians,* and mussels, *M. galloprovincialis,* the expression of HSP70 was significantly up-regulated from few hours up to more than 2 days after challenge with *V. anguillarum* (Song et al., 2006; Cellura et al., 2007). However, in agreement with our results, *V. splendidus* LGP32 did not affect the expression of HSP70 in mussels *M. galloprovincialis* (Cellura et al., 2006).

4.5.4. Pathogen-associated molecular pattern recognition and cell signaling

LPS and β-1,3-glucan-binding protein (LGBP) is a pattern recognition receptor (PRR) that recognize pathogen associated molecular patterns (PAMP) as non-self and binds LPS and β-1,3-glucan of bacteria and fungi, respectively. Commonly found in crustaceans (Lin et al., 2008), LGBP has also been identified in scallops *Chlamys farreri*

and was up-regulated by *Listonella* (*Vibrio*) *anguillarum* 24h after infection (Su et al., 2004; Wang et al., 2009). The possibility that *V. splendidus* LGP32-GFP is recognized by this receptor in *M. arenaria* haemocytes cannot be ruled out as LGBP up-regulation could have occurred earlier than 24h and might not detected.

Homologues to genes related to signaling pathways such as TLR-2 and IRAK-4 were significantly regulated. Toll-like receptors, homologues of *Drosophila* Toll, are PRR that initiate signal transduction cascades for inflammatory responses (reviewed in Takeda and Akira et al., 2004; Mogensen, 2009). Among bivalves, Toll receptor homologues have been identified in the scallop *Chlamys farreri* (Qiu et al., 2007). The *in vitro* expression of CfToll-1 was differentially regulated with different concentrations of LPS. In the shrimp, *Fenneropenaeus chinensis,* the Toll receptor gene FcToll was significantly up-regulated in the lymphoid organ 8 h to 23 h after infection with *V. anguillarum* (Yang et al., 2008). In this study, the highly significant down-regulation ($p < 0.01$) of the TLR-2 homologue only with *V. splendidus* LGP32-GFP, and not with 7SHRW, suggested a role in immunity of *M. arenaria* haemocytes. TLR-2 in humans is involved in recognition of several microbial components including peptidoglycan, a component found in Gram-positive and –negative bacterial cell walls (Mogensen, 2009).

IL-1 receptor-associated kinases (IRAK), homologue to *Drosophila* Pelle, are mediators of the signal transduction of the Toll/IL-1 receptor (TIR) domains of TLR (Li et al., 2002; Takeda and Akira, 2004). Over-expression of IRAK-4 can activate the NF-κB and mitogen-activated protein kinase (MAPK) pathways (Li et al., 2002). Among aquatic invertebrates, IRAK has only been identified in the sponge *Suberties domuncula* (Wiens et al., 2007). The very high significant up-regulation ($p < 0.001$) of IRAK-4 in

M. arenaria haemocytes, after challenge with *V. splendidus* LGP32-GFP, suggests an involvement of this kinase in immune responses against bacteria. Although not significant, *V. splendidus* 7SHRW also induced up-regulation of IRAK-4.

4.5.5. Host defense

Macrophage expressed gene (Mpeg) is a homolog of the mammalian protein perforin that has cytolytic properties and is involved in immune processes. It has been identified in marine gastropods (Mah, 2004; Wang, G-D. et al., 2008) and recently in oyster *C. gigas* (Roberts et al., 2009). In hepatopancreatic cells from *Haliotis diversicolor* challenged with *V. parahaemolyticus* up-regulation of Mpeg was found at 8h and 96h after infection, while the levels of expression at 24 h and 48 h were not significant (Wang G-D. et al., 2008). Since we measured the expression at a single time point it is difficult to know if a similar pattern occurs in *M. arenaria.*

Lysozymes are enzymes involved in digestion and host defense. Various forms of lysozymes with either function have been described in bivalves (Olsen et al., 2003; Matsumoto et al., 2006; Itoh et al., 2007; Xue et al., 2004, 2007; Itoh and Takahashi, 2007). There is little information however on gene expression of lysozymes induced by bacterial challenge. Costa et al. (2009) found increased gene expression of lysozyme in haemocytes of mussels, *M. galloprovincialis,* 24 h after infection with *V. anguillarum.* Conversely, using the same host species, Li et al. (2008) found significant down-regulation of lysozyme in haemocytes from 1h up to 48h after challenge with *V. splendidus* LGP32. Significant down-regulation of the lysozyme-2 homologue at 24 h after infection with both *V. splendidus* strains was observed in this study. As several

lysozymes have bactericidal properties a mechanism of immune subversion by *V. splendidus* could be speculated. Probably, environmental strain 7SHRW also has this capability, but to a lesser degree, to allow survival within the host.

Defensin is an antimicrobial peptide present in a wide variety of hosts including marine invertebrates (reviewed in Tincu and Taylor, 2004). Varied defensin expression patterns have been reported in bivalve haemocytes using different bacterial challenges. Mitta et al. (1999) reported a decreased tendency in the expression of MGD1 and MGD2 from few hours up to 48 h and 72 h after challenging mussels, *M. galloprovincialis,* with heat-killed *V. alginolyticus*. Conversely, using heat-killed bacterial challenges in the same bivalve species, Cellura et al. (2007) found significantly increased expression of defensin from 1h up to 24h after infection with *V. splendidus* LGP32 whereas no difference was found using *V. anguillarum*. In scallops, *Argopecten irradians*, Zhao et al. (2007) found increased expression of AiBD from 4 h to 32 h after infection with *V. anguillarum*. In oyster *C. gigas* haemocytes, Gonzalez et al. (2007) found a pronounced decrease in the expression of Cg-defh2 at 24 h and 48 h after challenge with a bath of heat-killed bacteria including *V. splendidus* and *V. anguillarum*. However, in mantle and gills there was an increased expression, explained by the migration of Cg-defh2 producing haemocytes (Gonzalez et al., 2007). Given the different characteristics of the challenge methods used previously, it is difficult to establish valid parallels with the present study. Moreover, the antibacterial activity of each isoform used in previous studies would play an important role in triggering different gene expression responses. In addition, the large variability of the values for defensin gene expression after infection with LGP32-GFP, the low power of non-

parametric tests (used in this case) and the absence of significant differences after infection with 7SHRW preclude us from suggesting a clear pattern in its expression.

The changes in gene expression (fold change) after challenge with LGP32-GFP and 7SHRW were compared (Figure 4.6). The up-regulation of γ-actin, down-regulation of TLR-2 and up-regulation of IRAK-4 was significantly higher after challenge with LGP32-GFP ($p < 0.001$, $p = 0.001$ and $p < 0.05$, respectively). These results suggest specific responses at a molecular level modulated by the bacterial strains. Induced regulation of immune effectors and/or blocking their signaling pathways are known pathogen strategies in humans (reviewed in Finaly and McFadden, 2006) and molluscs (Canesi et al., 2005; Travers et al., 2009). The ability of bacteria to modulate immune responses in molluscs has been attributed to the activation or interference of signaling pathways such as MAPK (Canesi et al., 2005; Travers et al., 2009). The expression of the γ-actin gene could reflect the cytoskeletal changes observed in the haemocyte. Changes in the cytoskeleton not only affect cell motility, adhesion and phagocytosis but also the activation of signal transduction pathways involving MAPKs and NF-κB (reviewed in Kustermans et al., 2008). Besides, TLR-2 and IRAK-4, whose homologues were significantly and differentially regulated in this study, are part of the signal transduction pathway that leads to the activation of the NF-κB, allowing the expression of several immune factors in mammals (Li and Verma, 2002). Homologues of NF-κB and some of the components of its signal transduction pathway have been found in bivalves which, due to its conserved nature, likely play an important role in immune response (Gueguen et al., 2003; Montagnani et al., 2004; Roberts et al., 2009; Green and Barnes, 2009).

In conclusion, the differential gene expression responses found, especially in γ-actin, TLR-2 and IRAK-4 homologues, demonstrate the capacity of *V. splendidus* strains to modulate *M. arenaria* haemocytes responses at the molecular level. It is difficult to establish parallels elsewhere due to the limited molecular responses reported on bivalves and the different methodology used. Moreover, the precise role of some of the gene homologues studied is presently unknown in bivalves. The probable capacity of *V. splendidus* strains to differentially regulate the NF-κB pathway in *M. arenaria* haemocytes requires further analysis. Future work should also investigate the kinetics of gene expression during challenge. The differential gene regulation found here could be used as a baseline for exploring markers of infection comparing the expression induced by other bacterial species in *M. arenaria* haemocytes.

Table 4.1. Characteristics of the gene homologues from the *Mya arenaria* cDNA library, identified using BlastX and used for gene expression assessment after challenge with *Vibrio splendidus* LGP32-GFP and 7SHRW.

Homologue gene identity	Species - accession number	e-value	Protein function
LPS and β-1,3-glucan-binding protein (LGBP)	*Pinctada fucata* (Mollusca, Bivalvia) – ACN76701.1	$2e^{-26}$	Pathogen-associated molecular pattern (PAMP) recognition
Toll-like receptor-2 (TLR-2)	*Homo sapiens* - ACS88978.1	$2e^{-10}$	Pathogen-associated molecular pattern (PAMP) recognition
Interleukin 1 receptor-associated kinase (IRAK-4)	*Eupryma scolopes* (Mollusca, Cephalopoda) - AAY27972.1	$2e^{-31}$	Cell signaling
Macrophage expressed gene (Mpeg)	*Haliotis diversicolor* (Mollusca, Gastropoda) – ABY87434.1	$5e^{-20}$	Host defence (bacterial killing)
Defensin	*Crassostrea gigas* (Mollusca, Bivalvia) – ACQ76287.1	$3e^{-18}$	Host defence (bacterial killing)
Lysozyme-2	*Mytilus galloprovincialis* (Mollusca, Bivalvia) – BAF63423.1	$2e^{-35}$	Digestion / host defence
Heat shock protein 70 (HSP70)	*Ostrea edulis* (Mollusca, Bivalvia) – CAC83010.1	$5e^{-22}$	Protein chaperone / immunity (antigen presentation)
Heat shock protein 90 (HSP90)	*Latemula elliptica* (Mollusca, Bivalvia) – ACF35426.1	$2e^{-28}$	Protein chaperone

Table 4.2. Primer sequences and expected amplicon size corresponding to gene homologues (housekeeping and target), from the *Mya arenaria* cDNA library, used for gene expression assessment after challenge with *Vibrio splendidus* LGP32-GFP and 7SHRW

Gene	Primer	Sequence (5'-3')	Tm	Amplicon (bp)
Gamma actin	Forward	GCGAAAATACTCCGTCTGGA	60	179
	Reverse	GCAGGTACGATCACAAGCAA		
Elongation factor 1α	Forward	GGTGGCTGTTGGTGTCATC	60	158
	Reverse	GGCCTAGGTGTTTTCCATGA		
Elongation factor 2	Forward	CTACAAGCCTGGCTCAAAGG	60	218
	Reverse	TGACAACTGGGCTGACAGAG		
Ribosomal protein S-18	Forward	AAGATTCCCGACTGGTTCCT	60	189
	Reverse	GCCGGTTGTCTTTGTATGCT		
Ubiquitin	Forward	GAACCAAAGGAGACGCCATA	60	194
	Reverse	GTTGCAGAATGGCCTTGATT		
LPS and β-1,3-glucan-binding protein (LGBP)	Forward	TTGCTCTTTAGCTTTCCCGA	60	193
	Reverse	CACGAGGAATGGACACCTTT		
Toll-like receptor-2 (TLR-2)	Forward	CACCCATGCTCACATAAACG	60	180
	Reverse	TCACCAACGGGACTTCCTAC		
Interleukin 1 receptor-associated kinase (IRAK-4)	Forward	GTCCACTCGTGTTGTCATCG	60	122
	Reverse	CGGCAGTAAGCATTTCCAGT		
Macrophage expressed gene (Mpeg)	Forward	GACCCAGTAATTTTTCGGCA	60	166
	Reverse	ACGGCATCCTTGACGTAATC		
Defensin	Forward	GTTTTCTTCGCGATGTTGGT	60	119
	Reverse	CGACACCCGATTGACTTACA		
Lysozyme-2	Forward	TGTTGTTTGCGCAAGTCTTC	60	146
	Reverse	TATCTGCAATGTGGAGTCGC		
Heat shock protein 70 (HSP70)	Forward	CTTCTGGATGTCACACCCCT	60	131
	Reverse	GGCTGGTTGTCGGAGTATGT		
Heat shock protein 90 (HSP90)	Forward	ACCTTGTGGCCGATAAAGTG	60	117
	Reverse	GGAATTCCAGCTGTCCCTCT		

Table 4.3. Percentages of rounded haemocytes 24h after injection of either FSSW (control) or 4.5 x 10^6 bacteria/g (treated) using *Vibrio splendidus* 7SHRW and LGP32-GFP

V. splendidus strain	% rounded haemocytes		*p* value		
	Control (mean ± sem)	Treated (mean ± sem)	Control vs. treated	Control vs. control	Treated vs. treated
7SHRW	10.1 ± 6.3	21.4 ± 5.2	> 0.05	> 0.05	0.001
LGP32-GFP	2.3 ± 0.7	98.9 ± 0.9	< 0.001		

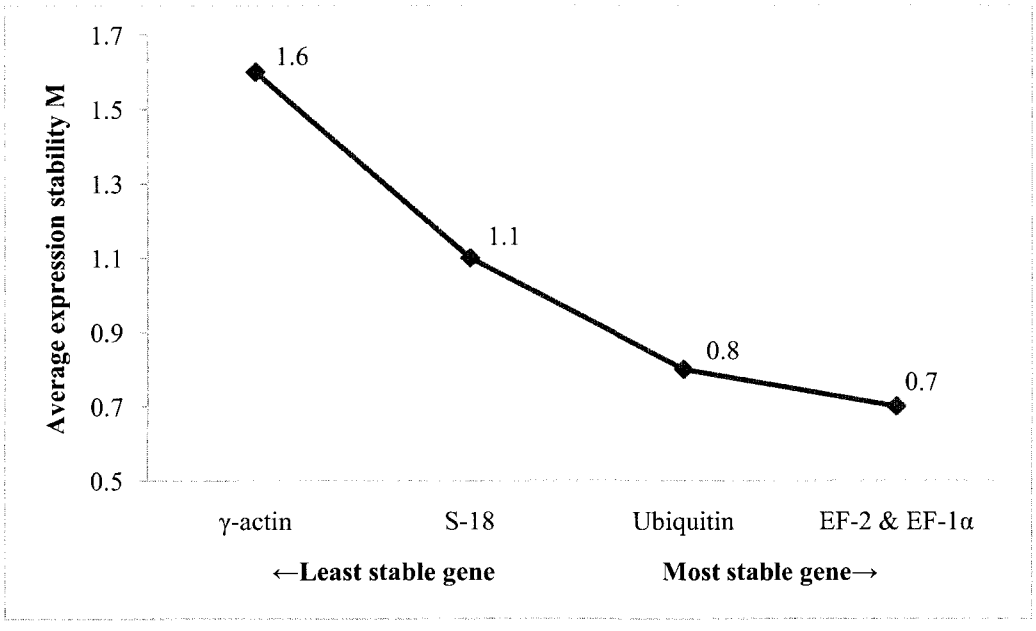

Figure 4.1.a. Average expression stability values of housekeeping genes in haemocytes of *Mya arenaria* after *in vivo* experimental infection with *Vibrio splendidus* LGP32-GFP obtained by geNorm software.

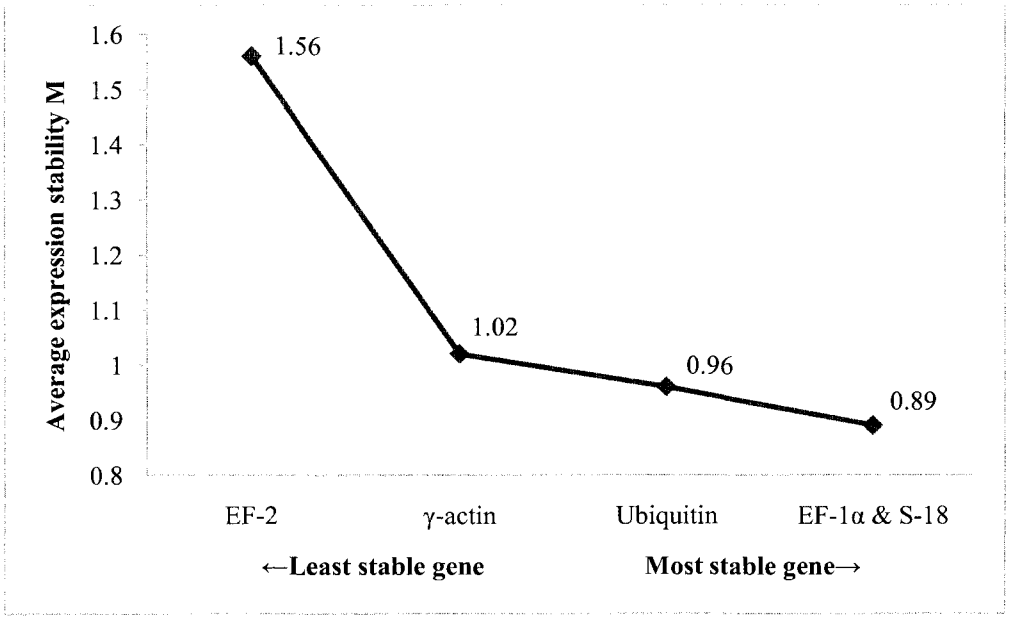

Figure 4.1.b. Average expression stability values of housekeeping genes in haemocytes of *Mya arenaria* after *in vivo* experimental infection with *Vibrio splendidus* 7SHRW obtained by geNorm software.

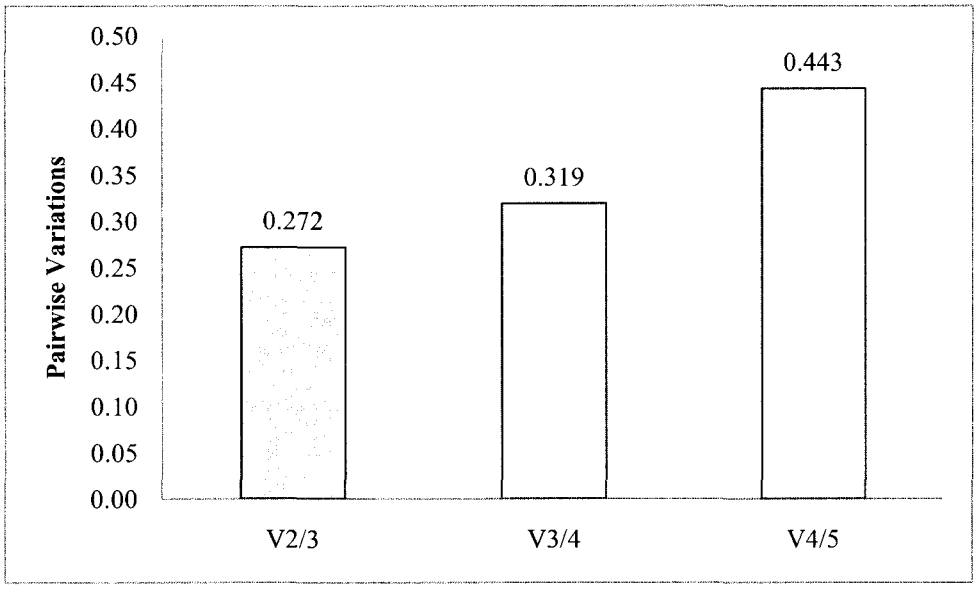

Figure 4.2. Pairwise variation analysis (V_n/V_{n+1}), obtained by geNorm, for determining a suitable number of housekeeping genes used as internal control for accurate normalization.

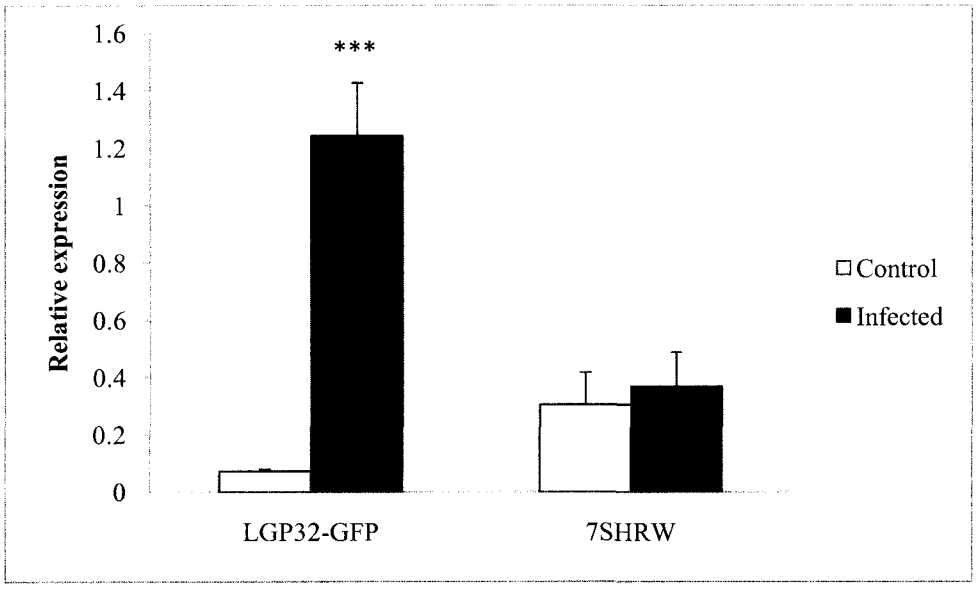

Figure 4.3. Relative expression (mean ± SEM) of γ-actin in haemocytes of *Mya arenaria* after *in vivo* experimental infection with *Vibrio splendidus* LGP32-GFP and 7SHRW (***: $p < 0.001$).

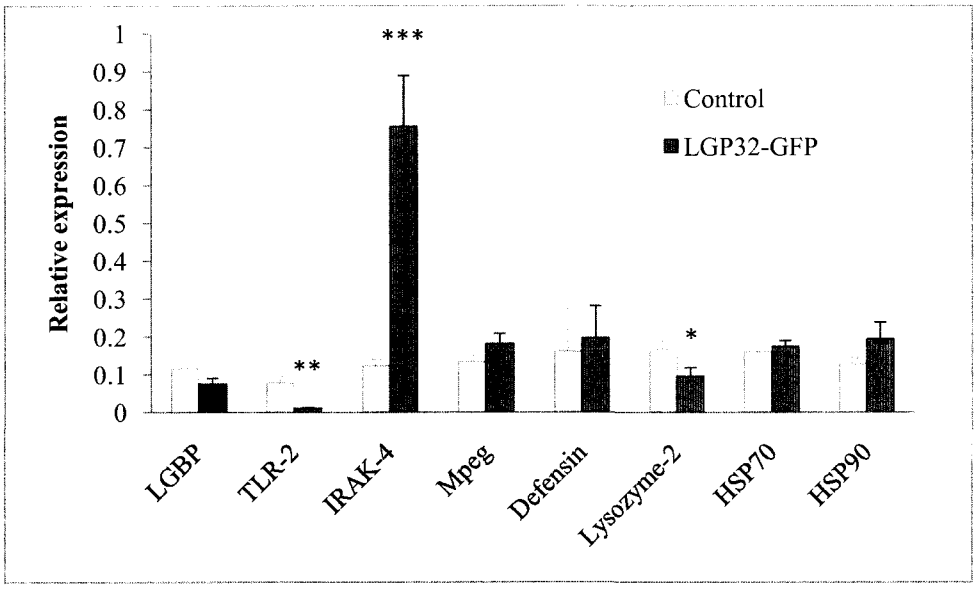

Figure 4.4. Relative expression (mean ± SEM) of candidate target genes of *Mya arenaria* haemocytes induced by *in vivo* experimental infection with *Vibrio splendidus* LGP32-GFP. Asterisks indicate significant difference in expression compared to controls (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

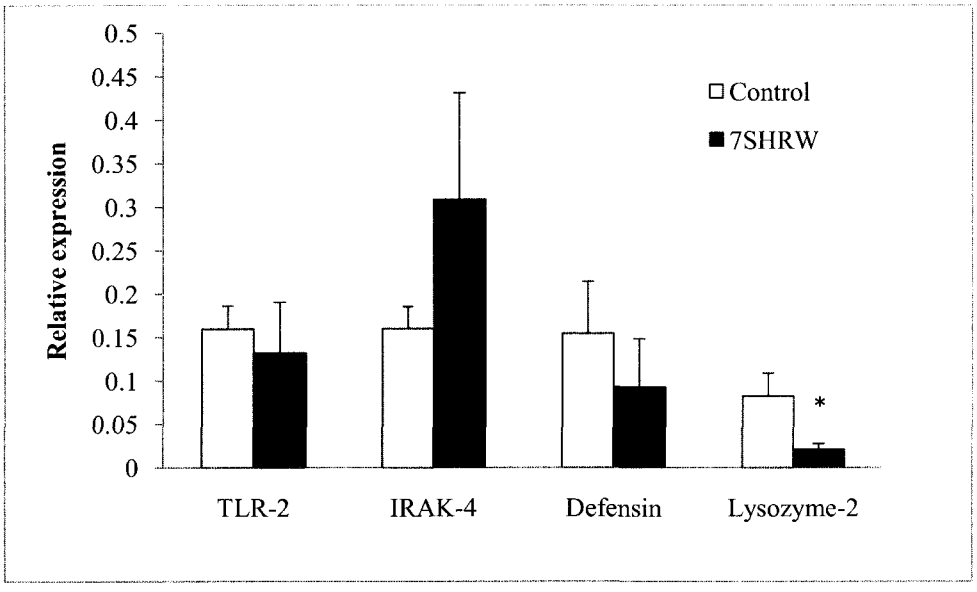

Figure 4.5. Relative expression (mean ± SEM) of target genes of *Mya arenaria* haemocytes induced by *in vivo* experimental infection with *Vibrio splendidus* 7SHRW. Asterisks indicate significant difference in expression compared to controls (*: $p < 0.05$).

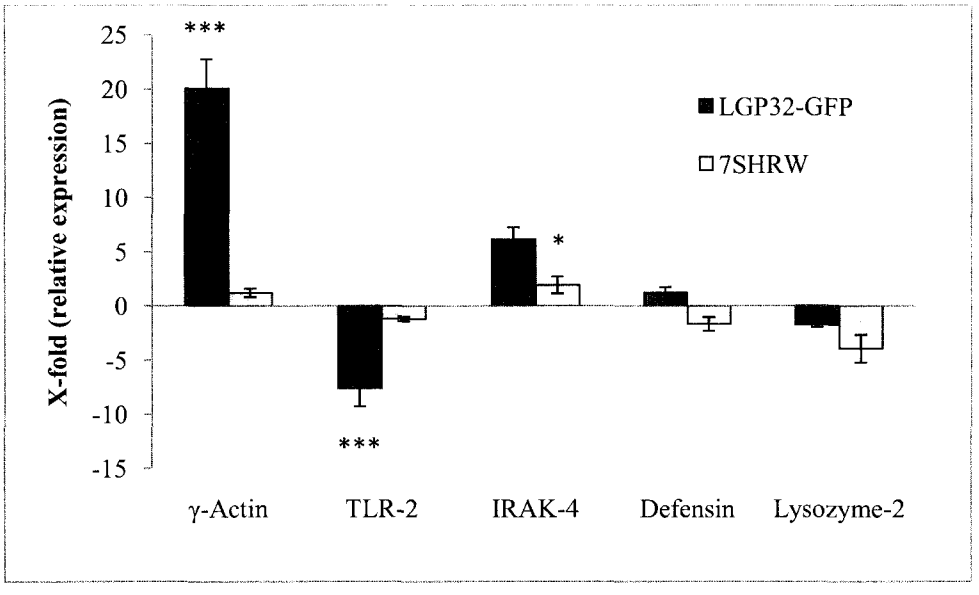

Figure 4.6. Expression level (mean ± SEM) of γ-actin and immune genes of *Mya arenaria* haemocytes induced by *in vivo* experimental infection with *Vibrio splendidus* LGP32 and 7SHRW. Asterisks indicate significant difference in expression between strains (*: $p < 0.05$; ***: $p \leq 0.001$).

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Chapter 5: Assessment of lysozyme activity in haemolymph of soft-shell clams, *Mya arenaria,* from Canada and Ireland after *in vivo* infection with *Vibrio splendidus* LGP32

5.2. Abstract

Lysozymes are hydrolytic enzymes that play an important role in defense responses. Bacterial challenge has been observed to activate the lysozyme activity in some bivalves, however, there is no information on *Mya arenaria* to this respect. Soft-shell clams from Canada and Ireland were challenged with *Vibrio splendidus* LGP32 and the concentration of lysozyme was measured in plasma and haemocytes. Clams from Ireland experienced a peak of significant increase ($p < 0.001$) of lysozyme activity in plasma and haemocytes at 12 h and those levels decreased but remained higher than controls at 24 h after challenge. Lysozyme levels returned to normal at 36 h in both haemolymph components and there was a significant decrease ($p < 0.05$) in haemocytes from challenged clams at 48h. Clams from Canada had much lower levels of lysozyme activity than those from Ireland. These levels were not significantly different between control and infected clams except for the lower levels in haemocytes from challenged clams at 12 h. Lysozyme levels in plasma and haemocytes were irregular without a clear pattern of higher content in any of the haemolymph components in clams from both origins. Increased lysozyme levels in plasma and haemocytes were a response to infection with LGP32 in clams from Ireland while the data from Canada is not conclusive. Lower lysozyme levels might have been the effect of either sample degradation during shipment, different virulence between LGP32 and LGP32-GFP or the nature of clam population from a different geographical area.

5.2. Introduction

An approach to unveil the mechanisms of host-pathogen interaction is to compare the responses induced by different microorganisms in the same host. In previous chapters, the focus has been the investigation of the specificity of responses in soft-shell clam haemocytes induced by 2 strains of *Vibrio splendidus*. Another helpful means to understand host-pathogen interactions is by comparing the responses of different hosts to the same pathogen. Even individuals of the same species are able to exert different response as stocks from different geographical origin have varied levels of sensitivity (or resistance) to certain pathogens (Cronin et al., 2001; da Silva et al., 2008a, b).

Lysozymes are a key component in the mollusc innate immune system (Canesi, 2002; Pruzzo, 2005). Different isoforms of lysozymes were characterized and classified into different types based on structural, catalytic and immunological criteria. Three families were identified: (1) c-lysozyme, initially isolated from chickens but has also been found in other birds, mammals, reptiles and some insects, (2) g-lysozyme isolated from goose but is also present in other birds (Jollès and Jollès, 1984) and (3) i-lysozyme characterized in invertebrates, including bivalve molluscs, Penaied crustaceans and some insects (Bachali et al., 2002). Recently, a goose-type lysozyme domain in a peptidoglycan recognition protein of Pacific oysters *C. gigas* haemocytes has been described (Itoh and Takahashi, 2009).

The most common modes of action of lysozyme are the ability to hydrolyse glycoside bonds of the peptidoglycan wall of Gram positive bacteria and the hydrolysis of chitin (Jollès and Jollès, 1984). More recently, the mechanism of some lysozymes to

affect Gram negative bacteria was revealed by structural studies. A helical hairpin or C-terminal helix allows lysozyme to cross the outer membrane *via* self-promoted uptake causing damage to the inner membrane through channel formation in a similar way as antimicrobial peptides (Ibrahim et al., 2001).

Lysozymes have been detected in several bivalves including mussels *M. galloprovincialis* (Caraballal et al., 1979), *M. edulis* (McHenery et al., 1979; Olsen et al., 2003), *M. modiolus*, oysters *C. virginica* (Chen and Rodrick, 1975; Rodrick and Ulrich, 1984), *C. gigas* (Itoh et al., 2007; Itoh and Takahashi, 2007; Xue et al., 2007), *O. edulis* (Cronin et al., 2001; Matsumoto et al., 2006), scallops *C. opercularis* (McHenery et al., 1979), *C. islandica* (Nilsen et al., 1999), and *C. farreri* (Xing et al., 2002), and clams *M. campechiensis*, *A. ovalis* (Rodrick and Ulrich, 1984), *M. arenaria* (Cheng and Rodrick, 1974; Rodrick, 1979; McHenery et al., 1979), *R. philippinarum* (Allam et al., 2000), *R. decussatus* (López et al., 1997). In *M. arenaria*, lysozyme has been mostly found in tissues such as gill, palps, and digestive glands (McHenery et al., 1979). Its activity was found at higher levels in plasma than haemocytes (Rodrick, 1979). The levels of lysozyme activity have been associated with infection. In clams *R. philippinarum*, *R. decussatus* and mussels *M. galloprovincialis* the lysozyme activity changed after bacterial infections (Allam et al., 2000, 2006; Ciacci et al., 2001), and in oysters *C. virginica* was associated with protozoan infection susceptibility (Chu et al., 1993).

This research aims to evaluate the lysozyme activity in haemolymph of soft-shell clams after bacterial infection. The activity of lysozyme in haemocytes and plasma separately was assessed in *M. arenaria* from locations in Canada and Ireland in order to

compare their responses against *V. splendidus* LGP32, a bacterial pathogen associated with mortalities of juvenile oysters *C. gigas* in France (Gay et al., 2004).

5.3. Materials and methods

5.3.1. Clams and infection

Soft-shell clams *M. arenaria* from Canada (n = 48) and Ireland (n = 74) were used in this experiment. Canadian clams were obtained from the Magdelen Islands (Gulf of Saint Lawrence, Quebec) while Irish clams were collected in Bannow Bay (Co. Wexford). Clams were acclimatized in tanks with re-circulating seawater for one week before challenge as described in previous chapters.

The bacterial strain *V. splendidus* LGP32 was used (Gay et al., 2004) for infection of both Canadian and Irish clams. Following the methodology of previous chapters, clams were infected by injecting into the posterior adductor muscle 200 µL of either FSSW or 4.5 x 10^6 bacteria/g of clam. Haemolymph samples (3-4 mL) were collected just prior to infection, (0 h) for Irish clams, and at 12, 36, 24 and 48 h after infection for both clam groups. After screening through an 80 µm mesh, to prevent aggregated haemocytes or debris, haemolymph was placed in glass tubes on ice. Haemocytes were collected after centrifugation (500 g, 4°C, 15 min) and both components of haemolymph (i.e. plasma and haemocytes) were frozen at -20°C. This procedure was done separately in the facilities of Atlantic Veterinary College and the Aquaculture and Fisheries Development Centre of the University College Cork and the samples were analyzed for lysozyme activity at the latter institution (samples from Canada were shipped by courier in a temperature isolating container). Samples from

Canada were kept stored for a month before analysis while samples from Ireland were stored for two days.

5.3.2. Standard curve construction and sample assessment

The methodology uses a modification of the earlier protocol set up by Shugar (1952) and is based on the ability to disrupt the bacterial wall of the Gram-positive bacteria *Micrococcus lysodeikticus*. The principle of the technique is based on the lysis of the bacterial walls which cause a decrease of the bacterial suspension turbidity. Consequently, the activity of the lysozyme is inversely correlated with the concentration of bacteria in the solution.

Plasma and haemocytes, re-suspended in 250 µL of 0.1 M phosphate buffer (pH 6.4), from Canadian and Irish clams were thawed and assessed for lysozyme activity using a slight modification of the methodology previously described (Shugar 1952; Cronin et al., 2001). An egg white lysozyme (Sigma) solution in 0.1 M phosphate buffer was prepared at a concentration of 20 µg/mL. From this concentration, serial dilutions of 60 µL were prepared to obtain 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078 µg/mL of egg white lysozyme in a 96 well microplate. Aliquots of 140 µL of *M. lysodeikticus* were placed in contact with each concentration to obtain a final volume of 200 µL per well. Likewise, the 60 µL of either plasma or haemocyte suspension of each sample were placed in wells and 140 µL of *M. lysodeikticus* was added. Duplicates were used to set up the standard curves and for sample analysis. Plates were read spectrophotometrically at a wavelength of 540 nm. Kinetic calculations were measured

5 times at 0, 1, 2, 3, 4 min and a standard curve was constructed by the software KC junior (Biotek). The results were expressed in concentration of lysozyme (µg/mL).

5.3.3. Statistical analysis

Statistical analysis was completed using the statistical software Minitab 15.1.0.0. To compare lysozyme concentrations between control and infected clams, data from Canadian clams was transformed to the base 10 logarithm and the one way general linear analysis of variance (ANOVA), followed by Bonferroni pairwise comparison was performed. Irish clam data was analyzed using the non parametric test Kruskal-Wallis followed by Mann-Whitney test for pairwise comparisons. To compare normalized lysozymes data (percentages) between plasma and haemocytes the Mann-Whitney test was used. A statistical significance level of 0.05 was considered for the differences in all tests.

5.4. Results

The lysozyme activity in plasma of Irish clams significantly increased ($p < 0.001$) at 12 h after infection (Fig. 5.1.a). The level of lysozyme activity was still increased at 24h but not significantly. At 36 h, it returned to normal levels and remained constant until 48h. In haemocytes, lysozyme activity increased and was significantly higher ($p \leq 0.001$) at 12 h where it reached its peak (Fig. 5.1.b). At 24h levels decreased with respect to 12h but were still significantly higher ($p \leq 0.001$) than controls. At 36 h the lysozyme activity returned to normal levels and continued decreasing at 48 h with significantly lower levels than controls ($p < 0.05$).

In general, Irish clams had higher concentrations of lysozyme in haemocytes than in plasma. Among control clams the concentration was significantly higher ($p < 0.001$) at 0, 36 and 48 h after infection (Fig. 5.2.a). Among infected clams this pattern was observed at all times (Fig. 5.2.b), and this difference was significant ($p < 0.001$) at 0, 36 and 48 h after infection.

The lysozyme activity in Canadian clams was much lower than those from Ireland. There were no significant differences of lysozyme activity in plasma (Fig. 5.3.a) and haemocytes (Fig. 5.3.b) between samples from control and infected clams. Only at 12 h the level of lysozyme activity in haemocytes was significantly lower ($p < 0.05$) than in controls.

Similarly to Irish clams, the levels of lysozyme concentration in Canadian clams tended to be higher in haemocytes than in plasma. This difference, however, was only significant at 12 h ($p < 0.05$) in control clams (Fig. 5.4.a), and at 24 h ($p < 0.01$) in infected clams (Fig. 5.4.b).

5.5. Discussion

The commercial value and importance as environmental bioindicator of soft-shell clams *M. arenaria* in North America have been reflected in many studies of health and disease aspects (McGladdery et al., 2001; Fournier et al., 2002; Gagnaire et al., 2007). In Europe, however, there are few studies on health aspects of *M. arenaria*. Nevertheless, studies of responses of *M. arenaria* from distant geographical zones are important for understanding immune responses of bivalves, especially considering that

genetic variability between American and European soft-shell clams has been reported (Strasser and Barber, 2008).

Lysozyme is an important component of bivalve immune response to bacterial infection. Isoforms of this enzyme have roles in digestion and host defense with both functions being associated with tissue location and antibacterial activity (Olsen et al., 2003; Xue et al., 2004, 2007; Itoh et al., 2007). Host defense lysozymes are found in haemolymph (haemocytes and plasma), extrapallial fluids, labial palps and mantle (McHenery et al., 1979; Allam et al., 2000; Itoh et al., 2007) and are active against both Gram positive and negative bacteria (Nilsen et al., 1999; Xue et al., 2004). Although lysozyme has been previously identified in *M. arenaria* (Chen and Rodrick, 1974; Rodrick 1979; McHenery et al., 1979), to the author's knowledge no analysis of its activity induced by bacterial challenge has been published.

In this study, increased lysozyme activity was observed in plasma and haemocytes of Irish clams at 12 and 24 h after infection. The lysozyme levels regained control levels by 36h and remained constant at 48h in plasma but in haemocytes decreased significantly. Similar trends have been reported for other bivalves. Ciacci et al. (2009) reported increased levels of lysozyme activity in plasma of mussels *M. galloprovincialis* after challenge with *V. splendidus* LGP32. The peak of activity was reported at 3h and was followed by progressive decrease until 48 h after infection, although still significantly higher than control levels. Allam et al. (2000) reported a small increase of lysozyme levels in plasma and haemocytes of *R. philippinarum* challenged with *V. tapetis* 6 h after infection followed by a decrease to control levels at 24 h after infection. The levels of lysozyme however increased again later and remained

elevated for several days. Using the same host species, Allam et al. (2006) reported an increase of lysozyme activity in haemolymph after challenges with *V. tapetis* and *V. anguillarum* from 2 h after infection, and the peak of activity was at 4 h after infection. This trend towards higher lysozyme activity after challenge suggests an increased immune response. However, this would be a general response to bacterial challenge as the enzyme activity was tested against a Gram-positive bacterial species while the challenge was done with Gram-negative bacteria. The outcome of the infection likely depends on the combination of different host defense factors, the virulence and dose of the bacteria.

In general, higher levels of lysozyme concentration were found in haemocytes than in plasma in control and infected clams from both origins. Varied patterns have been reported in previous reports. Lysozymes have been reported at higher levels in haemocytes than plasma in mussels *M. galloprovincialis* (Carballal et al., 1997) and oysters *O. edulis* (Cronin et al., 2001). Conversely in oysters, *C. virginica,* (Chen and Rodrick, 1975) and soft-shell clams, *M. arenaria,* (Rodrick, 1979) higher levels of lysozyme in plasma have been reported previously.

The results with Canadian clams are not conclusive. The similar lower levels of lysozyme activity found in Canadian clams may suggest that either: (1) the samples were not in optimal condition after shipment and storage, hence lysozyme was degraded, (2) the responses induced by *V. splendidus* in Canadian clams are intrinsically lower and, in particular lysozyme activity, might also be influenced by environmental factors, or (3) there are differences in the virulence of the bacterial strains used. It has been previously noticed that re-freezing and re-thawing samples affects lysozyme activity in

haemolymph (Cronin et al., 2001). However, the lysozyme levels on control clams from both origins had similar values (about 1 μg/mL) which would prevent assuming degradation of the samples lysozyme. A limit in the sensitivity of detecting of lysozyme (of 1 μg/mL) cannot argue against this possibility as levels as low as 0.085 μg/mL were detected in individual wells. Conversely, different environmental factors such as temperature and salinity have been associated with fluctuations of lysozyme levels in oysters *C. virginica* (Chu and La Peyre, 1989; Chu et al., 1993; Fisher et al., 1996). It is possible that a longer acclimation period was required to equilibrate the initial level of lysozyme activity between clams from both origins. Another possibility to explain the difference in lysozyme activity would be the bacterial strain virulence. In the infection of Irish clams the strain LGP32 was used instead of LGP32-GFP, used for Canadian clams. Although a clear pathogenic effect have been noticed before comparing phenotypic responses between LGP32-GFP and the environmental strain 7SHRW (see Chapter 2), it is possible that the strain LGP32 induce an even stronger effect against *M. arenaria* haemocytes than their genetically modified counterpart. Slightly attenuated virulence has been reported in GFP tagged *Yersinia ruckeri* compared with the wild type virulent strain assessed in trout tissues (Welch and Wiens, 2005).

In conclusion, the results from Irish clams suggest that one of the defense mechanisms actively involved against bacterial infection is the increased activity of lysozyme in the haemolymph of soft-shell clams. Since the activity of lysozyme was measured against *M. lysodeikticus*, a Gram-positive bacterium, and the challenge was performed with *V. splendidus*, a Gram-negative bacterium, it can be deduced that this is a general response against bacterial pathogens. Canadian clams showed lower levels of

lysozyme activity due to undefined reasons. The level of lysozyme concentration tended to be higher in haemocytes than in plasma in clams from both origins. This is the first study on bacterially induced responses in soft-shell clams from Ireland and also the first evaluation of lysozyme activity against bacterial pathogens in *M. arenaria*.

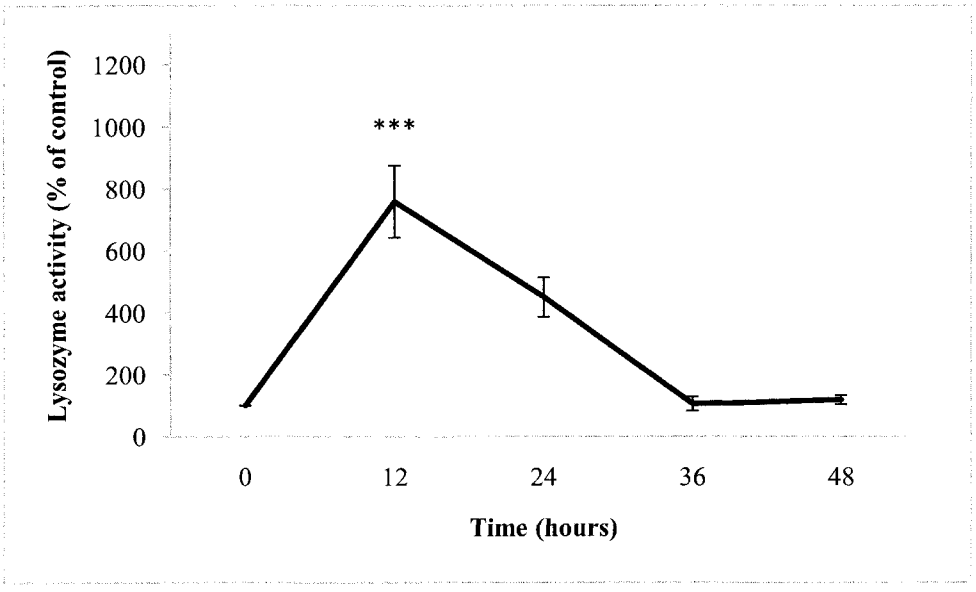

Figure 5.1.a. Lysozyme activity in plasma of *Mya arenaria* from Ireland after infection with *Vibrio splendidus* LGP32. Data are presented normalized with controls and expressed as mean ± SEM. Asterisks indicate the level of significant difference (***: $p < 0.001$) between samples from control and infected clams.

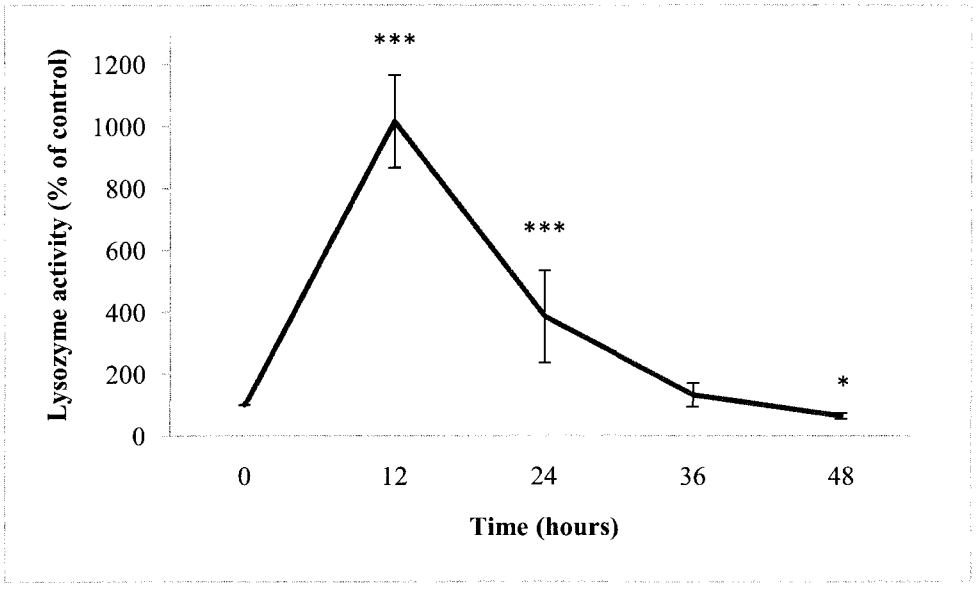

Figure 5.1.b Lysozyme activity in haemocytes of *Mya arenaria* from Ireland after infection with *Vibrio splendidus* LGP32. Data are presented normalized with controls and expressed as mean ± SEM. Asterisks indicate the level of significant difference (***: $p < 0.001$; *: $p < 0.05$) between samples from control and infected clams.

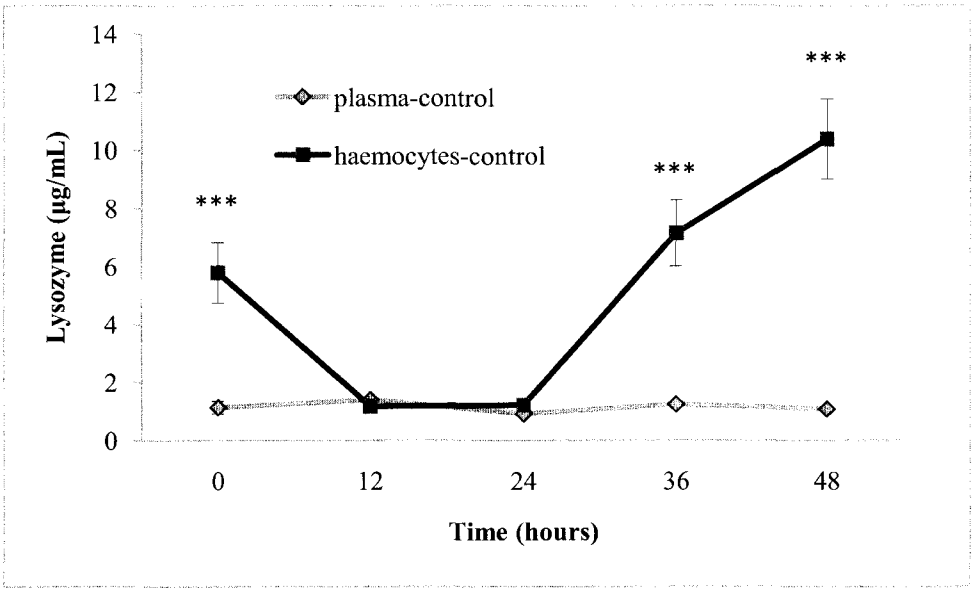

Figure 5.2.a Lysozyme activity in haemolymph of *Mya arenaria* from Ireland after injection with FSSW. Data are presented normalized with controls and expressed as mean ± SEM. Asterisks indicate the level of significant difference (*: $p < 0.05$) between plasma and haemocytes from infected clams.

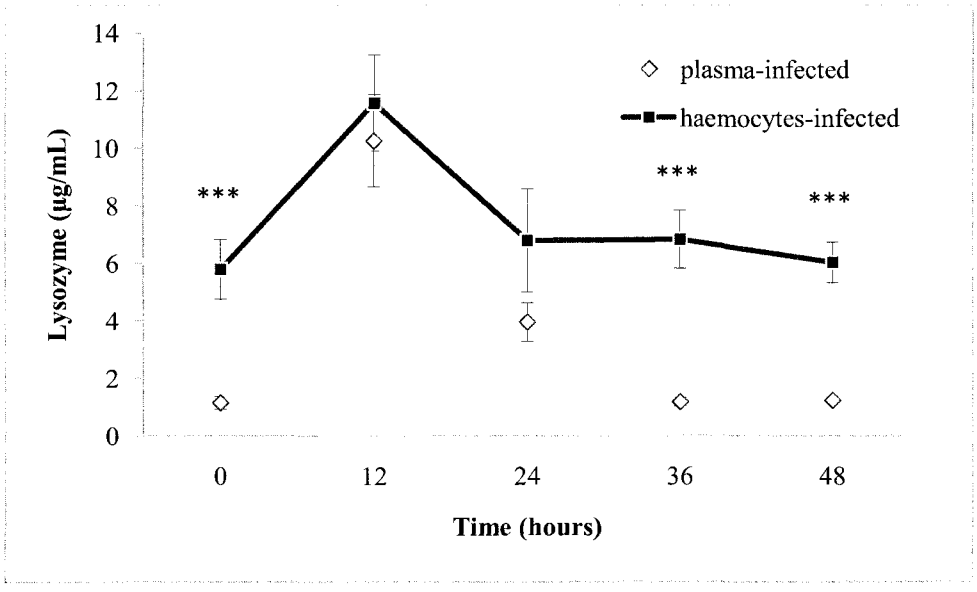

Figure 5.2.b. Lysozyme activity in haemolymph of *Mya arenaria* from Ireland after infection with *Vibrio splendiduds* LGP32. Data are presented normalized with controls and expressed as mean ± SEM. Asterisks indicate the level of significant difference (*: $p < 0.05$) between plasma and haemocytes from infected clams.

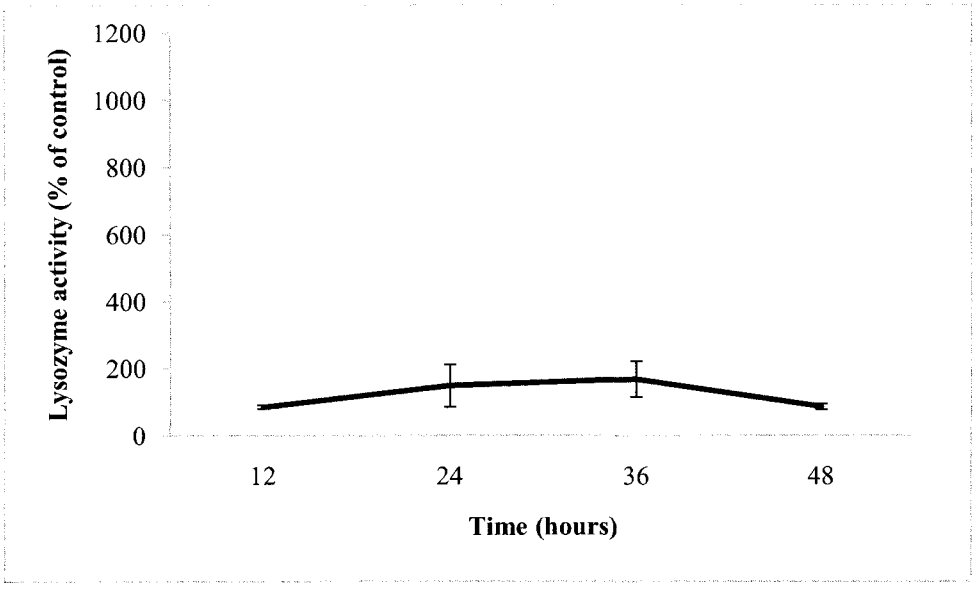

Figure 5.3.a. Lysozyme activity in plasma of *Mya arenaria* from Canada after infection with *Vibrio splendidus* LGP32-GFP. Data are presented normalized with controls and expressed as mean ± SEM.

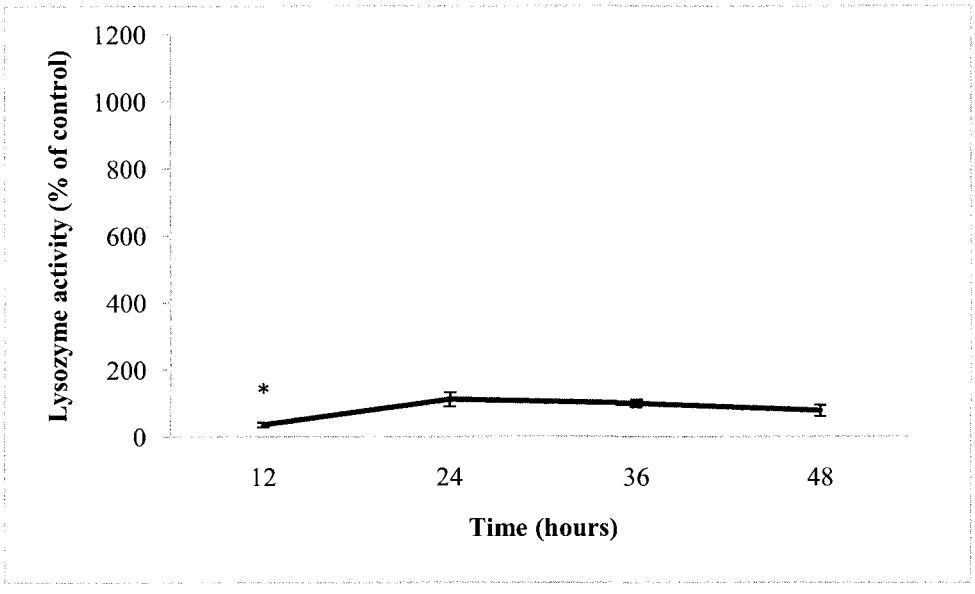

Figure 5.3.b. Lysozyme activity in haemocytes of *Mya arenaria* from Canada after infection with *Vibrio splendidus* LGP32-GFP. Data are presented normalized with controls and expressed as mean ± SEM. Asterisks indicate the level of significant difference (*: $p < 0.05$) between samples from control and infected clams.

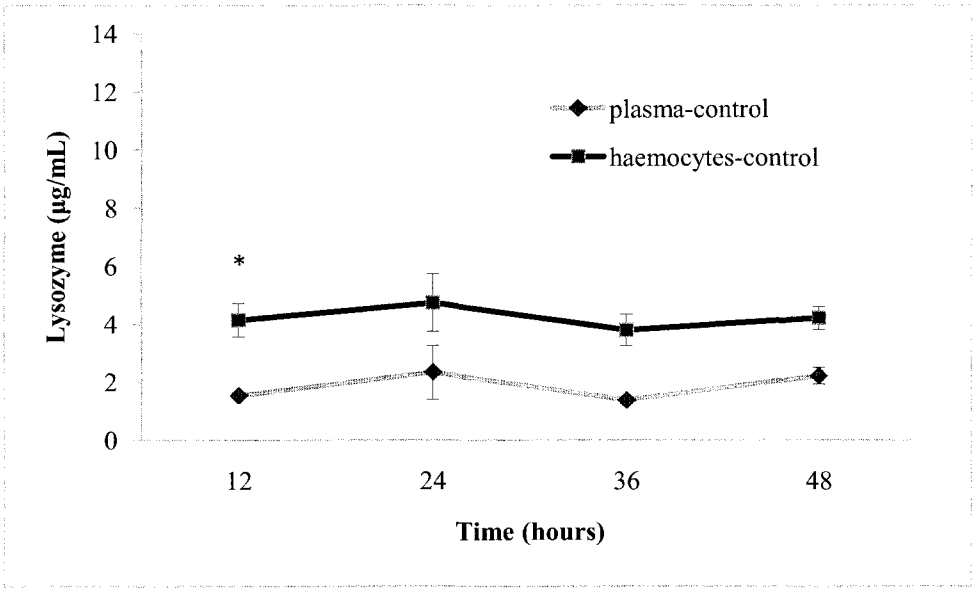

Figure 5.4.a. Lysozyme activity in haemolymph of *Mya arenaria* from Canada after injection with *Vibrio splendidus* LGP32. Data are presented normalized with controls and expressed as mean ± SEM. Asterisks indicate the level of significant difference (*: $p < 0.05$) between plasma and haemocytes from infected clams.

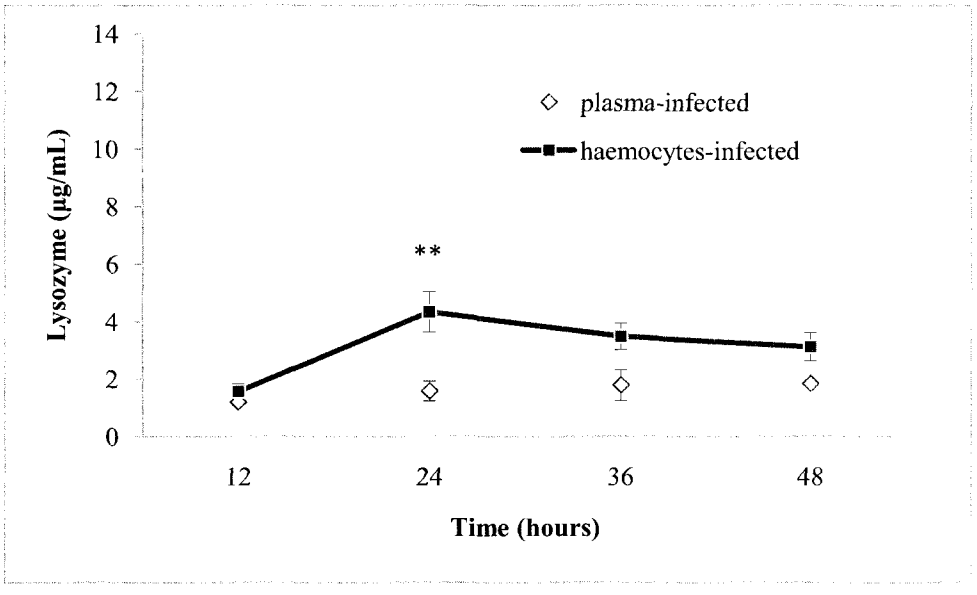

Figure 5.4.b. Lysozyme activity in haemolymph of *Mya arenaria* from Canada after infection with *Vibrio splendidus* LGP32. Data are presented normalized with controls and expressed as mean ± SEM. Asterisks indicate the level of significant difference (*: $p < 0.05$) between plasma and haemocytes from infected clams.

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Chapter 6. Preliminary study on the mechanisms involved in *Mya arenaria* haemocyte degranulation

6.1. Abstract

Regulated degranulation or exocytosis is a mechanism used by cells with immune functions. In bivalve haemocytes degranulation is triggered by bacteria but the mechanisms involved are unknown. In this study the degranulation mechanisms of *Mya arenaria* haemocytes were investigated using the β-hexosaminidase assay and various stimulants and inhibitors (in particular of G protein pathways). In addition, the expression of genes that might be activated by G proteins-mediated degranulation (NF-κB, defensin, and TNF) were assessed. The compound 48/80 triggered degranulation in haemocytes in a concentration-dependent fashion, which suggested the intervention of G proteins however this could not be confirmed as the inhibitors wortmannin and pertussis toxin did not prevent degranulation. None of the genes studied were significantly regulated at 1, 3, or 6h of haemocyte exposure to c48/80. A probable effect of stress in haemocytes during the assay could have accounted for the absence of gene regulation. Longer exposure for NF-κB, defensin, and TNF would also be necessary to detect gene regulation.

6.2. Introduction

The secretion of stored material (exocytosis) from intracellular vesicles or granules is a characteristic shared by many cell types involved in inflammatory responses and is a key process during innate immune response (Logan et al., 2003). Vesicles that secrete their contents into phagosomes are commonly referred to as lysosomes. Their degradative function is revealed by the presence of various hydrolases. Lysosomes that release their contents to the extracellular environment are called secretory lysosomes or lysosome-related organelles and are crucial in a variety of immune functions (Dell'Angelica et al., 2000; Holt et al., 2006).

In haemopoietic cells of the immune system, this process is tightly regulated (Stinchcombe and Griffiths, 1999) in opposition to the constitutive secretion characteristic of cells that secrete proteins without storing them in secretory granules (Burgess and Kelly, 1987). Stimulation of regulative secretion is carried out through specific ligands binding to cell surface receptors (e.g. antibodies bound to Fc receptors) (Stinchcombe and Griffiths, 1999). This activation is followed by the mobilization of vesicles to the plasma membrane in which membrane-associate proteins known as synaptosomal-associated protein receptors (SNAREs) and GTP-binding proteins play important roles (Watson, 1999; Logan et al., 2003; Holt, 2006). A transient rise of cytosolic Ca^{2+}, acting as a second messenger, and an increase of pH within lysosomes usually precede degranulation (Burgess and Kelly, 1987; Tapper, 1996).

Among the receptors involved in regulated exocytosis are Fc receptors, T cell receptors, collagen receptors and Toll-like receptors (TLR) (Stinchcombe and Griffiths, 1999; Janke et al., 2009). In vertebrate mast cells, exocytosis is mainly induced through

the high-affinity receptor for IgE (FcεRI) (reviewed in Rivera et al., 2006; Gilfillan and Tkaczyk, 2006). Degranulation mediated by FcεRI can be, however, regulated by other receptors such as the G protein-coupled receptors (GPCR) (Kuehn and Gilfillan, 2007) (Fig. 6.1). Upon binding to a variety of ligands, these receptors usually associate with heterotrimeric G proteins initiating signal transduction cascades that include the mitogen-activated protein kinase MAPK pathway (Kehrl, 1998; Bockaert and Pin, 1999; Kristiansen, 2004). The activation of G proteins results in the regulation of several enzymes, activation of ion channels and vesicle transport (Hamm and Gilchrist, 1996). In addition, G proteins have important roles in the activation of other cells with inflammatory functions through chemokine and chemoattractant receptors (Omann et al., 1987; Kehrl, 1998).

Aquatic invertebrates have a wide variety of defense factors as components of their immune system (reviewed in Iwanga and Lee, 2005). Many of these components have been localized in granules or lysosomes of haemocytes and upon stimulation are released during inflammatory responses (Cheng, 1983; Mitta et al., 2000; Bachère et al., 2004). In granular haemocytes of American and Japanese horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus* respectively, the signaling pathway of exocytosis can be triggered by LPS and occurs through G proteins (Solon et al., 1996; Ariki et al., 2004). Also, the antimicrobial peptide tachyplesin is able to induce exocytosis in horseshoe crab haemocytes through G protein cell signaling (Ozaki et al., 2005). However, the signaling pathways that lead to degranulation in bivalve molluscs remain to be revealed.

In order to shed light on possible mechanisms involved in degranulation of *Mya arenaria* haemocytes, this study aims: (1) to investigate the effect of stimulants and inhibitors of degranulation, commonly used in other cell types, and (2) to explore the molecular mechanisms of the degranulation process in molluscs by investigating the level of gene transcripts that would be related to the degranulation of *M. arenaria* haemocytes.

6.3. Materials and methods

6.3.1. Clams and haemolymph collection

Soft-shell clams *Mya arenaria* (approximately 5 cm), obtained from the Magdalen Islands (Gulf of Saint Lawrence, Quebec), were acclimatized for at least 1 week as indicated in previous chapters.

After acclimation, haemolymph samples (2-3 mL from each clam) were collected from the posterior adductor muscle of 5-15 clams, screened through an 80 µm mesh, to eliminate aggregated haemocytes or debris, pooled and placed in glass tubes on ice. Haemocyte concentrations were approximated using light microscopy (400x) and a haemocytometer.

6.3.2. Beta-hexosaminidase assay using 4 stimulants

The beta-hexosaminidase assay was a modification of a method previously reported (Kulka et al., 2007). Briefly, the assay consisted of placing 90 µL of haemolymph separately with 10 µL of each of four degranulation stimulants: compound 48/80 (c48/80, Sigma), calcium ionophore (Ca^{2+} ionophore, Sigma), lipopolysaccharide

(LPS, Sigma), and phorbol myristate acetate (PMA, Sigma) at 5 decreasing concentrations in wells (quadruplicated) of a 96 well microplate divided accordingly into 4 quadrants. Control wells were treated with 10 µL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. After the first aliquot of haemolymph, the microplate was centrifuged (1000 rpm, 5 min, 4°C) and, after disposing of the supernatant, a second aliquot was added to achieve approximately 4 x 10^4 cells/well (2.2 x 10^5 cells/mL). Microplates were incubated for 1h at 16°C and after centrifugation, 50 µL of supernatant was transferred to another microplate ("supernatant") while 50 µL of 0.1% Triton X-100 was added to the remaining haemolymph ("pellet"). Then, 50 µL of, the β-hexosaminidase substrate, p-nitrophenyl N-acetyl-β-D-glucosamine (pNAG, Sigma) was added to each well of both microplates and incubated for 2h at 37°C. Absorbance was read at A_{405} with reference at A_{570} and the percentage of release of β-hexosaminidase was obtained according to: 100 x supernatant/(supernatant + pellet).

6.3.3. Beta-hexosaminidase assay using c48/80

This assay, using only c48/80 with increasing concentrations as follows: 0.1, 1, 10, 100, 1000 µg/mL, was repeated 3 times in order to obtain a clearer pattern of degranulation.

In order to obtain sufficient RNA from haemocytes for cDNA synthesis this assay was performed with higher concentrations of haemocytes in a 24 well microplate with some modifications. An aliquot of 900 µL of haemolymph was placed with 100 µL of c48/80 at 1000 µg/mL. Each well had approximately 2 x 10^6 cells (10^6 cells/mL). Microplates were incubated for either 1, 3 or 6 h at 37°C.

6.3.4. Beta-hexosaminidase assay using c48/80 and inhibitors of degranulation

The methodology used during inhibitor experiments was as previously described with the addition of either of two degranulation inhibitors at different concentrations: Wortmanin (100, 10, 1, 0.1, 0.01, 0.001 μM) and pertussis toxin (100, 50, 10, 1, 0.1 nM). Ninety six well microplates were divided into four quadrants so that wells of each quadrant contained either 50 μL of cells + 50 μL of buffer; 50 μL cells + 10 μL c48/80 (100 or 1000 μg/mL) + 40 μL buffer; 50 μL cells + 10 μL of inhibitor (at decreasing concentrations) + 40 μL buffer; or 50 μL of cells + 10 μL of inhibitor (at decreasing concentrations) + 10 μL c48/80 (100 or 1000 μg/mL) + 30 μL of buffer.

6.3.5. Gene expression

Total RNA from haemocytes was extracted using RNeasy Micro kit (Qiagen) according to the manufacturer's protocol. RNA integrity was analysed using a Bioanalyzer (Aligent Technologies) and quantified using a Nanodrop spectrophotometer (ND-1000). Complementary DNA was synthesized using the SuperScript III Platinium Two-Step qRT-PCR kit (Invitrogen) according to the manufacturer's protocol as described in Chapter 4.

Expressed sequence tags (EST) of housekeeping gene candidates: EF-1α, EF-2, S-18 and ubiquitin, were obtained from a subtracted cDNA library of *M. arenaria* haemocytes (Siah, 2007). The two most stable genes were selected as internal controls to establish the relative expression of the target genes according to geNorm (Vandesompele et al., 2002) as described in Chapter 4. The target genes chosen were defensin, TNF and NF-κB, and were obtained from EST libraries generated from *M.*

arenaria haemocytes (Siah, unpublished). Primer design, quantitative PCR, and gene expression analyses were performed according to the procedure described in Chapter 4. The primer sequences of housekeeping and target genes are indicated in Table 6.1.

6.3.6. Statistical analysis

Statistical analysis was done using the statistical software Minitab 15.1.0.0. Data were transformed to the base 10 logarithm and then further analysed by one way general linear analysis of variance (ANOVA), followed by Bonferroni pairwise comparison. Data was analysed using the non parametric test Kruskal-Wallis followed by Mann-Whitney test for pairwise comparisons. A statistical significance level of 0.05 was considered for the differences in all tests.

6.4. Results

6.4.1. Beta-hexosaminidase assay

The percentage of release of β-hexosaminidase is shown in Figure 6.2. From the 4 stimulants, only c48/80 induced degranulation in haemocytes and it appeared to be dose dependent. After repeating this assay three times using only c48/80, a dose dependent pattern of degranulation was observed (Fig. 6.3). However, the percentage of β-hexosaminidase release induced by 0.1 and 1 μg/mL c48/80 was similar.

None of the inhibitors of degranulation was effective at preventing β-hexosaminidase release. Wortmanin was used at concentrations that ranged from 0.001 to 100 μM however a dose dependent association was not observed when haemocytes were exposed to 100 or 1000 μg/mL of c48/80 (Figs. 6.4.a and b). Similarly, pertussis

toxin did not inhibit β-hexosaminidase release, but instead seemed to stress haemocytes as they showed increased degranulation at 100 nM (Fig. 6.5).

6.4.2. Gene expression

The most stable housekeeping genes under this experimental condition were EF-1α and EF-2. These genes were selected as internal controls to estimate the relative expression of the target genes. By using these housekeeping genes it was found that there was no significant difference in the relative expression of any of the three genes analysed between control and haemocyte samples exposed to c48/80 (Fig. 6.6). There was, however, a time-related increase of NF-κB expression in both control and treated samples. This difference was significant between 1 and 6h for both sample groups and between 3 and 6h for treated samples ($p < 0.05$).

6.5. Discussion

In this study, the capacity of c48/80, Ca^{2+} ionophore, LPS and PMA to induce degranulation was investigated in haemocytes of soft-shell clams *Mya arenaria* by measuring the release of β-hexosaminidase. This lysosomal enzyme cleaves the glycosidic linkage of *N*-acetylglucosamine and *N*-acetylgalactosamine residues from glycoconjugates (Wendeler and Sandhoff, 2009). This characteristic has been applied in assays using different substrates in order to quantify the levels of cell degranulation (Mahuran et al., 1985).

Compound 48/80 is a cationic secretagogue composed of a mixture of phenylethylamine polymers cross-linked by formaldehyde (Ferry et al., 2002). The

compound 48/80 has the capacity to stimulate degranulation in vertebrate mast cells through activation of G proteins in a receptor-independent fashion (Mously et al., 1990; Kulka et al., 2007). This concept has, however, been challenged with the finding of lysophosphatidic acid receptor intervention (Palomäki and Laitinen, 2006). Calcium ionophore (Ca^{2+} ionophore) is a lipid-soluble molecule that transports Ca^{2+} across the lipid bilayer of cell membranes, therefore increasing the intracellular Ca^{2+} concentration of the cell. This molecule has been used to activate mast cells (Foreman et al., 1973), neutrophils (Omann et al., 1987; Lollike et al., 1995), and to induce exocytosis in lysosomes of fibroblasts and epithelial cells (Dell'Angelica et al., 2000). Phorbol myristate acetate (PMA) is a lipophilic molecule with the capacity to cross cell membranes and induce protein kinase C activation, similar to diacylglycerol (Castagna et al., 1982; Lunardi et al., 2006). PMA is commonly used to stimulate oxidative burst or degranulation of specific granules (DeChatelet et al., 1976; Omann et al., 1987; Welch et al., 1999) either through a mechanism that does not require calcium or that obtains it from intracellular stores (Lunardi et al., 2006). Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria and a member of the pathogen-associated molecular pattern (PAMP) family (Medzhitov and Janeway, 2000). LPS is able to induce cytokine release from macrophages (Galanos and Freudenberg, 1993) and mast cells (Supajatura et al., 2002; Varadaradjalou et al., 2003; Kirshenbaum et al., 2008) as well as nitric oxide from monocytes (He et al., 2008).

The compound c48/80 was able to induce degranulation in *M. arenaria* haemocytes in a dose dependent manner (Figs. 6.2 and 6.3), whereas LPS, Ca^{2+} ionophore and PMA failed. Although LPS is known to induce the production and release

of cytokines in inflammatory cells, it is not able to induce degranulation and Ca^{2+} mobilization in mast cells (Supajatura et al., 2002). Moreover, LPS was found to decrease β-hexosaminidase release in mast cells (Kirshenbaum et al., 2008). In horse shoe crabs *L. polyphemus* and *T. tricentatus* granulocytes, however, LPS could induce G-protein mediated exocytosis (Solon et al., 1996; Ariki et al., 2004). Exocytosis was triggered by a G protein-linked receptor, leading to IP_3 production which in turn binds to its receptor localized at the endoplasmic reticulum membrane, allowing the release of the Ca^{2+} from this organelle reticulum into the cytoplasm (Solon et al., 1996), and the proteolytic activity of the LPS-sensitive protease zymogen factor C in the granulocyte surface (Ariki et al., 2004). Given the similarity of function among invertebrate haemocytes, a similar mechanism was expected in soft-shell clams.

Since G proteins are known to be activated by c48/80 in mast cell degranulation (Mously et al., 1990; Chahdi et al., 2000; Ferry et al., 2002), the possibility of a G protein-mediated process of degranulation occurring in clam haemocytes was suggested. In order to confirm G protein involvement, pertussis toxin (PTX) and wortmannin were used as inhibitors of degranulation involving G protein activating pathways. Pertussis toxin catalyzes ADP-ribosylation on Gα subunits of heterotrimeric G proteins blocking the ability of the G protein to transduce signals to effectors, such as the inhibition of adenylyl cyclases (Gilman, 1995; Morris and Malbon, 1999; Watson, 1999; Kristiansen, 2004). Thus, PTX is often used to demonstrate the involvement of G proteins (Kehrl, 1998; Palomäki and Laitinen, 2006). Wortmannin is a, cell permeable, potent inhibitor of the phosphoinositide 3-kinase (PI-3 kinase). This specific toxin is used to investigate the dependency of further signals related to PI-3 kinase (Arcaro and Wymann, 1993;

Shpetner et al., 1996; Wymann et al., 1996; Morris and Malbon, 1999) also involved in G protein signaling (Stephens et al., 1997; Kehrl, 1998). In this study, neither of these two toxins inhibited degranulation (Figs. 6.4.a, b and 6.5). This would suggest that G protein is not involved in the degranulation haemocytes.

The gene expression levels in *M. arenaria* haemocytes after exposure to the basic secretagogue c48/80 were explored in this study. The levels of nuclear factor κB (NF-κB), tumor necrosis factor (TNF), and defensin transcripts were quantified in haemocytes of *M. arenaria* exposed to c48/80. NF-κB, TNF and defensin are involved, in different ways, in inflammatory responses. The transcription factor NF-κB, upon signal and by release from cytoplasmic sequestration, translocates into the nucleus and allows the up-regulation of the expression of cytokines essential to immune responses such as IL-1, IL-6, TNF-α, lymphotoxin, and IFN-γ (Ghosh et al., 1998). The cytokine TNF is involved in the regulation of immune responses, cell proliferation, differentiation and apoptosis in vertebrates (Betti et al., 2006). The highly conserved antimicrobial peptide defensin plays an important role in innate immunity among animals including bivalve molluscs (Mitta et al., 2000; Seo et al., 2005; Gueguen et al., 2006).

None of these three gene homologues were regulated in response to c48/80. Only, up-regulation of NF-κB was detected, but it was time- rather than treatment-dependent, and might be related to cellular stress. Probably, the activation of the expression of NF-κB, TNF and defensin is a process that requires longer incubation periods than was used with c48/80, after degranulation. It is known that the activation of G protein-coupled receptors results in stimulation of the mitogen-activated protein kinase (MAPK) pathway (Kehrl, 1998; Morris and Malbon, 1999; Kristiansen, 2004).

However, direct activation of MAPK through G proteins, stimulated by the basic secretagogue c48/80, has also been reported (Shefler et al., 1999). MAPK signaling pathways are involved in several cellular processes including inflammatory responses (Kaminska, 2005). Through crosstalk signals that involve NF-κB, MAPK can up-regulate the expression of inflammatory cytokines, including TNF-α (Campbell et al., 2004), and β-defensin (Jang et al., 2004). Moreover, the ability of c48/80, and some neuropeptides, to induce the production of several cytokines including TNF has been reported in mast cells (Kulka et al., 2007), suggesting the direct interaction of G proteins with external stimulants in the production of cytokines.

In conclusion, this study showed the capacity of c48/80 to induce degranulation. This suggested the direct activation of heterotrimeric G proteins initiating the cascades leading to degranulation. Unfortunately, the use of pertussis toxin and wortmannin, inhibitors of G protein subunits and the PI-3K respectively, could not confirm the involvement of the cascades generated by the activation of G proteins. None of the three genes: TNF, NF-κB and defensin tested were up-regulated in response to the treatment with c48/80. The lack of difference in the expression between treated and control samples might have occurred due to cellular stress under the experimental conditions. Further studies are required to understand the mechanisms leading to degranulation in haemocytes. For instance, as cells infected with bacteria seem to decrease in lysosome content (see Chapter 3) it would be interesting to study their response to c48/80. In addition, the study of expression genes directly involved in the degranulation process such as inostiol triphosphate receptor, and genes encoding for components of the heterotrimeric G-protein would reveal molecular mechanisms that occur in haemocytes.

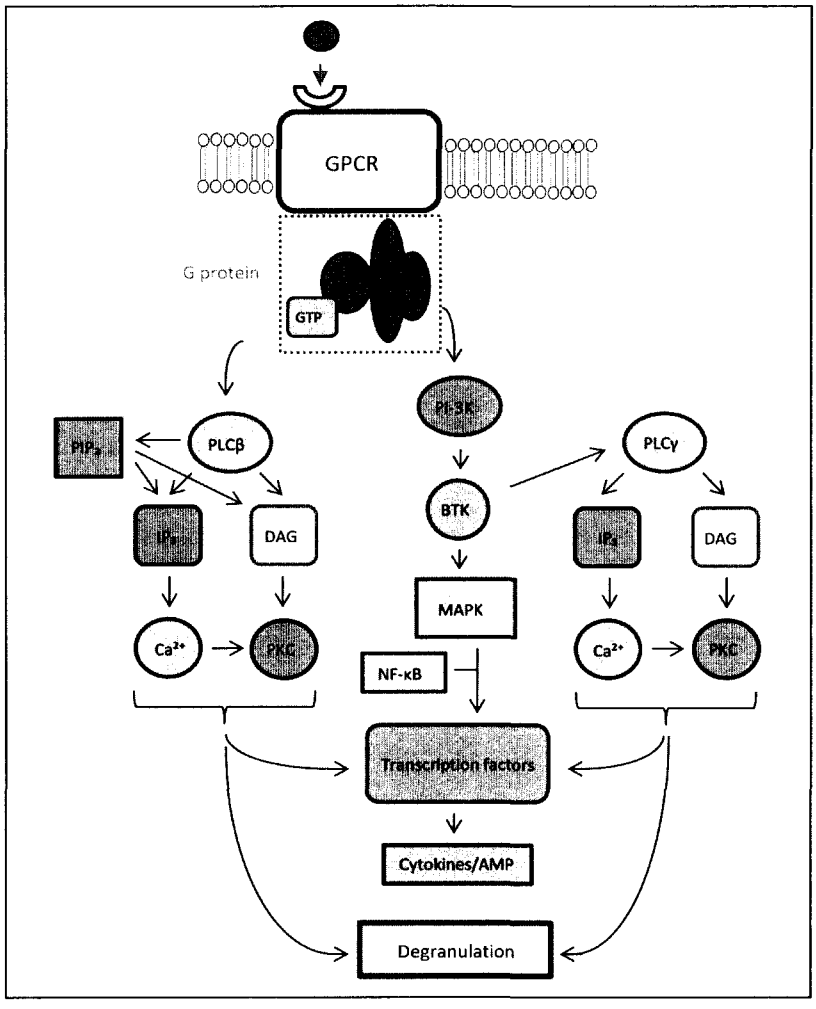

Figure 6.1. Signaling pathway of immune cell degranulation/cytokine production through G protein-coupled receptors (adapted from Shefler et al., 1999; Ferry et al., 2002; Gilfillan and Tkaczyk, 2006; Kuehn and Gilfillan, 2007). Immune cells have GPCR in their membranes that are linked to G proteins. G proteins are trimeric molecules composed by α, β and γ subunits. The α-subunit has a GDP molecule bound. When GPCR is activated GDP is replaced by a GTP and activate the G protein with subsequent separation of the α-subunit from the βγ-complex. The α-subunit then mediates the recruitment and activation of PLCβ whereas the βγ-complex activates PI-3K. PLCβ catalyzes the hydrolysis of PIP_2 and the liberation of IP_3 and DAG which allow the movement of Ca^{2+} from the endoplasmic reticulum and the activation of PKC, respectively. PI-3K contributes to maintain the calcium signal through the BTK enhancement of PLCγ activation. The release of Ca^{2+} and activation of PKC induce degranulation, and also the activation of transcription factors and the subsequent production of cytokines. This process is also induced by the activation of MAPK directly or through crosstalk with the NF-κB. BTK: Bruton's tyrosine kinase; DAG: diacylglycerol; GDP: guanosine diphosphate; GPCR: G protein-coupled receptor; GTP: guanosine triphosphate; IP_3: inositol (1,4,5)-triphosphate; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor κB; PI-3K: phosphoinositide 3-kinase; PIP_2: phosphatidylinositol (4,5)-bis phosphate; PKC: protein kinase C; PLC: phospholipase C.

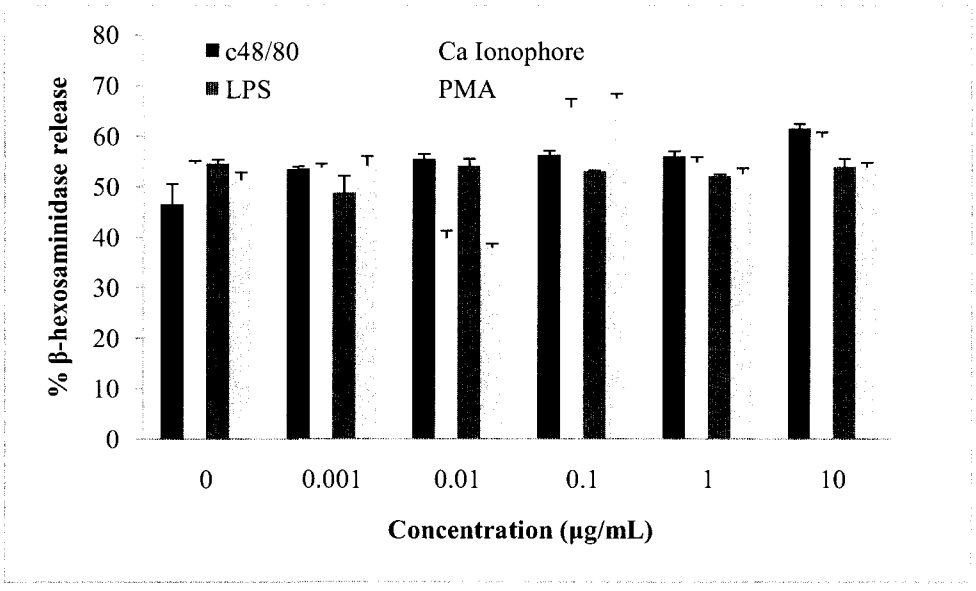

Figure 6.2. Percentage of β-hexosaminidase release in *M. arenaria* haemocytes exposed to compound 48/80, Ca^{2+} ionophore, LPS and PMA. Values are given as means ± SEM.

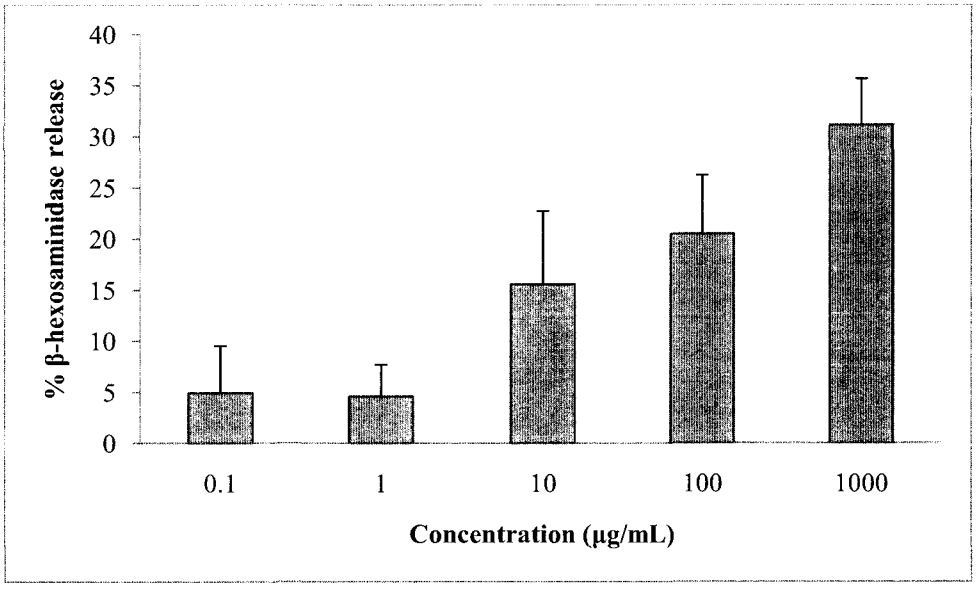

Figure 6.3. Percentage of β-hexosaminidase release in *M. arenaria* haemocytes exposed to compound 48/80. Values represent the mean (±SEM) of 3 assays.

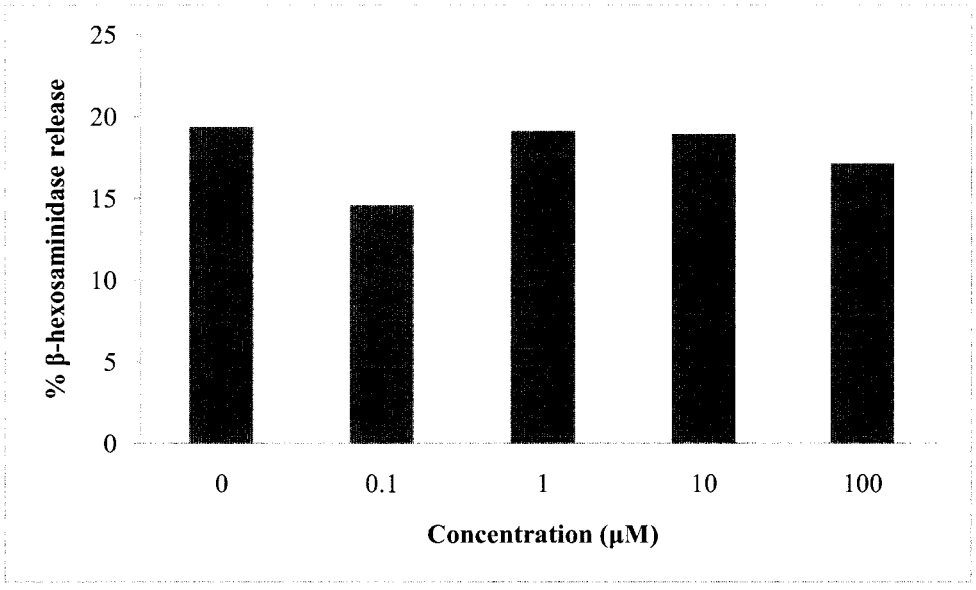

Figure 6.4.a. Percentage of β-hexosaminidase release by increasing concentrations of wortmannin in *M. arenaria* haemocytes exposed to 100 μg/mL of c48/80.

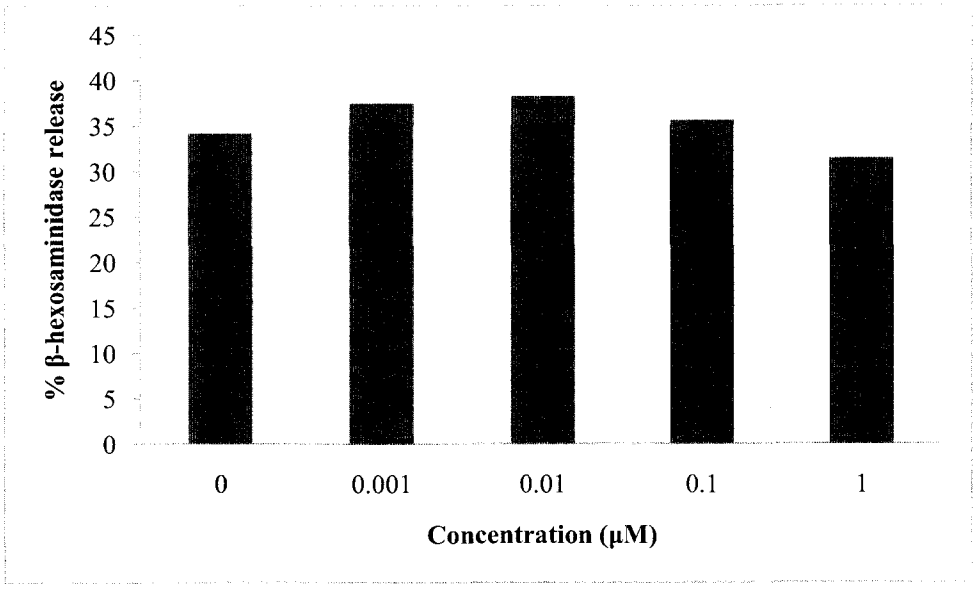

Figure 6.4.b. Percentage of β-hexosaminidase release by increasing concentrations of wortmannin in *M. arenaria* haemocytes exposed to 1000 μg/mL of c48/80.

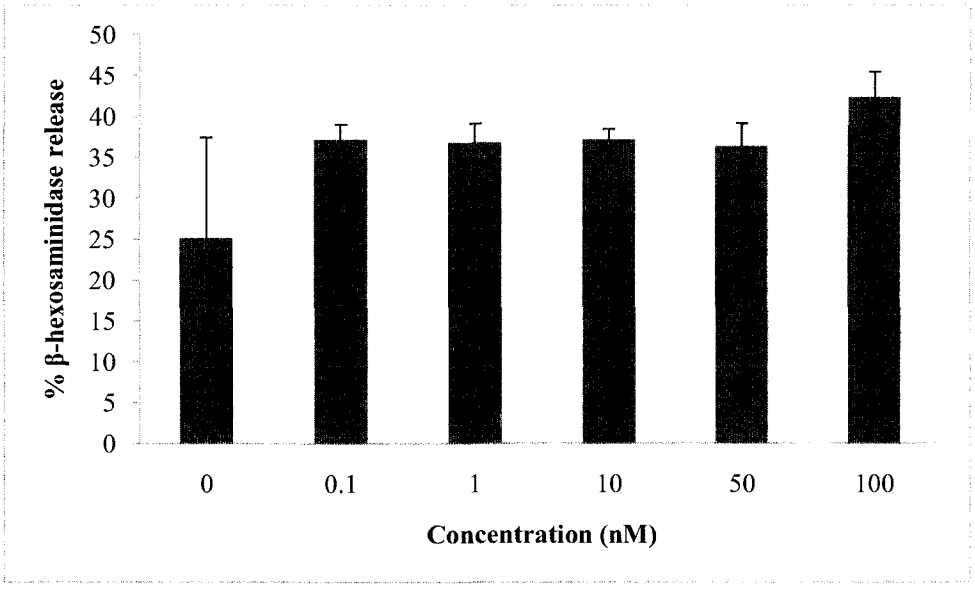

Figure 6.5. Percentage of β-hexosaminidase release by increasing concentrations of pertussis toxin in *M. arenaria* haemocytes exposed to 1000 μg/mL of c48/80. Values represent the mean (±SEM) of 3 assays.

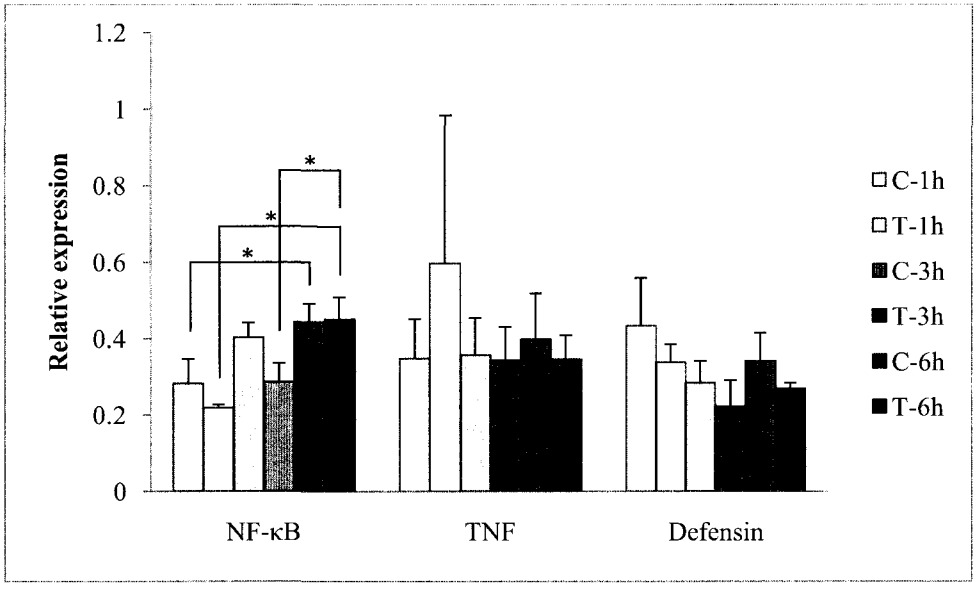

Figure 6.6. Relative expression of nuclear factor-κB, tumour necrosis factor and defensin gene homologues on control (C) and treated (T) haemocytes of *M. arenaria*, after exposure to buffer or compound 48/80, respectively for 1, 3 and 6h. Asterisks show significant difference ($p < 0.05$).

Table 6.1. Primer sequences and expected amplicon size corresponding to gene homologues (housekeeping and target), from the *Mya arenaria* cDNA library, used for gene expression assessment after exposure to compound 48/80

Gene	Primer	Sequence (5'-3')	Tm	Amplicon (bp)
Elongation factor 1α	Forward	GGTGGCTGTTGGTGTCATC	60	158
	Reverse	GGCCTAGGTGTTTTCCATGA		
Elongation factor 2	Forward	CTACAAGCCTGGCTCAAAGG	60	218
	Reverse	TGACAACTGGGCTGACAGAG		
Ribosomal protein S-18	Forward	AAGATTCCCGACTGGTTCCT	60	189
	Reverse	GCCGGTTGTCTTTGTATGCT		
Ubiquitin	Forward	TCGCTAAGGAGCTGGACATT	60	194
	Reverse	ACCGTCGCTCCTTGTACATC		
Nuclear factor κB (NF-κB)	Forward	AACGACGCTCTGTTCGTTTT	60	189
	Reverse	GACGTGGATTCTCGGACATT		
Tumor necrosis factor (TNF)	Forward	ACACCTGTTGCATTGTCGTC	60	181
	Reverse	TGTCCACTGTTCTCCGTCTG		
Defensin	Forward	GTTTTCTTCGCGATGTTGGT	60	119
	Reverse	CGACACCCGATTGACTTACA		

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Chapter 7. General Discussion

The use of models to study the interaction between hosts and bacteria is a useful approach to understand the immune responses on bivalve molluscs and the ability of pathogenic bacteria to modulate those responses. This research aimed to develop an *in vivo* model of interaction between soft-shell clam (*M. arenaria*) haemocytes and two strains of the marine bacteria *Vibrio splendidus*. Despite the constraints of developing an *in vivo* model using a wild bivalve (i.e. unknown previous health status and presence of environmental bacteria inside the host), the outcome of such models offer a more realistic panorama of host–pathogen interaction than *in vitro* models.

Many of the assessments of immune parameters in bivalves are, unfortunately, performed using non-standardized procedures. Besides the differences between *in vivo* and *in vitro* models, other variations occur in the dose of bacteria, the method of challenge, the method used to assess a specific parameter and the time points for reading the parameters under study. These variations in methodology preclude establishing valid parallels with results reported in the literature. This *in vivo* model was standardized using a dose of 4.5 x 10^6 bacteria/g of clam because of the notorious changes observed in the percentage of rounded haemocytes and the uniformity of the results achieved. With higher doses (e.g. 6 x 10^6 bacteria/g) there were clam mortalities in less than 24 h and by using lower doses (e.g. 3 x 10^6 bacteria/g) changes were minor and more individual variation between clams were obtained. An incubation time of 24 h, in seawater (at 16°C), was established because some clams would die on the second and third day after challenge (i.e. accumulative mortalities of 40% during the second day and 70% during the third day were observed). The survival during the first day was

mostly stable, having in most cases 100% survival (with several moribund clams), and occasional mortalities (8 - 13%). In addition, other *in vivo* studies assessed responses at 24 h or at different times that included a 24 h time point. Although injection is not a natural route for bacterial infection it offered a more uniform outcome and prevented the use of high quantities of bacterial culture if, for instance, challenge by immersion was chosen (i.e. only less than 5% rounded haemocytes in clams immersed in a bacterial suspension of approximately 5 x 10^8 bacteria/mL).

Nevertheless, the techniques used to assess the parameters described were as representative as possible with other published methods. Similar methods for assessing the change in the haemocyte cytoskeleton (i.e. percentage of rounded haemocytes) are described by Lane and Birkbeck (1999, 2000) in their *in vitro* assessment of bacterial toxicity. The method of assessing non-adherent haemocytes was similar to those described *in vitro* using bacteria (Choquet et al., 2003) or extracellular products (Labreuche et al., 2006a), and *in vivo* using bacteria (Labreuche et al., 2006b). The assessment of the number of haemocytes by flow cytometry was similar to that described using live (Labreuche et al., 2006b) and fixed haemocytes (Parisi et al., 2008). Similar analysis of the distribution of haemocytes using light scatter profiles were reported by Xing et al. (2002) with scallops *Chlamys farreri*, Tu et al. (2007) with clams *Meretrix lusoria*, and Parisi et al. (2008) with mussels *M. galloprovincialis*. Similarly, although using a logarithmic scale for the side scatter axis (complexity), haemocyte distribution was reported with oysters *C. virginica* (Aston-Alcox and Ford, 1998; Goedken and De Guise, 2004), and clams *R. philippinarum* (Allam and Ford, 2006), and using both forward (size) and side scatter in logarithmic scale was reported with oysters

C. gigas (Lambert et al., 2003), *S. glomerata* (Aladaileh et al., 2007), and mussels *M. galloprovincialis* (García-García et al., 2008). The methods used for quantification of cDNA to infer the mRNA expression of genes related to immune and physiological function (i.e. Reverse Transcriptase real time quantitative PCR) followed standard procedures (Bustin, 2000; Huget et al., 2005). Similar methodology has been applied for assessing expression of several genes involved in bivalve immune functions, including *HSP70* in *M. galloprovincialis* (Cellura et al., 2006, 2007), defensin in *C. gigas* (Gonzalez et al., 2007) and *Argopecten irradians* (Zhao et al., 2007), Toll (Qiu et al., 2007) and kazal-type serine protease inhibitor (Wang et al., 2008) in *C. farreri*, lysozyme in *C. virginica* (Itoh et al., 2007) and *M. galloprovincialis* (Li et al., 2008), superoxide dismutase, peroxiredoxin 6, IK cytokine, IκB in oyster *S. glomerata* (Green and Barnes, 2009) after challenge with bacteria or their components. Most of these studies have used only one housekeeping gene as an internal control (β-actin, 28S or elongation factor) as opposed to the model developed in this thesis that included more than one housekeeping genes which has become the recognized standard (Vandesompele et al., 2002). The assessment of lysozyme levels in haemolymph was accomplished using a widely accepted test for lysozyme activity used, with slight modifications, in several other studies on bivalves (Chen and Rodrick, 1974; Rodrick, 1979; Carballal et al., 1997; López et al., 1997; Cronin et al., 2001; Olsen et al., 2003; Xue et al., 2004, 2007; Itoh et al., 2007; Li et al., 2008; Ciacci et al., 2009) based on the method established by Shugar (1952).

In order to evaluate and compare the immune modulatory capacity that marine bacteria can have in *M. arenaria* haemocytes, the responses induced by two strains of *V.*

splendidus were assessed. *Vibrio splendidus* LGP32-GFP is derived from strain (LGP32) associated with juvenile oyster mortalities in France (Gay et al., 2004a,b), while 7SHRW is an environmental strain isolated from marine sediments in Prince Edward Island (Mateo, 2006). The responses assessed involved various phenotypic features including changes in haemocyte structure, adhesion, total numbers as well as granulocyte and hyalinocyte lysosome content. Molecular responses were also investigated to monitor changes in the expression of genes related to immune and physiological functions. These assessments showed differential responses induced by both LGP32-GFP and 7SHRW strains.

Various phenotypic responses were obtained through microscopy and flow cytometry (see Chapter 2). Significantly higher percentages of rounded haemocytes, higher total numbers of haemocytes and decreased adhesion capacity were observed in haemocytes challenged with LGP32-GFP. These data contrasted with moderate to insignificant changes induced by 7SHRW and illustrated the more virulent nature of the strain LGP32-GFP. Although viable (from observations using vital staining with trypan blue), rounded haemocytes and the concomitant diminished capacity to adhere likely would have affected the capacity of *M. arenaria* haemocytes to phagocytose. These alterations in cell structure and adhesion have been previously observed in other bivalve haemocytes after challenge with *Vibrio* species (Lane and Birkbeck, 1999; Choquet et al., 2003; Allan and Ford, 2006; Labreuche et al., 2006a, b) and are likely associated with the presence of toxins such as proteases and haemolysins (Nottage and Birkbeck, 1987; Borrego, 1996; Lane and Birkbeck, 1999). Similar phenotypes have been observed in human cells affected by *V. cholerae* toxin which depolymerises F-actin of

the cytoskeleton (Fullner and Mekalanos, 2000; Kudryashov et al., 2008). The recently described metalloprotease Vsm produced by *V. splendidus* LGP32 caused cytopathic effect in Bge cells (Binesse et al., 2008) and would be expected to have similar effects in *M. arenaria* haemocytes. Moreover, in this study, elevated percentages of rounded haemocytes coincided with significant up-regulation of actin gene expression after challenge with LGP32-GFP (see Chapters 2 and 4). Thus, the drastic changes in the cytoskeleton may have accounted for a compensatory response at the molecular level.

Increased numbers of haemocytes may account for the need to amplify the defensive response against infectious pathogens. Similar observations have been reported in other bivalves challenged with *Vibrio* species (Suresh and Mohandas, 1990; Oubella et al., 1993; Allam et al., 2001, 2006; Labreuche et al., 2006b; Parisi et al., 2008). Increased numbers however do not guarantee a successful outcome as has been observed in infections with the protozoan *Perkinsus marinus* (Oliver and Fisher, 1999). The increased haemocyte numbers appear to be a common response to different situations of stress (Malagoli et al., 2007; Jones et al., 1995) including pathogen infection (Ford and Tripp, 1996; Bachère et al., 2004). It is not clear if the increase in haemocyte numbers is due to activation of haematopoiesis and proliferation or the result of redistribution of the cells into different host compartments as haemocytes tend to migrate towards the focus of infection (Bachère et al., 2004). Although the differentiated haemopoietic tissue has not been clearly identified in bivalves, the connective tissue has been suggested as a possible location in oysters (Cheng, 2000). It was observed, however, that infected clams seemed to contain less volume of total haemolymph than

control clams, therefore the increase of cells may indicate an increase in cell density rather than numbers.

Responses at a cellular level, such as the study of the distribution of haemocytes after infection was assessed thorough flow cytometry light scatter profiles and using the fluorescent dye LysoTracker as an indicator of the presence of lysosomes (see Chapter 3). The cell size and complexity revealed two subpopulations of haemocytes in *M. arenaria*. The larger and more complex cells contained more lysosomes than the smaller and less complex cells. This suggested that *M. arenaria* possesses two distinct subpopulations: granulocytes and hyalinocytes, as previously reported using light scatter profiles without fluorescence (Brousseau et al., 2000; Fournier et al., 2001, 2002). After challenge, *M. arenaria* haemocytes experienced changes in their distribution that varied according to the bacterial strain, with the change being more drastic after challenge with LGP32-GFP than with 7SHRW. After infection, there was no distinction between hyalinocytes and granulocytes but instead, haemocytes appeared to form a single continuous group. Moreover, during infection a decrease in lysosome content was observed using LysoTracker in both hyalinocytes and granulocytes, as measured by flow cytometry. This decrease was more striking in granulocytes as revealed by the decrease of LysoTracker fluorescence in the subpopulation of haemocytes with larger size and complexity. The loss of lysosomes in granulocytes suggested degranulation as a consequence of the infection. Release of lysosome content is acknowledged as a common feature in bacterial infections in bivalves, including *M. arenaria* (Chen and Rodrick, 1974; Rodrick, 1979, Cheng, 1983, Chu, 1988). Reduced amounts of lysosomes in hyalinocytes, however, could indicate the occurrence of precursor cells

prematurely released to the circulation. This is a common pattern in mammals during severe infections, known as "left shift", and occurs upon inflammation and septic shock (Opdenakker, 2001). In several bivalves, precursor cells have been reported in circulation (Hine, 1999; Cima et al., 2000, Chang et al., 2005; Aladaileh et al., 2007; Matozzo et al., 2008). Moreover, Cima et al. (2000) found that the small and undifferentiated haemocytes of *R. philippinarum* (called "haemoblasts") reacted positively to anti-CD34 antibody known to identify haemapoietic cells in mammals. Since some of these haemoblasts have mitotic actin spindles, characteristic of mitotic cells, circulating haemoblast division was suggested to account for at least part of the new haemocytes (Matozzo et al., 2008). Further studies are necessary to establish if similar mechanisms account for the increased haemocyte numbers observed in *M. arenaria* (described in Chapter 2).

Responses at the molecular level, such the expression of several genes involved in immunological and physiological processes were investigated through RT real time qPCR (see Chapter 4). In an initial step, the evaluation of housekeeping genes was performed in order to select suitable internal controls under the experimental conditions of this study. While EF-1α and EF-2 were selected under challenge with LGP32-GFP, EF-1α and S-18 were selected with 7SHRW using the software geNorm (Vandesompele et al., 2002). Under challenge using both strains, γ-actin was among the least stable housekeeping gene candidates. These results confirm the need to evaluate traditionally used housekeeping genes under the specific experimental conditions for an accurate analysis as established for *M. arenaria* under *in vitro* bacterial infection (Araya et al., 2008) and different ploidy status analysis (Siah et al., 2008).

When compared to control clams, the expression of IRAK-4, TLR-2, defensin and lysozyme-2 was significantly different after challenge with LGP32-GFP. After infection with 7SHRW, only the expression of lysozyme-2 was significantly different. With the exception of defensin, a similar trend in gene regulation was found after challenge with both strains. While TLR-2 and lysozyme were down-regulated, the expression of IRAK-4 was up-regulated in both cases. However, down-regulation of TLR-2 and up-regulation of IRAK-4 was significantly different between both strains, showing that the effect of LGP32-GFP was more marked than 7SHRW. The expression of IRAK-4 after challenge with LGP32-GFP suggested the involvement of NF-κB, a transcription factor engaged in the expression of immune genes such as cytokines. Possible involvement of NF-κB in bivalve immune response pathways has been previously suggested (Gueguen et al., 2003; Montagnani et al., 2004; Roberts et al., 2009; Green and Barnes, 2009). These results reveal the ability of *V. splendidus* strains to modulate cell signaling through molecular pathways related to innate immunity in *M. arenaria* haemocytes. Other molecular pathways could be also activated by bacterial infections in bivalves. The activation of MAPK and other kinase cascades in bivalve haemocytes induced by bacterial infection have been suggested (Canesi et al., 2002; 2005; Cao et al., 2004; García-García et al., 2008; Roberts et al., 2009).

The responses observed at cellular and molecular levels induced by LGP32-GFP and 7SHRW implies that the bivalve immune system is differentially activated by specific bacteria. Responses could be interpreted either as an efficient activation of the immune system or, on the contrary, the effect of bacterial manipulation subverting the host defense mechanisms. The rounding of the cytoskeleton and the decreased adhesion

suggest bacterial manipulation to avoid phagocytosis and other cellular defense mechanisms. The increase of haemocyte numbers and degranulation likely has a negative impact on bacteria. While up-regulation of IRAK-4 suggests activation of the NF-κB, the effect of TLR-2 down-regulation is not clear. It would be more reasonable that TLR and IRAK genes be co-regulated as they are part of the same pathway. In human immune cells TLR-2 is activated by Gram-positive bacteria and TLR-4 by Gram-negative (Takeda and Akira, 2004). It could be hypothesized that the down-regulation detected corresponds to a host strategy to direct their resources to the expression of other genes involved in mechanisms against Gram-negative bacteria. Finding a TLR-4 gene homologue in clam haemocytes would shed light on the specificity of receptors used by *M. arenaria*. A Toll receptor has been characterized in scallop *Chlamys farreri* and its expression is regulated by LPS, a component of the outer membrane of Gram-negative bacteria, in a dose-dependent fashion (Qiu et al., 2007). It is, however, unknown if bivalves possess different TLR receptors with particular specificities. The possibility of other receptor genes that are up-regulated by the Gram-negative *V. splendidus* cannot be ruled out. The meaning of lysozyme down-regulation for the host is also unclear. Host defense lysozyme is mostly expressed in haemocytes. Assuming that lysozyme-2 is involved in host defense functions, its down-regulation could be the result of bacterial manipulation. This kind of mechanism is expected in a pathogenic strain such as LGP32-GFP but lysozyme-2 was also significantly down-regulated by 7SHRW. Probably, environmental bacteria exert some degree of host immune manipulation that allows them to survive in the host without causing harm. Nevertheless, it is important to notice the limitations of assessments at a single time-point. The phenotypic and

molecular responses observed might have varied before or after 24h. Thus, kinetic studies are necessary to unravel the pattern of the transcript levels in response to bacterial infections.

The study of responses at the protein level involved the assessment of lysozyme activity in clam haemolymph components after bacterial challenge (see Chapter 5). As mentioned above, lysozyme is an enzyme commonly used in defense mechanisms and its activity has been reported to be affected by bacterial challenge in bivalves (Allam et al., 2000, 2006; Ciacci et al., 2009). In this case, clams from two geographic origins: Canada and Ireland were challenged with *V. splendidus* LGP32 (with and without GFP, respectively). A clear increase in lysozyme activity was detected in plasma and haemocytes of clams from Ireland after challenge. The peak of increase occurred at 12 h after challenge and returned to levels, similar to control by 36 h. The levels of lysozyme were higher in haemocytes than plasma in clams from both origins. In clams from Canada, there were no clear differences in the levels of lysozyme between control and infected clams. Either deterioration of the samples, lower intensity in the responses of Canadian clams (probably influenced by environmental differences), or differences in the virulence of the bacterial strains used (wild-type vs. genetically modified) might have accounted for the detected outcome.

Finally, the mechanisms involved in haemocyte degranulation were studied using stimulants and inhibitors of degranulation (see Chapter 6). As pointed out above (Chapters 2 and 3), degranulation of haemocytes seemed to be triggered by bacterial challenge with *V. splendidus*. The compound 48/80 was able to induce degranulation in *M. arenaria* haemocytes in a dose dependent manner suggesting the involvement of G

proteins (Mously et al., 1990; Kulka et al., 2007). Unfortunately, this could not be confirmed by using inhibitors of molecules that play a role in G protein pathways such as pertussis toxin, which directly affects G proteins, or with wortmannin, an inhibitor of PI-3 kinase (Kehrl, 1998; Morris and Malbon, 1999). In addition, the expression of genes involved in immune responses, such as defensin, NF-κB and TNF, were assessed. None of these genes were regulated in response to c48/80 either after 1, 3 or 6 h. These results suggest that gene expression is not regulated during the degranulation process in haemocytes of *M. arenaria*. It is also possible that the expression of the genes involved in immune functions require longer activation times.

In conclusion, no previous studies exist that examine the immune responses of soft-shell clams *M. arenaria* to bacterial challenge. Most of the studies of immune parameters of *M. arenaria* haemocytes have been focused on responses against diverse pollutants (Fournier et al., 2001, 2002; Blaise et al., 2002; Gauthier-Clerc et al., 2006; Gagné et al., 2006, 2008). This study describes a series of *in vivo* immune responses, at both cellular and molecular levels induced by two *V. splendidus* strains on *M. arenaria* haemocytes. This study could be considered as the foundation *in vivo* model of soft-shell clam haemocyte interaction with marine bacteria that could be used in future studies to assess other immune responses. It reveals that the strain LGP32-GFP cause changes in the haemocyte cytoskeleton, possibly due to the presence of a toxin, such as the recently discovered metalloproteinase Vsm (Binesse et al., 2008). These changes in the cell structure were associated with the up-regulation of γ-actin gene which may be a compensatory mechanism to the cytoskeleton disarrangement. The striking changes in the haemocyte cytoskeleton were also reflected in the concomitant loss of adherence.

Higher numbers of total haemocytes induced by LGP32-GFP occur in parallel with a distortion on the distribution of haemocyte subpopulations observed in flow cytometry light scatter profiles. Distinctly distributed hyalinocytes and granulocytes merged into a single group after infection with a parallel drastic loss of lysosomes, especially in granulocytes, as revealed by LysoTracker. Low lysosome content in hyalinocytes suggests the occurrence of immature or precursor cells that likely account for the increased numbers of total haemocytes. In granulocytes, degranulation seems to explain the decreased lysosome content. Since degranulation could be induced by c48/80 the involvement of G proteins was suggested however this could not be confirmed with inhibitors of G protein pathways. Furthermore, the regulation of IRAK-4 and TLR-2 expression levels suggests activation of the NF-κB pathway, a common signaling cascade that probably leads to the expression of inflammatory cytokines, in haemocytes as a response to bacterial challenge. However, the down-regulation of lysozyme gene expression suggests a mechanism that *V. splendidus* has to subvert the defense responses of *M. arenaria* haemocytes to either proliferate, in case of the pathogenic strain LGP32-GFP, or to survive within the host, in the case of the environmental strain 7SHRW.

It is important to mention that the responses assessed are only a part of the whole defensive system of soft-shell clams and only represent a "snap shot" taken at 24 h after challenge. Studies on the kinetics of these haemocyte responses are necessary to have a more complete picture of the immune processes involved. As previously suggested (Adamo, 2004), the health status of an organism and the complexity of its immune mechanisms can only be approached by the study of multiple parameters and the inter connection among them.

Similar phenomena to "left shift" and "septic shock", which are common aspects in mammalian infections, have been proposed to occur in bivalves. These responses would account for the possible presence of precursor cells and the tissue damage and eventually death of the host, respectively. In addition, the novel use of LysoTracker to identify haemocyte subpopulations in bivalves, by their lysosome content, and their changes after bacterial infection has been established.

The phenotypic assessments performed in this study are of high reproducibility for an *in vivo* model. Although the genes studied do not encompass entire pathways of immune processes, their regulation offers an indication of possible mechanisms that are activated by *Vibrio* species in *M. arenaria* haemocytes. Nevertheless, the expression of these genes has potential to be used as markers at the early stage of infection in health assessments of soft-shell clam populations. The whole model of phenotypic and molecular responses could be applied to evaluate the health status of soft-shell clam populations.

Furthermore, the study of the capacity of *V. splendidus* strains to modulate the immune responses of soft-shell clam haemocytes was described at the cellular and molecular level. Some of the responses observed in *M. arenaria* appeared to be induced by LGP32-GFP and manipulated in order for the bacteria to subvert the host defenses and proliferate. This could be related to the presence of a toxin such as metalloprotease (Vsm). As expected, the environmental strain 7SHRW induced very mild responses in the host. This reveals the evolutionary co-existence of host and the bacteria in its local environment. Indeed, differential interaction between mollusc hosts and diverse bacteria has been observed previously between indigenous and non-indigenous bacteria and

between pathogenic and non-pathogenic bacteria. A higher ability to adhere and multiplicate of indigenous bacteria as a result of co-evolution with their host would explain their persistence in bivalves (Jones et al., 1991; Canessi et al., 2002). While pathogenic bacteria have the ability to establish themselves within the host by evading host immune factors or interfering with cell signaling pathways, non-pathogenic bacteria trigger host immune mechanisms leading to activate immune responses (Canessi et al., 2005; Pruzzo et al., 2005; Travers et al., 2009).

In this study, soft-shell clams from Ireland were tested for the first time for an immune indicator after bacterial challenge. The pattern of lysozyme activity seemed to be different between clams from Canada and Ireland, but was not conclusive. Further studies are required to confirm the pattern of activity observed after challenge. The involvement of G proteins in the degranulation mechanism in *M. arenaria* haemocytes also requires further investigation to reveal mechanisms that might be used by bacteria to activate or deactivate host defenses.

This model of interaction between *M. arenaria* haemocytes and *V. splendidus* contributes to our further understanding of host immune mechanisms during bacterial infections and the bacterial strategies to subvert host defenses. This *in vivo* model could be enriched with future immune assessments and kinetic studies. The application of this model could also be used to study the health status of soft-shell clams, or other bivalves, and their interactions with various other bacteria that might constitute health challenges to wild or cultured populations.

7.1. Future directions

The model of interaction is prepared to be exploited to further investigate the mechanisms of the immune system of *M. arenaria*, or other bivalves, and the extent of the ability of pathogenic bacteria to modulate the immune response of their host. Various marked phenotypic and molecular changes were detected after challenge with the strain LGP32-GFP. Except for the changes in adhesion capacity, that implied a direct impairment in the functionality of haemocytes, demonstration of immune functions caused by other responses is required. Additionally, the regulation of genes involved in immune responses suggested pathways of immune activation that require further gene expression assessments. In respect to the bacteria, the virulence factor(s) involved in the responses induced by the strain LGP32-GFP need to be explored.

Future investigations should include the following suggestions:

1. Metalloprotease Vsm- and outer membrane protein OmpU-deficient *V. splendidus* strains are available (Le Roux et al., 2007 and Duperthuy et al., 2010, respectively) and could be used to determine their involvement in virulence and in the responses observed. The same model could be used with LGP32 mutant and wild type bacteria in haemocytes of *M. arenaria*.
2. Similar responses could be investigated using extracellular products (ECPs) in order to establish the origin of the virulence factors (i.e. endotoxin vs. exotoxin)
3. The model could be used to identify the disruption of haemocyte functions such as phagocytosis, ROS and NO^- production, with and without metalloprotease. These assays could be approached using bacteria and ECPs.

4. The molecular mechanism pathways could be approached using an open source strategy such as microarray analysis. No microarray platform is currently available for *M. arenaria*. However, several studies generated EST libraries that could be combined into microarray tools. Using this tool, kinetic experiments using LGP32 with and without metalloproteinase could be performed.
5. In order to confirm haemocyte degranulation after bacterial infection, the β-hexosaminidase assay could be tried in haemocytes from challenged clams.
6. The use of a CD34 antibody in flow cytometry sorted hyalinocytes could help to confirm the presence of precursor haemocytes in *M. arenaria* after infection with LGP32-GFP.

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APPENDIX A

A.1. Assessment of phenotypic and molecular responses in *Mya arenaria* haemocytes, induced by infection with *Vibrio splendidus* LGP32-GFP and 7SHRW, expressed as *p* values after analysis with parametric and non-parametric statistical models. (1): Sign test; (2): Mann-Whitney test; (3): ANOVA; (4) Paired t-test; (5) Two-sample t-test; H: hyalinocytes; G: granulocytes; n.a.: not assessed.

	LGP32-GFP vs. control (or before infection*)	7SHRW vs. control (or before infection*)	LGP32-GFP vs. 7SHRW
Rounded cytoskeleton*	0.00 (1)	0.00 (1)	0.00 (2)
Decreased adhesion	0.00 (3)	0.09 (3)	0.00 (3)
Increased total numbers*	0.00 (4)	0.03 (4)	0.01 (5)
Decreased lysosome content	H: 0.00 (3) G: 0.00 (3)	H: 1.00 (3) G: 1.00 (3)	n.a. n.a.
γ-actin gene relative expression/x-fold	0.00 (5)	0.55 (5)	0.00 (5)
LGBP gene relative expression	0.13 (5)	n.a.	n.a.
TLR-2 gene relative expression/x-fold	0.00 (5)	0.17 (2)	0.00 (5)
IRAK-4 gene relative expression/x-fold	0.00 (5)	0.53 (5)	0.02 (5)
Mpeg gene relative expression	0.16 (5)	n.a.	n.a.
Defensin gene relative expression/x-fold	0.05 (2)	0.42 (5)	1.00 (5)
Lysozyme-2 gene relative expression/x-fold	0.04 (5)	0.02 (5)	0.07 (5)
HSP70 gene relative expression	0.13 (5)	n.a.	n.a.
HSP90 gene relative expression	0.19 (5)	n.a.	n.a.