1 **Immune responses to infectious diseases in bivalves**

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Abstract

Many species of bivalve mollusks (phylum Mollusca, class Bivalvia) are important in fisheries and aquaculture, whilst others are critical to ecosystem structure and function. These crucial roles mean that considerable attention has been paid to the immune responses of bivalves such as oysters, clams and mussels against infectious diseases that can threaten the viability of entire populations. As with many invertebrates, bivalves have a comprehensive repertoire of immune cells, genes and proteins. Hemocytes represent the backbone of the bivalve immune system. However, it is clear that mucosal tissues at the interface with the environment also play a critical role in host defense. Bivalve immune cells express a range of pattern recognition receptors and are highly responsive to the recognition of microbe-associated molecular patterns. Their responses to infection include chemotaxis, phagolysosomal activity, encapsulation, complex intracellular signaling and transcriptional activity, apoptosis, and the induction of anti-viral states. Bivalves also express a range of inducible extracellular recognition and effector proteins, such as lectins, peptidoglycan-recognition proteins, thioester bearing proteins, lipopolysaccharide and β 1,3-glucan-binding proteins, fibrinogen-related proteins (FREPs) and antimicrobial proteins. The identification of FREPs and other highly diversified gene families in bivalves leaves open the possibility that some of their responses to infection may involve a high degree of pathogen specificity and immune priming. The current review article provides a comprehensive, but not exhaustive, description of these factors and how they are regulated by infectious agents. It concludes that one of the remaining challenges is to use new “omics” technologies to understand how this diverse array of factors is integrated and controlled during infection.

Keywords: Immunity, Innate, Mollusk, Bivalve, Hemocyte

1. Introduction

Global production of bivalve mollusks reached over 13.2 million tons in 2012 representing a commercial value in excess of 16 billion US\$ (FAO, 2014). This growing economic importance of bivalves has been associated with an increased awareness of, and attention to, infectious diseases affecting these animals. Currently, there are eight infectious diseases impacting mollusks that are listed by the Office International des Epizooties (OIE, the World Organization for Animal Health), including two viral infections, a prokaryote infection and five infections caused by protistan pathogens. Six out of these 8 infections impact bivalves. In parallel, there was a growing interest in the study of bivalve immunity and in the exploration of mechanisms used by these organisms to fight and resist infectious agents (Bachère et al., 2004; Bayne, 1983; Cheng, 1981; Chu, 1988; Song et al., 2010a). This was driven by 3 interrelated reasons: (1) thrive for basic understanding of immunity in bivalves in a comparative framework among the invertebrates, (2) generation of information for the development of disease-resistant varieties of cultured bivalves, and (3) discovery of new bioactive compounds for biotechnological applications. Since the pioneering work of Cuénot (1914) over a century ago, a large body of information has accumulated on the characteristics of cellular and humoral factors mediating immunity in bivalves, even though current knowledge remains for the most part descriptive. Functional characterization of the components of the bivalve immune system remains fragmentary and, when available, often circumstantial. The truth is that it is often difficult to identify immune responses proper towards an infection versus changes in a particular defense-related factor as an indirect, general, stress response. In this paper, highlights of the bivalve immune responses to some of the important infectious diseases affecting this group will be described. Focus will be given to provide information in a comparative framework to assess immune response towards infectious agents ranging from viruses to metazoans.

2. General organization of the bivalve defense system

The bivalve defense system includes several layers of physical and biological barriers. The most obvious physical barrier is provided by the shell that supports and protects the soft tissue from biological and physico-chemical insults. The second physical barrier beyond the shell is provided by the skin and the mucosal layer that covers it and that entraps microbes facilitating their elimination via ciliary activity. Internal defense is ensured by the effectors of the innate

immunity. Like most invertebrates, bivalves have an open circulatory system populated by hemocytes (molluscan blood cells, previously and sometimes still referred to as amoebocytes) that circulate in hemolymph vessels and sinuses as well as throughout soft tissues. The circulation is mainly ensured by a systemic heart, which in bivalves is often located near the posterior adductor muscle, lodged in a pericardial coelom delimited by the pericardium. Most prior work on molluscan immunobiology targeted internal immune factors and activities associated with circulating hemocytes and dissolved humoral factors of the plasma which work in a complementary fashion to neutralize invading organisms. Invaders are detected via humoral and hemocyte-bound recognition factors, triggering the production of cytokines that mediate the recruitment of additional hemocytes, activation of phagocytosis and the production and/or release of a wide range of antimicrobial compounds. Recent research suggests a certain level of specific immune priming in mollusks (Wang et al., 2013; Zhang et al., 2014b). Even though precise mechanisms for "immune memory" have not been fully established in the invertebrates, they are thought to involve recognition factors such as thioester-containing proteins (Rodrigues et al., 2010), C-type lectins (Wang et al., 2013) or the Down syndrome cell adhesion molecule, Dscam (Ng et al., 2014).

In addition to internal defense ensured by circulating hemocytes and plasma factors, a growing body of evidence highlights the role of immune factors associated with mucosal surfaces in interactions with microbe. Peripheral compartments such as bivalve's extrapallial cavity (space between mantle and shell) have been shown to contain abundant hemocytes that contribute to the immune protection of these compartments (Allam et al., 2001; Allam and Paillard, 1998; Allam et al., 2000a) in addition to their role in biomineralization and shell deposition (Beedham, 1965; Fisher, 2004; Mount et al., 2004; Wilbur, 1964). Hemocytes have been also described in association with mucosal secretions covering pallial surfaces (Allam, 1998; Lau et al., 2013a; Lau et al., 2013b; Takatsuki, 1934). These "peripheral" hemocytes are functionally active as demonstrated by their ability to phagocytose biotic and abiotic particles and to secrete hydrolytic and antimicrobial compounds (Allam, 1998; Allam and Paillard, 1998; Lau et al., 2013b; Takatsuki, 1934). They can move bi-directionally via trans-epithelial migration (Allam, 1998; Lau et al., 2013a), providing the animal with a sentinel system similar to that played by dendritic cells in vertebrates. The extrapallial fluid has also been shown to contain hydrolytic enzymes

such as lysozyme and peptidases (Allam and Paillard, 1998) that contribute to pathogen neutralization. Similarly, humoral defense factors associated with bivalve pallial mucus have been suggested to represent the first line of immune defense in these animals extending the defensive role of mucus beyond that of a simple physical barrier. Mucosal secretions covering bivalve pallial organs were shown to contain a wide range on antimicrobial factors (Allam and Pales Espinosa, 2015; Pales Espinosa et al., 2014). Previous studies have shown that oyster pallial mucus contains agglutinins (a functional but generic term that encompasses proteins that agglutinate non-self entities) that interact with various bacterial species (Fisher, 1992). More recent work showed that some of these agglutinins are lectins that bind microbes through protein-carbohydrate interactions (Pales Espinosa et al., 2009; Xing et al., 2011). Previous studies also reported the presence in pallial mucus of hydrolytic enzymes that likely contribute to host protection, such as lysozyme (McDade and Tripp, 1967) and proteases (Brun et al., 2000). The contribution of these external immune factors to bivalve health is greatly under-investigated and very likely under-estimated. While a rich body of literature exists on host-pathogen interactions in mollusks once infection is established, the interactions of microbes and their hosts at these water-tissue interfaces during initial encounters remain poorly characterized. This lack of information is exacerbated by the fact that virtually all economically important infections affecting these animals initiate at mucosal surfaces.

3. Cellular components of the immune system

3.1. General description and kinetics of immune cells

Hemocytes represent the backbone of the bivalve immune system (Anderson and Good, 1976; Song et al., 2010a). They are multi-potent and contribute to several biological functions (reviewed by Cheng, 1996; Feng, 1988; Fisher and Brehelin, 1986). Of course, they play a major role in phagocytosis of invading microbes and encapsulation of larger or refractive invaders, but they are also involved in wound healing, food digestion and transport of nutrients, reproduction, excretion, shell formation, and production and secretion of humoral factors (Cheng, 1981; Cheng, 1996). Little is known about hemocyte formation in mollusks, and the hematopoietic organ (often dubbed the “amebocyte-producing organ”) has been identified only in a few classes including cephalopods and gastropods such as the medically-important snail *Biomphalaria*

glabrata (Lie et al., 1975). In bivalves, evidence suggests that hemocytes may be produced from differentiation of connective tissue cells (Cheng, 1996; Smolowitz et al., 1989). Molluscan hemocytes are generally classified based on morphological appearance and "granularity" of their cytoplasm adding to the ambiguity and lack of consistencies between different studies within the same species and across various species within and across taxa. The degree of complexity that may be understated in current hemocyte classification schemes is highlighted by Aladaileh et al. (2007), who studied the ultrastructure of hemocytes from the Sydney rock oyster, *Saccostrea glommerata*. They identified numerous sub-types of granular hemocytes based on their granular and vacuolar contents. Granulocytes could be classified as distinct basophilic, acidophilic and mixed forms in Giemsa/May–Grünwald stained preparations. Similar sub-types of granulocytes have been identified in Pacific oysters (*Crassostrea gigas*), mussels (*Mytilus edulis*), clams (*Ruditapes decussatus*) and pearl oysters (*Pinctada imbricata*) (Chang et al., 2005; Kuchel et al., 2010; Lopez et al., 1997; Pipe et al., 1997). In *S. glomerata* and *P. imbricata*, further distinction among granulocytes is evident at the ultrastructural level reflecting a substantial diversity in the morphology and content of granular inclusions (Aladaileh et al., 2007; Kuchel et al., 2010) (Figure 1). This diversity may reflect a broad array of functional capacities among granulocytes.

One important feature of hemocytes is their ability to perform chemotaxis towards microbial products (Cheng and Howland, 1979; Howland and Cheng, 1982). Chemotactic movement of hemocytes represents the first step in phagocytosis. This is followed by the attachment of hemocytes to the particles recognized as non-self, preceding phagocytosis. Phagocytosis can be undertaken by a range of hemocyte types. Granulocytes, however, are generally the most avidly phagocytic (Kuchel et al., 2010). Shortly after engulfment, membrane bound granules within the hemocyte migrate to the surface of the phagosome and fuse with the phagosomal membrane resulting in the deposition of granular contents onto the surface of the ingested material and a rapid decrease in the pH of the phagosomes. This process is likely to facilitate the neutralization of ingested microbes via a series of microbicidal processes, including the production of reactive oxygen species, nitric oxides, as well as release of a wide range of antimicrobial factors and hydrolytic enzymes packed in lysosomes (Anderson, 1994; Anderson et al., 1992; Buggé et al., 2007; Cheng, 1981; Gourdon et al., 2001; Kuchel et al., 2010; Pipe, 1992; Song et al., 2010a).

When phagocytosis fails or when particles are too large to undergo phagocytosis, hemocytes are recruited in large numbers to surround and encapsulate the invader (Figure 2) and to release cytotoxic products for extracellular killing (Dove et al., 2004; Loker et al., 1982). Encapsulation can lead to granuloma formation when hemocytes accumulate in thin layers surrounding the parasite as sometimes seen in hard clams *Mercenaria mercenaria* infected with quahog parasite unknown (QPX, Dove et al., 2004). Aladaileh et al. (2007) demonstrated that *S. glomerata* granulocytes aggregate around fungal hyphae *in vitro* and alter their morphology to form a capsule enclosing the hyphae. Similarly, Huehner and Etges (1981) showed that encapsulation of trematode parasites (*Aspidogaster conchicola*) in freshwater mussels is undertaken by “fibrocytes”. These cells appear to be morphologically differentiated granulocytes in the latter stages of encapsulation process.

While phagocytic activity of hemocytes is a hallmark of the innate immune system and has been well described in bivalves, the diverse activities of epithelial cells lining mucosal surfaces also contributes to microbial homeostasis at these pathogen portals of entry. Epithelial cells produce and secrete a wide range of bioactive molecules that are embedded in mucus. In addition, virtually all mucosal epithelia of bivalves are capable of endocytosing biotic and abiotic particles and colloids, including epithelial cells lining the external (extrapallial) and internal (pallial) surfaces of the mantle (Bevelander and Nakahara, 1966; McLean, 1980; Nakahara and Bevelander, 1967), the gill (George et al., 1976; Johnson and Le Pennec, 1995), the foot (Grenon and Walker, 1982; Ryder and Bowen, 1977), or epithelia lining different sections of the digestive gland and gut (Yonge, 1926; Yonge, 1928; Yonge, 1935). Phagocytic activity performed by these epithelia provides a dual nutritional/defense function by enhancing the uptake and digestion of food particles and by keeping pathogens in check and limiting infections. Particles phagocytosed by epithelial cells are then exposed to toxic reactive oxygen species produced by the host cells during the respiratory burst associated with phagocytosis and are neutralized by antimicrobial compounds and hydrolytic enzymes present in phagosomes/phagolysosomes. Microbes that inhibit, or that are able to resist, intracellular killing and digestion can initiate infection in epithelial cells. This is typically the case for members of the Chlamydiales and Rickettsiales that infect mucosal epithelial cells throughout various taxa, including mollusks (Fryer and Lannan, 1994).

3.2. Interactions of immune cells with infectious agents

Attraction of hemocytes to infectious agents is considered as the first step in phagocytosis. Bivalve hemocytes display positive chemoattraction towards products released by infectious agents ranging from trematodes (Cheng et al., 1974) to various bacterial species (Cheng and Howland, 1979) and bacterial products such as lipopolysaccharides (Howland and Cheng, 1982). This response is not generic since some microbes do not induce any chemotactic response (Howland and Cheng, 1982) while others induce a chemokinetic (increase in random -not directional- movement) response (Alvarez et al., 1995; Schneeweib and Renwranztz, 1993). The motility of oyster (*Crassostrea virginica*) hemocytes is enhanced following exposure to cells or cell lysates of its alveolate parasite *Perkinsus marinus* (Garreis et al., 1996), and this motility was reported by Lau et al. (2013a) to be directional (chemotaxis). Recruitment of hemocytes to infection sites (focal inflammation) is a common, but not systematic (e.g. lack of focal inflammation in some viral or trematode infections), cellular response in bivalve mollusks even though it remains unclear if hemocytes primarily migrate to products released by infectious agents, intact and damaged hemocytes and other host cells already on site (via release of cytokines and other cellular products), or, most likely, both. This can result in a dynamic and sometimes temporary change in the number of circulatory hemocytes and a general increase in hemocyte infiltration in affected tissues as shown in typically focal infections such as *Roseovarius* Oyster Disease (ROD; formerly Juvenile Oyster Disease; Maloy et al., 2007) in *C. virginica* and Brown Ring Disease (BRD) in the clam *Ruditapes philippinarum*. In both of these two shell diseases, infection initiates in the periostracum and at the external surface of the mantle and hemocyte counts display a transient increase in hemolymph before a more consistent increase in the extrapallial fluid and in the connective tissue underlying the affected mantle epithelia (Allam et al., 2000a; Ford and Borrero, 2001; Paillard et al., 1996; Perkins, 1996). Similar localized hemocyte infiltrations have also been documented in other focal infections affecting bivalve connective tissues such as QPX disease in the hard clam *Mercenaria mercenaria* where massive hemocyte recruitment can be visible (Figure 2). In contrast, general invasive infiltration of hemocytes is usually seen in response to typically systemic infections such as those caused by *Perkinsus* spp. (Villalba et al., 2004), *Haplosporidium nelsoni* or *Bonamia ostreae* (Balouet et al., 1985; Cochenne-Laureau et al., 2003; Ford et al., 1993b), even

though lack of hemocyte response was also reported in bivalves severely infected with some systemic pathogens such as a *Haplosporidium* sp. in the oyster *Saccostrea cucullata* (Hine and Thorne, 2002). Overall, focal and systemic responses of hemocytes can result in changes in total and differential hemocyte counts in the circulatory system as a combined result of mobilization of hemocytes from and to various compartments, hematopoiesis, degranulation or lysis and degradation of hemocytes in animals heavily infected with some pathogens such as intrahemocytic parasites (e.g. *Bonamia ostreae*, *B. exitiosa* or *Perkinsus* spp.). There are numerous studies reporting changes in hemocyte counts and composition in response to infection and contradicting results within and between studies were often highlighted as a likely result of physiological differences between animals from various genetic/environmental backgrounds or at different stages of infection.

Recruited hemocytes interact with pathogenic agents either directly through membrane bound receptors or indirectly via dissolved recognition factors present in the surrounding environment (plasma, extrapallial fluid, mucus). The studies by Vasta et al. (Vasta et al., 1984; Vasta et al., 1982) were the first to detect the presence of a lectin associated with hemocyte membrane in bivalves (the oyster *C. virginica*) and proposed this to serve as a membrane receptor for non-self entities. Lectin is a relatively generic term encompassing a very diverse group of carbohydrate-binding proteins widely distributed in nature, some of which are implicated in host-pathogen interactions in several taxa (reviewed by Vasta et al., 2007). Hemocyte surfaces also contain lectin- receptors that were suspected to contribute to host resistance to infection. For instance, Cheng and Manzi (1996) reported the presence on *C. virginica* hemocytes of a ligand dubbed "lathyrose" (because it binds the *Lathyrus odoratus* lectin) that correlated well with oyster resistance towards MSX. Other membrane-bound molecules known to serve as pathogen recognition receptors (PRR) include Toll-like receptors (TLR). These are transmembrane receptors characterized by the presence of extracellular leucine-rich repeats and are highly expressed in sentinel immune cells such as dendritic cells and macrophages, as well as mucosal epithelia (e.g. TLR4 and TLR5 in the gut, Ortega-Cava et al., 2003; Vijay-Kumar et al., 2010) of higher vertebrates. They are able to selectively recognize and initiate responses against a large number of varied and complex microbe-associated molecular patterns (MAMP) (Arancibia et al., 2007). Initially discovered to play a major role in *Drosophila* immunity (Lemaitre et al., 1996),

the Toll pathway has been consequently shown to be highly conserved in metazoans and TLRs have already been identified in several molluscan species (Perrigault et al., 2009b; Tanguy et al., 2004) with up to 23 and 27 TLRs identified in the mussels *Mytilus galloprovincialis* (Toubiana et al., 2013) and *M. edulis* (Philipp et al., 2012), respectively. In mollusks, TLRs have been shown to be highly expressed in circulatory hemocytes and were reported to have a wide tissue distribution (possibly as an outcome of the systemic distribution of hemocytes), even though previous studies show TLRs to be also highly expressed in pallial organs (in particular the mantle) which are in direct contact with environmental microbes (Perrigault et al., 2009b; Zhang et al., 2011). All TLR-related studies in bivalves so far were based on a combination of mRNA/DNA quantification/sequencing activities and bioinformatic analysis. There is no direct evidence for the involvement of TLRs in bivalve interactions with pathogens. Despite this lack of functional investigations of bivalve TLRs, accumulated evidence strongly suggest the central role of the TLR pathway in initiating cellular responses to infections. For instance, several studies showed significant regulation in various members of the pathway in response to experimental and natural infections by microorganisms ranging from viruses to protists (Green and Montagnani, 2013; Perrigault et al., 2009b; Renault et al., 2011; Tanguy et al., 2004; Toubiana et al., 2013).

Host cells can also interact with microbes indirectly via recognition factors dissolved in the surrounding liquid environment (discussed in more details under "*Immune recognition factors*" section below). There are numerous studies showing the presence in bivalve plasma of proteins that recognize and bind microbial cells facilitating their uptake by hemocytes (opsonization). Hardy et al. (1977) demonstrated the opsonic activity of agglutinins present in oyster (*C. gigas*) plasma that enhance bacteria uptake by hemocytes. Similarly, Renwrautz and Stahmer (1983) showed that lectin-like agglutinins from *M. edulis* can act as opsonins, promoting phagocytosis by hemocytes. Several studies also reported the opsonic effect of whole bivalve plasma on the phagocytic activity of hemocytes (reviewed by Canesi et al., 2002). The study by Bulgakov et al. (2004) reported the presence in Manila clams (*R. philippinarum*) of a lectin that binds the hypnospores of *P. olsenii*. Similarly, Tasumi and Vasta (2007) identified a galactose-binding lectin (galectin) they called CvGal (later becoming CvGal1; Feng et al., 2013) that is produced and secreted by *C. virginica* hemocytes into the surrounding plasma. CvGal was shown to bind

carbohydrates present on a varieties of microbial cells including *P. marinus* as well as hemocyte plasma membrane, therefore serving as a bridge facilitating the uptake of *P. marinus* by hemocytes (Feng et al., 2013; Tasumi and Vasta, 2007). Additional studies reported the presence of galectins with microbe-binding activities in other bivalve species such as the clam *R. philippinarum* (Kim et al., 2008) and the scallop *Argopecten irradians* (Song et al., 2011). Beside its ability to agglutinate both Gram-positive and -negative bacteria, the galectin identified by Song et al. (2011) was also shown to bind hemocytes and to mediate encapsulation of non-self material by hemocytes.

Dissolved and/or membrane surface bound factors determine hemocyte behavior towards microbes, with some avidly phagocytosed while others are avoided. Bivalve hemocytes phagocytose and digest a wide range of opportunistic microbial pathogens such as various bacterial species (reviewed by Canesi et al., 2002). The process of phagocytosis has been described in detail by Kuchel et al. (2010), who investigated the ultrastructural interactions between hemocytes from *S. glomerata* and the protozoan parasite, *Marteilia sydneyi*, which causes fatal QX disease. They reported a typical phagocytic process, in which hemocyte pseudopodia extend around *M. sydneyi* leading to engulfment of the parasite within a phagosome. In contrast, some obligate parasites seem to have developed refined means to control neutralization by host immune cells. The interactions between *C. virginica* hemocytes and *P. marinus* and *H. nelsoni* represent a pertinent case study showing various strategies for the avoidance of phagocytosis and intracellular killing and highlighting the specificity of the crosstalk between host cells and invading microbes. In this case, *P. marinus* cells are readily phagocytosed by oyster hemocytes, while *H. nelsoni* is avoided. The elegant study by Ford et al. (1993a) showed that avoidance of phagocytosis by oyster hemocytes is, at least in part, mediated by *H. nelsoni* cell surface components and that chemical modification of these epitopes can lead to phagocytosis. The involvement of microbial cell surface components in evasion of phagocytosis by bivalve hemocytes has also been reported for several bacterial agents including *V. vulnificus*, *V. parahaemolyticus* and *E. coli* (Genthner et al., 1999; Harris-Young et al., 1995). Interestingly, phagocytosis avoidance of MSX was also reported in *C. gigas* which was also shown to be susceptible to MSX, but not in the resistant ribbed mussel *Geukensia demissa* (Ford et al., 1993a). While the avoidance of phagocytosis by hemocytes may represent an efficient

defense strategy of some pathogens (including *H. nelsoni*), other microbes have developed alternative means allowing them to survive phagocytosis and pursue development inside phagocytes. Intracellular survival implies the parasite's ability to control the maturation of the phago(lyso)somal compartment. It also provides major advantages since parasites become protected from the various antimicrobial agents present in the plasma. Again, in the case of *P. marinus* interactions with *C. virginica* hemocytes, previous work reported the ability of this parasite to inhibit intracellular killing and digestion. In this case, uptake of *P. marinus* by phagocytes favors the spread of the infection using a Trojan horse strategy that is common among well-adapted, obligate, parasites including other bivalve pathogens (e.g. *Bonamia ostreae* and *B. exitiosa*, Balouet et al., 1985; Chagot et al., 1992; Engelsma et al., 2014; Hine and Wesney, 1994). In many instances, the resistance of intracellular parasites to digestion may be linked to their ability to inhibit lysosome-phagosome fusion as shown in the case of *Bonamia* sp. interactions with oyster hemocytes (Hine and Wesney, 1994). The regulation of host reactive oxygen species (ROS) production as a protective means seems to represent another strategy used by bivalve intracellular parasites. ROS regulation has been studied in detail during *P. marinus* infection of oyster hemocytes. In this case, the parasite showed high resistance to superoxide anion and hydrogen peroxide (first 2 intermediates in the ROS cascade) while it was susceptible to hypochlorite ions (Schott et al., 2003). Further investigations strongly suggested that this resistance is linked to *P. marinus* antioxidant arsenal that is directed against ROS produced by phagocytes. In particular, *P. marinus* was shown to produce several iron superoxide dismutases (SOD) that are thought to suppress ROS production via dismutation of superoxide radicals produced by phagocytes (Ahmed et al., 2003; Schott and Vasta, 2003). Similar conclusions were also reached in the case of *B. ostreae* (Morga et al., 2009; Morga et al., 2011).

The similarity in the strategies used by *P. marinus* and *B. ostreae* to regulate oyster defense processes is striking and seem to include, in addition to ROS modulation, the regulation of apoptosis in infected cells to favor parasite survival (Goedken et al., 2005; Hughes et al., 2010; Morga et al., 2009; Morga et al., 2012; Sokolova, 2009; Sunila and LaBanca, 2003). Apoptosis represent an ultimate defense response for an infected cell that is unable to clear the infectious agent via other, less dramatic, means. The mechanisms of regulation of hemocyte apoptosis by *P. marinus* have been investigated and were shown to be caspase-independent, possibly involving

endonuclease G and apoptosis-inducing factors (Hughes et al., 2010). Interestingly, recent investigations suggest that the regulation of ROS production and apoptosis are interrelated since the inhibition of parasite superoxide dismutases was shown to neutralize the anti-apoptotic activity of live *P. marinus* cells (Allam, unpublished). These results are in line with the current knowledge on the involvement of ROS in the initiation of caspase-independent apoptosis, possibly via lysosomal and/or mitochondrial damage (Bröker et al., 2005; Fleury et al., 2002; Kroemer and Martin, 2005).

The hijacking of host immune cells provides intracellular parasites with major advantages facilitating their survival and spread in host tissues. This is particularly relevant for early host-pathogen interactions during initial acquisition of infections at mucosal interfaces. The occurrence of two-way movements of hemocytes across mucosal epithelia (see above) raises the possibility for these cells to serve as vehicles allowing the acquisition of adapted microbes that are capable of surviving phagocytosis. Previous investigations showed the ability of *P. marinus* to take advantage of transepithelial migration of pallial hemocytes to gain access to the internal tissues of its oyster host (Allam and Parvez, 2007). Furthermore, exposure of naive oysters to *P. marinus* was shown to increase transepithelial migration of hemocytes likely resulting in higher infection rates (Lau et al., 2013a). In contrast, exposure of oysters to the opportunistic bacteria *Vibrio alginolyticus* did not cause any change in transmigration suggesting that change in transepithelial migration rates is not a generic response to microbial exposure. A better understanding of hemocyte-microbe interactions at mucosal interfaces may help identify biological bases for infection refraction (and possibly resistance) in some bivalve-pathogen associations and will likely help unravel possible pathways for the transmission of information from these interfaces to the internal defense system.

Given the critical role of hemocytes in the bivalve immune system, it is not surprising that changes in hemocyte morphology and function have been correlated with resistance against a number of bivalve pathogens. For instance, Ford et al. (1993b) showed that resistance to *H. nelsoni* in *C. virginica* is associated with a circulating sub-population of granulocytes. Resistant oysters were found to have significantly higher relative frequencies of large granulocytes. The authors concluded that these large granulocytes improved oyster survivorship through enhanced

tissue repair and debris removal. Allam et al. (2001) also found that resistance and susceptibility to brown ring disease in Manila clams, caused by *Vibrio tapetis*, is associated with the relative frequencies of granular hemocytes. Resistant clams had relatively more granular hemocytes in their body fluids, in particular in the extrapallial fluid, resulting in increased phagocytic capacity. Cochenne-Laureau et al. (2003) reported a decrease in the proportion of granulocytes in oysters *O. edulis* infected with *B. ostreae* and suggested that hyalinocytes may favor parasite survival. Da Silva et al. (2008) also suggested a better resistance against *B. ostreae* in oysters displaying high percentage of granulocytes. Finally, Butt and Raftos (2008) identified significantly higher phagocytic and phenoloxidase activities among hemocytes from Sydney rock oysters (*S. glomerata*) selected for resistance to QX disease relative to non-selected stocks. They also found that hemocytes from QX disease resistant oysters were larger than those from wild-type oysters. Even though these findings suggest a link between hemocyte composition and activity, and resistance to some infections, the evidence remains circumstantial and require further validation given the multitude of factors affecting general hemocyte parameters.

3.3. Melanization and biomineralization as cellular defense responses

Melanization of pathogens and damaged tissues is a major innate defense process in invertebrates, especially arthropods (Cerenius et al., 2010). Melanin biosynthesis is highly regulated (phenoloxidase cascade) and is mediated by a diverse group of enzymes designated under the generic name of phenoloxidases that encompass several metalloproteins including tyrosinases, catecholoxidases and laccases (Walker and Ferrar, 1998). Hemocytes can promote the formation of melanotic nodules that limit the spread of infectious microorganisms or damaged tissues. The phenoloxidase system, which is primarily an extracellular effector mechanism in other invertebrate taxa, may also represent a key intracellular killing system in bivalves. Intracellular phenoloxidase activities have been reported in a number of bivalve species, including *S. glomerata*, *P. imbricata*, *R. philippinarum*, *Chamelea gallina* and *Tapes decussatus* (Aladaileh et al., 2007; Kuchel et al., 2010; Muñoz et al., 2006). In *S. glomerata*, encapsulation of fungal hyphae is followed by melanization both within the encapsulating hemocytes and in the extracellular matrix of the capsule (Aladaileh et al., 2007). Furthermore, phenoloxidase activity has been implicated in the phagolysosomal destruction of the parasite *M. sydneyi* by *S. glomerata* hemocytes (Butt and Raftos, 2008; Kuchel et al., 2010) (Figure 3).

Phenoloxidase deposition was evident in phagosomes containing engulfed parasites. Ingested, melanized *M. sydneyi* were also detected *in vivo* among hemocytes from infected oysters. Similar phenoloxidase activity has been implicated with intracellular phagolysosomal processes in *M. edulis* (Renwrantz et al., 1996), *M. galloprovincialis* (Carballal et al., 1997) and *Scapharca inaequivalvis* (Holden et al., 1994), even though none of these earlier studies was in the context of interactions with specific pathogens.

Melanization is a normal process during shell formation in mollusks (Waite and Wilbur, 1976) in addition to being an important mechanism involved in wound healing and encapsulation of non-self entities. All pallial epithelia in mollusks has been shown to support melanization, in particular the external epithelium of the mantle. The melanization response of the mantle is a major trait of several microbial infections affecting this organ. This is the case for the response of eastern oysters (*C. virginica*) to *Roseovarius* Oyster Disease (ROD, caused by *Roseovarius crassostreae*) or that of Manila clams (*R. philippinarum*) to Brown Ring Disease (BRD, caused by *Vibrio tapetis*). In both cases, mantle tissues and secretions (extrapallial fluid and hemocytes contained within) produce copious amounts of melanin to wall off the pathogens, leading to a major rearrangements of shell matrix deposition (Ford and Borrero, 2001; Paillard, 2004). Several tyrosinases have been identified in mollusks and are considered to represent key enzymes in the melanogenic pathway that catalyzes the production of melanin. For instance, a cDNA sequence encoding a putative tyrosinase (OT47) was detected in the pearl oyster *Pinctada fucata* and was shown (by PCR and *in situ* hybridization) to be expressed only in the epithelial cells of the mantle edge (Zhang et al., 2006), a strategic area for both defense against pathogens and shell formation even though no functional investigations were performed on this transcript. In parallel, previous studies showed a regulation in the expression of melanogenic enzymes in response to infections. This was the case for a laccase that was shown to be up-regulated in the mantle of clams affected by BRD (Allam et al., 2014).

Melanization of foreign bodies in mollusks is sometimes followed by a biomineralization mechanism that embeds melanized invaders in new calcified shell layers. This is the mechanism of the nacrezation process during pearl formation. There has been many studies focusing on the mechanisms of pearl formation in mollusks (Addadi and Weiner, 1997; Bevelander and

Nakahara, 1969), but rarely from an immune perspective. Nacrezation and pearl formation is a common response in mollusks to irritants (including parasites and pathogens as well as abiotic irritants) affecting pallial organs, in particular the mantle. Good examples include infections caused by trematodes in different bivalve species (Jameson and Nicoll, 1913; Stunkard and Uzman, 1958). Mantle tissues and secretions are heavily involved in this process and previous studies have shown significant changes in the expression of biomineralization-related proteins in response to infection. For example, several genes involved in calcium binding and biomineralization were regulated in mantle tissues and extrapallial hemocytes in Manila clams affected by BRD (Allam et al., 2014). These included several perlucin-like transcripts (which contain C-type lectin-like domains) that were up-regulated in diseased clams. Perlucin nucleates the growth of calcium carbonate crystals in mollusks (Blank et al., 2003; Weiss et al., 2000) and was suggested to play a dual role as an organic support for biomineralization and as a potential defense molecule against pathogenic microorganisms (Wang et al., 2008).

4. Immune recognition factors

Complete genome sequences have revealed a comprehensive suite of pattern recognition receptors (PRRs) in bivalves that are commonly associated with host defense responses against infection. The first and most comprehensively annotated genome – of the Pacific oyster, *C. gigas* - contains numerous homologues of C-type lectins, fibrinogen-related proteins (FREPs), complement homologues, Toll-like receptors (TLR), scavenger receptors (SRCR), lipopolysaccharide and β -1,3-glucan-binding proteins (LGBP), and peptidoglycan-recognition proteins (PGRPs) (Zhang et al., 2012a). Many of these genes seem to be highly expressed in the digestive gland, suggesting that the filter feeding life history of *C. gigas* (and other bivalves) may have biased pattern recognition toward the digestive system as a first line of defense. Overall, based on the molecular diversity of these putative recognition molecules, the repertoire of dissolved factors that could mediate recognition and microbial uptake by hemocytes is likely larger than reported so far. Some authors boldly propose a functional diversity of dissolved plasma factors to match that provided by the molecularly diverse antibodies in vertebrates (Fisher and Dinuzzo, 1991).

There are also numerous high throughput transcriptomic studies showing that the expression of typical PRRs is up-regulated after bivalves are challenged with pathogens, suggesting that PRRs are associated with immune responses rather than other biological processes (Allam et al., 2014; De Lorgeril et al., 2011; McDowell et al., 2014; Moreira et al., 2012; Venier et al., 2011; Zhang et al., 2014a). For instance, de Lorgeril, et al. (2011) used next generation transcriptomics to demonstrate that TLR and lectin homologues were up-regulated in *C. gigas* responding to either virulent or avirulent species of *Vibrio*. Similarly, Kang et al. (2006) found that C-type lectins were among the most common expressed sequence tags (ESTs) identified in clams (*R. philippinarum*) infected with *Perkinsus* parasites.

4.1. Lectins

Lectins are perhaps the best studied group of humoral PRRs in bivalves, primarily due to their long history of investigation. Many lectins were initially identified by their capacity to agglutinate mammalian erythrocytes and other particles (e.g. Olafsen et al., 1992; Tripp, 1992). Numerous studies have gone on to demonstrate the role of bivalve lectins in immune responses. While evaluating oyster defense factors against *P. marinus*, La Peyre et al. (1995) reported higher agglutination activity in resistant *C. gigas* as compared to susceptible *C. virginica* and subsequent studies showed this activity to increase in the former species following *P. marinus* challenge (Romestand et al., 2002). Olafsen et al. (1992) identified the link between lectins and host defense by showing that the activity of a lectin from *C. gigas* (Gigalin E) was increased when oysters were challenged with either living or heat-killed *V. anguillarum*. Song et al. (2010b) have also shown that expression of a galactoside-binding lectin from scallops (*A. irradians*) increased by 1.5- to 3-fold after challenge with *V. anguillarum* and *Micrococcus luteus*.

Most bivalves appear to express a range of immunologically active lectins. For instance, seven distinct lectin ESTs were identified in *Perkinsus*-infected *R. philippinarum* (Kang et al., 2006). Interestingly, different subsets of the seven *R. philippinarum* lectins were expressed in *Perkinsus*-infected clams when compared to those with *Vibrio* infection. Similarly, Zhang et al. (2010b) have shown that expression of a mannose binding lectin (Cflec-5) from the scallop, *Chlamys farreri*, could be rapidly induced by the microbe associated molecular patterns

(MAMPs) lipopolysaccharide (LPS) and β -glucan, but not by peptidoglycan (PGN). These data suggest that bivalve lectins have a degree of selectivity in their target specificities. Finally, Xing et al. (2011) characterized a C-type lectin they designated CvML (for *C. virginica* mucocyte lectin) that was solely expressed in mucocytes lining oyster pallial organs. Interestingly, CvML mRNA levels increased significantly in oysters exposed to *V. alginolyticus* but only following bath exposure and not bacterial injection into the circulatory system showing that this lectin responds to external cues associated with the presence of pathogens at the water-tissue interface and highlighting its role in mucosal immunity.

Galectins are among the best characterized groups of extracellular bivalve lectins (see Vasta, 2012 for an extensive review). They are β -galactoside binding proteins that are found throughout the animal kingdom and in fungi. Galectins seem to have a variety of roles in development and immune responses. Bivalve galectins include defensive PRRs from pearl oysters, *Crassostrea* species, scallops and clams (Kim et al., 2008; Song et al., 2010b; Tasumi and Vasta, 2007; Yamaura et al., 2008). They can have relatively complex structures, as exemplified by a galectin from the bay scallop, *Argopectens irradians*, designated AiGal1 (Song et al., 2010b). AiGal1 polypeptides comprise 549 aa incorporating four galectin CRDs, each of which has two consensus ligand-binding motifs. One of the most interesting findings with regard to galectins is that their defensive activity seems to be subverted by some pathogens to facilitate infection (Tasumi and Vasta, 2007; Vasta, 2012). Feng et al. (2013) suggest that the protozoan parasite, *P. marinus*, has mimicked the surface carbohydrates to which the *C. virginica* galectin, CvGal1, is specific. They believe that this mimicry facilitates phagocytosis and infection of oyster hemocytes by *P. marinus* (see "Interactions of immune cells with infectious agents" section above). Coincidentally, Wang et al. (2010) have reported that the expression of CvGal1 is significantly down-regulated during *P. marinus* infections, implying that oysters may be attempting to control parasite uptake and proliferation by decreasing expression of its co-opted receptor. Overall, galectin transcription levels were shown to be highly regulated in response to infections as reported, for example, in Manila clams *R. philippinarum* naturally (Allam et al., 2014) or experimentally (Jeffroy et al., 2013) infected with *V. tapetis*.

4.2. Peptidoglycan-recognition proteins (PGRPs)

PGRPs are PRRs with at least one carboxy-terminal PGRP domain (approximately 165 aa) that is homologous to bacterial type 2 amidases. In bivalves, they have been identified and characterized in oysters and scallops (Itoh and Takahashi, 2008; Itoh and Takahashi, 2009; Ni et al., 2007). All of the bivalve PGRPs characterized so far appear to be the short form of the protein, with some variants incorporating additional goose-type lysozyme or defensin-like domains. The additional domains imply functions broader than MAMP recognition. Itoh and Takahashi (2009) suggest that the lysozyme domain in *C. gigas* PGRP (CgPGRP-L) may confer bacteriolytic activity in addition to the peptidoglycan binding capacity of the PGRP domain. These authors also showed that the expression of CgPGRP-L was enhanced after challenging oysters with *Marinococcus halophilus* and *V. tubiashii*, and that the polypeptides incorporate a signal sequence for secretion. Similarly, Ni et al. (2007) found that expression of the PGRP in bay scallops (*A. irradians*) could be induced *in vitro* by exposing hemocytes to PGN, but not LPS.

4.3. TEPs and other complement-like molecules

Thioester bearing proteins (TEPs) are encoded by a large gene family homologous to mammalian complement components (particularly the central complement component, C3) and the protease inhibitor, α 2-macroglobulin (Nonaka, 2011). They are characterized by a number of highly conserved amino acid motifs, critically GCGEQ, which forms the highly reactive thioester binding site. TEPs have been characterized in carpet shell clams (*R. decussatus*) and Zhikong scallops (*C. farreri*) (Prado-Alvarez et al., 2009; Zhang et al., 2007; Zhang et al., 2009b). The scallop TEP incorporates a typical GCGEQ motif, as well as a putative catalytic histidine and proteolytic cleavage sites. These features suggest that it is more closely related to the immunologically-active C3 branch of the TEP gene family (as opposed to the protease α 2-macroglobulin lineage). Expression of the *C. farreri* TEP is up-regulated by microbial challenge, again suggesting that it is involved in host defense rather than proteolysis.

In addition to TEPs, homologues of other components of the vertebrate complement cascade are also frequently identified in studies of bivalve immune responses. These are primarily C1q domain-containing proteins (C1qDC and their sialic-acid binding lectin relatives), which have been shown to represent a highly diverse group of molecules playing an important role as

pathogen recognition receptors and providing tailored responses to different pathogens (Gerdol et al., 2011; Gestal et al., 2010; Li et al., 2011; Wang et al., 2012b). For instance, previous studies reported the presence in the mussel *M. galloprovincialis* of 168 different transcripts of C1qDC, many of which showing differential expression following challenge with Gram-positive or -negative bacteria (Gerdol et al., 2011; Gestal et al., 2010; Moreira et al., 2013). Similar findings were also reported in clams *R. decussatus* infected with *P. olseni* (Leite et al., 2013) and in *R. philippinarum* infected with *V. tapetis* (Allam et al., 2014). Functional confirmation of the involvement of C1qDC proteins as diversified PRR are however limited in bivalves, and most studies focused on the functional characterization of one protein from each species (He et al., 2011; Zhang et al., 2008a). Two forms of C1qDC were identified in the scallop *A. irradians* (AiC1qDC-1 and -2) and were shown to have sequence and structural similarities with immunologically active C1qDCs from other species (Kong et al., 2010; Wang et al., 2012a). Expression of both AiC1qDCs was significantly increased after challenge with fungi (*Pichia pastoris*), bacteria (*M. luteus* and *V. anguillarum*) and/or MAMPs. Recombinant AiC1qDC-2 could also bind a range of different MAMPs suggesting that it may act as a broad spectrum PRR, whilst AiC1qDC-1 could agglutinate *P. pastoris*. Overall, these studies showed that different bivalve C1qDC are able to bind different repertoires of bacteria and yeast and enhance their phagocytosis by hemocytes. A better characterization of molluscan C1qDC and other complement proteins can shed light on the evolution of this pathway.

4.4. Lipopolysaccharide and β -1,3-glucan-binding protein (LGBP)

Examples of LGBPs or β -glucan binding proteins (β GBPs) characterized in bivalves include LGBPs from pearl oysters, clams and scallops and β GBPs from *C. gigas* (Itoh et al., 2010a; Liu et al., 2014; Su et al., 2004; Zhang et al., 2010a). The pearl oyster LGBP (poLGBP) incorporates a predicted polysaccharide-binding motif, a glucanase motif, and a LPS-binding site. Its expression can be up-regulated by both bacterial challenge and LPS (Zhang et al., 2010a). Similar structural and functional characteristics have been reported for LGBPs from the clam *Meretrix meretrix* (Liu et al., 2014) and the scallop *C. farreri* (Su et al., 2004).

Two β GBPs (Cg- β GBP-1 and Cg- β GBP-2) have been characterized in *C. gigas* (Itoh et al., 2010a). The two proteins differ structurally, in that one (Cg- β GBP-1) incorporates two integrin

recognition motifs. This might explain the functional differences between Cg- β GBP-1 and Cg- β GBP-2. Recombinant Cg- β GBP-2, but not Cg- β GBP-1, can enhanced phenoloxidase activity in hemocyte suspensions. The authors suggested that Cg- β GBP-1 may mediate hemocyte-related functions via integrin, whilst Cg- β GBP-2 mediates phenoloxidase activation (Itoh et al., 2010a).

4.5. FREPs

Fibrinogen-related proteins (FREPs) are lectin-like proteins that incorporate fibrinogen and immunoglobulin superfamily related domains (Adema et al., 1997; Hanington and Zhang, 2010; Zhang et al., 2008b; Zhang and Loker, 2004). They have been most extensively studied in gastropod molluscs, specifically the pond snail, *Biomphalaria glabrata*. In that species, there is strong evidence that FREPs play a major role as opsonins and favor the encapsulation of parasites such as *Schistosoma mansoni* or *Echinostoma paraensei* (Hanington et al., 2010). FREPs have gained considerable attention because they exhibit extremely high levels of molecular variability generated by somatic mutation and gene conversion. Moreover, different FREP variants seem to respond to different parasites, suggesting a high degree of selectivity in their recognition capacity (Mone et al., 2010). These characteristics of FREPs and some other invertebrate recognition molecules are beginning to blur the distinction between pattern recognition receptors and hypervariable recognition molecules (Dishaw and Litman, 2013; Raftos and Raison, 2008).

Among bivalves, FREP-like molecules have been characterized in various species including bay scallops (*A. irradians*), various mussel species (*M. galloprovincialis*, *M. edulis* and *M. californianus*), and Pacific oysters (*C. gigas*) (Gorbushin and Iakovleva, 2011; Romero et al., 2011; Yang et al., 2014; Zhang et al., 2009a; Zhang et al., 2012b). These molecules bear many of the hallmarks of their gastropod homologues, although they may lack immunoglobulin superfamily domains (Zhang et al., 2009a). FREPs in mussels are encoded by multigene families, with 2 genes in *M. edulis*, four in *M. californianus* and seven in *M. galloprovincialis* (Gorbushin and Iakovleva, 2011). Five types of FREP (CgFREP-1 to CgFREP-5) comprising three sub-families have been identified in *C. gigas* (Zhang et al., 2012b). CgFREPs exhibit similar hypermutation to those of gastropods. This is characterized by frequent single nucleotide polymorphisms and insertions/deletions (indels) that may arise from recombination between

alleles or somatic diversification. Similar diversity has been reported among FREPs from the mussel *M. galloprovincialis* (Romero et al., 2011). At a functional level, Yang et al. (2014) found that the expression of one class of FREP from *A. irradians* (AiFREP-2) was significantly up-regulated in hemocytes exposed to a range of different MAMPs. Recombinant AiFREP-2 could bind these MAMPs, as well as Gram-negative bacteria (*V. anguillarum*), Gram-positive bacteria (*S. aureus*) and fungi (*P. pastoris* and *Yarrowia lipolytica*). These data suggest that bivalve FREPs may be highly diversified molecules involved in pathogen recognition.

5. Anti-viral immunity

Anti-viral responses remain the “black box” of the bivalve immune system, largely because tractable systems in which to study responses to viral infection have only recently become available. However, it has been known for some time that bivalves do respond to viral infections and have antiviral effector activities. Anti-viral activities have been reported in clams (*Mya arenaria*; Li and Traxler, 1972) and *C. gigas* (Bachère et al., 1990; Olicard et al., 2005). Moreover, gene expression analyses have indicated that bivalves mount extensive anti-viral responses at the transcriptional level. Fleury and Huvet (2012) identified 10 immune response genes that were differentially expressed among *C. gigas* responding to “summer mortality”, which occurs as a result of opportunistic Ostreid Herpes Virus 1 (OsHV1) and *Vibrio* infections. The genes included a Toll-like receptor (TLR), a superoxide dismutase-like molecule, a complement C1q homologue, two inhibitors of NF- κ B and a suppressor of cytokine signaling. A subsequent study by Renault et al. (2011) identified a more extensive suite of genes that responded to experimental exposures of *C. gigas* hemocytes to OsHV1. Suppression subtractive hybridization (SSH) identified 304 clones that were differentially expressed in virus-challenged hemocytes. These included genes with known immunological functions in other species (macrophage expressed protein, multicopper oxidase), apoptosis related genes (Bcl-2) and anti-viral cell signaling molecules (IK cytokine, glypican, myeloid differentiation factor 88, interferon-induced protein 44). Anti-viral responses are also evident at the level of protein activity. Xing et al. (2008) showed that infection of the scallop, *C. farreri*, with the acute virus necrobiotic virus (AVNV) was accompanied by alteration of acid phosphatase, superoxide

dismutase (SOD), myeloperoxidase (MPO), and phenoloxidase (PO) enzymatic activities in hemocytes.

Mechanistically, there is new evidence to support the existence of a coordinated antiviral immune system in bivalves that is mediated by pattern recognition receptors initiating an interferon-like antiviral response. Zhang et al. (2014c) have shown that the expression of a RIG-I like pattern recognition receptor in *C. gigas* is specifically up-regulated in response to poly(I:C) double stranded RNA, which is a virally-associated MAMP. Similarly, Green and collaborators (Green and Barnes, 2009; Green et al., 2014; Green and Montagnani, 2013) demonstrated that the expression of numerous receptors, intracellular signaling molecules and effectors that are commonly associated with anti-viral immune responses in other species is up-regulated in oysters responding to either poly(I:C) or OsHV1. In *C. gigas*, the expression of genes homologous to putative virus recognition receptors (TLR & MDA5), MyD88, interferon regulatory factor (IRF) and protein kinase R (PKR) were up-regulated in response to poly(I:C), whilst the expression of MyD88, IRF, PKR, and anti-viral effectors (viperin and Mpeg1) increased in response to OsHV-1 infection. Green and Montagnani (2013) concluded that these patterns of gene expression are similar to anti-viral responses in mammals that are mediated by type I interferons. They also showed that inoculation with viral MAMPs, such as poly(I:C), can induce protective antiviral immunity. *C. gigas* injected with poly I:C one day before infection with OsHV-1 had significantly reduced viral loads at 48 h post-infection when compared to controls primed with seawater. Immune-priming with *V. splendidus* did not induce comparable protection, suggesting that the response is specific to viral MAMPS.

6. Antimicrobial peptides (AMP)

With their pioneering work in mussels, Charlet et al. (1996) and Mitta et al. (Mitta et al., 1999a; Mitta et al., 1999b) were the first to identify and characterize AMP in bivalves. To date, the most comprehensive characterization of antimicrobial peptides (AMP) has been made in mussels (namely *M. edulis* and *M. galloprovincialis*; Charlet et al., 1996; Gerdol et al., 2011; Mitta et al., 1999a; Mitta et al., 1999b; Rosani et al., 2014; Rosani et al., 2011; Venier et al., 2011) and oysters (*C. gigas*; Schmitt et al., 2010; Schmitt et al., 2012). In *Mytilus*, four groups of AMP

were described including 3 groups of cationic cystein-rich AMP (defensin-like peptides, myticins and mytilins) and an anti-fungal AMP called mytimycin. Subsequent investigations in this genus reported a large diversity of these AMP with up to 23 forms in *M. galloprovincialis* alone (6 mytilin variants, 7 myticins, 5 defensins and 5 mytimycins; Venier et al., 2011). Overall, most studies concur that defensins represent the most widespread group of AMP among bivalves. Defensins have been shown to represent a potent animal defense against a wide range of bacteria and fungi (Lehrer and Ganz, 2002). Several defensins have been reported in bivalves with up to 19 variants in the oyster *C. gigas* (Gonzalez et al., 2007a; Gueguen et al., 2006; Rosa et al., 2011; Schmitt et al., 2010) and 7 in the clam *R. philippinarum* (Allam et al., 2014) some of which are present in epithelial cells associated with mucosal tissues. Gueguen et al. (2006) characterized a defensin mRNA (subsequently called *Cg-Defm*) from the oyster *C. gigas*, specifically expressed, and present as a mature protein, in the mantle edge. Further characterization of *Cg-Defm* showed that this defensin is highly diverse displaying up to 9 variants. In addition to the defensins, a proline-rich AMP family (dubbed *Cg-Prp*) was also identified in *C. gigas* with up to 24 variants (Schmitt et al., 2010).

Bivalve antimicrobial arsenal also includes other antimicrobial proteins such as histones (Dorrington et al., 2011), lysozymes (discussed below) and larger molecules with potent antibacterial activity such as the bactericidal/permeability-increasing protein (BPI, discussed below). There is little doubt that the repertoire of antimicrobial compounds is larger than previously reported, particularly since most studies so far used similarity searches to identify antimicrobial compounds produced by bivalves. The study by Seo et al. (2013) is a good illustration of the limitations of this approach. These authors isolated and characterized a new AMP (designated *cgMolluscidin*) that is highly expressed in *C. gigas* mucosal tissues and that has no homology with any known peptide. Interestingly, *cgMolluscidin* was also present in unidentified expressed sequence tags from *O. edulis* infected with *B. ostreae* and *B. exitiosa* (Seo et al., 2013).

Despite the increasing information about the distribution and diversity of AMP in bivalves, data on their effective role in fighting infections remain relatively limited. However, a significant number of studies infer a prime role for these compounds in host defense based on changes in the

levels of corresponding transcripts following experimental challenge, sometimes with microbes that are not infectious to bivalves under natural environmental conditions. The extremely limited number of bacterial diseases affecting adult bivalves (reviewed by Paillard et al., 2004) may be an indication of the efficiency of these compounds in neutralizing invaders. The expression of several AMP (particularly those associated with mucosal tissues) has been shown to remain unchanged following microbial challenge suggesting these are constitutively expressed offering a baseline layer of defense against waterborne microbes (Gueguen et al., 2006). In contrast, upregulation in the expression of other AMP in response to natural infections or experimental challenge has been reported in different bivalve taxa (Adhya et al., 2012; Allam et al., 2014; Perrigault et al., 2009b; Rosa et al., 2011; Zhao et al., 2007). In some instances, an increase in the expression of antimicrobial peptides has been reported in naturally infected bivalves while these molecules have no direct effect on the primary etiologic agent of these infections. For example, the histone protein H4 was reported to be present in enriched libraries obtained from *C. virginica* infected with *P. marinus* but further testing showed that, even though recombinant H4 displays a strong antibacterial activity, it has no activity against *P. marinus* itself (Dorrington et al., 2011). Similarly, the expression of defensins was up-regulated in clams *M. mercenaria* affected by QPX even though there is no evidence for the effect of these AMP on parasite cells (Perrigault et al., 2009b). These findings suggest that the main function of bivalve AMP is to maintain microbial homeostasis and to provide a wide-range protection against opportunistic bacterial infections.

7. Antimicrobial proteins, hydrolytic enzymes and protease inhibitors

Enzymes (e.g. lysozyme, proteases) that can degrade specific substrates comprising microbial cell walls (e.g. bacterial peptidoglycan) represent integrative components of the bivalve defense system in addition to antimicrobial peptides. Several studies reported the inhibitory effect of bivalve plasma and tissue extracts on the *in vitro* growth of pathogens (Anderson and Beaven, 2001; Perrigault et al., 2009a). In many instances, there is evidence that this activity involves proteins other than AMP. For example, Anderson and Beaven (2001) reported that the anti-*P. marinus* activity they measured in mussel (*M. edulis* and *Geukensia demissa*) and oyster (*C. virginica*) was not correlated with antibacterial activity, and involved proteins larger than 10kDa.

The bactericidal/permeability-increasing (BPI) proteins represent an important family of antimicrobial proteins. These are cationic proteins (about 50 KDa, Elsbach, 1998) and are known to display a high selective cytotoxicity towards Gram-negative bacteria (Boman, 1995) even though their activity against bivalve protistan pathogens has never been evaluated (vertebrate BPI are active against some protistan parasites; Khan et al., 1999). A cDNA sequence similar to BPI proteins was identified and characterized in the oyster *C. gigas* (Cg-BPI, Gonzalez et al., 2007b). The recombinant Cg-BPI protein was able to bind LPS, to modify bacterial membrane integrity and to kill the Gram-negative *E. coli*. *In situ* hybridization showed that Cg-BPI is transcribed in various epithelia of the oysters, and most specifically those in contact with the external environment (mantle, gills, gut and digestive gland). The authors concluded that this antimicrobial protein may provide a first line of defense against potential bacterial invasion. Up to 24 variants of Cg-BPI have been identified (Schmitt et al., 2010).

Another group of potent antimicrobial compounds that display a wide activity spectrum are members of the lysozyme families (N-acetylmuramide glycanhydrolase). McDade and Tripp (1967) described the presence of lysozyme activity in oyster mantle mucus and suggested that this formed an antimicrobial defense. McHenery and collaborators (McHenery et al., 1986; McHenery et al., 1979) reported the presence of lysozyme activity in several bivalve species and hypothesized lysozyme to play a dual role in immunity and digestion. Several lysozymes have also been reported in the oysters *C. gigas* and *C. virginica*, including some mainly expressed in epithelial cells of the digestive gland and other mucosal tissues (Itoh et al., 2010b; Itoh and Takahashi, 2007; Xue et al., 2007; Xue et al., 2010). Bivalve lysozymes have been shown to be active against Gram-positive and -negative bacteria and to increase the antibacterial activity of AMP (Haug et al., 2004; Smith et al., 1995). Interestingly, a lysozyme isolated from *C. virginica* has also been found to display activities against *P. marinus* (La Peyre et al., 2004). Mantle tissues and secretions were also shown to be particularly rich (in terms of diversity and quantity) in lysozyme activity in various bivalve species including clams (Allam and Paillard, 1998) and oysters (Itoh et al., 2010b). Lysozyme activity is widely thought to protect bivalves from microbial invasions, and has been shown to increase in plasma and mantle secretions (extrapallial fluid) in response to pathogen exposure (Allam et al., 2000a; Allam et al., 2000b;

Chu and La Peyre, 1993). These conclusions were also supported by the findings of Perrigault et al. (2009b) who reported a significant increase in lysozyme transcription levels in the mantle of hard clams following exposure to the thraustochytrid pathogen QPX. A significant increase in lysozyme transcription levels was also measured in mantle and hemocytes from the extrapallial fluid of clams *R. philippinarum* infected with *V. tapetis* (Allam et al., 2014). In contrast, there are numerous studies showing that lysozyme levels in bivalve plasma may respond to environmental cues rather than, or in parallel to, pathogen exposure (Chu and La Peyre, 1989; Paillard et al., 2004). Overall, there is strong evidence that the repertoire of lysozymes is diverse in mollusks with most members mainly expressed in mucosal tissues (mantle, gills, digestive gland) helping the degradation of microbial food particles and providing a "generic" immune protection from environmental microbes.

The pioneering work of Cheng (reviewed in Cheng, 1983) was the first to describe the presence of protease activity in bivalve plasma and the regulation of the production and release of proteases in response to *in vivo* and *in vitro* challenge with microbes. Current information highlights the presence of proteolytic activities in different bivalve tissues and secretions. This is not surprising given the molecular and functional diversity of proteases. Interestingly, mucus covering the gills of oysters (*C. virginica*) was shown to contain several proteases including a putative acid protease (96 kDa), a zinc metalloprotease (64 kDa), and a serine protease (33 kDa) (Brun et al., 2000). The authors noted a change in the mucus protease profile in response to infection of the gills by the turbellarian worm *Urastoma cyprinae*. Similarly, significant regulation was reported for a member of the cathepsin L family of proteases in mantle tissues from clams *R. philippinarum* infected with *V. tapetis* (Allam et al., 2014). Members of this group of cysteine proteases were suggested to play an important role in immunity after they have been shown to be up-regulated following bacterial exposure in several molluscan species including pearl oysters (Ma et al., 2010) and razor clams (Niu et al., 2013).

Together with proteases, bivalve tissues and secretions also contain a wide range of protease inhibitors, including serine and cysteine protease and metalloprotease inhibitors (McDowell et al., 2014; Moreira et al., 2012; Nikapitiya et al., 2013). The involvement of protease inhibitors in bivalve defense remains largely unexplored even though previous studies suggested that they

may provide protection against proteases produced by invading microorganisms (La Peyre et al., 2010; Xue et al., 2006; Yu et al., 2011). The widely accepted scenario is that these inhibitors inactivate pathogen proteases, directly limiting their proliferation and growth rates (pathogen proteases serve primarily for food acquisition) or indirectly facilitating their neutralization via host immune proteins. Protease inhibitors may also protect the host from the deleterious effect of endogenous protease activities over-expressed in response to pathogen exposure (Phillips et al., 2004). Most progress on the unraveling of the role of protease inhibitors in self-defense was made in the oyster *C. virginica*, where serine protease inhibitors were suggested to represent and integrative component of the anti-*P. marinus* arsenal (Faisal et al., 1998; Oliver et al., 2000). In this species, two serine protease inhibitors (dubbed cvSI-1 and cvSI-2) were purified from plasma (Xue et al., 2009; Xue et al., 2006). Exposure of *P. marinus* cells to cvSI-1 inhibits *in vitro* proliferation suggesting that inhibitor may contribute to oyster protection *in vivo* (La Peyre et al., 2010). Finally, genotyping of *P. marinus* -resistant and -susceptible oysters before and after disease outbreaks showed that resistance of *C. virginica* to *P. marinus* is correlated with a mutation in the promoter region of cvSI-1 (Yu et al., 2011). Recent molecular investigations showed that the regulation of protease inhibitors in bivalves in response to microbial challenge is not systemic. For example, several transcripts matching serine protease inhibitors were up-regulated in the extrapallial fluid and mantle of clams infected with BRD while they were strongly down-regulated in the hemolymph of the same individuals. This highlights a differential response to the infection across different tissues (Allam et al., 2014).

8. The promises and limitations of new technologies

Recent progress in "omics" technology provides the resources and tools needed to make significant progress in the understanding of the mechanisms and effectors of bivalve immunity. There are currently six more or less complete molluscan genomes that are publically available: the pearl oyster *Pinctada fucata*, the Pacific oyster *C. gigas*, the Mediterranean mussel *Mytilus galloprovincialis*, the owl limpet *Lottia gigantea*, the snail *Biomphalaria glabrata*, and the California sea hare *Aplysia californica*. This number is expected to increase dramatically in the coming years. In parallel, there is a growing number of sequence read archives and assembled transcriptomes of varying qualities available in public databases that have been barely exploited.

As assessed earlier by Loker (2010), a synthetic comparative interpretation of the molluscan immunome is currently lacking and should be addressed. This is still the case 5 years later. Nevertheless, combined with new developments in high throughput genomic and proteomic tools, this resource opens the way to thorough, in depth, investigations of the nature and the dynamics of the effectors of bivalve immunity. There are several research venues that can greatly benefit from some of the latest technological advances. For example, comparative, large scale, assessment of the nature, dynamics and specificity of immune effectors can now be performed providing useful information on the diversity (e.g. C1qDC proteins) and putative function of immune molecules in mollusks. Similarly, transcriptome sequencing and protein profiling of various hemocyte types (separable and in sufficient quantities using modern flow cytometry methods) can shed light on hemocyte ontogeny and maturation processes and research activities along these lines are currently ongoing (Gary Wikfors, NOAA, personal communication). The understanding of the contribution of epigenetic regulation in immune performances is also an area that can greatly benefit from the usage of modern genomic tools given the importance of DNA methylation in the regulation of inducible genes (Gavery and Roberts, 2010; Roberts and Gavery, 2011). High throughput genomics tools can also be used for concomitant sequencing of host and parasite transcriptomes from tissue lesions (e.g. QPX nodules in clam tissues) to gather information on host-parasite molecular cross-talk. Finally, the use of modern proteomics can also provide much needed information on post translational regulation of immune proteins.

While promising, current approaches using "omics" techniques do have major limitations, in particular their limited usefulness for the discovery of new bioactive molecules. The study of Seo et al. (2013, discussed under the "Antimicrobial peptides" section above) is a good example showing the continuous usefulness of targeted biochemical and bioactivity testing studies. Many transcriptomic studies probing bivalve responses to natural infections or experimental pathogen challenge result in up to 80% of transcripts (contiguous sequences) that have no functionally-characterized match in public databases. While these facts represent a clear reminder of the limitations of our current knowledge on immune effectors and their regulation in mollusks, available genomic information represents a gold mine that calls for exploitation.

9. Conclusions and perspectives

Significant progress has been made in the understanding of bivalve responses to infections at the cellular level and a growing body of information is being generated on the molecular mediators of immunity in these animals. While bivalves lack the mechanisms conferring adaptive immunity in vertebrates, recent research demonstrates that they possess components known or thought to offer a certain level of immune specificity and immune memory (Ng et al., 2014; Rodrigues et al., 2010; Wang et al., 2013; Zhang et al., 2014b). This is an area that deserves to be tackled via targeted research because of its basic and applied implications. Another area that requires research investment is the identification and functional characterization of mediators involved in hemocyte recruitment and activation mechanisms. This can shed light on the strategies used by some parasites to evade the immune system. Similarly, the contribution of symbiotic microflora to host protection, whether directly (e.g. direct killing of bacteria by phages, Barr et al., 2013) or indirectly (production of compounds that can become integrative part of host's antimicrobial arsenal, Desriac et al., 2014), requires more attention.

As mentioned at the beginning of this paper, it is sometimes difficult to differentiate immune responses from general stress responses. Most recent molecular investigations targeting bivalve immune responses to infection lack the functional validation of targeted immune effectors or pathways. This is particularly critical for ground-truthing putative immune effectors identified via similarity searches using heterologous systems or databases. This is a rewarding but tedious endeavor, particularly given the lack of bivalve cell lines and the unavailability of valid methods for the production of genetic mutants. A much easier endeavor to achieve, but that can also help differentiate specific immune responses from general stress responses, is to better probe focal vs. systemic responses to infections wherever possible. For example, Allam et al. (2014) evaluated Manila clam (*R. philippinarum*) responses to brown ring disease (affects the edge of the mantle) and reported significantly different transcriptomic profiles (including for putative immune-related genes) between hemolymph (systemic response) and the extrapallial fluid and mantle (focal response). Similar trends have been also observed in QPX infection foci in the hard clam *M. mercenaria* (Wang et al., 2014) allowing the identification of the players in the host-pathogen warfare at the infection site.

911 Considering the ensemble of information available on bivalve immune responses to infections,
912 one should keep in mind the dynamic nature of host-pathogen interactions in general and in
913 aquatic invertebrates in particular. There are numerous studies highlighting the effect of
914 environmental factors on immune responses to infection in bivalves. These aspects were not
915 covered in this paper because that topic deserves a manuscript on its own given the major effect
916 of the environment on the physiology of these poikilotherm osmoconformers. Clearly, any study
917 of bivalve immunity needs to give a consideration of the genetic, physiological and
918 environmental (including micro-environmental, e.g. position of the bivalve in the intertidal zone,
919 Letendre et al., 2009) background of the bivalves under investigation.

920
921 Arguably, most research on bivalve immunity has been done on the cellular and humoral factors
922 present in the hemolymph, which represents the last defense layer in these organisms. The
923 quantity of microorganisms reaching the circulatory system represents an extremely small
924 fraction of that bathing mucosal tissues. Suspension-feeding bivalves are exposed to an
925 amazingly large number of waterborne microbes through their basic physiological activities
926 (oyster filtration rate was estimated to exceed $10 \text{ L h}^{-1} \text{ g}^{-1}$ dry weight (Dupuy et al., 2000)
927 equating over 25,000 microbes/second considering a 1 g oyster and 10^4 microbes ml^{-1} seawater -
928 microbial concentrations in estuarine areas are often orders of magnitude higher). Most of these
929 microbes are stopped at mucosal interfaces of the pallial cavity and the digestive tract and
930 evidence supports the major role of the mucosal barriers in limiting the spread of opportunistic
931 infections. For example, bivalves can overcome very large numbers of microbes if exposed via
932 "natural means" (bath exposure) but succumb to a much smaller numbers of the same microbes if
933 exposed by direct injection into tissues (Allam et al., 2002; Tubiash, 1971; Tubiash et al., 1965).
934 Of course, some of that protection is secured by the physical barrier made by mucosal secretions
935 and epithelial cells, but evidence is accumulating on the role of defense factors associated with
936 mucosal interfaces in fending microbial invasions (Allam and Pales Espinosa, 2015). Evidence
937 shows that the regulation of immune factors associated with mucosal tissues can be made
938 independently of those associated with the circulating hemolymph (Allam et al., 2014; Xing et
939 al., 2011). In other words, mucosal tissues can respond to local cues using locally produced
940 immune effectors without noticeable contribution from the circulatory system. Finally, blood
941 cells associated with mucosal interfaces represent the first alert system across many taxa. In that

regard, the role of hemocytes identified at bivalve tissue-water interfaces in sampling and deciphering the microbial landscape and transmitting relevant information to the internal immune system (Lau et al., 2013a; Lau et al., 2013b; see section "General organization of the bivalve defense system") requires further investigation.

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Figure legends

Figure 1. Differential interference contrast and fluorescence micrographs of live hemocytes from the Sydney rock oysters, *Saccostrea glommerata*. (A) hemoblast-like cell, (B) hyalinocyte, (C) large granulocyte, (D) small granulocyte, (E and F) large granulocytes. Scale bar = 5 μ m. From Aladaileh et al., 2007.

Figure 2. Massive hemocyte infiltration in mantle connective tissue of the hard clam, *Mercenaria mercenaria*, in response to QPX infection (arrows point to parasite cells). Scale bar = 20 μ m.

Figure 3. Phenoloxidase cytology of granular hemocytes from the Sydney rock oyster (*S. glomerata*) mixed with sporonts of the QX disease agent, *Marteilia sydneyi*. (A) Once *M. sydneyi* are ingested by hemocytes, intense phenoloxidase activity (pink staining) is directly associated with sporonts. (B) As melanization continues, entire hemocytes, including the internalized sporont(s), become melanized (black). Scale bar = 5 μ m. From Butt and Raftos, 2008.

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1631

Figure

A

B

C

D

E

F

Figure

Figure

A

B

Humoral factors in
plasma and
mucosal secretions

Immune cells
(hemocytes,
mucosal epithelia)

Recognition (TLR,
Lectins, PGRP, TEP,
C1qDC, LGBP, FREP)

Chemotaxis

Phagocytosis

Apoptosis

Encapsulation

Melanization

**Extracellular and
intracellular
neutralization** (AMP,
BPI, Hydrolytic
enzymes, Protease
inhibitors, ROS, RNS)

Viruses

Bacteria

Protists

Metazoan
parasites