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Abstract	The study of molluscan immune systems, and in particular those of bivalve molluscs (clams, oysters, scallops, mussels, etc.) has experienced great growth in recent decades, mainly due to the needs of a rapidly growing aquaculture industry to manage the impacts of disease and the application of -omic tools to this diverse group of invertebrate organisms. Several unique aspects of molluscan immune systems highlighted in this chapter include the importance of feeding behavior and mucosal immunity, the discovery of unique levels of diversity in immune genes, and experimental indication of transgenerational immune priming. The development of comparative functional studies using natural and selectively bred disease-resistant strains, together with the potential but yet to be fully developed application of gene-editing technologies, should provide exciting insights into the functional relevance of immune gene family expansion and molecular diversification in bivalves. Other areas of bivalve immunity that deserve further study include elucidation of the process of hematopoiesis, the molecular characterization of hemocyte subpopulations, and the genetic and molecular mechanisms underlying immune priming. While the most important aspects of the immune system of the largest group of molluscs, gastropods (e.g., snails and slugs), are discussed in detail in Chap. 12, we also briefly outline the most distinctive features of the immune system of another fascinating group of marine molluscs, cephalopods, which include invertebrate animals with extraordinary morphological and behavioral complexity.

Keywords (separated by " - ")	Mollusca - Bivalves - Oyster - Mussel - Scallop - Clam - Cephalopods - Aquaculture - Lectins - Opsonization - Pathogen recognition - Immune signaling - Antimicrobial peptides - Apoptosis - Complement system - Immune priming - Phagocytosis - Prophenoloxidase - Neuroendocrine immunomodulation

Q4

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Q4 This chapter has been lightly edited for clarity, correctness and house style. Please check it carefully to make sure the intended meaning has been preserved. If the intended meaning has been inadvertently altered by the editing changes, please make any corrections needed.

Immunity in Molluscs: Recognition and Effector Mechanisms, with a Focus on Bivalvia

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An Introduction to Bivalve Molluscs

Evolution and Life Cycle

The phylum Mollusca includes eight taxonomic classes comprising more than 85,000 living species, and 60,000 additional species documented by fossil records (Fig. 1). This ranks molluscs as the second most abundant phylum of animals after arthropods and before chordates (Ponder and Lindberg 2008). Molluscs are successful invertebrates characterized by a broad morphological

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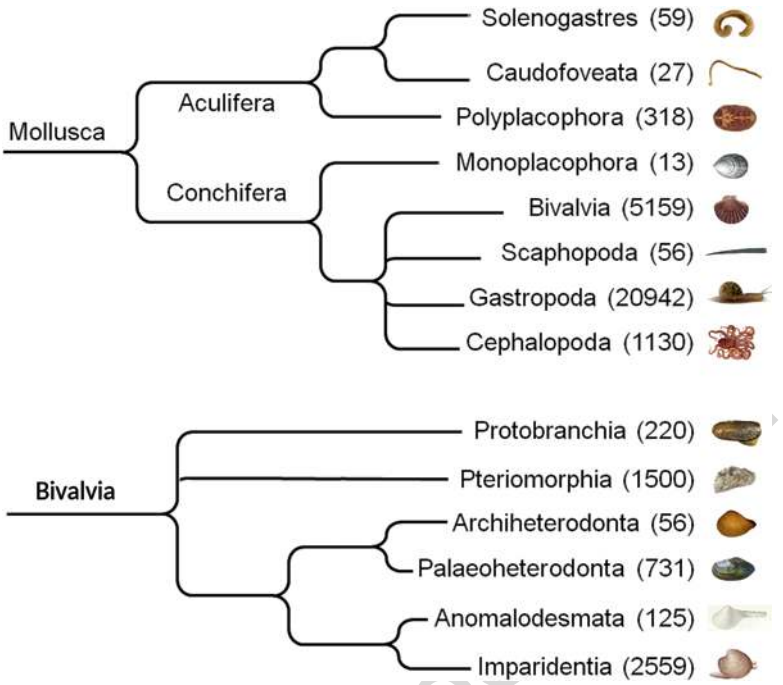

Fig. 1 Simplified tree of life of molluscs (above) and bivalves (below), based on Bieler et al. (2014) and the Tree of Life web project (http://tolweb.org/Mollusca/2488). The number of species currently registered in the NCBI Taxonomy database for each taxon (data retrieved in December 2017) is displayed between brackets

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and physiological diversity. They are extraordinarily well adapted to adverse environmental conditions and, starting from the early radiation that occurred in the Late Cambrian era, they have colonized almost all ecological niches: from terrestrial habitats over 3000 meters above sea level to deepsea hydrothermal vents, coping with extreme levels of heavy metals, pH, temperature, CO_2, methane, and sulfide (Plazzi and Passamonti 2010)

Bivalvia represent the second largest class within the phylum Mollusca, with over 5000 recognized species, mostly adapted to marine environments. Although the phylogenetic relationship among the different groups of bivalves and, more generally, of all molluscs have been the subject of debate for decades (Kocot et al. 2011; Smith et al. 2011; Sigwart and Lindberg 2015), recent studies tried to reorganize the bivalve tree of life into six major lineages, as shown in Fig. 1 (Bieler et al. 2014). Briefly, the authors recognized the primitive and relatively small group of Protobranchia, the large groups of Pteriomorphia (comprising oysters, mussels, and scallops, among others), Palaeoheterodonta (mostly freshwater clams and mussels), Imparidentia (the largest and most diverse group of bivalves, comprising over 2500 clam species), and two additional small groups with peculiar morphological features, i.e., Archiheterodonta and Anomalodesmata.

Bivalves can be protandric hermaphrodites (oysters in the genera *Magallana* and *Crassostrea*), simultaneous hermaphrodites (scallops in the genus *Pecten*), and rhythmical consecutive hermaphrodites (oysters in the genus *Ostrea*). As exemplified in Fig. 2, the general life history of the majority of molluscan bivalve species starts during the main spawning season when adult animals with mature gonads release oocytes and spermatozoa in the water column and external fertilization occurs (Pechenik 2010). Bivalve larvae are planktonic (free-living) and remain in the water column for days to weeks, depending on the species and the environmental conditions. During larval development, the molluscan embryo becomes a

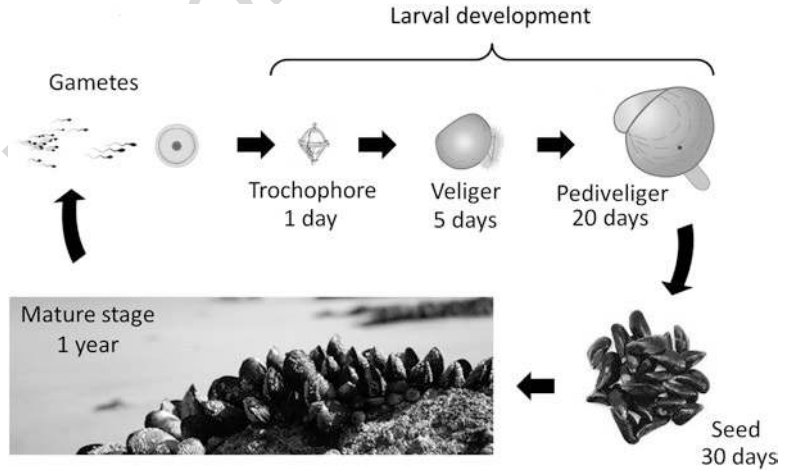


Fig. 2 Life cycle of a bivalve, as exemplified for the Mediterranean mussel, *Mytilus galloprovincialis*

planktonic (free swimming) trochophore larva. The late trochophore is the phylotypic stage, defined as the ontogenetic stage, characterized by maximum similarity among the species within a phylum (Xu et al. 2016a). After a few days, the primordium of the shell appears and the bands of cilia used by larvae to feed and swim develop into the velum, a characteristic organ of the veliger stage. Then, larvae develop a foot, characteristic of the pediveliger stage, and undergo metamorphosis. Once metamorphosis is complete, their body plan and physiological aspects resemble those of the adult form and the larvae will settle out of the water column where, depending on the species, they might attach to a substrate, lie on a substrate and swim, or bury themselves in sediments (Balseiro et al. 2013). When adults become mature, gametogenesis occurs, with modalities that depend on the species, geographic region, water depth, and season (Shumway and Parsons 2006).

Anatomy and Physiology of Bivalves

Although the adult anatomy of molluscs can greatly differ from one taxon to another, they share a general basic plan derived from a hypothetical shared ancestor (Fig. 3). This includes a soft oval body with bilateral symmetry, a muscular foot, a mantle—which secretes the shell (absent or internalized in some groups) or the spicules—and a feeding organ formed by chitinous sharp structures, called radula (absent in bivalves).

Overall, this shared body plan results in a great morphological diversity of bivalve groups adapted to different ecological niches, as shown in Fig. 4 (Ruppert et al. 2004). Bivalve shells consist of two, sometimes symmetric, hinged valves.

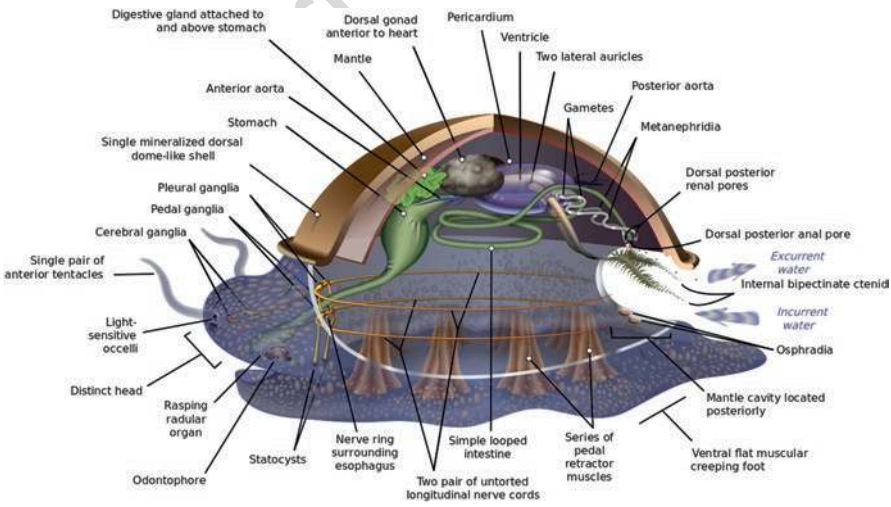


Fig. 3 Anatomy of the hypothetical common ancestor of all molluscs. (Author: KD Schroeder—Archimollusc-en.svg from Wikimedia Commons—License: CC-BY-SA 3.0)

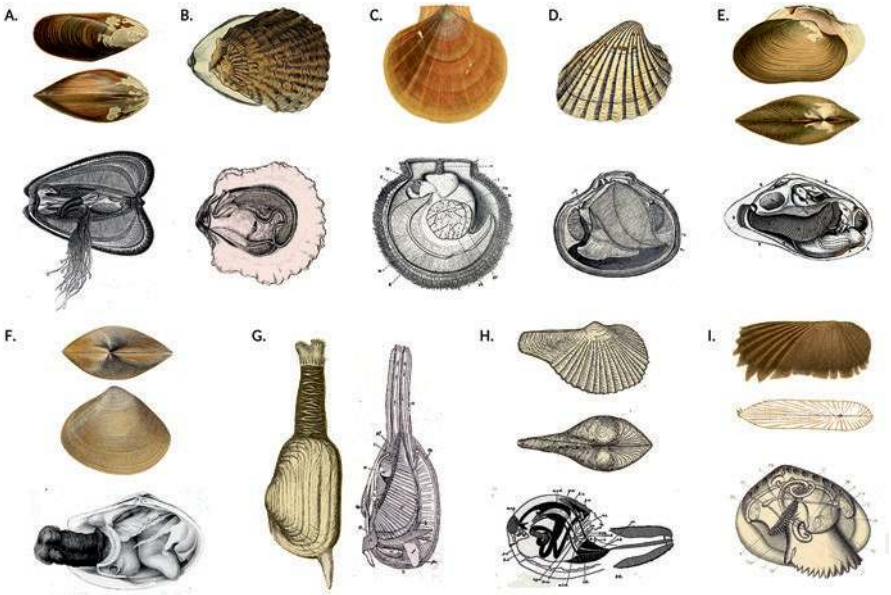


Fig. 4 Examples of diversity in the basic anatomy of different bivalve lineages. (**a**) Anatomy of Mytiloida (Pteriomorphia): *Mytilus uguiculatus* (external) and *Mytilus galloprovincialis* (internal). (**b**) Anatomy of Ostreoida (Pteriomorphia): *Ostrea edulis*. (**c**) Anatomy of Pectinoida (Pteriomorphia): *Placopecten magellanicus*. (**d**) Anatomy of Archiheterodonta: *Cardites floridanus* (external) and *Astarte borealis* (internal). (**e**) Anatomy of Palaeoheterodonta: *Anodonta cygnaea*. (**f**) Anatomy of Mactroidea (Imparidentia): *Mactra antiquate* (external) and *Tresus capax* (internal). (**g**) Anatomy of Myida (Imparidentia): *Mya arenaria*. (**h**) Anatomy of Anomalodesmata: *Cardiomya reticulata* (external) and *Laternula elliptica* (internal). (**i**) Anatomy of Protobranchia: *Solemya velum* (external) and *Ennucula delphinodonta* (internal). To better show anatomic internal details, in most cases one of the valves and the mantle have been removed. (The anatomic tables have been taken from multiple sources, kindly provided by the Biodiversity Heritage Library)

The shell is produced by secretory cells in the epithelium of the mantle or pallium, with contributions from the hemocytes (blood cells) (Mount et al. 2004). Bivalve shells are formed mainly of conchiolin, which is composed of protein-hardened calcium carbonate (aragonite or calcite) and has three layers: the outer layer (periostracum), a middle layer, and the inner layer, which is often nacreous and in some cases has exceptional economic value. The mantle encloses a chamber surrounding the bivalve body called the mantle or pallial cavity, which is in direct contact with the environment when the shell is open. Organs that have direct contact with the pallial cavity include the gills (or ctenidia), the osphradia (chemical sensors), and the openings of the nephridia, gonads, and digestive system. The space between the mantle and the shell constitutes the extrapallial cavity (Ruppert et al. 2004).

The movement of shell valves is controlled by one, two, or (rarely) three adductor muscles that control shell closure and keep it tightly shut when needed, and by an elastic ligament that acts as a spring, allowing the shell to open when muscles are relaxed. Some bivalves also possess a pair of siphons (inhalant and exhalant) used

in the exchange of water. These systems ensure the flow of water into the pallial cavity for feeding and respiration.

The gills divide the mantle cavity into distinct chambers and their cells possess cilia, which produce a laminar flow of water that facilitates feeding and enhances respiratory gas diffusion and exchange. Gills also exhibit osmoregulatory, ion transport, homeostasis, and sensorial functions (Moreira et al. 2015). Gas exchange occurs mainly in the center of the gill filament, where the hemocytes circulate through hemolymph vessels. Most bivalves absorb oxygen directly from water through their tissues and oxygen-carrying molecules such as hemocyanin have been identified in only a few genera. As coelomates, bivalves have another characteristic cavity, the coelom, a small pericardial cavity enclosing the heart. Hemolymph is pumped throughout the body by the heart, which receives oxygenated blood from the gills and pumps it into the main blood vessel, a short artery that opens directly into the hemocoel. Bivalve molluscs have an open circulatory system, with the hemolymph reaching all of the organs by passive diffusion aided by the pumping effect of the heart, which also has excretory functions. A pair of nephridia connected to the coelom extracts any reusable materials from the coelomic cavity, dumps additional unwanted products into it, and then excretes all of the materials into the mantle cavity. In bivalves, gonads are located within the connective tissue at the edge of the mantle, with spawning occurring directly in the mantle cavity (Ruppert et al. 2004).

Depending on the species, bivalves feed on suspended particles in the water column, using an inhalant opening or siphon and ctenidia (e.g., *Magallana* and *Crassostrea* spp. oysters); on deposits or particles on top of sediments, using an inhalant siphon and ctenidia (e.g., *Macoma* spp. clams); or on deposits in the sediments, using proboscides (e.g., *Yoldia* spp. clams). Many bivalves are able to pump large volumes of water while feeding. In bivalve species that use the ctenidia to feed, food particles (mainly phytoplankton) are selectively trapped in a thick layer of mucus covering the gills, transported with the aid of the cilia, sorted, and directed to the outer labial palps, where particles are further sorted on the basis of size and other physical and chemical characteristics. Some particles are then transferred to the mouth by the inner palps, while other particles are rejected in pseudofeces released into the pallial space. Mucus and cilia facilitate particle movement toward the stomach, where there is further sorting and selection of particles (Ward and Shumway 2004), leading to the prostyle, a mass of food and mucus. The prostyle is extracellularly digested by the action of the enzymes produced by the digestive gland. In most bivalve species, phagocytic cells have been evidenced in the tubules of the digestive diverticula, where they contribute to intracellular digestion of the selected particles reaching this organ. The remaining particles are excreted via the nephridia or via the gut and finally reach the mantle cavity through the anus (Ruppert et al. 2004).

Although mostly a sedentary group in their adult life stages, some bivalve species are able to move. Most bivalves rely on the foot, a muscular organ with sensorial abilities achieved through balance receptors, the statocysts (Williamson 1993). Larval pediveligers use the foot to sense and locate appropriate substrate for

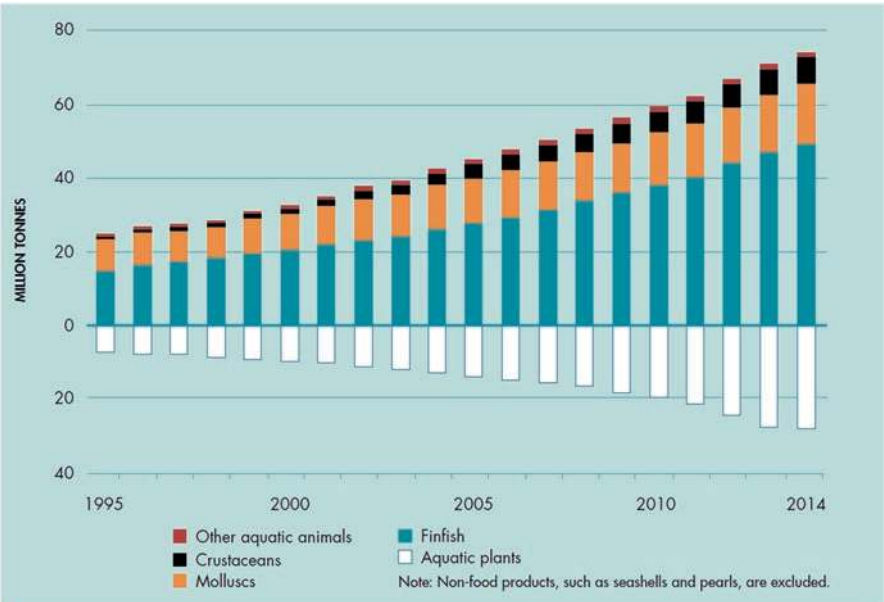
settlement. In burrowing species such as clams, the foot is used by adults to burrow into the sediments. In mussels, the foot is linked to the production of byssus, an extremely resistant extracellular protein used to attach to the substrate (Carrington et al. 2015). Some species of bivalves (e.g., scallops) are also able to swim by rapidly opening and closing the two valves of the shell (Ruppert et al. 2004).

The nervous system of bivalve molluscs has a simple structure, organized in paired ganglia connected by nerve commissures within them and nerve cords along them in a "rope ladder structure." The visceral cords innervate the internal organs and the pedal cords innervate the foot. The ganglia are divided in two groups: (1) cerebral, pleural (absent in bivalves), and visceral above the esophagus; and (2) the pedal ganglia below. These two differentiated parts are connected by the collar nerve, which surrounds the esophagus (Ruppert et al. 2004).

Ecological and Economical Roles

Bivalve molluscs cover multiple important roles, from both ecological and socioeconomic points of view. Ecologically, bivalves have a key role in the environmental energy flux, in the maintenance of water quality by filter feeding and, for reef-building species such as oysters, in providing substrates and habitats for other species (Zu Ermgassen et al. 2012). Several bivalve species, and mussels in particular, have been used worldwide as sentinels for environmental pollution because of their sedentary and cosmopolitan nature in coastal waters, ease of sampling, ability as filter feeders to concentrate pollutants, and commercial use as an important food staple (Campos et al. 2012; Farrington et al. 2016; Burgos-Aceves and Faggio 2017). Bivalves can also concentrate pathogens and marine toxins, reaching harmful levels for consumers (Visciano et al. 2016). Moreover, as exemplified in Fig. 5, bivalves constitute a major sector of world fishery and aquaculture production, with more than 16 million metric tons with a value of almost US$18 million produced in 2015, representing 15% of total aquaculture production (FAO 2016).

The main purpose of the molluscan aquaculture industry is to produce food, although this industry also has other applications such as ecosystem restoration, extraction of pharmaceutical and industrial products, and ornamentation (aquaria, nacre, pearls). The most important cultured species of molluscs are bivalves such as oysters, mussels, clams, cockles, and scallops, hence the focus of this chapter on these species. The culture process generally starts with the "conditioning" of broodstock in hatcheries by feeding them nutrient-rich cultured microalgae. Spawning is initiated by manipulation of environmental conditions (i.e., temperature, food availability) or, in some cases, gametes are surgically harvested. Fertilization is achieved by mixing of sperm and eggs. Larvae are kept in the hatchery while being fed cultured microalgae until they undergo metamorphosis and settle, and the small juveniles (also called spat) are moved out of the hatchery to a nursery and/or grow-out facility in open water to take advantage of the natural food supply. Grow-out culture technology varies depending on the species and location but can include the use of rope culture (mussels), cages/bags (oysters), and planting in natural beds (clams).



		INLAND AQUACULTURE	MARINE AND COASTAL AQUACULTURE	TOTAL
			(Tonnes)	
World	Finfish	43 559 260	6 302 631	49 861 891
	Molluscs	277 744	15 835 450	16 113 194
	Crustaceans	2 744 537	4 170 536	6 915 073
	Other animals	520 850	372 718	893 568
	TOTAL WORLD	47 102 391	26 681 334	73 783 725

Fig. 5 World aquaculture production from 1995 until the present day. (FAO 2016)

Feeding relies on natural phytoplankton production at the site, and most of the labor involves predator and biofouling control.

Major Infectious Diseases Affecting Bivalve Molluscs

The commercial importance of many bivalve molluscs and efforts to manage diseases that severely impact the aquaculture industry have driven much of the research in the immunology of these species. Bivalve aquaculture has been severely impacted in recent years by infectious diseases and toxins from harmful algal blooms causing morbidity and mortality, as well as closures of the industry due to the accumulation of toxins and pathogens affecting the health of human consumers (GLOBEFISH 2017). The relevance of these diseases is highlighted by the fact that the World Organization for Animal Health (most commonly known as the OIE) lists six diseases affecting bivalve molluscs among those with major relevance for animal

protection (OIE 2017). While pathologies caused by viruses, bacteria, and parasites have been documented in nearly all major molluscan classes, in this chapter we will present an overview of the pathological agents that have so far been relevant causes of concern for marine aquaculture activities and most commonly used as models in the study of bivalve immunity, leaving a discussion of infectious agents targeting other molluscs to the section "An Overview of Infectious Agents with Which Molluscs Must Contend" in Chap. 12.

Many diseases affecting bivalves result from an accidental side effect derived from the transfer of aquaculture species, leading to naïve hosts (indigenous or introduced) being exposed to new pathogens. Disease dynamics are heavily influenced by environmental factors, mainly temperature and salinity (Carella et al. 2015; Lafferty and Hofmann 2016; Stentiford et al. 2017), which are remarkably influenced by human activities, as thoroughly discussed in the section "Challenges for Molluscs in the Anthropocene Epoch" in Chap. 12. The study of bivalve immunology has benefited from many decades of research on host–pathogen interactions, the identification of species displaying natural resistance to diseases, the development of disease-resistant strains through selective breeding, and the recent application of -omic tools to bivalve research (Allam and Raftos 2015; Gómez-Chiarri et al. 2015). Most of the research has been focused on pathogens that can be cultured (Fernández Robledo et al. 2014).

Major Viral Diseases of Marine Bivalves

Although the characterization of viral diseases in bivalves has been hampered by the lack of cell lines from marine molluscs, recent advances in sequencing and the development of challenge models and disease-resistant strains have resulted in a better understanding of viral pathogenesis and immunity in several commercially important marine molluscs (Arzul et al. 2017). The best-characterized viral disease of bivalves is caused by oyster herpesvirus 1 (OsHV-1) and its variants (OsHV-1 Var and several microvariants, µVar). Massive mortalities of bivalve larvae and/or juveniles due to OsHV-1 infection have seriously impacted the oyster industry in Europe, but also in Mexico, the USA, Australia, New Zealand, China, Japan, and Korea. These infections are recurrent in Pacific oysters (*Magallana gigas*), but other species of oysters, clams, mussels, and scallops are affected as well (Arzul et al. 2017). As shown for other diseases, some strains and species of bivalves appear to be resistant to or tolerant of the disease, such as the Sydney rock oyster, the eastern oyster, and mussels (Masood et al. 2016). Susceptibility to the disease also varies with age, size, and genetics within a species, and several selectively bred lines of Pacific oysters with increased resistance have been developed (Dégremont et al. 2015). In contrast to herpesviruses infecting vertebrates, both inter- and intraspecies horizontal transmission of OsHV-1 have been shown, with more tolerant individuals or species acting as disease carriers and reservoirs (Arzul et al. 2017).

Morphological and genomic characterization has led to the classification of this large enveloped virus as a member of the *Malacoherpesviridae* (Mushegian et al. 2018). The function of most of the 124 ORFs found in the OsHV-1 viral genome is unknown, mostly because of lack of homology with sequences with known function (He et al. 2015; Arzul et al. 2017). Infection of oysters with OsHV-1 causes reduced feeding and swimming in larvae. High levels of viral replication are observed mainly in connective tissues, leading to changes in tissue and cellular architecture, including dilation of the digestive tubules, nuclear chromatin margination and pycnosis, and damage to the cytoskeleton and organelles. The disease is also characterized by massive infiltration of hemocytes. High levels of mortality occur within 48 h postinfection in susceptible animals (He et al. 2015; Young et al. 2017).

Exposure of oysters to the virus through experimental challenges indicates that the viral particles infect the host through the digestive gland and/or other mucosal surfaces, probably exploiting hemocytes to reach target tissues (Segarra et al. 2016; Morga et al. 2017). The virus is able to rapidly (within 1 h) infect and initiate replication in hemocytes. The formation of viral particles has not been observed in hemocytes, however, suggesting that these cells impede completion of the viral cycle, as observed in vertebrate macrophages infected with other herpesviruses (Morga et al. 2017). Viral infection leads to activation of the integrin pathway in the host cells, followed by activation of the actin pathway, indicating that the virus exploits these pathways to enter the cell and eventually deliver the viral genome into the nucleosome. Proteomic and metabolomic studies in challenged oysters show that OsHV-1 causes substantial alterations in central carbon metabolism and glycolysis (Warburg effect) in the host, as well as alterations in lipid metabolism and a characteristic fatty acid signature indicative of lipolysis. These metabolic alterations increase the availability of substrates for virion synthesis and assembly. They can also lead to increased inflammation and pathology through the activation of immune-responsive gene 1 protein/*cis*-aconitic acid decarboxylase (IRG1/CAD), a protein linking cellular metabolism with immunity, activation of the respiratory burst, increased permeabilization of the mitochondrial membrane, and reduced ATP production (Corporeau et al. 2014; Young et al. 2017).

Major Bacterial Diseases of Marine Bivalves

With a few exceptions (detailed below), mass mortalities caused by bacterial pathogens in bivalves are observed in larvae and, less often, in juveniles in hatcheries and nurseries (Travers et al. 2015). Experimental challenges with bacterial pathogens, however, are commonly used to study immune responses in bivalves because of the ability to perform culturing and ease of isolation and characterization (Gómez-Chiarri et al. 2015). A wide variety of *Vibrio* spp., including several belonging to the *V. splendidus, V. harveyi,* and *V. tubiashii/coralliilyticus* clades, have been isolated from outbreaks in bivalve hatcheries. In general, early signs of infection of bivalve larvae by pathogenic vibrios include decreased feeding and damage to the velum, followed by widespread necrosis of tissues and rapid mortality (Travers et al. 2015).

Strains of *V. aestuarianus, V. splendidus, V. crassostreae,* and others are often detected during summer mortality events in juvenile and adult Pacific oysters, also associated with infection with OsHV-1. Mass mortalities are, in general, seen during the spawning season and other conditions of stress (De Decker et al. 2011). The genomes of many of these pathogenic vibrios have been sequenced, facilitating the identification of mechanisms of virulence (Travers et al. 2015; Gómez-Chiarri et al. 2015). Examples of virulence factors involved in vibriosis include a variety of metalloproteases, hydrolases, cytotoxins, siderophores, the type III secretion system, and an OmpU from *V. tasmaniensis* LGP32, which is involved in internalization of the bacteria into *M. gigas* hemocytes (Travers et al. 2015; Le Roux et al. 2016).

Two bacterial pathogens of bivalves—*Aliiroseovarius crassostreae* and *Vibrio tapetis*—are notable for their ability to colonize the periostracal lamina of the inner side of bivalve shells. These pathogens cause Roseovarius Oyster Disease (ROD, also called Juvenile Oyster Disease) in the eastern oyster *Crassostrea virginica* and Brown Ring Disease in *Ruditapes* spp. clams, respectively. Susceptible bivalves respond to the presence of the pathogen in the inner side of the shell and the pallial cavity by producing conchiolin mixed with melanin and other quinones with antimicrobial action, resulting in pathognomonic brown deposits that surround the edge of the mantle (Travers et al. 2015). Little is known about mechanisms of virulence in ROD, but it is likely that formations of polar fimbriae and biofilm on the shell of oysters by *A. crassostreae* are involved in the disease (Boardman et al. 2008). Virulence factors identified in the genome of *A. crassostreae* include a hemolysin/cytotoxin and a putative type IVA secretion system (T4ASS) (Kessner et al. 2016). The metabolic demand of the chronic infections derived from an unsuccessful immune response in susceptible animals may contribute to mortality (Paillard et al. 2014; McDowell et al. 2014).

A few selected bacterial pathogens have been associated with sporadic episodes of mortality in adult bivalves, most notably *Nocardia crassostreae* and several intracellular Rickettsia-like organisms (RLOs). Little is known, however, about mechanisms of virulence and host immunity in these diseases (Travers et al. 2015; Zannella et al. 2017).

Major Parasitic Diseases of Marine Bivalves

Haplosporidian Parasites

Protistan parasites constitute the largest cause of adult bivalve morbidity and mortality. Among the most devastating groups of protozoan parasites of bivalve molluscs are several parasites belonging to the phylum Haplosporidia (Arzul and Carnegie 2015). In particular, the haplosporidians *Bonamia ostreae, B. exitiosa,* and *Haplosporidium nelsoni* have been well known for decades for causing significant economic and ecological losses, mainly in Europe and the USA. The growth of the bivalve aquaculture industry has led to the recent identification of many other haplosporidian parasites affecting a variety of bivalves. Most of the outbreaks caused

by the best-known representatives of this phylum, *B. ostreae* and *H. nelsoni,* have been observed in adult oysters. While species from the genus *Bonamia* are only known to affect oysters, have a direct mode of transmission, and are mostly intracellular, other haplosporidian taxa have representatives affecting a wide variety of bivalve hosts, are transmitted through intermediate hosts, and are typically extracellular. Many aspects of the life cycle of these parasites are unknown, as they cannot be maintained in culture. However, it is presumed that infective stages of *H. nelsoni* enter the host through the epithelial lining of the gill, developing into multinucleated plasmodia, which are seen in all tissues in heavily infected oysters. Depending on the haplosporidian species, sporulation occurs in the epithelium of the digestive diverticula or in connective tissues of the host, leading to the development of sporocysts, which are thought to eventually burst upon death of the host, releasing spores into the environment. Sporulation of *H. nelsoni* has rarely been observed in *C. virginica,* indicating that this oyster may be an atypical host. Oysters that have survived outbreaks of *H. nelsoni* and *B. ostreae* show increased resistance to these diseases, a fact that has been exploited in the development of selectively bred disease-resistant strains (Arzul and Carnegie 2015; Morga et al. 2017).

Cercozoan Parasites

Several *Marteilia* spp. (Cercozoa, Paramyxida) have been responsible for flat and Sydney rock oyster epizootics in Europe and Australia. These parasites affect a diversity of molluscan hosts, including oysters, clams, and mussels, and disease pathogenesis varies depending on the *Marteilia* spp. and the host. Clinical signs of the disease may include nodules (a gross manifestation of an encapsulation response) and, in many of the species, necrotic damage to the digestive gland. As other Paramyxean parasites, *Marteilia* spp. show a characteristic cell-within-cell development by budding. Therefore, most aspects of their complex life cycle, pathogenesis, mechanisms of virulence, and modes of transmission remain a mystery, since efforts to culture these parasites or transmit the disease using cohabitation challenges have been unsuccessful (Carrasco et al. 2015).

Perkinsozoan Parasites

Perkinsosis is caused by a variety of species belonging to the genus *Perkinsus* (phylum Perkinsozoa, superphylum Alveolata). The first *Perkinsus* spp. to be characterized, *Perkinsus marinus,* was identified in the 1940s as the cause of mass mortalities of eastern oysters in the Gulf of Mexico. As is the case for haplosporidian parasites, many other species have been described with the growth of the bivalve aquaculture industry, including *P. olseni, P. chesapeaki, P. mediterraneus, P. beihaiensis, P. honshuensis,* and *P. qugwadi*. While the geographic range of *P. marinus* seems to be limited mainly to that of *C. virginica* in North America, other *Perkinsus* spp., such as *P. olseni,* have a wider geographic and host range. Therefore, *Perkinsus* spp. affect oysters, clams, scallops, cockles, and mussel species in Australia, New Zealand, Asia, America, and Europe (Reece et al. 2017). These parasites have a direct life cycle with four described life stages: trophozoites, hypnospores (or prezoosporangia), zoosporangia, and biflagellated spores (Soudant et al. 2013). The

disease is transmitted horizontally, infecting the host through the epithelia of the digestive tract and mantle after the parasites are brought into the pallial cavity and ingested through feeding. Although *Perkinsus* spp. can cause relatively rapid mortality with few clinical signs in the most susceptible individuals within a population, it is most frequently manifested as a chronic disease in adult bivalves. Signs of disease are characterized by severe hemocytic infiltration of tissues, a decrease in gametogenesis and the condition index and, in some individuals, death by occlusion of vascular sinuses, tissue necrosis, and/or emaciation. In some host species, such as *Ruditapes* spp. clams infected by *P. olseni,* the chronic response is characterized by granuloma-like formations, which can be visibly detected as nodules at the base of gills. Parasites are transmitted to other hosts after being released to the water through diapedesis, in feces, or at the death of the host (Soudant et al. 2013; Ruano et al. 2015). Clonal cultures of most *Perkinsus* spp. are available, allowing for the characterization of putative virulence factors through genetic, genomic, and proteomic studies (Gómez-Chiarri et al. 2015; Hasanuzzaman et al. 2016; Fernández-Boo et al. 2016). Some interesting examples of mechanisms of virulence potentially contributing to the ability of *P. marinus* to survive within the hemocytes of the eastern oyster (Alavi et al. 2009) include antioxidant enzymes, such as superoxide dismutases (Schott and Vasta 2003; Schott et al. 2003; Asojo et al. 2006; Fernández-Robledo et al. 2008) and ascorbate-dependent peroxidases (Schott et al. 2003), and a natural resistance–associated macrophage protein (NRAMP) (Lin et al. 2011). Exposure of *P. marinus* to oyster tissue homogenates or pallial fluid in vitro modulates the production of serine proteases and the expression of genes coding for anti-apoptotic proteins, heat shock proteins, and proteinase inhibitors (Soudant et al. 2013; Pales Espinosa et al. 2014). Another interesting feature of *Perkinsus* spp. may be the presence of a relic plastid with no photosynthetic capabilities (Fernández Robledo et al. 2011) and the ability to secrete several fatty acids, including arachidonic acid (Soudant et al. 2013). Differences in resistance to or tolerance of infection by *Perkinsus* spp. have been documented within and between bivalve species, and selectively bred lines with moderate resistance to or tolerance of *P. marinus* are available (Proestou et al. 2016).

Quahog Parasite Unknown

The protist Quahog Parasite Unknown (Labyrinthulomycetes, Stramenopiles), better known as QPX, causes an opportunistic disease in the quahog *Mercenaria mercenaria* in the northeast and mid-Atlantic regions of the USA (Burge et al. 2013). The disease caused by QPX is characterized by the presence of areas of massive focal inflammation, visibly manifested as nodules commonly observed at the edge of the mantle or the base of the siphon. Differences in susceptibility to QPX infection have been observed between clam populations from different geographic locations (with clams originating south of Virginia being more susceptible than northern clams) and lines of clams derived from survivors of disease outbreaks. Resistance is probably due to a combination of factors, including adaptation to local conditions, as well as selection for molecules involved in more effective immune responses against the parasite (Wang et al. 2016b). QPX is a saprophyte that secretes a thick

mucus layer while in tissues of the clam that appears to protect the parasite from the immune response of the host. Putative virulence factors include a variety of hydrolytic enzymes and proteases, antioxidants, polysaccharide production, and factors involved in recognition, such as lectins. The expression of many of these putative virulence factors—in particular, genes that may be involved in the formation of the protective mucus layer—are significantly regulated by temperature (Rubin et al. 2017).

Metazoan Parasites

Some metazoan parasites have been documented in marine bivalves, including the copepod *Mytilicola intestinalis* (a parasite of mussels) and the trematode *Schistosoma mansoni* (a parasite of humans that also infects snails). Trematode infections are common in molluscs, which act as intermediate hosts. This complex host–parasite interplay is modulated by pattern recognition and effector molecules, as thoroughly reviewed by other authors (Zhang and Loker 2004; Adema et al. 2010; Pila et al. 2017) and discussed in detail in the section "Disease-Transmitting Snails" in Chap. 12.

A General Overview of Bivalve Immunity

Feeding: An Aspect Not to Be Overlooked

Invertebrates, including molluscs, lack the acquired response in a narrow sense (Criscitiello and de Figueiredo 2013), but they possess a potent and efficient cellular and humoral innate immune system, physical barriers such as the shell and the mucus, and behavioral avoidance. This innate response involves, as its major players, circulating hemocytes and a broad range of diverse molecular effectors. A general overview of immune defenses in bivalves is depicted in Fig. 6. One of the first lines of defense of bivalves against pathogens derives from their ability to sense the environment and sort particles during feeding (Ben-Horin et al. 2015). As described in the section "Anatomy and Physiology of Bivalves", bivalves are filter feeders, and the surfaces of the mantle and the gills are exposed to large volumes of water containing microbes and plankton. Bivalves are able to distinguish non-nutritious or potentially harmful particles on the basis of size, physical, and chemical cues, and reject (expel) these particles using mucociliary mechanisms. Bivalves are also able to shut down feeding and keep the valves tightly closed under unfavorable environmental conditions (e.g., low oxygen or blooms of an undesirable phytoplankton species). Although the specific roles of sensing and behavioral responses in disease resistance and immunity have not been well studied, some recent evidence indicates that these may be an interesting avenue for further study. For example, it is thought that oysters accumulate relatively less domoic acid (a toxin produced by the harmful algae *Pseudo-nitzschia* spp.) than mussels, in part because oysters ingest fewer algal cells (Mafra et al. 2010). There is also evidence that feeding behavior is responsible for increased resistance to the parasite *P. marinus* observed in some selectively bred

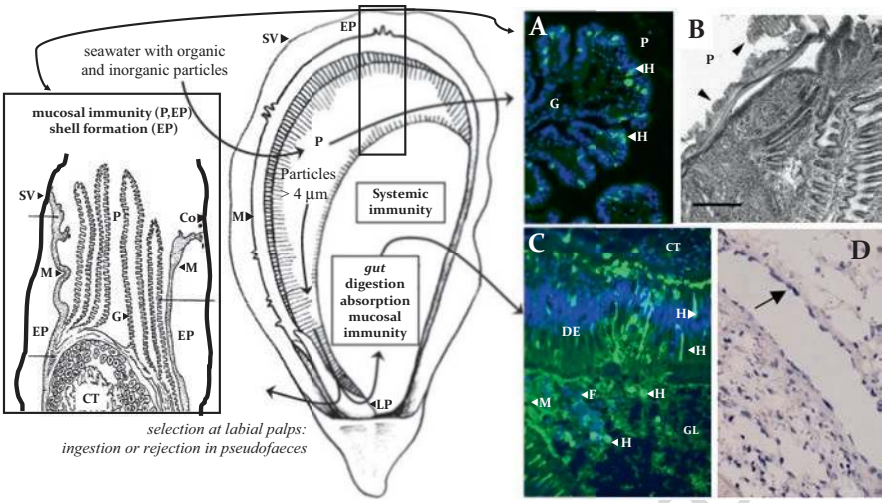


Fig. 6 Overview of immune responses in a representative bivalve (an oyster). *Center:* View of oyster tissues on top of one of the valves, illustrating the flow of water and particles during feeding (Troost 2010). Left: Lateral view of the ventral side of an oyster, showing the pallial (P) and extrapallial (EP) cavities. Right: Micrographs illustrating examples of cellular responses in different immune compartments. (**a**–**c**) Examples of mucosal immune responses. (**d**) Example of a systemic immune response (see below for more details). When the two shell valves (SV) characteristic of bivalves open to allow for feeding, water is pumped through the gills (G) and particles are selected to be either rejected or brought into the gut (central panel). Cells in the mucosal epithelium of the gills and mantle (M) secrete mucus and other effectors. The mantle is also responsible for sealing the edge of the shell valve from the environment (left panel) and producing conchiolin (Co, in the drawing on the right and the arrowhead in (**b**). Hemocytes (H) can migrate into the pallial and extrapallial cavities (**a** and **b**), the gut (**c**), and the blood sinuses (**d**) to recognize, capture, and digest particles and pathogens. (**a**) Immunofluorescence image of a section of oyster gill (G) tissue, showing hemocytes labeled in green (H). Shown in blue are cell nuclei stained with Hoestch. (**b**) H&E-stained sections of a challenged oyster showing degeneration and erosion of the mantle associated with hemocytic infiltration (arrows) and the presence of conchiolin (arrowheads) (scale bar = 100 µm) (Gomez-Leon et al. 2008). (**c**) Immunofluorescence image of a section of oyster gut showing the digestive epithelium (DE), with hemocytes labeled in green (H). The presence of mucus (M) and algal food (F) can be observed in the gut lumen (GL). Shown in blue are cell nuclei stained with Hoestch. (**d**) Big-defensin labeling in hemocytes (arrow) at the edge of a blood vessel in Pacific oysters challenged with *V. anguillarum* (Rosa et al. 2011)

families of eastern oysters, with oysters from resistant families removing (filtering) fewer algal cells from the water when mixed with *P. marinus* than susceptible oysters (Ben-Horin and Proestou personal communication).

Mucosal Immunity: An Important Yet Understudied Topic

Mucosal immunity constitutes the next barrier to infection on those tissue surfaces in contact with the external environment, while maintaining tolerance of nonharmful commensal microbes and innocuous substances. Mucosal immunity represents

an important, but understudied, first line of immune defense, extending the defensive role of mucus beyond that of a simple physical barrier (Allam and Pales Espinosa 2016) in all molluscs, as detailed in the section "Molluscan Immunity Begins at the Mucosal Surface, an Immunologically Active Site That Remains Understudied" in Chap. 12. This aspect seems to be of primary importance in bivalves, as their life is tightly linked to aquatic environments. Indeed, bivalves can overcome an experimental pathogen challenge by bath exposure but cannot overcome experimental challenge with smaller amounts of the same pathogen if exposed by injection. Pathogens able to bypass these initial barriers to infection (either by surviving inside phagocytic cells or by directly migrating through epithelial junctions) then trigger a systemic immune response. In general, for both mucosal and systemic immunity, the recognition of nonself (in the form of microbe-associated molecular patterns (MAMPs)) by lectins and other pattern recognition receptors (PRRs) and opsonins in hemolymph (see section "Recognition, Agglutination, and Opsonization"), and by sentinel cells (most probably hemocytes), present in the tissues, triggers signaling transduction cascades and the release of cytokines (see section "Signaling and Regulatory Pathways"), leading to humoral immune responses (see section "Humoral Immune Effectors") and cellular immune responses (see section "Cellular Immune Responses") that vary according to the nature and location of the immune stimuli. A fine regulation of the immune response is achieved through the neuroendocrine immunomodulation (NEI) regulatory network (see section "Connections with the Neuroendocrine System"), a cross talk between the nervous, endocrine, and immune systems that maintains homeostasis and tunes innate immune response in all animals.

In particular, mucosal immune responses include (a) the production of humoral defense factors secreted into the mucus covering the epithelium of tissues in either the pallial or the extrapallial space; (b) chemotaxis and the transepithelial migration of hemocytes into the pallial and extrapallial spaces, followed by phagocytosis and intracellular killing; (c) phagocytosis and intracellular digestion by cells in the digestive epithelium; and, if needed, (d) an encapsulation response in the extrapallial cavity characterized by the secretion of conchiolin and antimicrobial products and activation of the prophenoloxidase cascade (see section "The Phenoloxidase Cascade") (Allam and Raftos 2015; Allam and Pales Espinosa 2016; Zannella et al. 2017). Systemic immune defenses include (a) recognition, opsonization, phagocytosis, and intracellular killing by circulating hemocytes and other, yet to be identified, phagocytic cells within tissues; (b) killing in plasma through secretion of humoral effectors and activation of an ancient complement system and the phenoloxidase system; and, if needed, (c) an encapsulation response that leads to granuloma-like formations, grossly visible as nodules in extreme cases.

Hemocytes: Key Cellular Players in Bivalve Immune Response

Hemocytes are a key component of the bivalve immune system. These cells are present in all cavities of bivalves, circulating in the hemolymph (which bathes all

tissues) and migrating into the pallial and extrapallial spaces. Different types of hemocytes have been described in molluscs on the basis of morphological characteristics (see section "A Short Journey in the 'Immune System' of Cephalopods" for a brief comparative overview between bivalve and cephalopod hemocytes and the section "Hemocytes Play a Central Role in Molluscan Immune Responses: Some Basics Regarding Their Morphology and Origins" in Chap. 12 for a broader discussion), and their roles in both physiological processes (e.g., digestion and shell formation) and immune functions (e.g., phagocytosis, synthesis of immune effectors, and modulation of immune responses) are well known (Cheng 1984; Ordás et al. 2000; Goedken and De Guise 2004; Costa et al. 2009b; Wang et al. 2017c; Ivanina et al. 2017).

The lack of specific cell markers, however, has so far prevented detailed characterization of the functionality and mechanism of action of specific cell populations; thus, recent efforts dedicated to the development of these markers are particularly exciting (Donaghy et al. 2009; Sekine et al. 2016; Allam and Pales Espinosa 2016). Moreover, the location of the hematopoietic organ and the process of hematopoiesis and maturation into distinct hemocyte populations are still controversial topics (Pila et al. 2016; Dyachuk 2016). While the hematopoietic organ in gastropods is the amoebocyte-producing organ (Jeong et al. 1983) and that in cephalopods is the white gland (Cowden and Curtis 1973), a variety of tissues in different species and developmental stages have been proposed as hematopoietic organs in bivalves. These include an irregularly folded structure in the gills (Jemaà et al. 2014) and unspecified locations within the mantle and gills (Song et al. 2016) of adult oysters, the mantle edge of mussel larvae (Balseiro et al. 2013), the connective tissues and gill epithelium of recently settled larvae from the flat oyster *Ostrea edulis* (Xue and Renault 2001), and a ring structure around the dorsal side of the embryo in oyster trochophore larvae (Song et al. 2016).

Expansion and Molecular Diversification: The Bivalve Immune System Is Not as "Simple" as We Thought

Exploration of molluscan genomes has revealed massive expansion and functional divergence of gene families involved in immune recognition and opsonization (detailed in section "Recognition, Agglutination, and Opsonization"), adhesion (syndecan, protocadherin), acute phase responses (hsp70), signal transduction (see section "Signaling and Regulatory Pathways"), cytokine production (see section "Production of Cytokines"), apoptosis (see section "Apoptosis and Autophagy"), or oxidation and antioxidation (cytochrome p450, superoxide dismutase) (Zhang et al. 2012a; Simakov et al. 2013; Albertin et al. 2015; Murgarella et al. 2016; Sun et al. 2017; da Silva et al. 2017; Mun et al. 2017; Du et al. 2017). Many of these immune gene family expansions are lineage (bivalve) specific (Zhang et al. 2015; McDowell et al. 2016). The mechanisms (i.e., gene duplications, rearrangements, polymorphism, etc.) and functional relevance of these gene expansions and divergence are still being studied, but there are indications that gene diversity may be responsible

for a certain level of species specificity in bivalve immune responses (see Chap. 12, section "Expansion and Diversification of Innate Immune Gene Families" for a comparative overview of a few specific cases).

Evidence of "Immunological Memory" in Bivalves

The plasticity of bivalve immune responses is also evidenced by indications that the immune system can be primed, leading to short-term memory. For example, scallops and oysters showed enhanced pathogen-specific phagocytosis upon a secondary challenge and upregulation of expression of genes involved in phagocytosis and hematopoiesis (Zhang et al. 2014d; Wang et al. 2015b; Green et al. 2015; Pinaud et al. 2016; Wang et al. 2017a). Recent experiments have further indicated that experimentally infected juvenile oysters can mount a long-lasting antiviral immune memory, persisting for at least 5 months, which protects them from subsequent viral infections (Lafont et al. 2017). Furthermore, transgenerational immune priming has been demonstrated in bivalves (Green et al. 2016). The specific mechanisms involved in these two types of priming are still unclear, but the switch from cellular to humoral response and epigenetic regulation are believed to play crucial roles. An in-depth discussion of the relevance of this poorly understood phenomenon in molluscs is provided in the section "Immune Priming" in Chap. 12. The role of maternal transfer has been also studied as a part of the innate immune response in molluscan larvae, making transgenerational immune priming possible. Bivalve oocytes possess significant antibacterial, lysozyme, and agglutinating activities against pathogens, and several immune factors have been identified in embryos (Wang et al. 2015b).

How Do Environmental Factors Affect the Bivalve Immune Response?

Bivalves are poikilotherm species living in highly diverse and variable environments. Consequently, immune responses are heavily affected by environmental conditions, such as temperature, salinity, dissolved oxygen, pH, and pollution. Therefore, an extensive body of knowledge has been built about the potential effect of environmental stress and pollution on immune parameters in these organisms and other molluscan groups—in particular, in connection with human activities, as discussed in detail in the section "Challenges for Molluscs in the Anthropocene Epoch" in Chap. 12. For example, exposure of bivalves to environmental toxins of natural origin, like those derived from harmful algal blooms or toxic cyanobacteria, has been shown to affect the phagocytic responses of bivalves, generally leading to immunosuppression (Hégaret et al. 2011; Soudant et al. 2013; Queiroga et al. 2017). Exposure of oyster hemocytes to pollutants such as TBT in vitro and in vivo reduces their production of ROS and phagocytic activity (Soudant et al. 2013), and exposure of bivalve hemocytes in vitro to nanomaterials leads, in general, to decreased

phagocytic activity, increased antioxidant levels, and increased apoptosis, indicating immunotoxicity (Rocha et al. 2015). The effects of environmental stressors on bivalve immunity, however, depend on the evolutionary history of the bivalve species and the history of exposure to different environmental conditions between populations within a species.

Recognition, Agglutination, and Opsonization

The Role of Lectins in Immune Recognition

A critical step of innate immune responses against an infectious challenge is the immediate recognition of the "nonself" carbohydrate moieties on the surface of potential pathogens and parasites, such as viral envelope glycoproteins, bacterial lipopolysaccharides and exopolysaccharides, and various surface glycans on eukaryotic parasites (Boehm 2012). These surface structures encode vast information that is "decoded" by the hosts' carbohydrate-binding proteins (lectins) (Vasta and Ahmed 2008) which, upon binding to the recognized ligand, can immobilize the infectious agents and activate downstream signaling pathways, leading to their uptake and intracellular killing by phagocytic cells. Furthermore, lectin-mediated activation of the complement system can also promote phagocytosis and killing of potential pathogens (Fujita et al. 2004; Vasta et al. 2007) (see section "Evidence of an Ancient Complement System in Bivalves?"). Thus, lectins are critical components of innate immune mechanisms as both recognition and effector factors—functions that are facilitated by the oligomerization of lectin peptide subunits, leading to increased avidity for the multivalent glycan ligands typically found on the microbial surface (Taylor and Drickamer 2003; Vasta et al. 2007). On the basis of the identification of unique amino acid sequence motifs and the structural fold of the carbohydrate recognition domain (CRD), and the requirement of divalent cations or a reducing environment for ligand binding, lectins have been classified into several major families. These include C-type lectins (CTLs), FTLs, RTLs, HTLs, PTLs, XTLs, I-type lectins, pentraxins, galectins (formerly S-type lectins), ficolins, and others (Vasta et al. 2007). Members of several lectin families such as CTLs, RTLs, FTLs, peptidoglycan-binding proteins, ficolins, pentraxins, and galectins have been implicated in immune surveillance and homeostasis (Vasta and Ahmed 2008) (Fig. 7).

Unlike immunoglobulins (Igs) and Ig superfamily members such as DSCAM (Yue et al. 2016) and FREPs (Zhang et al. 2004), which generate recognition diversity by genetic mechanisms, lectins are typically described as "hard wired" in the germline (Vasta et al. 2007). Therefore, given the great diversity of potential infectious agents present in the aquatic or terrestrial environments that molluscs inhabit, how their innate immune systems are able to cope with these infectious challenges is an outstanding question that remains to be fully addressed (Harvell et al. 1999). However, the complexity of the lectin repertoires in organisms that lack the typical Ig-mediated adaptive immunity, such as molluscs, strongly suggests that a wide variety of molecular topologies can be effectively recognized in surface carbohydrate

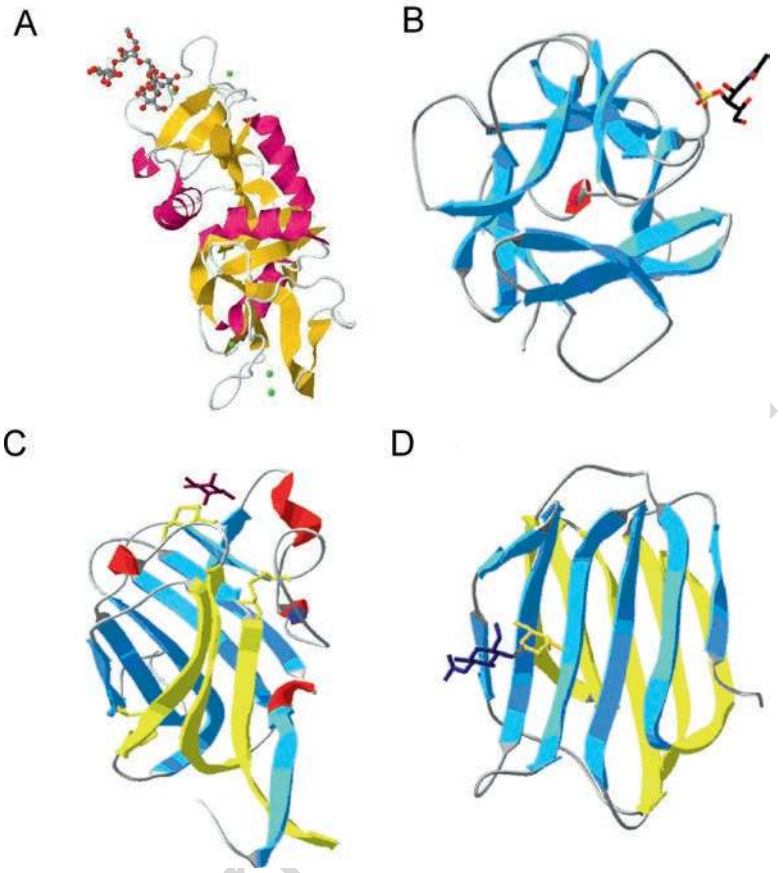


Fig. 7 Typical structural fold of four of the most important lectin families with functions in immune recognition in bivalve molluscs. (**a**) C-type lectin with bound carbohydrate ligand (PDB accession ID: 2MSB). (**b**) R-type lectin with bound 4-sulfated GalNAc (PDB accession ID: 1DQ0). (**c**) F-type lectin with bound fucose (PDB accession ID: 1 K12). (**d**) Galectin with bound LacNAc (PDB accession ID: 1KJL)

moieties common to diverse microbial pathogens, leading to activation of effector mechanisms that can kill and eliminate them for successful innate immune protection (Vasta et al. 2007, 2012a; Vasta and Ahmed 2008). A discussion of the best-characterized lectin families identified in molluscs follows below.

C-Type Lectins

Together with the S-type lectins (currently known as galectins; see below) C-type lectins (CTLs) were the first two families to be rigorously defined by the presence of unique sequence motifs in their CRDs (Drickamer 1988). CTLs are characterized by the CTL-like domain (CTLD) of the unique structural fold and the requirement of Ca^{2+} for ligand binding. The CTLD can be structurally diversified and associated

with a variety of lectin and nonlectin domains constituting "mosaic" or "chimeric" proteins endowed with multiple functional properties (Zelensky and Gready 2005; Pees et al. 2016). In mammals, this highly heterogeneous lectin family is currently subdivided into 17 groups based on their domain organization (Zelensky and Gready 2005; Vasta and Ahmed 2008; Pees et al. 2016). CTLs participate not only in the initial step of pathogen recognition via the CRD but also in various antimicrobial effector functions, including pathogen recognition, opsonization, and activation of the complement cascade (Vasta et al. 2007). In invertebrate taxa, CTLs are also key factors in carbohydrate-mediated recognition of the infectious challenge, but also in effector roles such as immobilization, phagocytosis, clearance, and encapsulation of the infectious agent. Furthermore, they have also been implicated in nodule formation, in the activation of the prophenoloxidase/melanization cascade, and in other functions, including direct antimicrobial activity and regulation of antimicrobial peptide (AMP) expression (Vasta et al. 2007; Vasta and Ahmed 2008; Wang et al. 2014b; Pees et al. 2016; Zhao et al. 2016b). Numerous studies have been conducted in various mollusc species, aimed at investigating the potential role of CTLs in immune defense, and their roles in recognition, agglutination/immobilization, and opsonization of bacterial pathogens have been firmly established (Zheng et al. 2008; Zhu et al. 2008; Jing et al. 2011; Huang et al. 2013a; Zhang et al. 2014b; Mu et al. 2014; Martins et al. 2014; Chovar-Vera et al. 2015; Huang et al. 2015b; Yang et al. 2015). In general, the CTL repertoire in any single species appears to be highly diversified and complex, and the temporospatial expression and localization of CTLs includes hemocytes, plasma, and pallial mucus, as well as organs and tissues relevant to immune responses such as the mantle, gills and gut. Additionally, infectious challenge experiments have revealed that in most cases their expression is modulated by exposure to potential pathogens (Zhu et al. 2008; Mu et al. 2014; Martins et al. 2014; Chovar-Vera et al. 2015). The report that molluscs can express components of the complement system (see section "Evidence of an Ancient Complement System in Bivalves?") (Li et al. 2015a; Wang et al. 2017b) has suggested that CTLs may function not only as pathogen agglutinins and opsonins but also in activating the complement cascade with further antimicrobial activity.

R-Type Lectins

The R-type lectins (RTLs) are lectins characterized by a CRD of unique structure, consisting of three lobes arranged around a threefold axis CRD (β-trefoil), in which each lobe may contain a carbohydrate-binding site (Cummings and Schnaar 2017). This structure is found in RTLs from higher plants as well as in hydrolases from prokaryotes, mammalian glycosyltransferases, and macrophage mannose receptors (Cummings and Schnaar 2017). RTLs with binding preference for α-D-galactose/GalNAc moieties and a very similar amino acid sequence have been isolated from the mussels *Crenomytilus grayanus* (CGL) (Jakób et al. 2015; Chernikov et al. 2017a, b), *Mytilus galloprovincialis* (Mytilectin-1) (Hasan et al. 2016; Terada et al. 2016), *Mytilus trossulus* (MTL) (Chikalovets et al. 2016), and *Mytilus californianus* (García-Maldonado et al. 2017). The RTL known as MytiLec-1 displays the typical β-trefoil structure (Terada et al. 2016), whereas two additional isoforms (MytiLec-2

and -3) identified in the same mussel species contain an additional pore-forming aerolysin-like domain (Hasan et al. 2016; Terada et al. 2016). The structure of CGL was resolved recently and shows a similar β-trefoil structure (Jakób et al. 2015). RTLs from mussels can recognize and agglutinate both Gram-positive and Gram-negative bacteria in a carbohydrate-dependent manner, display bacteriostatic activity, and also show antifungal activity by binding to and inhibiting hyphal growth (Jakób et al. 2015; Hasan et al. 2016; Terada et al. 2016; Chernikov et al. 2017a, b). It is noteworthy that mytilectins and CGL also show immunomodulatory activity for mammalian macrophages, and proapoptotic/antitumoral activity by binding to globotriose [Gb3; Galα(1,4)Galβ(1,4)Glcα1] on the cell surface glycolipids such as globotriaosyl ceramide (Chernikov et al. 2017a, b)—properties that have revealed their promise as effective diagnostic and therapeutic agents and have already led to the computational design of an artificial lectin named Mitsuba-1 (Terada et al. 2017).

F-Type Lectins

F-type lectins (FTLs) are the most recent lectin family to be identified (Odom and Vasta 2006), and they are characterized by a fucose recognition domain (F-type lectin domain; FTLD) that displays a novel β-barrel jellyroll fold ("F-type" fold), and unique carbohydrate- and calcium-binding sequence motifs (Bianchet et al. 2002). FTLs may exhibit single, double, or greater multiples of the FTLD and are widely distributed in nature (Bianchet et al. 2002; Odom and Vasta 2006; Bianchet et al. 2010). Like the CTLs, FTLs may display FTLDs combined with other structurally and functionally distinct domains, yielding lectin subunits of pleiotropic properties even within a single species (Bianchet et al. 2002; Odom and Vasta 2006; Bianchet et al. 2010; Vasta et al. 2012a). Although the F-type fold is distinctive for FTLs, it is not unique to these lectins, as other proteins with various functions also display the FTLD fold (Bianchet et al. 2002). Interestingly, although a phylogenetic analysis of FTLD sequences from viruses to mammals has revealed consistency with the taxonomy of extant species, the surprisingly discontinuous distribution of FTLDs within each taxonomic category suggests not only an extensive structural/functional diversification of FTLs along evolutionary lineages but also that they have been subject to frequent gene duplication, secondary loss, lateral transfer, and functional co-option (Bianchet et al. 2002; Bishnoi et al. 2015).

In addition, FTLs are unique in the extraordinary sequence variability (isoforms) that can be expressed in a single individual as a result of genetic mechanisms of diversification in ligand recognition, characterized in detail in the so-called bindins, proteins involved in gamete recognition in the Pacific oyster, *M. gigas* (Springer et al. 2008; Moy et al. 2008; Moy and Vacquier 2008). In addition to their roles in gamete recognition, oyster FTLs also mediate microbial recognition in innate immune responses. FTLs can display single or tandemly arrayed CRDs of distinct specificity in a single subunit (Odom and Vasta 2006; Bianchet et al. 2010), and can potentially cross-link the recognized pathogens to the endogenous glycans on the surface of the host's phagocytic cells (Odom and Vasta 2006). In this regard, the expression of CvFBL4 in *C. virginica* hemocytes is dramatically upregulated upon LPS challenge, suggesting that FTLs may function in pathogen recognition in the

oyster's innate immune response (Saito and Vasta unpublished data). Moreover, PmF-lectin from the pearl oyster (*Pinctada fucata martensii*) is an FTL highly expressed in the hemocytes and gill that is significantly upregulated by experimental challenge with *Vibrio* sp. (Wang et al. 2011a). The identification of FTLs in both the shell matrix and mantle tissue proteins of the blunt-gaper clam, *Mya truncata*, has led to the proposal that during the shell biomineralization process, FTLs secreted by the mantle may carry out immune defense functions and are later incorporated into the shell matrix (Arivalagan et al. 2016). It is noteworthy that the highly diversified FTL repertoire found in the common periwinkle (*Littorina littorea*), a gastropod, has been rationalized as an immune defense system (Gorbushin and Borisova 2015). However, in contrast to other expanded lectin and lectin-like gene families, this connection has not been hypothesized yet in bivalves.

H-Type Lectins

H-type lectins (HTLs) are lectins initially identified in gastropods such as the Roman snail *Helix pomatia* as abundant proteins in the albumin gland secretion that coats the fertilized oocytes before the eggs are laid underground (Uhlenbruck and Prokop 1966). This unique localization as perivitelline active factors, their presence in the snail's hemolymph, and their strong binding to several streptococci strains and other potentially pathogenic bacteria led to the proposal that their role was to protect the snail eggs and adults from infection, as part of the innate immune defense (Uhlenbruck and Prokop 1966). Their shared specificity for N-acetylgalactosamine (GalNAc) and the human blood group A led to their use as typing reagents (Uhlenbruck and Prokop 1966). Recent structural studies revealed that HTLs are characterized by hexameric organization of peptide subunits that display a β-sandwich fold. Although other snail species from the genus *Helix* and the garden snail *Cepaea hortensis* also produce similar lectins (Sanchez et al. 2006), to date, no functional information has been collected yet about HTLs in bivalves, other than the fact that they do not represent an expanded gene family (Gerdol 2017).

Galectins

Galectins are β-galactosyl-binding lectins that require a reducing environment for binding activity but, unlike CTLs and some FTLs, do not require Ca^{2+} (Vasta and Ahmed 2008; Vasta et al. 2012b). Although galectins are structurally conserved and taxonomically widely distributed, they display a remarkable functional diversity by participating in developmental processes, cell adhesion and motility, regulation of immune homeostasis, and recognition of glycans on the surfaces of viruses, bacteria, and protozoan parasites (Vasta 2009). On the basis of their primary structure and subunit organization, mammalian galectins are classified as "proto," "chimera," and "tandem-repeat" types (Vasta and Ahmed 2008; Vasta 2009; Vasta et al. 2012b). Prototype galectins contain one CRD per subunit and are usually homodimers of noncovalently linked subunits. The chimera-type galectins have a single C-terminal CRD, like the prototype, and a non-CRD N-terminal domain that mediates the formation of trimers and pentamers. In contrast, the tandem-repeat galectins, in which two CRDs are joined by a linker peptide, are monomeric.

Molluscan galectins are less diversified that those in mammals but also show different domain organizations, carbohydrate specificity for blood group oligosaccharides, and upregulation of expression by infectious challenge, a feature that supports their proposed role in innate immune responses (Tasumi and Vasta 2007; Feng et al. 2013, 2015; Kurz et al. 2013; Vasta et al. 2015). In contrast to vertebrates, the identification and characterization of galectins in aquatic molluscs has been relatively recent, with most of the studies being aimed at the identification of their transcripts or proteins in diverse tissues and cell types, including hemocytes, and the assessment of their expression upon environmental or infectious challenge (Yamaura et al. 2008; Yoshino et al. 2008; Song et al. 2010, 2011; Zhang et al. 2011a; Bao et al. 2013; Dheilly et al. 2015; Bai et al. 2016). In the eastern oyster, *C. virginica*, however, the galectins CvGal1 and CvGal2 have been characterized in their detailed molecular, structural, and functional aspects (Tasumi and Vasta 2007; Feng et al. 2013, 2015; Kurz et al. 2013). As a result, unique features of the galectin repertoire of aquatic molluscs have become apparent, such as their domain organizations, as well as structural and functional aspects (Vasta et al. 2015). CvGal1 and CvGal2 carry four canonical galectin CRDs (Tasumi and Vasta 2007; Feng et al. 2013, 2015), a domain organization that does not conform to any of the galectin types described in vertebrates (Vasta and Ahmed 2008; Vasta et al. 2012b). Since then, galectins have been identified in an increasing number of aquatic mollusc species, including both bivalves and gastropods, and can be classified, in the vast majority of cases, into the 2-CRD and 4-CRD types (Vasta et al. 2015). As revealed by a phylogenetic analysis, these galectin types are ancient, as they were already present in the most recent common ancestor of both bivalves and gastropods (Vasta et al. 2015). From the functional standpoint, CvGal1 can recognize microbial pathogens and parasites and promote their phagocytosis, but it can also selectively bind to phytoplankton components, suggesting its participation in uptake of microalgae (Tasumi and Vasta 2007). Furthermore, recent studies suggest that the protozoan parasite *P. marinus* has adapted to subvert the oyster's innate immune/feeding recognition mechanisms to gain entry into the host cells by being preferentially recognized by CvGal1 and CvGal2 over algal food or bacterial pathogens (Tasumi and Vasta 2007; Feng et al. 2013, 2015; Kurz et al. 2013; Vasta et al. 2015).

Fibrinogen-Related Domain–Containing Proteins

A class of proteins containing a C-terminal fibrinogen-related domain (FReD), and similar to vertebrate ficolins, has gained a significant amount of attention in molluscs. Because of their important role in the resistance of the snail *B. glabrata* to trematode infection, together with their somatic sequence diversification (Adema et al. 1997; Adema 2015; Gordy et al. 2015), a subclass of FReD-containing proteins (which also contain one or two immunoglobulin-like domains), named fibrinogen-related proteins (FREPs), have been studied as one of the first examples in support of immune memory in invertebrates (Milutinović and Kurtz 2016). Unlike fibrinogen chains, these lectin-like molecules are primarily involved in

immune recognition and are not linked to coagulation (Hanington and Zhang 2011). While these immune properties have been extensively documented in snails since the 1990s (as reported in detail in the section "Expansion and Diversification of Innate Immune Gene Families" in Chap. 12), the first studies of FReD-containing proteins in bivalve molluscs are quite recent.

The first indications pointing toward an involvement of bivalve FReD-containing proteins in immune recognition came from the upregulation of AiFREP in the scallop *Argopecten irradians* in response to *V. anguillarum* but not to *Micrococcus luteus* infections. The recombinant protein could agglutinate Gram-negative and Gram-positive bacterial cells, confirming AiFREP as a reasonable soluble PRR candidate (Zhang et al. 2009b). Years later, AiFREP-2 was functionally characterized in the same species, confirming and to some extent even extending the marked recognition properties of these two scallop proteins (Yang et al. 2014). Very similar results were obtained in *Magallana hongkongensis*, where the recombinant protein ChFCN could selectively bind different bacterial species, agglutinate *Escherichia coli* cells, and enhance hemocyte phagocytosis in vitro (Xiang et al. 2014b). Purified *M. galloprovincialis* transcripts encoding FReD-containing proteins were upregulated in mussels by multiple challenges and could similarly improve the phagocytic rate of hemocytes (Romero et al. 2011). Indirect indications supporting the immune involvement of FReD-containing proteins have been also collected from transcriptomic studies in QPX-infected *M. mercenaria* (Wang et al. 2016b) and *V. splendidus*–infected *Mytilus edulis* hemocytes (Tanguy et al. 2013).

Early sequence database mining approaches revealed that FReD-containing proteins are part of a large multigene family in *Mytilus* spp. (Gorbushin and Iakovleva 2011), and it is now well recognized that the genome of several bivalve species encodes more than 100 such genes, which are, for the most part, expressed in the hemocytes, gills, and digestive gland (Zhang et al. 2015; Huang et al. 2015a; Gerdol and Venier 2015). Bivalve FReD-containing proteins are characterized by a simpler domain organization than snail FREPs, as they lack N-terminal immunoglobulin domains, which are thought to play a fundamental role in somatic mutation (Gerdol 2017). Comparative genomics analyses have further revealed that the Ig–FReD domain combination is exclusively found in heterobranch gastropods (Gorbushin et al. 2010). In most cases, bivalve proteins contain a single FReD associated with a coiled coil region, which probably allows oligomerization (Skazina and Gorbushin 2016). In addition, while the process of somatic mutation in snail FREPs is supported by experimental evidence, no data have been provided yet to sustain a similar mechanism in bivalve FReD-containing proteins, which are however characterized by a relevant sequence diversity. This topic has been investigated in detail in *M. gigas*, where the occurrence of polymorphisms in five of these transcripts was originally attributed to allelic recombination or somatic diversification (Zhang et al. 2012b). However, the large number of FReD genes in bivalves suggest that some of these variants might be the result of recent duplications or interindividual sequence variability, mirroring the evolutionary patterns observed for C1q domain–containing (C1qDC) proteins and other expanded PRR families (Huang et al. 2015a).

The remarkable immune properties of FReD-containing proteins, together with their remote sequence similarity with vertebrate ficolins, suggest that these secreted

PRRs are somehow involved in the lectin pathway of the bivalve complement system (see section "Evidence of an Ancient Complement System in Bivalves?") (Gerdol and Venier 2015; Wang et al. 2017b). However, definitive proof in support of this hypothesis remain to be collected, in particular for what concerns the identification of mannose-binding protein-associated serine proteases (MASPs)—essential mediators of the complement system, which have not been identified yet in molluscs.

C1q Domain–Containing Proteins

Some Insights into the Massive Gene Family Expansion of C1q Domain–Containing Proteins

Although the outstanding binding potential of the C1q domain allows high functional versatility in the recognition of different ligands, no metazoan taxa seem to have exploited these properties to the same extent as bivalve molluscs. The genomes of these animals encode several hundred secreted proteins containing this conserved domain at their C-terminal end, collectively known as C1q domain–containing (C1qDC) proteins. The immune properties of the C1q domain, whose structural fold is exemplified in Fig. 8, have been well documented from the study of the vertebrate

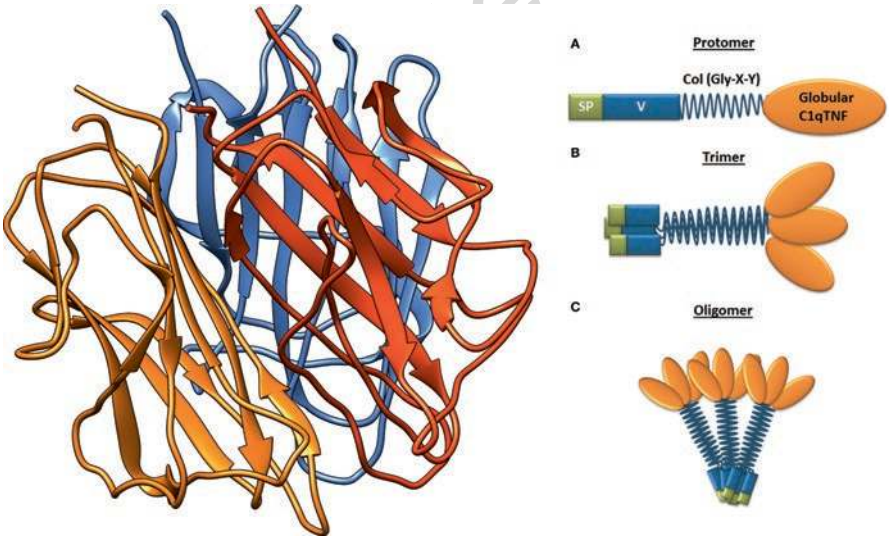


Fig. 8 Left: Three-dimensional structure of the three chains of the human C1q globular head (PDB accession ID: 2WNU; C1qa, C1qb, and C1qc chains are colored in orange, red, and blue, respectively). Right: Prototypical organization of vertebrate C1qDC proteins: **a** single protomer, comprising a signal peptide (SP), followed by a variable region (V, which might be absent in bivalve molluscs), a collagen region (usually replaced by a coiled coil domain in bivalve molluscs), and the globular C-terminal C1q domain. Promomers can assemble into trimers (**b**) and form higher-order bouquet-like structures (**c**). (Source: Thanasupawat et al. 2015)

complement system, where it is the major structural unit in the three chains of the C1q complex. However, the first indications pointing toward a similar role in molluscs only surfaced in 2004, with the isolation of a sialic acid–binding lectin from the garden snail *Cepaea hortensis* (Gerlach et al. 2004).

In bivalves, C1qDC proteins were first tentatively linked to pathogen recognition because of their high sequence diversity, exemplified by the identification of 168 different transcripts in *M. galloprovincialis* which, for the most part, strikingly displayed hemocyte specificity (Gestal et al. 2010; Gerdol et al. 2011), and the presence of over 300 genes in the Pacific oyster genome (Gerdol et al. 2015b). While most vertebrate C1qDC proteins, including those involved in the complement system, contain a central collagen region required for oligomerization (Fig. 8), about half of the oyster C1qDC proteins contain a coiled coil region, possibly exerting a function homologous to that of collagen. A relevant number of the other members of this gene family, however, lack oligomerization motifs and contain only an N-terminal signal for secretion followed by a globular head C1q domain, identifying the sgC1q subfamily. Surprisingly, just a few gene products have shown an association with additional domains; among these, the most notable example is provided by proteins containing multiple consecutive C1q domains (Gerdol et al. 2015b).

Another interesting finding was that such a massive expansion and diversification event occurred in Pteriomorphia and Heterodonta but not in the two other major subclasses, Palaeoheterodonta and Protobranchia, which possess only a few C1qDC genes, like most other protostomes (including nonbivalve molluscs). This lineage-restricted expansion event might have had important biological implications in mussels, clams, oysters, and scallops, providing these marine organisms with an unparalleled array of recognition molecules to be potentially used in microbe-associated molecular pattern (MAMP) recognition (Gerdol et al. 2015b). Another key piece in the puzzle of the evolution of bivalve C1qDC proteins was provided by the genome of the Manila clam, *Ruditapes philippinarum*. Indeed, most of the sequences 1589 C1qDC genes found in this clam appear to be unrelated to those found in oyster, thereby suggesting that the astounding molecular diversity in the two species derives from independent evolution (Mun et al. 2017).

Functional Studies Are Progressively Revealing the Immune Functions of C1q Domain–Containing Proteins

Genomic investigations are, however, insufficient in the absence of a functional characterization to link this expansion event to improved immune functions. Confirmations, in this sense, have been provided by different experimental approaches, i.e., gene expression studies that have evidenced the upregulation of oyster C1qDC transcripts in response to Rickettsia-like organisms and revealed their implication in the response to Brown Ring Disease, *P. olseni,* and QPX infections in clams (Xu et al. 2012; Leite et al. 2013; Allam et al. 2014; Wang et al. 2016b). Experimental challenges have further demonstrated that many bivalve C1qDC genes are induced by infection with various Gram-positive and

Gram-negative bacteria, as well as by fungi (Kong et al. 2010; Gestal et al. 2010; Li et al. 2011a; Gerdol et al. 2011; Jiang et al. 2015), but also by direct stimulation with LPS, PGN, β-glucan, and polyI:C (Wang et al. 2012a, b, 2015a; Yang et al. 2012), altogether reinforcing their role as PRRs. The indications collected from gene expression studies were later confirmed by the binding properties of C1qDC recombinant proteins toward LPS, PGN, polyI:C, mannan, β-1,3-glucan, and yeast glucan (Wang et al. 2012a, 2015a; Jiang et al. 2015) as well as toward live bacteria (Wang et al. 2015a; Zhao et al. 2016a; Huang et al. 2016).

From a functional point of view, an oyster recombinant C1qDC protein was capable of significantly inhibiting the growth of Gram-positive and Gram-negative bacteria (He et al. 2011), and others displayed strong agglutinating activity toward Gram-positive bacteria, Gram−negative bacteria, and fungi, with a certain degree of selectivity (Kong et al. 2010; Wang et al. 2012a). Some studies have tried to better elucidate the mode of action of bivalve C1qDC proteins and their connection with other molecular components of the immune system. For example, the bactericidal properties of mussel hemolymph appear to be mediated by a C1qDC serum opsonin that binds bacterial D-mannose, promoting the phagocytic action of hemocytes (Pezzati et al. 2015). Similarly, a protein isolated from the scallop *Azumapecten farreri* is capable of enhancing the phagocytosis of invading *E. coli* cells (Wang et al. 2012b), and an oyster LPS-binding C1qDC protein could sensibly boost this activity toward *E. coli* and *V. splendidus* (Jiang et al. 2015). Furthermore, other recombinant proteins are able to interact with heat-aggregated human IgGs and IgMs (Wang et al. 2015a), providing novel and stimulating insights into the possible involvement of these components in the activation of the prototypical complement system of bivalve molluscs (see section "Evidence of an Ancient Complement System in Bivalves?").

Although bivalve C1qDC proteins were initially considered as hemocyte-specific products, it is now clear that they are broadly expressed in all main tissues, with a particular prevalence in the gills or in the digestive gland (Gerdol et al. 2015b), leaving some open questions concerning their involvement in functions other than immune recognition. In fact, the extreme diversification and binding properties of these proteins would allow, in line of principle, additional physiological functions, which are progressively starting to emerge.

Evidence of an Ancient Complement System in Bivalves?

A Brief Description of the Complement System

Despite the highly divergent evolutionary strategies adopted by metazoans to develop an efficient immune system in highly diverse life environments, complex molecular machinery of the utmost importance in pathogen recognition and clearance is surprisingly conserved in nearly all animals. This protein complex, able to enhance recognition and removal of microbial cells by recruiting the main players of the vertebrate immune system (phagocytic cells and immunoglobulins), has been named the "complement" system.

The complement system can be potentially activated by different biochemical pathways, which involve components of both innate and adaptive immunity, and has thereby been defined as a functional link between these two major branches of the immune system (Dunkelberger and Song 2009). In vertebrates, the different routes that can lead to complement activation involve either the binding of C1q to antigen-complexed M or G immunoglobulins (the classical pathway), the recognition of MAMPs by mannan-binding lectins (MBLs) and ficolins (the lectin pathway), or the direct recognition of MAMPs by C3b following spontaneous C3 hydrolysis (the alternative pathway) (Fig. 9). Overall, complement activation triggers, through a proteolytic cascade, the opsonization of invading microbes, their lysis by the action of the membrane attack complex (MAC), and the recruitment of phagocytic cells for their final elimination.

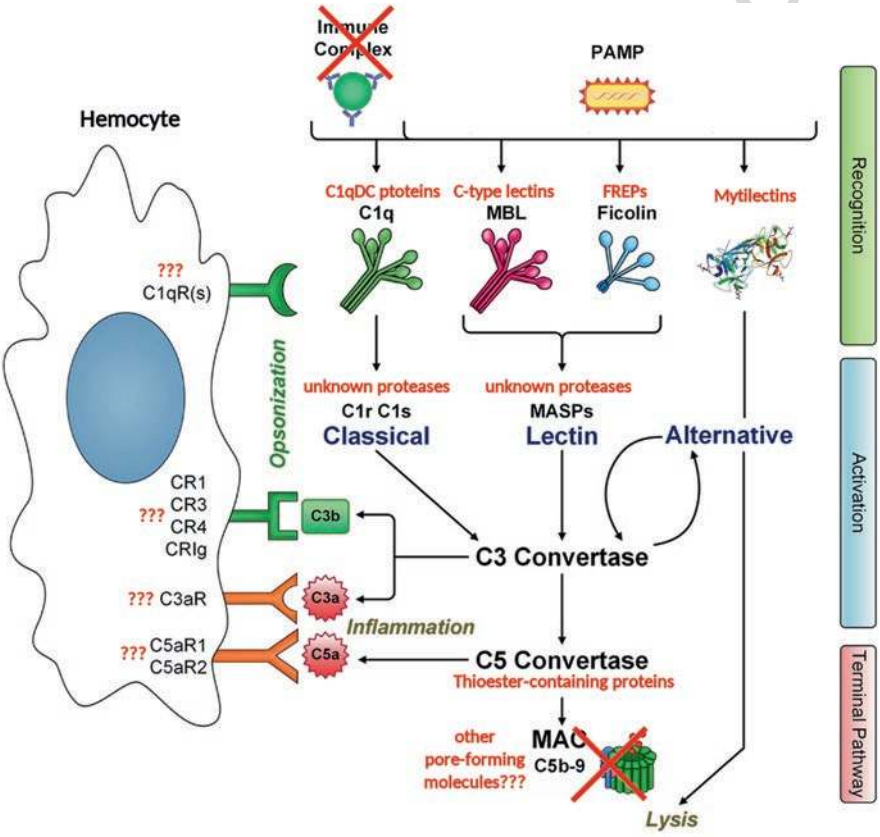


Fig. 9 Overview of the complement system in bivalves and comparison with vertebrates. The vertebrate molecular players are shown in black and the bivalve homologous components are indicated in red, whenever needed. Components that are absent in bivalves (namely, the membrane attack complex and antigen-complexed immunoglobulins) are struck through. (Edited from Bohlson et al. (2014))

The Conserved "Core Components": C2 and C3

With the exception of Ecdysozoa, the near universal conservation of two core molecular components of the complement system—C3 and C2/factor B—suggest that a prototypical complement system was present in the common ancestor of all metazoans (Smith et al. 1999; Pinto et al. 2007). Accordingly, genes encoding these two highly conserved elements are also readily identifiable in most bivalve genomes and transcriptomes (Moreira et al. 2012a; Zhang et al. 2014c; Gerdol and Venier 2015). Their first formal description was provided in the grooved carpet shell, *Ruditapes decussatus* (Prado-Alvarez et al. 2009). The C3 component of the razor clam *Sinonovacula constricta* was strongly upregulated in hemocytes and digestive gland upon bacterial challenges. In addition, the serum of *S. constricta* was activated by LPS and bacteria, confirming that the function of the bivalve protein was highly homologous to vertebrates (Peng et al. 2016). Further confirmation was recently provided by the use of polyclonal antibodies directed toward three distinct fragments of the Pacific oyster C3 protein, homologous to the α, β, and γ chains obtained in vertebrates from the proteolytic cleavage of the C3 precursor. The observation of a single band recognizable in serum under non-reducing conditions, as opposed to the presence of three distinct bands of 110, 60, and 30 KDa under reducing conditions, pointed out that bivalve C3 molecules are processed by serum proteases in a similar fashion to what happens in animals with a canonical complement system (Wang et al. 2017b).

The bivalve complement system might also involve thioester-containing proteins (TEPs), accessory complement proteins that share a high degree of similarity with C3/C4/C5 and promote opsonization of invading microbes and their elimination by phagocytosis in other invertebrates (Blandin et al. 2008; Bou Aoun et al. 2010). TEPs have been functionally characterized only in the scallop *A. farreri*, where they possess a highly variable central region produced by the alternative splicing of six mutually exclusive exons. This sequence variation appears to cover a key role in the specificity of the immune response to be triggered, as the amount of the isoforms produced largely varies on the basis of the type of challenge and the sex of the specimens (Zhang et al. 2009c). A very recent study went into the subject in depth, evidencing that like C3, scallop CfTEP undergoes fragmentation due to the action of endogenous serum proteases (Xue et al. 2017b).

Present Uncertainties and Future Directions

The absence of immunoglobulins rules out the existence of the classical pathway of the complement system in animals lacking an adaptive immune system, which include bivalve molluscs. At the same time, the remote homology between vertebrate C1q, ficolins, and MBLs, and similar sequences in invertebrate organisms, further complicates the interpretation of the functional overlap between the lectin pathway of the complement system between vertebrates and invertebrates. However, the high diversification of C1qDC proteins might potentially provide a very broad potential of recognition toward MAMPs, even in absence of immunoglobulins. At the same time, while no bona fide sequence that is homologous to vertebrate MBLs or ficolins is present in molluscs, both C-type lectins and FReD-containing proteins (see sections "The Role of Lectins in Immune Recognition" and "Fibrinogen-Related

Domain (FReD)-Containing Proteins") underwent massive expansion and diversification events similar to C1qDC proteins. This further reinforces the idea that bivalves possess an astoundingly complex arsenal of soluble PRRs, which are possibly part of a complement lectin pathway. However, it is presently unclear how their recognition signals would converge to C3, as no clear homologs to MASP-1, MASP-2, C1r, and C1s serine protease, required for downstream activation of C3 in vertebrates, are present in bivalves (Gerdol and Venier 2015).

Altogether, these reports support the existence of a prototypical complement system in bivalve molluscs, therefore expanding the taxonomic distribution of this ancient immune defense system to Lophotrochozoa, in addition to echinoderms, horseshoe crabs, tunicates, and amphioxus. However, many uncertainties remain about the modes of activation of this system, and some of the hypothetical molecular players that are expected to be involved still remain to be identified. The mechanism of regulation of the complement system in oysters in response to LPS has been hypothesized in a recent study. The authors suggested that 12 serine protease domain–containing proteins might somehow play a key role in complement activation, and they further identified some possible C3 receptors containing integrin α/β domains and similar to ascidian C3 receptors (Wang et al. 2017b).

Finally, it is presently difficult to assess whether the final outcome of this process is simply the opsonization of pathogenic cells, which would facilitate their elimination by the recruitment of phagocytic cells, or whether it also involves lytic components functionally homologous to the membrane attack complex. As will be discussed in section "Lysozymes, BPIs and Other Pore-Forming Molecules," while the constituents of the terminal pathway of the complement system appear to have been specifically developed in the vertebrate lineage, it is possible that other divergent pore-forming molecules function in a similar manner, sometimes combining MAMP-sensing and pore-forming properties within the same protein precursor.

Toll-Like Receptors

Structure and Function of Toll-Like Receptors

Toll-like receptors (TLRs) are metazoan immune receptors, which have found major evolutionary success. Because of their ability to recognize a broad range of ligands, TLRs are important players of the innate immune system of both vertebrate and invertebrate animals, functioning as MAMP sensors either on the plasma membrane or in endosomal compartments. The recognition properties of TLRs are provided by several extracellular leucine-rich repeats (LRRs), which can be organized either in a single cysteine cluster (scc) or in a multiple cysteine cluster (mcc) configuration, whereas the transduction of the immune signal occurs thanks to an intracellular TIR (Toll–interleukin receptor) domain (Fig. 10). This conserved signaling module is separated from the extracellular LRRs by a short transmembrane α-helical domain, which anchors TLRs to cell membranes.

The prototypical Toll protein of the fruit fly *Drosophila melanogaster*, after which all TLRs are named, is a multifunctional protein, acting both as a primary

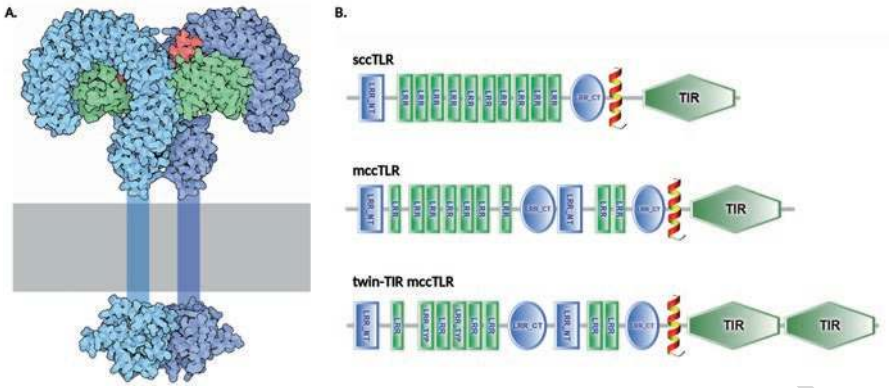


Fig. 10 (**a**) Structure of the human Toll-like receptor 4 dimer (blue) bound to bacterial lipopolysaccharide (red) through its extracellular LRR domains. The transmembrane region is shown schematically. (Image courtesy of RCSB PDB, http://pdb101.rcsb.org/motm/143). The intracellular TIR domain is shown on the inner side of the cell membrane. (**b**) Schematic domain organization of single cysteine cluster (scc), multiple cysteine cluster (mcc), and twin-TIR mcc Toll-like receptors found in bivalve molluscs

determinant of embryonic dorsal–ventral polarity and as the receptor for the proinflammatory cytokine Spätzle. However, most of the TLRs described so far in vertebrates function exclusively as immune receptors by directly recognizing LPS, PGN, foreign nucleic acids, and other MAMPs without the mediation of cytokine-like molecules. While the organization of TLRs has long been considered to be similar to that of *Drosophila*, genomic studies have progressively unearthed some important peculiarities that strikingly differentiate arthropods from all other animals. In particular, echinoderms have developed an arsenal of immune receptors that are potentially capable of recognizing a very broad range of invading microorganisms (Buckley and Rast 2012).

The Emerging Role of Toll-Like Receptors in Bivalve Molluscs

Besides echinoderms, the massive expansion of the TLR repertoire by gene duplication involved other phyla, including molluscs (Gerdol et al. 2017), as most notably evidenced by the identification of 83 TLR genes in the genome of the Pacific oyster (Zhang et al. 2015). However, the genomic expansion of the bivalve TLR gene family occurred independently from that of sea urchins, as it mostly targeted a group of phylogenetically distinct genes. Because of the high molecular diversification of bivalve TLR sequences, a novel uniform nomenclature has been recently suggested to avoid confusion in the discussion of the functional properties of these receptors (Zhang et al. 2015; Gerdol et al. 2017). Thus, it has been suggested that bivalve receptors should be categorized as P-type, sPP-type, or twin-type (in the case of mccTLRs), or as V-type or sP-type (in the case of sccTLRs) (Fig. 10). V-type TLRs, present in hundreds of members in the sea urchin genome, include only a few sequences in bivalve molluscs, where most TLRs are ascribable to the sP-type expanded group (Gerdol et al. 2017).

CfToll-1 was the first TLR to ever be described in bivalve molluscs, providing the first pieces of evidence in support of the possible involvement of these receptors in bivalve immune recognition. Indeed this TLR, identified in the scallop *A. farreri* and pertaining to the P-type subfamily, is mildly upregulated by LPS challenges, pointing out a role in the detection of Gram-negative bacteria (Qiu et al. 2007). Following this initial report, several gene expression studies have implicated TLRs in the immune response to different types of microbes and associated pathologies. For example, a single TLR was strongly modulated in QPX-infected *M. mercenaria* (Perrigault et al. 2009) and in *P. marinus*–infected *C. virginica* oysters (Tanguy et al. 2004). Finally, TLRs have been also reported to be upregulated in response to *V. alginolyticus* challenges in different marine clam and mussel species (Moreira et al. 2012b; Martins et al. 2014).

These observations encouraged the design of targeted functional experiments aimed at identifying the microorganisms recognized by bivalve TLRs and their possible ligands. The most significant studies have been carried out in (1) *M. gigas*, where a TLR was found to be strongly induced by *V. anguillarum* challenges (Zhang et al. 2011c) and a second one (CgTLR6) displayed binding ability toward Gram-positive and Gram-negative bacteria, further revealing affinity to LPS and PGN but not to mannan (Wang et al. 2016b); (2) *Hyriopsis cumingii*, where three different TLRs, responsive to distinct microbial challenges, have been identified, pointing out a remarkable functional specialization (Ren et al. 2013, 2014; Zhang et al. 2017); (3) the noble scallop, *Mimachlamys nobilis*, where an sccTLR responded to *V. parahaemolyticus*, LPS, and PolyI:C challenges in hemocytes (Lu et al. 2016); and (4) *M. galloprovincialis*, where the upregulation of the P-type TLR MgTLR-i could be observed in response to *Vibrio* spp. and *M. luteus* but not to *Fusarium oxysporum* injection (Toubiana et al. 2013). The high selectivity of TLRs, in terms of both transcriptional responsiveness and binding potential, has been further confirmed by the transcriptional analysis of the entire complement of oyster TLR genes, which often responded to just a single pathogenic challenge in a highly specific manner (Zhang et al. 2015).

One of the most praiseworthy studies aimed at clarifying the placement of these receptors in the molecular networks of immune signaling targeted four different sccTLRs in *M. gigas* and permitted demonstration of their participation in the activation of nuclear factor kappa B (NF-κB). The finding that oyster sccTLRs are localized both on the plasma membrane and in late endosomal vesicles was equally important, as it revealed a possible role of TLRs also in the modulation of immune response upon phagocytosis of invading microbes (Zhang et al. 2013a). Although only little effort has so far been put into the identification of the effector molecules whose production is controlled by TLRs, preliminary results clearly point toward a key role of TLR signaling in the regulation of AMP and lysozyme production through a MyD88-dependent pathway (see section "Canonical TLR Signaling").

The experimental data collected so far confirm that the fundamental role of TLRs in the bivalve immune response to invading microorganism appears to be supported by overwhelming evidence. However, one might wonder whether this large family of receptors has acquired additional physiological roles due to neofunctionalization,

as has been suggested for other bivalve recognition protein families. While evidence in support of this hypothesis still remains scarce, some reports hint that TLRs might be modulated by other stimuli, i.e., biotoxins (Detree et al. 2016b), abiotic stress (Zhang et al. 2015), and variations of pH (Xing et al. 2017).

Other Membrane-Bound Immune Receptors

Peptidoglycan Recognition Proteins

Peptidoglycan recognition proteins (PGRPs) are a class of well-characterized PGN-binding molecules that, in the fruit fly *D. melanogaster*, comprises both membrane-bound and secreted members. Membrane-bound PGRPs are directly involved in MAMP recognition during infections by Gram-negative bacteria and activate the Immune deficiency (IMD) signaling cascade (Royet and Dziarski 2007). On the other hand, secreted PGRPs cooperate with Gram-negative Binding Proteins (GNBPs) in the extracellular environment, triggering the prophenoloxidase cascade, which leads to the activation of Toll signaling (see section "Canonical TLR Signaling") and melanization (see section "The Phenoloxidase Cascade"). While PGRPs are also present in vertebrates, they are not anchored to the plasma membrane and they mostly exert bactericidal/bacteriostatic activity in the extracellular environment (Montaño et al. 2011).

PGRPs have been functionally characterized in detail in arthropods and vertebrates, but nearly no information is available for the other major animal phyla. In bivalve molluscs, genome and transcriptome screenings show the presence of both membrane-bound and secreted PGRPs, even though large margins of uncertainty remain about their functional overlap with arthropods and vertebrates. First, there is no evidence in support of an extracellular pathway homologous to that of the *Drosophila* prophenoloxydase proteolytic cascade, and the absence of Spätzle-like proteins make it highly doubtful that secreted PGRPs participate in TLR activation in bivalves (see section "The Phenoloxidase Cascade"). Second, the high sequence divergence between bivalve PGRPs and those from other organisms does not allow similarity-based functional inference (Gerdol and Venier 2015).

The first report of PGRPs in bivalve molluscs, in the form of a short secreted protein, dates back to 2007, when an inducible gene product was identified in the scallop *A. farreri* following Gram-positive and Gram-negative bacterial challenges (Su et al. 2007). This finding was later confirmed in *M. galloprovincialis*, *Bathymodiolus azoricus* (Martins et al. 2014), and *H. cumingii*, where broad-spectrum antibacterial activity and lytic activity toward both Lys-PGN and DAP-PGN were demonstrated (Yang et al. 2013c). Furthermore, another study reported the modulation of the expression of two secreted short PGRPs in *Solen grandis*, in particular, in response to PGN but not LPS (Wei et al. 2012), confirming previous results concerning PGN specificity obtained in the bay scallop (Ni et al. 2007). Finally, another secreted PGRP molecule from *M. gigas* displays a unique domain architecture, as it combines the PGN-binding domain with a G-type lysozyme domain, which could potentially enable the coexistence of bacterial recognition and

killing properties in the same molecule (Itoh and Takahashi 2009) (see section "Lysozymes, BPIs and Other Pore-Forming Molecules"). Overall, the vast majority of the studies that have targeted bivalve secreted PGRPs so far are seemingly concordant in attributing to them functional properties more similar to those of vertebrate PGRPs than to those of arthropods. Their cooperation with GNBPs and their involvement in the activation of TLRs seem unlikely at this point.

Interestingly, while no membrane-bound PGRP has been functionally characterized yet in bivalves, at least two proteins of this type are present in the Mediterranean mussel transcriptome. Together with the contemporary identification of some conserved intracellular mediators, this prompted researchers to hypothesize the possible existence of an IMD-like pathway (see section "Other Immune Signaling Pathways") (Gerdol and Venier 2015). While this hypothesis still awaits experimental confirmation, a recent study carried out in *B. azoricus* identified five paralogous PGRP genes, which were connected to the regulation of bacterial endosymbiosis in gills (Détrée et al. 2017).

Recently Discovered Receptors

Besides TLRs and PGRPs, only a very few other cases of PRRs anchored to the extracellular surface of bivalve immune cells have been studied so far. The most relevant are the Nimrod-like receptor (CgNimC) and LRR and Ig domain–containing proteins (LRRIGs), both identified in *M. gigas*. The former receptor has been implicated in the recognition of Gram-negative bacteria because of its relevant upregulation in response to *Vibrio* spp. challenges and LPS binding. Further functional assays established that CgNimC plays a fundamental role in regulating the phagocytic rate of hemocytes toward invasive Gram-negative bacteria (Wang et al. 2015d). On the other hand, the two LRRIgs genes identified in the genome of *M. gigas* encode large proteins bearing extracellular LRRs (like TLRs), coupled with an immunoglobulin-like domain, a transmembrane domain, and a short uncharacterized cytosolic C-terminal domain. Immunoglobulin-like domains are abundant in bivalve genomes, and their marked immunological properties have been well defined in vertebrates and, partly, also in invertebrates (e.g., gastropod FREPs; see Chap. 12, section "Defense-Associated Humoral Components"). LRRIGs can bind a broad range of MAMPs and are upregulated in hemocytes in response to various types of challenges. Furthermore, they can modulate the expression of cytokine-like factors (i.e., TNF and IL-17) and promote hemocytic phagocytosis of *Vibrio* cells, thereby reinforcing their position as key regulators of immune response in oysters (Wang et al. 2017a; Huang et al. 2018).

Cytosolic Pattern Recognition Receptors

In comparison with the impressive amount of literature produced about soluble and membrane-bound PRRs, it is perhaps surprising that only a handful of studies have so far taken into account the possible involvement of cytosolic receptors in the immune system of bivalves. Most of the molecular players described below have

been identified just at the sequence level and therefore emerge as interesting targets for future functional investigations.

Different intracellular PRRs are potentially capable of recognizing MAMPs present in the cytosol. These receptors have a dual function in: (1) directly detecting the presence of pathogens (e.g., viruses) in the cellular space; and (2) indirectly detecting microbes in the extracellular environment from their degradation products (e.g., peptidoglycan components). In summary, this system works in a synergistic manner with membrane-bound PRRs, thereby reinforcing the immune response through the combination of converging signaling routes derived from the intracellular and extracellular environments.

NACHT–Leucine-Rich Repeat Proteins and Bacterial Sensing

NACHT–leucine-rich repeat (NACHT-LRR) proteins (NLRs) act as sensors of the two major peptidoglycan-derived bacterial components, muramyl dipeptide (MDP) and γ-D-Glu-meso-diaminopimelic acid (iE-DAP) in the cytosol (Fritz et al. 2006). These MAMPs can be translocated inside the cytoplasm whenever bacteria present in the extracellular environment are attacked by antimicrobial effectors, or they can be released as a consequence of the digestion of phagocytosed bacterial cells. Activated NLRs oligomerize, recruiting adaptor molecules that can modulate immune response, cell death, or survival. Vertebrate NLRs are also responsible for the assembly or inflammasomes—large macromolecular complexes involved in the modulation of inflammation—which are however unlikely to exist in invertebrate animals (Latz et al. 2013).

In spite of the great expansion of NLRs in many metazoans, no such receptor has ever been functionally characterized in molluscs. The typical tripartite domain architecture of NLRs comprises C-terminal leucine-rich repeats required for ligand binding, a central NACHT domain, which regulates oligomerization, and an N-terminal death fold domain (DFD), whose type (DEATH, DED, CARD, or PYD) determines the recruitment of specific downstream signaling adaptors. Although the single NLR-like protein identified in *M. galloprovincialis* displays a CARD/NACHT/LRR domain combination, it bears limited sequence homology with bona fine vertebrate NLRs, leaving its possible involvement in immunity a matter of speculation (Gerdol and Venier 2015).

RIG-Like Receptors: Fundamental Receptors of Viral Infection

While NLRs are mainly employed in bacterial sensing, a series of other receptors collectively known as RIG-like receptors (RLRs) cover an analogous function in the sensing of viruses. Upon activation, these helicase-like molecules trigger the antiviral response through the their N-terminal caspase recruitment domain (CARD) (Yoneyama and Fujita 2007). RLRs are capable of recognizing a broad range of dsDNA viruses, thanks to the mediation of DNA-dependent RNA polymerase III, which uses viral DNA as a template for the generation of 5′ triphosphate single-stranded RNAs, which are efficiently recognized by the helicase domain of RLRs.

Consistently with the expected rapid evolution of antiviral defense mechanisms in the continuous race to arms between the host and the pathogen, this

molecular machinery diverged significantly among animal groups (Paro et al. 2015). Bona fide RLRs were long thought to be exclusively present in vertebrates. However, following early reports of RLR-like genes in the genomes of cnidarians (Zou et al. 2009), a RLR highly responsive to poly(I:C) stimulation was also identified in *M. gigas* (Zhang et al. 2014e). Definitive proof about the involvement of RLRs in antiviral immunity was provided in a study demonstrating that the RLR CgRIG-I-1 was upregulated in response to OsHV-1 infection in Pacific oyster larvae, and that it could directly bind poly(I:C). The identification of the key adaptor protein IPS-1/MAVS (see section "Other Immune Signaling Pathways"), brought convincing evidence in support of the existence of an RLR-mediated signaling pathway activated in response to dsDNA viruses, closely matching that of vertebrates.

Another important aspect in the context of viral sensing is the possible involvement of Dicer, the main antiviral molecule in the cytosol of insect cells, which lack RLRs. In particular, only one out of the two Dicer gene copies present in the genome of *Drosophila* (Dicer-2) can process dsRNAs to produce siRNA (Lee et al. 2004), whereas the single mammalian Dicer gene is mostly involved in the production of miRNAs and only in some cell types can it generate siRNAs (Maillard et al. 2013). While the preferential substrates of this catalytic helicase in bivalves are presently unknown, all molluscs bear a single-copy Dicer gene (Rosani et al. 2016).

Stimulator of Interferon Genes: A Major Hub for Microbial Sensing in the Cytosol

The third major intracellular sensor of microbial infections is the Stimulator of Interferon Genes (STING). Unlike NLRs and RLRs, STING is a multifunctional protein, which can act either as a direct MAMP sensor or as a signaling adapter collecting infection signals derived from several pathogenic agents (Burdette and Vance 2013). This broad spectrum of recognition is guaranteed by the interaction with different cytosolic cofactors, whose presence in molluscs is mostly unconfirmed and sometimes even unlikely due to lineage-specific gene losses and high sequence divergence (Gerdol and Venier 2015).

In vertebrates, the dimerization and migration of STING from the endoplasmic reticulum membrane to the perinuclear region is a fundamental step for the subsequent activation of interferon response and inflammation (Ishikawa et al. 2009) (see section "Lysozymes, BPIs and Other Pore-Forming Molecules"). Although only a few reports have documented the existence of STING in bivalve molluscs (Gerdol and Venier 2015; He et al. 2015), the peculiar domain architecture of this molecule suggests a different subcellular localization and mode of action. Indeed, all lophotrochozoan STING molecules lack transmembrane domains and present a duplicated STING globular domain associated with a TIR domain; this structure could potentially enable self-dimerization upon ligand binding and the activation of downstream immune signaling through TIR–TIR heterotypic interactions. At the same time, it might imply important functional differences in comparison with vertebrates, including the interaction with different and presently unknown alternative MAMP cosensors.

In any case, the main functional property of the STING globular domain, i.e., the ability to bind cyclic dinucleotides in the cytosol, is expected to be retained. The most relevant ligands of STING are cyclic diguanylate (c-di-GMP) and cyclic guanosine monophosphate–adenosine monophosphate (cGAMP). While the former is a second messenger directly produced by bacteria, the latter is synthetized by cyclic GMP–AMP synthases (cGAS) whenever foreign DNA is detected in the cytoplasm, playing a fundamental role in the detection of both bacterial and viral nucleic acids (Ablasser et al. 2013). Although the importance of the cGAS/STING complex in activating the antiviral response has been only recently uncovered, it is certainly noteworthy that bivalve genomes display a significant expansion of cGAS-like genes in comparison with gastropods, which would suggest improved competence for viral detection (Gerdol 2017).

Signaling and Regulatory Pathways

MAMPs of various natures, such as glycoproteins, components of cell walls and membranes, and exogenous nucleic acids can be recognized by the broad array of bivalve PRRs described in the previous sections, activating a cascade of intracellular events that eventually result in cell response to the perceived stimulus. Multiple signal transduction pathways, mostly based on protein–protein interactions and modifications (e.g., kinase-mediated phosphorylation), regulate the timing and intensity of the immune response, as well the cellular fate (death or survival).

Canonical Toll-Like Receptor Signaling

The Essential Role of MyD88 in Immune Signal Transduction

The main signal transduction pathway reported to mediate the immune responses of bivalve species is TLR/NF-κB signaling (Fig. 11), which is deeply intertwined with other accessory networks that will be described in the section "Other Immune Signaling Pathways." The recognition of ligands by the extracellular LRR domains of TLRs leads to their dimerization, which in turn activates key transcription factors, enabling the production of AMPs, lysozymes, interleukins (ILs), and other immune effectors against bacterial, fungal, and viral pathogens. The first essential step of TLR-mediated signal transduction involves the recruitment of TIR-DC adaptor proteins, which in vertebrates are primarily the myeloid differentiation primary response protein 88 (MYD88) and the TIR-domain-containing adapter-inducing interferon-β (TRIF) (O'Neill and Bowie 2007).

Because of the lack of a TRIF homolog, the TLR signaling in bivalves is essentially a MyD88-dependent pathway, even though the possible involvement of alternative evolutionarily conserved TIR-DC adapters cannot be excluded (Gerdol et al. 2017). The fundamental signaling mediator MyD88 is characterized by an N-terminal death domain, required for perpetrating signal transduction, and by a C-terminal TIR domain that interacts upstream with the cytosolic TIR domain of

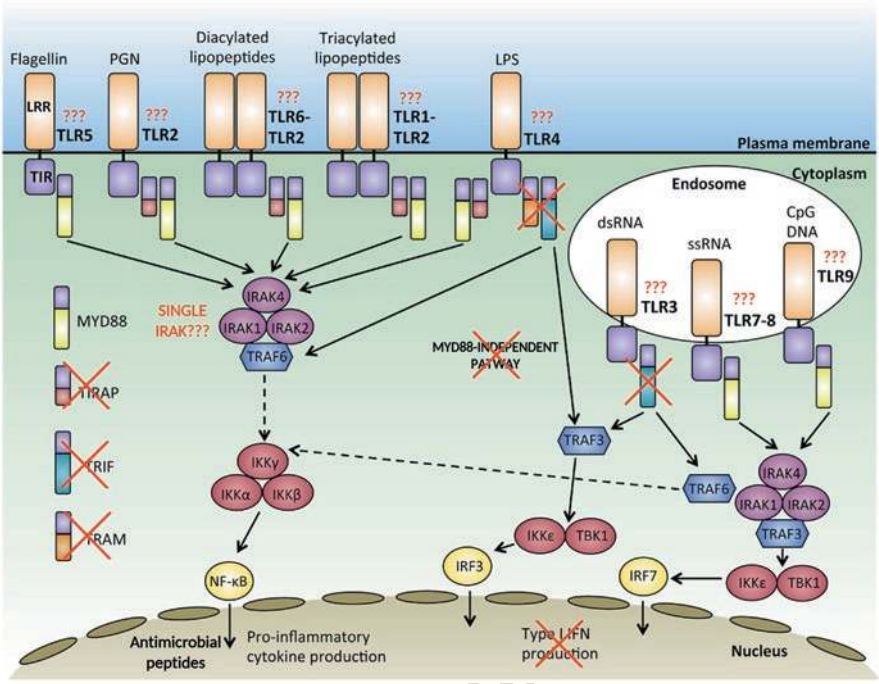


Fig. 11 Vertebrate canonical Toll-like receptor (TLR) signaling and comparison with that of bivalve molluscs. Unidentified components in bivalves are struck through and elements whose presence is uncertain are indicated by question marks. In particular, the low similarity between vertebrate and molluscan TLRs leaves the binding specificity of bivalve TLRs, for the most part, unknown. The homo- or heterodimerization of TLRs following ligand binding, either in the extracellular environment or in the endosomal compartment, recruits adaptor proteins, which propagate immune signals. Only MyD88, among the vertebrate adaptors, has been identified so far in bivalves. The recruitment and activation of IRAK kinases and the IKK complex results in the migration of the NF-κB and possibly IRF transcription factors to the nucleus, where they regulate the production of proinflammatory cytokines and antimicrobial peptides. (Edited from Wang et al. 2014c)

TLRs. The upregulation of MyD88 transcripts has been documented in different bivalve species in response to various bacterial MAMPs (Toubiana et al. 2013; Ren et al. 2016; Xin et al. 2016a) and OsHV-1 infection in oysters (Renault et al. 2011; Du et al. 2013). The multiple MyD88 genes identified in the genomes of *M. gigas* and *M. yessoensis* indicate an expanded gene family (Zhang et al. 2015; Ning et al. 2015), possibly linked with the diversification of TLRs (see section "Toll-Like Receptors"). Some MyD88-like proteins lack the N-terminal death domain and are therefore thought to function as negative regulators (Xu et al. 2015b), together with the sterile alpha and armadillo motif containing protein (SARM), an evolutionarily conserved negative regulator of TLR signaling, as well as an intermediary of apoptosis and antiviral innate response (Belinda et al. 2008; Panneerselvam and Ding 2015).

Toll-Like Receptor–Mediated Signal Transduction: From the Cell Membrane to the Nucleus

All of the expected elements of canonical MyD88-dependent TLR signaling have been identified in the transcriptomes of *M. gigas* (Zhang et al. 2011c), *M. galloprovincialis* (Toubiana et al. 2014), and *Saccostrea glomerata* (Ertl et al. 2016), and even physically mapped to *A. farreri* bacterial artificial chromosomes by fluorescence in situ hybridization (Wang et al. 2011b; Zhao et al. 2015). These approaches highlighted a remarkable similarity with the immune signaling system of deuterostomes and a less significant overlap with arthropods. The immune role of such molecules has been confirmed by the assessment of their upregulation following immune stimulation trials and a detailed functional characterization in several bivalve species. While many accessory factors take part in this elaborate signaling network, either as positive or negative regulators, or as molecular switches to activate connected pathways, we will discuss below only the main molecular players (Fig. 11).

The second intracellular step of the MyD88-dependent TLR signaling involves the interaction between MyD88 and the *Interleukin-1 receptor-associated kinases* (IRAK)-1/-4 complex, with the subsequent recruitment of the *TNF receptor-associated factor 6* (TRAF6). The two IRAK proteins identified in mussels (both homologous to IRAK-4) were strongly overexpressed in hemocytes following bacterial challenges (Toubiana et al. 2014), similarly to the soft shell clam *Mya arenaria* IRAK-4-like transcript, responsive to *V. splendidus* challenges (Mateo et al. 2010). The turnover of IRAK kinases is regulated by the Toll interacting protein TOLLIP, characterized as an acute phase protein in *M. yessoensis* (Zhang et al. 2015) but present with steady expression levels in *M. galloprovincialis* (Toubiana et al. 2014). TRAF6 is one of the key components of the pathway, as it regulates the activation of the IKK complex together with the *Transforming growth factor activated kinase-1* (TAK1). TRAF6 responds to Gram-positive and Gram-negative, as well as to LPS challenges in the scallop *A. farreri* and in the mussel *M. galloprovincialis* (Wang et al. 2011b; Toubiana et al. 2014). Very limited functional information has been collected so far about TAK1, the associated proteins TAB1/2, and the components of the *Inhibitor of kappa-B kinase* (IKK) complex, in bivalves. Most notably, an IKK-like sequence has been characterized in oyster and connected to the activation of NF-κB (Escoubas et al. 1999). As a major difference with vertebrates, only a single IKKα/β homolog is present in *M. galloprovincialis*. The IKK complex finally phosphorylates the *Inhibitor of nuclear factor kappa-B* (IKβ), which is then ubiquitinated and targeted for proteasomal degradation. This process allows the entering of the NF-κB or Rel transcription factors in the nucleus, ultimately enabling the transcription of the target effector genes.

After the initial characterization of an IKβ homolog in *M. gigas* (Montagnani et al. 2008), three paralogous genes were identified in this species. All of them were positively regulated by MAMP and heat-killed bacteria stimulation (Zhang et al. 2011e; Xu et al. 2015a). Similarly, *M. galloprovincialis* possesses at least two IKβ genes, which both experienced moderate to strong upregulation in response to bacterial challenges (Toubiana et al. 2014). IKβ homologs were also found to be

responsive to various types of challenges in *A. farreri*, *Cyclina sinensis*, *Meretrix meretrix*, *P. fucata*, *R. philippinarum*, *S. glomerata*, and *S. grandis* (Zhang et al. 2009a; Green and Barnes 2009; Wang et al. 2011b; Yang et al. 2011b; Moreira et al. 2012a; Lee et al. 2013; Liu et al. 2014; Gao et al. 2016). In this respect, a contrasting result was obtained in *A. irradians*, as IKβ was downregulated following *V. anguillarum* challenges (Mu et al. 2010). The consensus of studies further seems to indicate widespread expression of these inhibitors in all adult tissues, even though most experimental studies have been focused on expression dynamics in hemocytes.

Nuclear Factor Kappa B: A Key Regulator of Immune Response

Nuclear factor kappa B (NF-κB) family members, sharing a domain architecture similar to human p100/p105 or to p65, have been identified in multiple bivalve species, where they are present as single-copy genes (Li et al. 2015b). The first functional confirmation of the involvement of bivalve NF-κB homologs in immune response came from the observation that the overexpression of the oyster gene in *Drosophila* cell lines was able to induce the expression of a NF-κB reporter gene (Montagnani et al. 2004). This molecule could be further placed within the TLR-mediated MyD88-dependent circuitry thanks to RNAi studies in *C. sinensis* (Gao et al. 2016). Furthermore, the *A. farreri* homolog controls the expression of AMPs, providing direct evidence in support of its involvement in the production of effector molecules (Oyanedel et al. 2016). Overall, compelling evidence demonstrates the MyD88-dependent inducibility of NF-κB in the acute phase of response to various bacterial and viral MAMPs in bivalves, supporting the role of these transcription factors in regulating the expression of proinflammatory factors, effector molecules, and cytokines involved in fundamental aspects of bivalve immunity (Wang et al. 2011b; Huang et al. 2012; Toubiana et al. 2014; Li et al. 2015b; Gao et al. 2016). However, significant differences in the magnitude of this response exist among species which might, to some extent, even explain the different interspecies susceptibility to disease, as evidenced by the comparative analysis of shallow-water and deepsea mussels (Martins et al. 2014).

Other Immune Signaling Pathways

Role of the Mitogen-Activated Protein Kinase Cascade in Immune Signaling

While the processes outlined above cover the main signaling pathway from MAMP sensing to the activation of nuclear factors, some components of the TLR/NF-κB signaling found in vertebrates and invertebrates alike represent a bridge to other signaling pathways (O'Neill and Bowie 2007; Brown et al. 2011). Most notably, TRAF6 can interact with MEKK1 thanks to mediation by the *Evolutionarily conserved signaling intermediate in Toll pathways* adapter (ECSIT), which is also found in bivalves (Toubiana et al. 2014; Lin et al. 2017), activating the mitogen-activated protein kinase (MAPK) cascade. In essence, the MAPK signaling is a phosphorylation cascade activated by many immune and nonimmune signals (e.g.,

growth factors, cytokines, bacteria, viruses, oxidative stress), which modulates various cell processes. This important signaling cascade activates classical MAP kinases (ERK, p28, JNK), whose concerted action can determine alternative cellular fates, including cell survival and proliferation, differentiation, or death. The successful use of commercial antibodies targeting MAPK components evidenced the remarkable conservation of this pathway in all animals (Canesi et al. 2002; Bettencourt et al. 2009). Sequences denoting MAPK proteins have been identified in different mussel and oyster species (Martins et al. 2014; Zou et al. 2015; Gerdol and Venier 2015; Wang et al. 2017a) and p38, JNK, and ERK kinases in particular have been specifically linked to bivalve immune response (Sun et al. 2016; Qu et al. 2016, 2017a). Ultimately, MAPK signaling results in the activation of AP-1, a heterodimeric transcription factor composed of Jun and Fos subunits. The immune role of bivalve AP-1 has been so far mostly inferred from gene expression data collected in *C. hongkongensis* and *R. philippinarum* (Xiang et al. 2014a; Wu et al. 2015; Qu et al. 2015a). Regardless of the alternative activation of the IKK complex or of the MAPK cascade downstream of MyD88, the two signaling branches extensively communicate with each other, as TAK1 can phosphorylate (and activate) MAPKs, and MEKK1 can phosphorylate (and activate) the IKK complex (Moustakas and Heldin 2003).

Interferon-Responsive Factors

Another alternative signaling route potentially activated upon the interaction between TLRs and intracellular adaptors would lead to the activation of *Interferon-Responsive Factors* (IRFs), a class of transcription factors that enable the expression of interferons and other proinflammatory cytokines. However, this typical vertebrate pathway implies the mediation of TRIF (instead of MyD88) and RIP kinase 1 (instead of IRAKs) which both lack convincing homologs in bivalves (Meylan et al. 2004). Bivalve IRFs have been linked to resistance to infections in *H. cumingii* (Wang et al. 2013a) and to the transcriptional activation of genes with ISRE elements in the pearl oyster *P. fucata* and the mussels *Bathymodiolus platifrons* and *Modiolus modiolus* (Huang et al. 2013b, 2017a). However, since the existence of MyD88-independent TLR signaling seems unlikely in bivalves, these IRF-like molecules are probably related to other signaling routes originated from cytosolic PRRs, which will be described in detail below.

Is an Immune Deficiency–Like Pathway Present in Bivalve Molluscs?

The possible presence of a bivalve immune deficiency (IMD)–like pathway involved in the recognition of Gram-negative bacteria and homologous to that found in *Drosophila* (Lemaitre and Hoffmann 2007) has been long hypothesized. In this case, the immune signals would originate from membrane-bound PGRPs and be transduced in the cytosol by signaling molecules that are partially shared with the vertebrate tumor necrosis factor receptor (TNFR) signaling pathway. These include dFADD and DREDD/Caspase-8, which are both present in bivalves (Gerdol and Venier 2015), but also the IKK complex and MAPK pathway, which can be activated by the cross talk between TNFR and TLR signaling. Crucially, however, the

key IMD adaptor molecule is lacking and no functionally homologous component has been identified yet in bivalves (Gerdol and Venier 2015). Taking into account the relevant sequence divergence between the intracellular domain of arthropod and molluscan membrane-bound PGRPs (see section "Other Membrane-Bound Immune Receptors"), the identity of the hypothetical key mediator of the IMD-like pathway in these animals remains presently unknown.

Signaling Pathways Activated in Response to Microbial Sensing in the Cytosol

The interconnected signaling pathways presented so far act at the crossroads with the cytosolic PRRs described in section "Cytosolic Pattern Recognition Receptors," which share several signal transducers with the TLR/NF-κB/MAPK/IRF circuitry, thereby resulting in the activation of the same transcription factors and in the production of similar effector molecules. Among these, the signaling by NLRs would hypothetically involve the mediation of *receptor-interacting serine/threonine protein kinase 2* (RIPK2) for the recruitment of TAK1 and the consequent activation of the IKK complex (Nembrini et al. 2009). However, the lack of a bivalve RIP2K homolog points out that a bivalve NLR-based cytosolic MAMP–sensing system, if it exists, should be based on molecules that are divergent from their vertebrate functional homologs.

In vertebrates, STING stimulates the phosphorylation of IRF3 through the action of the TANK-binding kinase 1 (TBK1) (Tanaka and Chen 2012), the gene of which has been recently characterized in *M. gigas*. The oyster homolog was strongly upregulated in response to *V. alginolyticus* and OsHV-1 infections and, most importantly, its direct interaction with STING was demonstrated by co-IP studies, thereby confirming a mode of signal transduction similar to those in vertebrates (Tang et al. 2016).

RLRs, key sensors of viral nucleic acids (see section "Cytosolic Pattern Recognition Receptors"), require the *IFN-beta promoter stimulator* (IPS-1, also known as *CARD adaptor inducing IFN-beta*, or CARDIF, and *Virus induced signaling adaptor*, or VISA) to induce the expression of interferon and inflammatory cytokines via IRFs or NF-κB (Fredericksen et al. 2008). This adapter has remained elusive for a long time in invertebrates, until the very recent discovery of the *M. gigas* homolog CgMAVS. The functional characterization of the oyster protein confirmed its primary role in antiviral response, as (1) CgMAVS could be strongly upregulated in response to viral infections; (2) the interaction between the CARD domain of CgRIG-I-1 and CgMAVS was demonstrated by yeast two-hybrid and co-IP; (3) an interaction was similarly demonstrated with the downstream signaling adapter TRAF6; and (4) the inactivation of CgMAVS by RNAi in infected oyster spat determined a remarkable increase in mortality (Huang et al. 2017b). The demonstrated interaction with TRAF6 would imply the activation of NF-κB. However, the most important MAVS interactor in vertebrates is another member of the TRAF family, TRAF3, which can recruit TBK1, activating IRF3. The first molluscan TRAF3 homolog was recently identified in the freshwater mussel *Anodonta woodiana*. Although the physical interaction with MAVS and RLRs has not been

demonstrated yet, bacterial and viral challenges triggered the overexpression of this molecule, supporting its involvement in RLR-mediated signaling (Qu et al. 2017c).

Altogether, these functional studies, supported by the identification of nearly all of the required signaling molecules in sequence databases (Philipp et al. 2012; Green et al. 2015; Ren et al. 2017b), as well as by the observation of their significant upregulation in response to experimental OsHV-1 infection in oysters (He et al. 2015), highlight that bivalve molluscs are equipped with a well-developed molecular system for viral sensing in the cytosol.

Production of Cytokines

Elusive Regulators of the Molluscan Immune System

The complex signaling machinery described in detail in the previous sections ultimately leads to the production of effector molecules that are used to kill or to reduce the pathogenicity of invading microbes (see section "Humoral Immune Effectors") or to regulate immune response at a cellular level (see section "Cellular Immune Responses") and at a systemic level. Cytokines are small glycoproteins with regulatory immune functions, which are the most important regulators of metazoan immunity, as they activate signaling elements leading to the expression of other cytokines, antiviral effectors, and other immune-related genes. Their action is very fast and powerful in the amplification of the immune response despite an extremely low concentration in body fluids. Furthermore, many cytokines have a pleiotropic effect and a somewhat redundant function (Nicola 1994). Despite the essential and long-known role of cytokines in vertebrates, their existence in invertebrate animals was long debated until the first molecules with a cytokine-like activity were first identified (Beschin et al. 2001; Herpin et al. 2004). Moreover, as explained in section "Other Membrane-Bound Immune Receptors," one of the most studied cytokines in the *D. melanogaster* model, Spätzle (Parker et al. 2001), is not present in bivalves and therefore TLRs are likely to act in a vertebrate-like fashion, by directly binding MAMPs with their extracellular LRR domains. Despite the availability of genomic sequence data, interferon-like factors remain elusive in all invertebrates, seemingly supporting the idea that vertebrate and invertebrate cytokines have a different evolutionary origin, despite sharing a similar mode of action and a quite conserved intracellular signaling machinery. For the most part, molecular studies on molluscan cytokines are limited to evolutionarily conserved factors, readily identifiable by sequence similarity.

Structurally Conserved Cytokines: Interleukin-17, Macrophage Migration Inhibitory Factor, and Allograft Inflammatory Factor-1

The first bivalve cytokine to be identified was interleukin-17, produced at significant levels in oyster hemocytes in response to bacterial exposure (Roberts et al. 2008). IL-17 sequences have been subsequently isolated in many bivalve species or detected as highly responsive transcripts to bacterial challenges and abiotic stimuli

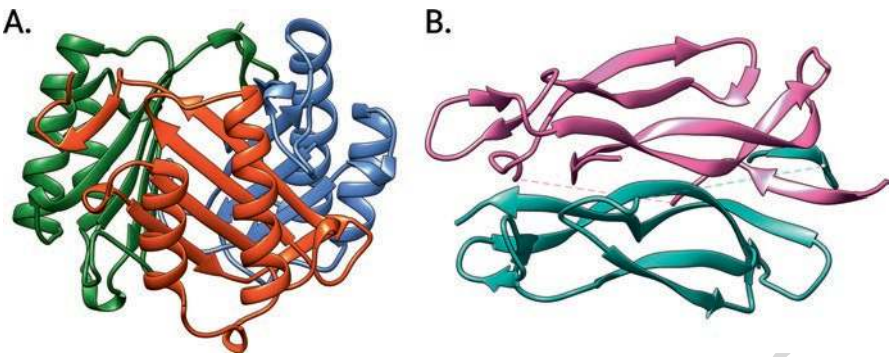


Fig. 12 Structure of two of the evolutionarily conserved cytokines found in bivalve molluscs. (**a**) Human macrophage migration inhibitory factor (MIF) trimer (PDB accession ID: 1MIF). (**b**) Human interleukin-17 dimer (PDB accession ID: 4HR9)

(Wu et al. 2013; Moreira et al. 2014; Xin et al. 2015, 2016b). Genomic studies have further revealed that oyster IL-17 proteins are the product of a multigenic family, which comprises at least five members (Li et al. 2014). Although IL17 signaling requires further study in bivalves, homology-based inference suggests that because of its conserved structure (Fig. 12), the binding of IL17 to its receptor stimulates downstream CIKS/CIKSL proteins via SEFIR–SEFIR domain interactions and, subsequently, TRAF proteins related to both MAPK and NF-κB signaling (Rosani et al. 2015).

The macrophage migration inhibitory factor (MIF) and the allograft inflammatory factor-1 (AIF-1) are two other proinflammatory cytokines that have been identified in bivalves by sequence similarity. The former is a CD74 ligand, which stimulates the acute phase response. Despite the clear difference between bivalve and vertebrate circulating immune cells, the *M. galloprovincialis* MIF displays a well conserved three-dimensional fold (Parisi et al. 2012) (Fig. 12). In contrast with expression data collected in mussels, the *A. farreri* MIF sequence was upregulated upon bacterial challenges in a study that also provided an important confirmation about the functional conservation this molecule, as the recombinant protein could induce fibroblast migration (Li et al. 2011b). In addition, single nucleotide polymorphisms of MIF have been connected with increased resistance to *Vibrio* spp. infections in *M. meretrix* (Zou and Liu 2016). AIF-1, on the other hand, is activated in macrophages upon tissue injury. In *O. edulis*, AIF-1 was upregulated in the hemocytes and mantle of oysters affected with heavy bonamiosis (Martín-Gómez et al. 2014), and its expression could be induced in *M. gigas* with multiple immune challenges (Zhang et al. 2013b). From a functional point of view, the similarity between vertebrate and bivalve AIF-1 proteins is remarkable. Indeed, the oyster homologs could stimulate phagocytosis in the granulocyte hemocyte subpopulation and a clear involvement in tissue damage could be also established (Li et al. 2013a).

Tumor Necrosis Factor-α: A Cytokine Acting at the Crossroads Between Immunity and Apoptosis

Following the identification of a *tumor necrosis factor* α (TNF-α) in disk abalone (De Zoysa et al. 2009), this multifunctional immune modulator was also described in *M. gigas*, *C. hongkongensis*, and *O. edulis* (Martín-Gómez et al. 2014; Sun et al. 2014; Qu et al. 2017b). Oyster TNF-α transcripts are upregulated in response to immune challenges and bonamiosis and modulate phagocytosis and apoptosis in hemocytes. Furthermore, TNF-α recombinant proteins could induce the expression of NF-κB reporter genes in human cell lines. In bivalve molluscs, the conserved function of this cytokine, which acts at the crossroads between the immune system and the apoptotic machinery, is supported by the identification of conserved accessory factors, i.e., TTRAP (Yang et al. 2011a) and *lipopolysaccharide-induced TNF factor* (LITAF), a positive regulator of TNF-α transcription (Zhu and Wu 2012; Yang et al. 2013a). As mentioned in section "Other Immune Signaling Pathways," TNF-α would exert its function through a signaling pathway partially shared with the arthropod IMD pathway, which includes the key evolutionarily conserved components dFADD and DREDD (Gerdol and Venier 2015). The transduction of immune signal inside the cell is enabled by the binding of TNF-like molecules to their receptors, collectively known as TNFRs. Functional tests carried out in many bivalve species support the involvement of bivalve TNFRs in the establishment of immune response, despite their limited homology with vertebrate receptors (Li et al. 2009; Su et al. 2011; Xing et al. 2016; Xiang et al. 2016). Another cytokine involved in the regulation of cell death, the *TNF-related apoptosis-inducing ligand* (TRAIL), is ubiquitously expressed in various tissues in *H. cumingii* and *Magallana ariakensis*. The few experimental pieces of evidence collected so far point toward the involvement of the MAPK pathway in the activation of this cytokine and also suggest the involvement of caspase 3 as a downstream effector (Yang and Wu 2010; Yang et al. 2013b).

New Opportunities for Cytokine Studies in Bivalves

Many divergent molecules with a cytokine-like function in bivalve molluscs have only been recently identified or still remain to be uncovered. An important example is provided by myticin C, a long-known mussel antimicrobial peptide, which has also been shown to bear chemotactic properties, stimulating hemocyte migration and morphological changes (Balseiro et al. 2011). The discovery of a class II helical cytokine in *M. gigas* with remote homology with vertebrate IFN-like molecules further stimulates research efforts directed at the discovery of novel cytokines in bivalves. CgIFNLP was upregulated in response to poly(I:C) stimulation and the recombinant protein could sensibly enhance both apoptosis and phagocytosis in oyster hemocytes (Zhang et al. 2015).

In the vertebrate IFN signaling, the activation of IFN receptors stimulates the activity of downstream Janus kinases (JAK) and, consequently, the migration of the Signal transducer and activator of transcription (STAT) to the nucleus, with the consequent expression of *IFN-stimulated genes* (ISGs). This signaling pathway, whose presence in bivalves had been already assessed by a number of transcriptomic studies

(Philipp et al. 2012; Green et al. 2014, 2015), has been conclusively implicated in the regulation of immune response by CgIFNLP through its newly isolated receptor (Zhang et al. 2016b).

Connections with the Neuroendocrine System

The neuroendocrine immunomodulation (NEI) regulatory network encompasses the complex cross talk between the nervous system, the endocrine system, and the immune system to maintain homeostasis and to modulate innate immune response in all animals (Fig. 13). Although NEI appears to be simpler in invertebrates than in vertebrates, it is highly conserved and represents an efficient regulatory mechanism (Hartenstein 2006). From this point of view, molluscs are of particular interest, as they are the most primitive animals with a complete NEI system and there is evidence that points to hemocytes as a connecting link between the immune and the nervous system (Liu et al. 2017b). While cephalopods have long been considered as privileged molluscan models for the study of NEI because of their well-developed nervous system and amenability for laboratory research (Di Cosmo and Polese 2016), in recent years bivalve molluscs have been the subject of an increasing number of studies (Song et al. 2015; Wang et al. 2017a). The main components of the NEI are the cholinergic, catecholaminergic, and nitric oxidase systems, together with the action of the neuropeptides.

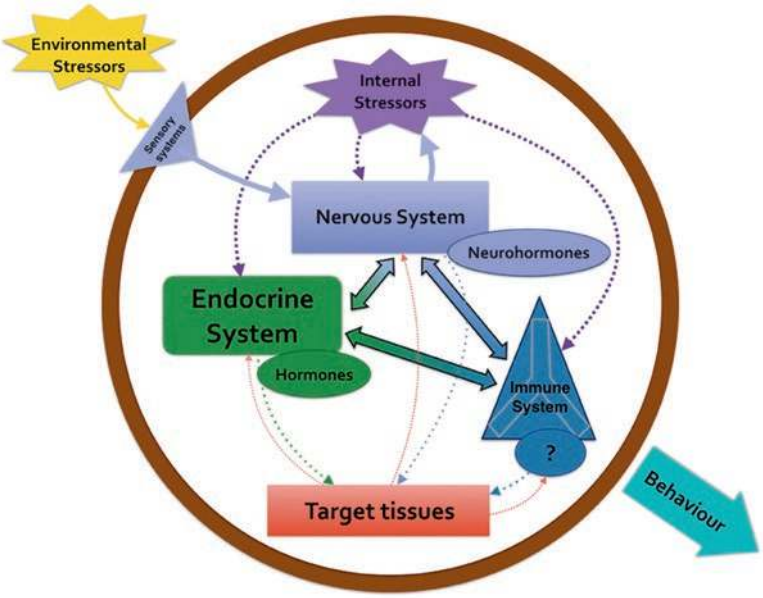


Fig. 13 Cross talk between the nervous, endocrine, and immune systems in response to an external stimulus. (Original Source: Di Prisco and Polese 2015)

The Cholinergic and Catecholaminergic Neuroendocrine Systems

The cholinergic neuroendocrine system can be activated by pathogens and tends to negatively regulate the immune response on a long time scale. The main component of the cholinergic nervous system is acetylcholine (ACh), whose concentration has been shown to significantly increase in the hemolymph of scallops upon stimulation with LPS or TNF-α (Shi et al. 2014). Acetylcholinesterase-like enzymes and muscarinic receptors of Ach have been detected in the hemocytes and other tissues of bivalve molluscs. Strikingly, the *A. farreri* acetylcholinesterase is thought to contribute to the rebalancing of the immune system following immune response in *A. farreri* (Shi et al. 2012). As a further confirmation in support of the existence of the cholinergic anti-inflammatory pathway in this animal group, the expression of a novel muscarinic acetylcholine receptor was regulated by LPS stimulation in *M. gigas*. The activation of this receptor seems to be crucial for the production of TNF and for the regulation of apoptosis in hemocytes (Liu et al. 2016b). Moreover, the subunits of the nicotinic acetylcholine receptor of *A. farreri* were subjected to a similar induction in response to LPS and TNF-α (Shi et al. 2015).

The catecholaminergic neuroendocrine system is mainly composed of catecholamines (dopamine, norepinephrine and epinephrine), their metabolic enzymes, and receptors. Catecholamines are among the first neurotransmitters to appear during the ontogenesis of molluscs to regulate cell proliferation, differentiation, and neurogenesis. In adults, the synthesis and release of catecholamines has been reported in the hemocytes, mantle, and gills. The first important evidence supporting the involvement of this system in the modulation of both the cellular and humoral immune response was provided by the observation of the induction of the alpha-1 norepinephrine receptor in response to LPS in *M. gigas*. This receptor could in turn modulate the expression of TNF and induce phagocytosis and apoptosis of hemocytes (Liu et al. 2016c). Furthermore, the catecholaminergic system is markedly activated after acute heat and bacterial stress in oyster larvae (Liu et al. 2017a).

Nitric Oxide, Neuropeptides, and Open Challenges in Neuroendocrine Immunomodulation Studies

NO synthase (NOS) is a fundamental enzyme for the production of nitric oxide (NO), a key signaling molecule involved in multiple processes, including immune defense. Unlike vertebrates, molluscs display only a single NOS isoform, pointing toward the existence of a unique prototypical enzyme that combines the functions of the three vertebrate isoforms. Recently, the mutual modulation between norepinephrine and nitric oxide during immune response has been demonstrated in scallops (Jiang et al. 2014), showing the intimate linkage among all of these regulatory systems.

Neuropeptides include a diverse class of cell signaling molecules. These molecules are produced and released by neurons, and their mechanism of action occurs through a regulated secretory pathway. As in vertebrates, various neuropeptides identified in molluscs could potentially play important roles in immune regulation. Although 74 possible neuropeptide genes have indeed been identified in the oyster

genome (Zhang et al. 2012a), neuropeptide studies in the context of immunity are still lacking in bivalves.

As a final consideration about the regulation of NEI function in molluscs, the action of microRNAs also needs to be taken into account. In fact, several miRNAs (named NeurimmiRs) are highly responsive to acetylcholine and norepinephrine stimulation in oyster hemocytes. The in silico–predicted targets for NeurimmiRs comprise over 300 genes with functions in cell death, immunity, and response to stimulus, which might therefore explain the observed decrease in phagocytosis and late apoptosis/necrosis in stimulated hemocytes (Chen et al. 2015). One of the identified miRNAs was subjected to further studies, which evidenced its role in repressing acetylcholine production and choline uptake in hemocytes (Chen et al. 2016).

Humoral Immune Effectors

Antimicrobial Peptides

Because of their fundamental role as a first line of defense in the molluscan innate immune system and potential biotechnological applications, antimicrobial peptides (AMPs) have been the subject of a considerable number of molecular studies. The first pioneer studies, targeting the hemolymph of mussels, provided the impetus for the characterization of novel antimicrobial compounds, using classical biochemical methods. This field of research is growing thanks to the application of in silico data-mining approaches, and bivalves have been one of the most extensively exploited sources of AMPs in the animal kingdom over the past 20 years.

Defensins, Mytilins, and Myticins: Main Players in Hemocyte-Mediated Immune Response

The story of antimicrobial research in bivalve molluscs dates back to 1996, when several novel cysteine-rich peptides similar to arthropod defensins were extracted from the active fraction of hemolymph in the marine mussels *M. edulis* and *M. galloprovincialis* (Hubert et al. 1996; Charlet et al. 1996). Two novel peptides, containing eight cysteine residues arranged in a slightly different pattern, were named mytilins and displayed significant activity mostly directed against Gram-positive bacteria (Charlet et al. 1996). Mytilins and defensins exert their antimicrobial action following the recruitment of a specialized subpopulation of circulating hemocytes to the site of infection, where they are intracellularly released from granules (Mitta et al. 2000b, c). Although these AMPs are clearly involved in the intracellular killing of bacterial cells phagocytosed by hemocytes, they also appear to secondarily participate in the systemic immune response when released in the hemolymph (Mitta et al. 2000d). A few years later, a new class of AMPs named myticins was identified in *M. galloprovincialis* plasma and hemocytes. These peptides displayed only limited antimicrobial properties in comparison with defensins and mytilins but shared eight conserved cysteine residues and high hemocyte

specificity (Mitta et al. 1999). Although the antimicrobial activity of myticins is rather weak and it can be only attained at acidic pH (Martinez-Lopez et al. 2013; Domeneghetti et al. 2015), they might have alternative potential roles both as antiviral agents and as chemokine/cytokine–like molecules (Balseiro et al. 2011; Novoa et al. 2016).

Molecular and genetic studies revealed that these mussel AMPs are produced as secreted pre-propeptides. The highly cationic charge of the central mature peptide region is balanced by an acidic C-terminal extension of the precursor protein, which is likely removed after its release from hemocyte granules. It was also revealed that these AMPs pertain to multigenic families that share a similar architecture, as they all comprise four exons and three introns, with fixed exon/intron boundaries (Mitta et al. 2000a). An aspect of mussel hemocyte-specific AMPs that has revealed somewhat counterintuitive patterns concerns unpredictable fluctuations in gene expression in response to bacterial challenges (Mitta et al. 2000a) and significant intraspecific variation, suggesting that genome–environment interactions play a major role in regulating AMP production (Li et al. 2010).

A few years after the original discovery of AMPs in mussel hemocytes, defensin-like AMPs with eight cysteines were also identified in circulating immune cells in the Pacific oyster, together with a second isoform mainly expressed in the mantle edge (Gueguen et al. 2006; Gonzalez et al. 2007a). Over the years, many other sequences labeled as "defensin" or "defensin-like" AMPs have been isolated in different bivalve species. Besides their structural differences, summarized by the presence of either three or four disulfide bonds, these AMPs are also often characterized by different spectra of activity, preferential tissues of expression, and accessory functions. For example, a foot-specific defensin-like peptide has been linked to byssogenesis in zebra mussels (Xu and Faisal 2010), whereas a gill-specific peptide with marked activity against Gram-positive bacteria has been isolated from gills extracts of *C. virginica* (Seo et al. 2005). Clam and freshwater mussel defensins display a spectrum of activity and tissue specificity similar to those of *Mytilus* AMPs, but they are reportedly upregulated following bacterial challenges (Peng et al. 2012; Wang et al. 2015c). These reports suggest that different cysteine-rich peptides currently classified with the same label could have slightly different biological properties depending on the species of origin.

From a structural point of view, all of the aforementioned defensin-like AMPs (including mytilins and myticins) share a common structural motif, the cysteine-stabilized α-helix β-sheet (CS-αβ) fold (Fig. 14). This conserved and successful compact domain consists of an α-helix and two antiparallel β-sheets, whose orientation and reciprocal position in the 3D space are fixed by intramolecular disulfide bridges (Yang et al. 2000; Gueguen et al. 2006). Crystallographic studies reveled that, in spite of a negligible primary sequence homology and a slightly different position of cysteine residues, defensins and mytilins share not only the same structural fold but also similar hydrophobic and hydrophilic areas (Roch et al. 2008). Although the 3D structure of myticins has not been experimentally determined yet, modeling approaches have unequivocally evidenced that they are also likely to adopt a CS-αβ fold (Domeneghetti et al. 2015).

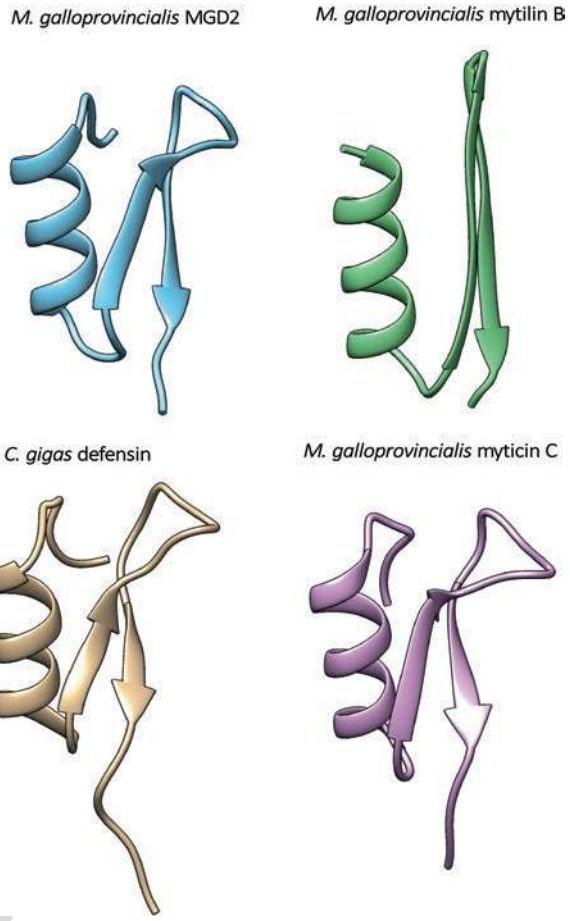


Fig. 14 Experimentally determined three-dimensional structures of *M. galloprovincialis* MGD2 defensin, mytilin B, and *M. gigas* defensin. The in silico–predicted structure of *M. galloprovincialis* myticin C is also reported. The conserved cysteine-stabilized α-helix β-sheet fold, comprising an α-helix followed by two antiparallel β-sheets, is easily detectable

Other Cysteine-Rich Antimicrobial Peptide Families

In recent years, data-mining approaches have led to the identification of macins, an additional group of bivalve AMPs in the CS-αβ peptide superfamily. Originally identified in other metazoan phyla, macins were first described as a multigenic family in *M. galloprovincialis* (Gerdol et al. 2012) and later reported in other bivalve species. Although the functional significance of the complex cysteine array of macins is still poorly understood, these peptides are of great interest because of their role in wound healing, in addition to bacterial killing, and their widespread expression across all main tissues.

In comparison with canonical defensins, big defensins pertain to a structurally different but evolutionarily widespread class, also comprising vertebrate β-defensins. The characterizing six-cysteine array of big defensins is located in the C-terminal domain of these AMPs, and it is coupled with an N-terminal α-helical domain whose presence is also required for antimicrobial action. Big defensins have been isolated in many different bivalve species and, while all studies have evidenced the

inducible expression of these AMPs, contrasting reports have been produced concerning the main tissues of expression (Zhao et al. 2010; Rosa et al. 2011; Gerdol et al. 2012; Li et al. 2012; Wang et al. 2014a; Yang et al. 2016). A more precise indication concerning the localization of big defensins has been provided by immunofluorescence studies carried out in *A. irradians*, which have evidenced a prominent abundance in the gill and mantle epithelia, strongly implicating a role in mucosal immunity (González et al. 2017).

The remarkable diversity of bivalve cysteine-rich AMPs is not limited to peptides with a known structure but also involves novel cysteine arrays and unknown disulfide connectivities. The first example is that of mytimycin, an antifungal peptide identified in mussel hemolymph extracts (Charlet et al. 1996). Like the other AMPs stored in granules, this peptide is produced as an inactive precursor, whose C-terminal extension contains an EF-hand domain. The mature peptide region can vary in terms of both the number and the arrangement of cysteine residues (Sonthi et al. 2011). More recently, three additional plausible AMP families—myticusins (Liao et al. 2013), mytichitins (Qin et al. 2014), and CRP-I (Gerdol et al. 2015a)—have been identified in *Mytilus* spp. but promising preliminary results still await a detailed functional characterization.

Improved Strategies Are Required to Discover Novel Antimicrobial Peptide Families

Although different molecules with heterogeneous evolutionary origins, amino acid compositions, and three-dimensional structures can act as antimicrobial agents, nearly all known bivalve AMPs pertain to a single large category, i.e., AMPs rich in cysteine residues engaged in disulfide bonds. This reflects the overwhelming prevalence of the scientific literature on the subject, as very scant information is available about AMPs devoid of disulfide bonds in Bivalvia. As a striking example, no AMP with an amphipathic α-helical secondary structure has ever been isolated, despite their widespread distribution and the important role these peptides cover in the innate immune system of other protostomes (Giangaspero et al. 2001). While it is possible that this lack of information mirrors a major shift toward the use of cysteine-rich AMPs in molluscs compared with other metazoans, other explanations are possible. For example, in silico similarity-based discovery methods are biased toward conserved disulfide arrays, whereas α-helical or linear AMPs do not necessarily present a primary sequence similarity significant enough to allow BLAST- or profile-based detection.

Some evidence supporting the involvement of peptides enriched in particular amino acids in bivalve immune response first surfaced with the report of short, secreted proline-rich peptides (CgPrp), which were found to be coexpressed with defensins in circulating hemocytes in *M. gigas*, synergistically enhancing their activity (Gueguen et al. 2009). A second, unrelated AMP was constitutively expressed in multiple tissues of the same species, and it was named molluscidin. This cationic peptide, similar to an AMP isolated in abalones, contained a series of dibasic repeats and exhibited broad-spectrum antimicrobial activity (Seo et al. 2013). The third and most recent case of linear cationic AMPs comprises myticalins and modiocalins from marine mussels pertaining to the *Mytilus* spp. and *Modiolus*

spp. genera, respectively. These AMPs, identified thanks to an in silico approach, display a broad spectrum of activity against Gram-positive and Gram-negative bacteria. Myticalins are produced as pre-propeptides and display a gill-specific pattern of expression, suggesting a possible function as modulators of the microbial communities associated with this important filtrating tissue (Leoni et al. 2017).

The last major category of AMPs comprises peptides generated by fragmentation of larger precursors with various nonantimicrobial functions. Two important examples are provided by an antibacterial peptide isolated from *Anadara kagoshimensis*, which is a fragment of hemoglobin I (Chen et al. 2017b) and by the N-terminal highly cationic fragment of the histone H2B (named molluscin), which appears to modulate the bacterial community in the gills of oysters and possibly other bivalves (Seo et al. 2011). Histone H4 may also have a role in bivalve immunity (Nikapitiya et al. 2013).

Sequence Hyperdiversity as an Effective Weapon to Fight Microbial Infection

In addition to interspecies variability, several bivalve AMPs are characterized by an unusually high degree of intraspecific diversity. For example, the diversity of myticin C was first observed by denaturing gradient gel electrophoresis (DGGE), because of the presence of unique characteristic band patterns in individual mussels (Costa et al. 2009a). It was later found out that this variability also matched nucleotide variation at the mRNA level and that about 8% of the codons within the myticin C sequence evolved under strong positive selection (Pallavicini et al. 2008; Padhi and Verghese 2008). This high level of polymorphisms has been also observed in other (but curiously not in all) mussel AMPs with targeted massive parallel sequencing (Rosani et al. 2011). Similar considerations are also valid for oyster and clams defensins, whose sequence variability can be linked to relevant directional selection pressures (Schmitt et al. 2010; Wang et al. 2015c). It is still not entirely clear whether this remarkable sequence diversity is due to a high number of paralogous genes, high allelic variability, RNA editing, or all of these factors combined. Furthermore, evidence collected from both oysters (Rosa et al. 2015) and mussels (Leoni et al. 2017) strongly hints that complex phenomena of gene presence/absence variability might partially explain the extreme diversification of antimicrobial effectors. Certainly, the presence of such a diversified arsenal of AMPs, apparently driven by selective forces, suggests that amino acid variations might have been evolutionarily exploited to broaden the spectrum of action of these molecules, endowing bivalve populations with effective weapons to face the challenge of microbial infection.

Lysozymes, Bactericidal/Permeability-Increasing Proteins, and Other Pore-Forming Molecules

Lysozymes

The term "lysozymes" is used to collectively describe a group of heterogeneous and widespread proteins involved in the animal innate immune system, which display

strong lytic action against bacteria. Although all lysozymes share a similar structural fold, they largely diverge in their primary sequence, which can therefore be used for classification purposes within three main classes: chicken-type (C-type), goose-type (G-type), and invertebrate-type (I-type) lysozymes (Callewaert and Michiels 2010). From a genomic perspective, it is now clear that genes encoding all three major lysozyme types can be simultaneously present in the same species, sometimes with several different variants, which might cover slightly different biological functions (Gerdol and Venier 2015). In spite of their remarkable primary sequence divergence, all lysozymes share the same glycoside hydrolase enzymatic activity, which catalyzes the hydrolysis of peptidoglycan and, to a lesser extent, chitin. As PGN is a main component of the bacterial cell wall in Gram-positive bacteria but not in Gram-negative bacteria, lysozymes display stronger activity against the former.

The first studies on bivalve lysozymes were conducted on I-type sequences, with the purification of chlamysin in the Arctic scallop, *Chlamys islandica* (Nilsen et al. 1999). Highly similar sequences, implicated either in immune response or in digestive processes, were later reported in several other bivalve species (Matsumoto et al. 2006; La Peyre et al. 2010; Yue et al. 2011; Ren et al. 2012). The isolation of the complete gene sequence of bivalve I-type lysozymes allowed in-depth phylogenetic analyses, which revealed a remote homology between this class of enzymes and vertebrate C-type lysozymes, hinting at an evolutionary origin from a common ancestor (Bachali et al. 2002). The discovery that different I-type paralogous genes in hydrothermal vent mussels play a crucial role not just in antimicrobial response but also in the management of symbiotic communities (Detree et al. 2016a) is one of the most significant recent developments in bivalve lysozyme research.

In comparison, bivalve C-type lysozymes have been the subject of little scientific attention, with only a handful of studies reported so far. Following its initial identification in *M. galloprovincialis* (Venier et al. 2009), this enzyme was characterized as an inducible gene product, capable of targeting a broad range of bacteria (Wang et al. 2013c).

The presence of G-type lysozymes, previously thought to be taxonomically restricted to vertebrates, was demonstrated in 2007 in the scallop *A. farreri* (Zhao et al. 2007). In the following years, G-type lysozymes have been genetically and partly also functionally characterized in scallops and mussels (He et al. 2012a; Wang et al. 2013c; Li et al. 2013b), evidencing that paralogous gene copies might have acquired a specialized function in either digestive or immune functions. As a unique known case in nature, a chimeric protein combining a C-terminal G-type lysozyme domain with an N-terminal PGRP domain has been identified in *M. gigas*. This protein, which might combine bacteria binding and lytic properties, was inducible in hemocytes in response to *Marinococcus halophilus* and *V. tubiashii* exposure (Itoh and Takahashi 2009).

More recently, a fourth type of lysozyme was identified in veneroid clams. This novel antibacterial protein surprisingly shared significant similarity with lysozymes produced by bacteriophages to break the PGN chains of the infected bacterial cell walls and release mature phages (Ding et al. 2014). An interesting comparative

study shed some light on the origin of this gene, revealing its co-option from viruses by horizontal gene transfer in two major bivalve groups, Heterodonta and Palaeoheterodonta. Following this event, the newly acquired sequences underwent complex genomic rearrangements, which overall contributed to increased antibacterial potential (Ren et al. 2017a).

Bactericidal/Permeability-Increasing Proteins

While lysozymes mainly target Gram-positive bacteria, a similar antibiotic action is exerted toward Gram-negative bacteria by Bactericidal/permeability-increasing proteins (BPIs), strong pore-forming agents found in nearly all metazoans. The specificity of action of BPIs is given by the recognition of LPS. The biological properties of *M. gigas* BPI (reminiscent of its vertebrate homologs) and its pattern of expression (broad distribution in different epithelia) suggested a role as a first line of defense in oyster mucosal immunity (Gonzalez et al. 2007b). Further genetic investigations revealed the presence of a second oyster gene copy, which displayed a slightly different expression pattern and functional specialization (Zhang et al. 2011d). Although the expression of BPIs can be positively regulated by LPS and bacterial challenges in oysters and ark shells (Zhang et al. 2011d; Mao et al. 2013), the molecular networks underlying this mechanism are still unknown. However, they are likely to be dissimilar to those involved in the production of lysozymes, which appear to be mostly downregulated under the same experimental conditions (Li et al. 2008; Ren et al. 2012), with some notable exceptions (He et al. 2012a; Wang et al. 2013c).

Might Pore-Forming Molecules Provide a Connection with the Complement System?

The possible connections with MAMP sensing by secreted and membrane-bound PRRs and maybe even with the primitive bivalve complement system remain to be fully elucidated. Because of the absence of convincing homologs of the molecular components of the terminal lytic pathway of the complement system, other pore-forming molecules are likely to cover a similar function in bivalve molluscs. While both lysozymes and BPIs could be involved, other options remain to be investigated.

A fascinating possibility is provided by different recently described cases. The first one, described so far only in the Mediterranean mussel, involves a protein containing a Membrane Attack Complex/Perforin (MACPF) domain structurally similar to that of C6/C7/C8/C9 proteins (Estévez-Calvar et al. 2011). Despite the negligible primary sequence similarity with these complement components, its upregulation strongly suggested an involvement in innate immune response. This observation gained even more importance with the report of over a dozen different similar gene products in the mussel transcriptome, which in some cases encode proteins where the perforin-like domain is associated with a PGN-binding ApeC domain (Gerdol and Venier 2015). The second class of molecules that might act as functional homologs to the complement terminal pathway are mytilectins (see section "The Role of Lectins in Immune Recognition"). Indeed, some mytilectins display a C-terminal aerolysin-like

pore-forming domain, which could be employed in the lysis of microbial cells (Gerdol and Venier 2015). While both the Ricin B/aerolysin and ApeC/MACPF domain combinations could potentially result in highly efficient and concerted recognition and killing of invading pathogens, further functional assays will clearly be needed to investigate the possibility that these molecules are involved in pathogen recognition and clearance in mussels and other bivalve species.

Proteases and Protease Inhibitors

An Overview on the Role of Proteases and Their Inhibitors in the Bivalve Immune System

Several important immune processes are regulated by the concerted action of proteases and their inhibitors, which might act either on endogenous proteins, by cleaving regulatory subunits and enabling their biological activity of their targets, or on exogenous proteins produced by invading microbes and parasites, leading to their inactivation and degradation. Some of the fundamental immune processes described in other sections, such as the complement system (see section "Evidence of an Ancient Complement System in Bivalves?"), the prophenoloxidase cascade leading to melanization (see section "The Phenoloxidase Cascade"), and apoptosis (see section "Apoptosis and Autophagy"), are essentially governed by a cascade of proteolytic activations, initially triggered by the recognition of MAMPs by PRRs. Although the molecular players involved in such cascades have been comprehensively characterized in some invertebrates, such as in the case of melanization in insects (Tang 2009) or hemocyte clotting in horseshoe crabs (Iwanaga et al. 1998), the nature of such proteases has not been entirely clarified in bivalve molluscs.

This can be partly explained by the lack of specific studies on the subject, but also finds a justification in the fact that these molecules pertain to large and multifunctional families of proteases involved in a multitude of other cellular processes, often not linked with immune response. As an example, while the core components of the bivalve complement system, as well as a remarkable number of lectin-like molecules, have been characterized in bivalves, no MASP-like proteases has been identified with certainty (see section "Evidence of an Ancient Complement System in Bivalves?"), leaving a huge gap of knowledge about the link between MAMP recognition in the extracellular environment and the activation of C3, even though several similar uncharacterized serine proteases are present in bivalve genomes (Wang et al. 2017b). Similarly, the nature and specificity of action of the bivalve prophenoloxidase-activating enzymes (see section "The Phenoloxidase Cascade") and the identity of the proteases involved in the process of activation of AMPs (see section "Antimicrobial Peptides") still remain uncertain. Big defensins, CRP-I, mytimycins, and myticalins, for example, possess a dibasic cleavage site, which could be potentially cleaved off by proprotein convertases (Gerdol et al. 2012, 2015a; Leoni et al. 2017). However, other mussel AMPs such as defensins, mytilins, and myticins lack a clear consensus motif for propeptide cleavage and are therefore expected to be the substrates of other, still unknown, proteases.

Cathepsins

While all of the aforementioned proteases mainly exert their biological action in the extracellular environment, others are typically present in lysosomal compartments, where they aid the phagocytic processing of heterophagic and autophagic material. Among these, cathepsins have been the subject of multiple studies and inked to immune functions in bivalves, consistently with the well-known role these proteases have in the regulation of vertebrate immune and cell death processes (Zavasnik-Bergant and Turk 2006; Repnik et al. 2012). In particular, multiple cathepsins have been characterized in the Chinese razor clam, *S. constricta*, where B-, C-, and L-type cathepsin were upregulated following *V. anguillarum* challenges in the mantle and, in particular, in the digestive gland (Niu et al. 2013a, b, 2014). Similar observations concerning tissue specificity and responsiveness to bacterial challenges have been also collected for a cathepsin L in *Cristaria plicata* (Hu et al. 2014), in contrast with a report from the Sidney rock oyster *S. glomerata*, where cathepsin B and L transcripts were mostly detected in hemocytes (Ertl et al. 2016).

Serine Protease Inhibitors: The Case of Oyster Perkinsosis

The infection process of many animal pathogens is also aided by a number of proteases, which target and inactivate host defense proteins and sometimes have more profound effects on the modulation of the host immune system (Armstrong 2006; Donnelly et al. 2011). In bivalve molluscs, this system has been best characterized in response to the parasite *P. marinus*, which produces proteases that specifically target defense plasma proteins, thereby impairing the immune response and creating favorable conditions for the establishment of infections by bacterial pathogens (Oliver et al. 1999; Tall et al. 1999). As a consequence, many bivalve species have developed large gene families of protease inhibitors to counteract the action of exogenous proteases produced by protozoans and other parasites (Romestand et al. 2002).

The serine protease inhibitors of the eastern oyster, *C. virginica* (CvSI) (Xue et al. 2009), pertain to the I84 family of serine protease inhibitors. These molecules have been implicated in resistance to *P. marinus* infections because of their high activity in oysters selected for increased survival in comparison with susceptible specimens (La Peyre et al. 2010) and their ability to inhibit the perkinsin pathogenic protease (Xue et al. 2006). Furthermore, a polymorphism located in the promoter region of the CvSI-1 gene was conclusively linked to its increased transcription and, consequently, to improved resistance to *P. marinus* (He et al. 2012b), and the expression levels of CvSI could also explain the interspecies differences in susceptibility to infection between *C. virginica* and the more resistant oyster species *Crassostrea corteziensis* (Gutiérrez-Rivera et al. 2015). Altogether, I84 serine protease inhibitors are part of a highly expanded and still rapidly evolving molluscan gene family (Xue et al. 2017a).

Kazal-Type Serine Protease Inhibitors and Tissue Inhibitors of Metalloproteinases

Kazal-type serine protease inhibitors are another large and widespread class of molecules that have been connected to immune functions in marine bivalves. These

molecules were reportedly upregulated in the hemocytes of the scallop *A. irradians* following tissue injury and bacterial challenges (Zhu et al. 2006). Another Kazal-type protease inhibitor from *A. farreri* contained 12 tandemly repeated Kazal domains and was upregulated upon *V. anguillarum* challenges (Wang et al. 2008), and two similar but shorter proteins could be similarly induced in the hepatopancreas of *R. philippinarum* and in multiple tissues of the clam *Mesodesma donacium* under similar experimental conditions (Maldonado-Aguayo et al. 2013; Yu et al. 2017). Like I84 inhibitors, Kazal-type inhibitors are produced by a multigenic family, whose members display different substrate specificity and sensitivity to stimulation (Zhang et al. 2014a).

The third large class of immunity-related protease inhibitors that has been studied in bivalves comprises the tissue inhibitors of metalloproteinases (TIMPs). Cg-TIMP, first identified in *M. gigas* because of its accumulation in hemocytes following shell injury and bacterial challenges (Montagnani et al. 2001), is activated through a DAMP-dependent pathway and is possibly regulated by NF-κB binding elements located in its promoter (Montagnani et al. 2007). The immune properties of TIMPs have not been investigated in other bivalve species, with the exception of the blood cockle *Tegillarca granosa*, where TgTIMP-4 is responsive to LPS, PGN, and *V. parahaemolyticus* challenges (Wang et al. 2012c).

These and other protease inhibitors might be involved in the management of microbial infections, as suggested by multiple reports of their upregulation from transcriptomic studies (Feng et al. 2010; Moreira et al. 2012a; Allam et al. 2014; Nikapitiya et al. 2014). However, the mode of action of just a few of these molecules has been properly functionally characterized. Therefore, protease inhibitors remain attractive targets for the study of host–pathogen interactions, in particular in the context of viral infections.

The Phenoloxidase Cascade

The recognition of MAMPs by PRRs, as well as various types of environmental stress, can trigger an extracellular proteolytic cascade, which leads to the conversion of prophenoloxidases (ProPO) to their active form, phenoloxidases (PO), copper-binding metalloproteins that catalyze the oxidation or hydroxylation of phenols. Different enzyme classes (tyrosinases, catecholases, and laccases) with low substrate specificity and similar activity exist in invertebrates, leading to a certain confusion in their unambiguous identification by biochemical tests on tissue extracts (Luna-Acosta et al. 2017). However, the activity of PO leads to the synthesis of the melanin pigment. This process, unique to a few invertebrate phyla, including arthropods and molluscs, enables the deposition of melanin on invading microbes, limiting the spread of infection. While the molecular players involved in the regulation of the melanization proteolytic cascade have been extensively studied and characterized in arthropods (Christensen et al. 2005; Tang 2009), limited information is available in molluscs (Luna-Acosta et al. 2017).

Secreted PGRPs are the main PRRs responsible for the activation of the ProPO cascade in *Drosophila* and other arthropods (Schmidt et al. 2008). However, as explained in section "Other Membrane-Bound Immune Receptors," while extracellular proteins with an N-acetylmuramoyl-L-alanine amidase domain are encoded by molluscan genomes, they seem to share closer similarities to those of vertebrates, where they play a direct bactericidal role. This divergence is in line with the major differences between arthropods and molluscs, which involve the interconnected TLR (with the lack of Spätzle; see section "Canonical TLR Signaling") and IMD pathways (see section "Other Immune Signaling Pathways").

In bivalves, the melanization process has been known for a very long time as a normal physiological process linked to shell deposition in pallial mantle epithelia (Waite and Wilbur 1976). However, increased melanization, usually followed by a massive rearrangement of extracellular matrix deposition and alterations in shell mineralization, is also among the most distinctive features of some common pathologies of the bivalve mantle tissue (see section "Major Infectious Diseases Affecting Bivalve Molluscs") (Ford and Borrero 2001; Paillard 2004). Further evidence supports the involvement of the ProPO cascade in response to parasitic, bacterial, and viral infection, as PO activity appears to be strongly altered in *M. sydneyi*–infected Sydney rock oysters (Raftos et al. 2014; Luna-Acosta et al. 2017). Melanization is probably not merely an extracellular event, as it might also be implicated in the intracellular killing of encapsulated microbes (Butt and Raftos 2008). Moreover, the different rates of inhibition of PO activity in the hemocytes of *M. gigas* and *Geukensia demissa* in response to *P. marinus* infections could be linked to the different degree of susceptibility of the two species to infection (Jordan and Deaton 2005). These observations support the important role of the ProPO cascade as a system of defense against microbial infections in bivalve molluscs.

The existence of an extracellular ProPO cascade linked to components of the hemolymph has been conclusively demonstrated in *M. gigas* and *Perna viridis*, where it could be induced by LPS, zymosan, and laminarin (Asokan et al. 1997; Hellio et al. 2007). However, a proper functional characterization of POs is still lacking in most bivalve species and the sequences of very few PO genes have been identified. This is ascribable in part to the broad distribution of PO activity in different tissues and life stages, including the digestive gland, the mantle and shell, and the foot, where POs are likely to cover specific functions that are yet to be fully unveiled (Luna-Acosta et al. 2011b). For example, tyrosinases pertain to a gene family which underwent significant expansion in bivalves and has been implicated in the shell mineralization process (Huang et al. 2017c; Chen et al. 2017a). However, a tyrosinase-like protein significantly contributes to PO activity in *S. glomerata* hemocytes (Aladaileh et al. 2007) and a tyrosinase-like transcript whose expression level was significantly overexpressed in response to bacterial challenges has been reported in *A. farreri* (Zhou et al. 2012). In the same species, a 576-kDa protein with PO activity, selectively inhibiting the growth of *Vibrio* spp. and *Aeromonas salmonicida*, has been purified from hemocytes (Xing et al. 2012). Interestingly, a protein with a similar molecular weight (555 kDa), displaying

p-diphenoloxidase activity, has been obtained from the hemocytes of a different scallop species, *A. irradians* (Jiang et al. 2011). Other studies have identified the hemocyte-specific PO enzyme as a laccase in *M. gigas* (Luna-Acosta et al. 2010, 2011a) and *R. philippinarum*, where only minor tyrosinase-like activity could be detected (Le Bris et al. 2013).

While the function of the ProPO cascade in the bivalve immune response has been fully established in relation to different diseases, this topic has been the subject of limited molecular studies and therefore still awaits detailed investigations to clarify which PRRs enable the melanization of invading microbes, both in the extracellular matrix and within phagocytic cells.

Cellular Immune Responses

Phagocytosis

Hemocytes Are the Main Cell Type Involved in the Phagocytic Process

Phagocytosis, encapsulation, and cell-mediated cytotoxicity have been extensively described in bivalves at a functional level and, more recently, at a genomic level (Schmitt et al. 2012; Soudant et al. 2013; Allam and Raftos 2015; Zannella et al. 2017; Schultz and Adema 2017) (Fig. 15).

During the early 1900s, the pathologist Metchnikoff used marine organisms, among other models, to describe and hypothesize the role of phagocytosis in digestion, immune defenses, and clearing of damaged cells (Gordon 2016; Schultz and Adema 2017). A dual role for bivalve hemocytes in digestion and immunity may be especially important during larval stages in bivalves, as suggested by evidence of phagocytic activity in early stages of larval development (Song et al. 2016). Moreover, hemocytes concentrate particulate material in the connective tissues surrounding the digestive glands in bivalve larvae (Dyachuk 2016). A more specific role for phagocytosis and encapsulation in disease resistance in bivalves has been hypothesized for Brown Ring Disease in clams, summer mortality in Pacific oysters, and QX disease (*M. sydneyi*) in Sydney rock oysters, based on in vitro observations of increased phagocytic function and/or upregulation of transcripts for genes putatively involved in phagocytosis in resistant bivalves compared with susceptible individuals (Allam and Ford 2006; Samain et al. 2007; Kuchel et al. 2010; Raftos et al. 2014).

Hemocytes are, by far, the best-studied phagocytic cells in bivalves. Flow cytometry has allowed for the development of high-throughput assays for the evaluation of hemocyte immune parameters in bivalves, including characterization of the populations of cells involved in phagocytosis of inert and biological particles and the subsequent stimulation of the oxidative burst response. Of the two major types of hemocytes described in bivalves on the basis of morphology, granulocytes in general seemed to be responsible for the majority of the phagocytic response and production of radical oxygen/nitrogen species (ROS/RNS), but this is highly dependent

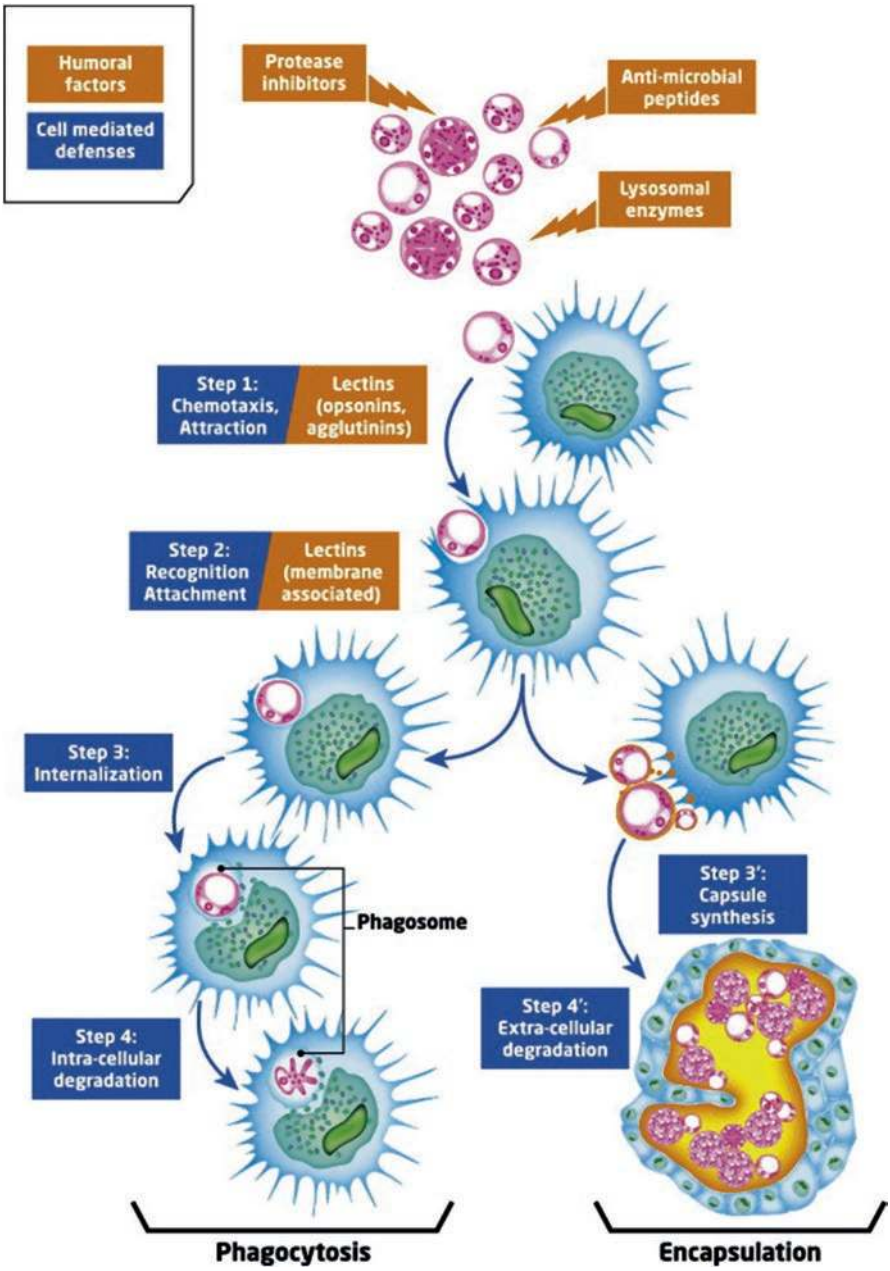


Fig. 15 The main humoral and cellular components of the bivalve immune response to microbial infection. The different steps of phagocytosis and encapsulation are shown in blue. Invading pathogens are indicated in purple, and humoral effectors (see section "Humoral Immune Effectors") are shown in green. (Source: Soudant et al. 2013)

on the bivalve species and the nature of the stimuli (Schmitt et al. 2012; Soudant et al. 2013; Allam and Raftos 2015; Zannella et al. 2017; Schultz and Adema 2017). Moreover, differences in the timing of phagolysosome fusion between eosinophilic and basophilic hemocytes in deepwater mussels indicate that these two types of granulocytes may play different roles in phagocytosis, suggesting further definition of phagocytic capabilities within hemocyte populations (Tame et al. 2015). An additional type of hemocyte, a hemoblast-like cell, may be involved in phagocytosis, composing a small percentage of all phagocytic cells in a hemocyte population and showing low levels of oxidative burst and lysosomal enzyme activity. Differences in the rates of phagocytosis by hemocytes also depend on the source of hemocytes within an individual (i.e., circulating hemocytes versus those present in the pallial or extrapallial spaces). Hemocytes have the ability to migrate through the epithelia into these cavities and then go back into the tissues, and those collected from the pallial cavity appear to have higher phagocytic activity than circulating hemocytes (Allam and Pales Espinosa 2016). These observations indicate that different populations of hemocytes may respond to selected stimuli and show different mechanisms of action (Evariste et al. 2016; Bettencourt et al. 2017; Vieira et al. 2017).

Other cells thought to have phagocytic capabilities are epithelial cells, with an ability that may be exploited by intracellular bacteria such as the Chlamydia- and Rickettsia-like organisms commonly seen in the gill and mantle epithelia of marine bivalves and gastropods (Allam and Pales Espinosa 2016). Development of specific cell markers will help us to understand if differences in phagocytic activity between cell populations within bivalves are due to the presence of specialized cell populations and/or the context in which these responses are occurring.

Phagocytosis in Detail: Chemotaxis, Opsonization, and Endocytosis

The process of phagocytosis involves the steps of chemotaxis, opsonization, endocytosis, formation of phagosomes, phagosome–lysosome fusion, respiratory burst, and exocytosis. Upon infection and injury, hemocytes migrate to the site of injury through the process of chemotaxis. Examples of bivalve pathogens causing massive focal infiltration of hemocytes at the site of infection include *V. tapetis* (Brown Ring Disease), *P. marinus,* and QPX. A chemotactic and/or chemokinetic response of hemocytes has been observed in response to several PAMPs, including bacterial endotoxins and extracts from trematodes and *P. marinus*. The nature of the chemotaxis/chemokinetic response depends on the type of PAMP (Schmitt et al. 2012; Soudant et al. 2013; Allam and Raftos 2015; Zannella et al. 2017; Schultz and Adema 2017).

Chemotaxis is followed by opsonization and phagocytosis. Transcriptomic analysis of Pacific oysters in response to LPS and other immune stimuli indicates that phagocytosis is promoted by a variety of opsonins (Zhang et al. 2012a). Several PRRs have been functionally demonstrated to mediate phagocytosis induction by immune stimuli through several signaling pathways (see sections "Recognition, Agglutination, and Opsonization" and "Signaling and Regulatory Pathways"). For example, an extracellular superoxide dismutase (Cg-EcSOD), highly abundant in oyster cell-free hemolymph, induces phagocytosis mediated by a β-integrin

(Duperthuy et al. 2011). Lectins from Manila clams (MCL and MCL4) stimulate the opsonization of *P. olseni* parasite and *V. tubiashii* bacterial cells and subsequent phagocytosis by clam hemocytes in vitro (Soudant et al. 2013; Zannella et al. 2017). Competitive inhibition of a sialic acid–binding immunoglobulin-type lectin (CgSiglec-1) inhibits the stimulation of phagocytosis and apoptosis by LPS in oyster hemocytes, consistent with the role of siglecs as regulators of immune responses (Liu et al. 2016a). Expression of genes involved in signaling pathways associated with integrin signaling and phagocytosis (PI3K, Rho J, MAPPK, PKC), phagosome maturation (Rab32), and respiratory bursts (NADPH oxidase) were upregulated upon secondary exposure to live *V. splendidus* after a primary challenge with killed *V. splendidus* (Zhang et al. 2014d).

Phagocytosis in Detail: Respiratory Burst and Exocytosis

The process of phagosome–lysosome fusion has been functionally observed in deepwater mussels (Tame et al. 2015). After phagosome–lysosome fusion, a respiratory burst ensues, followed by secretion of antimicrobial proteins (see section "Antimicrobial Peptides") (Soudant et al. 2013). On the basis of studies using enzyme activity measurements and the use of inhibitors, it appears that the mechanisms for production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are in general homologous to the ones observed in vertebrates (Soudant et al. 2013; Schultz and Adema 2017). The timing and extent of the respiratory burst in bivalve hemocytes, however, differs from those of the respiratory burst in vertebrate models. Moreover, bivalves and other marine invertebrates also show some differences from vertebrates in terms of the basal (not pathogen stimulated) generation of ROS as part of energy metabolism in organelles such as the mitochondria, endoplasmic reticulum, and peroxisomes (Donaghy et al. 2015). Sequencing studies indicate that, in addition to NADPH oxidase, bivalves contain genes similar to dual oxidase (DUOX, involved in immunity in *Drosophila*), which are upregulated in response to pathogenic vibrios. Bivalve hemocytes also show myeloperoxidase (MPO) activity (Schmitt et al. 2012; Donaghy et al. 2015). Radical nitrogen species, such as nitric oxide and peroxinitrite, also have an important role against pathogens in bivalves (Villamil et al. 2007). Nitric oxide also acts as an immune regulator (see section "Connections with the Neuroendocrine System"), enhancing phagocytosis, antibacterial activity, and apoptosis in bivalve hemocytes (Song et al. 2015). Expression of the single nitric oxide synthase (NOS) described in bivalve molluscs is modulated by immune stimuli (Song et al. 2015). In oyster hemocytes stimulated with zymosan, the NOS pathway is more active in hyalinocytes, while NADP oxidase activity is more prevalent in granulocyes (Lambert et al. 2007).

Antioxidant and detoxification enzymes are produced to protect cells from the toxicity of ROS and maintain redox homeostasis. Genome and transcriptome studies have led to the identification of the genes for five superoxide dismutases (SODs) in the Pacific oyster genome (He et al. 2015), two functional catalase genes in the oyster *M. hongkongensis,* and the genes coding for several glutathione peroxidases (GPxs) and gluthathione transferases (GSTs) (Sui et al. 2017; Wang et al. 2017a). Of the six known groups of superoxide dismutases, only manganese and copper/zinc

have been characterized so far in bivalves. Little is known, however, about the specific roles of these enzymes in immunity and disease resistance. An extracellular SOD from Pacific oysters, CgEcSOD, a major component of oyster plasma, shows both antioxidant and PRR activities and is able to promote the phagocytosis of the bacterial pathogen *V. splendidus* (Wang et al. 2017a). The expression of Mn and Cu/Zn SODs is upregulated with both viral and bacterial challenge, and alleles in the intracellular and extracellular Cu/Zn SOD have been associated with disease resistance to *Vibrio* infection in bay scallops (Wang et al. 2013b; Song et al. 2015; Wu et al. 2017).

Accessory Factors and Mechanisms of Regulation of Cell-Mediated Cytotoxicity

Other molecules shown to be involved in intracellular killing in the phagolysosome in bivalves include hydrolytic enzymes (β-glucuronidase, esterases, phosphatases, sulfatases, lipases), including unique versions of lysozymes showing tissue-specific patterns of gene expression (see section "Lysozymes, BPIs and Other Pore-Forming Molecules") and other antimicrobial molecules (phenoloxidases, antimicrobial peptides; see section "Antimicrobial Peptides") (Tanguy et al. 2013; Zannella et al. 2017). Phagocytosis and encapsulation are also aided by the prophenoloxidase system, a complex biochemical cascade occurring mainly in the hemolymph of bivalves, which is activated by microbial MAMPs, exogenous proteases, and environmental stress, leading to the formation of the antimicrobial molecule melanin (see section "The Phenoloxidase Cascade")

Little is known about the process of regulation of cell-mediated cytotoxicity in bivalves. A potential regulator of hemocyte function, thymosin beta-4, has been characterized in the oysters *M. hongkongensis* and *M. gigas,* and in the gastropod *Haliotis discus discus*. Treatment of oysters with recombinant protein led to increased numbers of circulating hemocytes, increased bacterial clearing, reduction of ROS production, and increased production of antioxidant enzymes, suggesting a potential role in wound healing (Li et al. 2016a). Dysregulation of the oxidative burst, on the other hand, may be involved in the pathogenesis of several diseases affecting marine bivalves. For example, oxidative stress resulting from a strong oxidative burst response, characterized by a strong upregulation of oxidase genes and downregulation of antioxidant genes, may contribute to the pathology seen in larval and juvenile oysters experimentally challenged with OsHV-1 µVar (He et al. 2015; Young et al. 2017) or infected with the bacterial pathogen *A. crassostreae* (McDowell et al. 2014).

Mechanisms of Evasion Adopted by Invading Pathogens

Several pathogenic and nonpathogenic vibrios, *Chlamydia* and Rickettsia-like organisms, and the protozoan parasites *B. ostreae*, *P. marinus*, and *P. olseni* appear to have evolved mechanisms to evade cell-mediated cytotoxicity in bivalves, exploiting that ability to survive within host tissues. Potential mechanisms used to evade phagocytosis and encapsulation include dysregulation of immune signaling through phosphorylation of p38-MAPK and induction of apoptosis of hemocytes

(Ciacci et al. 2017; Burgos-Aceves and Faggio 2017). Other microbes can avoid intracellular killing by respiratory burst pathways in bivalve molluscs (Schmitt et al. 2012; Soudant et al. 2013; Allam and Raftos 2015). The enzymes arginase, alkaline phosphatase, ascorbate-dependent peroxidase, and superoxide dismutase are several of the factors potentially involved in the ability of *P. marinus* to inhibit ROS production in oyster hemocytes and survive in vitro exposure to ROS (Schott and Vasta 2003; Schott et al. 2003; Fernández-Robledo et al. 2008) (Fig. 16). The parasite is also resistant to high concentrations of nitric oxide (Villamil et al. 2007). The natural resistance–associated macrophage protein (NRAMP) in *P. marinus,* involved in iron uptake in *P. marinus* trophozoites, is hypothesized to deplete iron in hemocytes, limiting the ability of hemocytes to mount an effective respiratory burst (Lin et al. 2011). Moreover, the wall of parasites such as *P. olseni* appears to be resistant to proteolysis (Montes et al. 2002). Extracellular products from a pathogenic strain of *V. splendidus* inhibit phagocytic activity in mussel *M. edulis* hemocytes, while those of a nonpathogenic strain do not (Ben Cheikh et al. 2016). Some metazoan parasites such as the digenean trematodes *Bucephalus* sp. and

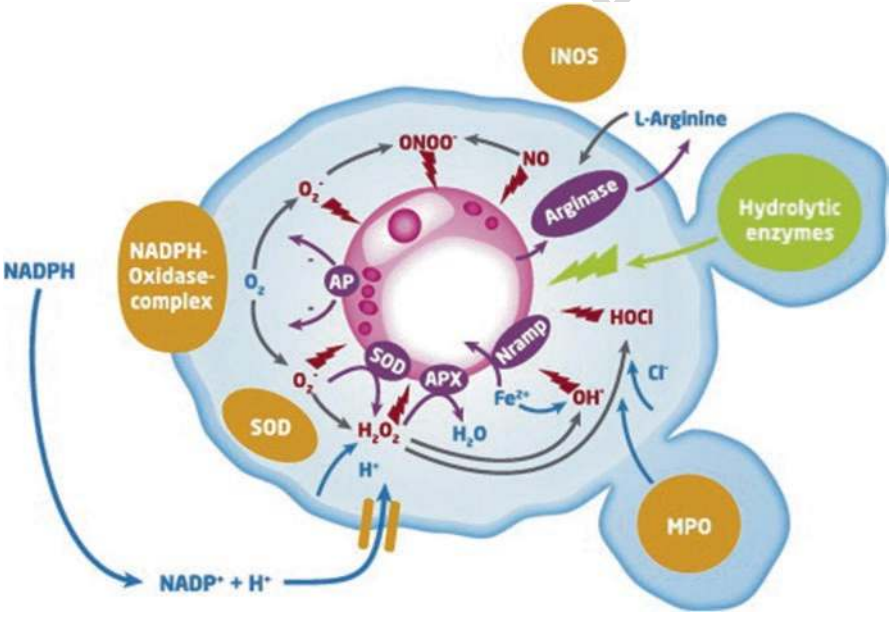


Fig. 16 Interaction between prooxidant (orange) and antioxidant (purple) activities in the phagosome of an hemocyte from the eastern oyster, *Crassostrea virginica* (blue), upon phagocytosis of the protozoan parasite *Perkinsus marinus* cell (purple). Prooxidant activities are exerted by hemocytes to kill the invading microbe by exposure to ROS (red), whereas antioxidant activities are used by *P. marinus* to escape these defensive measures. AP acid phosphatase, APX ascorbate-dependent peroxidase, HOCl hypochloride, iNOS inductible nitric oxide synthase, MPO myeloperoxidase, NO nitric oxide, Nramp Natural Resistance–Associated Macrophage Protein, O2− superoxide anion, ONOO− peroxynitrite, SOD superoxide dismutase. (Source: Soudant et al. 2013)

Proctoeces maculatus may also modulate hemocyte function in bivalve hosts, leading to decreased hemocytic infiltration in infected tissues (Carella et al. 2015).

Encapsulation and Granuloma Formation

The processes of encapsulation and granuloma formation occur when particles or pathogens are too large to be engulfed by hemocytes (e.g., in infection by trematodes) or the phagocytosis response is unsuccessful (e.g., in infection by *Perkinsus* spp. or *Nocardia* spp.). In the process of encapsulation, hemocytes recruited to the site of infection surround and encapsulate the invading pathogen, secreting extracellular matrix products to prevent dissemination of the pathogen to other tissues and a variety of lysosomal enzymes and antimicrobial molecules to attempt to kill it (Soudant et al. 2013; Allam and Raftos 2015; Carella et al. 2015). This process can occur within the tissues, leading to granuloma-like formation, or within the extrapallial space between the mantle and the inner side of the bivalve shell, leading to conchiolin or pearl formation (Carella et al. 2015). Examples of diseases leading to granuloma formation include trematode infestations, Perkinsosis in *Ruditapes* clams, QPX in the quahog *M. mercenaria*, and fungal infections in Sydney rock oysters (Soudant et al. 2013; Allam and Raftos 2015). Diseases characterized by conchiolin formation include Roseovarius or Juvenile Oyster Disease and Brown Ring Disease in *Ruditapes* clams (Allam and Pales Espinosa 2016). On the basis of morphological differences it has been hypothesized that specialized populations of hemocytes may be responsible for encapsulation (Allam and Raftos 2015). In *Ruditapes* clams infected by *P. olseni*, granulocytes secrete (from membrane-bound granules) a polypeptide named p225, which surrounds encapsulated parasites and restricts parasite proliferation (Montes et al. 2002). Consistent with the importance of hemocytic infiltration in diseases characterized by granuloma-like formations, transcriptomic studies have shown differential expression of genes involved in hemocyte migration, pathogen recognition and binding, and inflammation (McDowell et al. 2014; Allam et al. 2014; Wang et al. 2016a, b).

The process of shell formation aids in encapsulation in the extrapallial cavity, playing an important role in immune defenses by preventing the penetration of pathogens through the mantle of bivalves. The process of shell formation in bivalves involves the secretion of organic molecules by secretory cells in the epithelium of the mantle outer fold, which provide a matrix for the deposition of calcium carbonate in a variety of structures, depending on the bivalve species. Hemocytes also play an important role in shell formation. A population of granulocytes containing calcium carbonate stored in granules migrate into the extrapallial space upon shell injury, forming aggregates at the biomineralization edge, which are incorporated into the shell as it forms (Mount et al. 2004; Zhang et al. 2012a; Li et al. 2016a). The fact that about 45% of the domains identified in the shell proteome of bivalves are related to immune function indicate the importance of the shell in bivalve immune defenses (Arivalagan et al. 2017). Among the organic compounds (1–5% of the total shell) that are embedded in the calcium carbonate structure that makes the shell, many immune-related molecules are worthy of mention, including PRRs such as galectin, scavenger receptor and C1q-related proteins, and effectors such as

phenoloxidases, proteases, and protease inhibitors (Zhang et al. 2012a; Arivalagan et al. 2017; Calvo-Iglesias et al. 2017). Moreover, genes coding for the shell proteins are differentially expressed in oysters challenged with *A. crassostreae* and in Manila clams infected with *V. tapetis*. These two bacterial pathogens preferentially attach to the inner side of the shell in bivalves, and the diseases they cause are characterized by the formation of conchiolin (McDowell et al. 2014; Allam et al. 2014).

Apoptosis and Autophagy

The Profound Implications of Apoptosis in Bivalve Physiology and Pathology

Apoptosis, a form of programmed cell death, is a highly evolutionarily conserved process involving two major distinct but converging pathways, the death-receptor-mediated pathway (an extrinsic pathway) and the mitochondrial pathway (an intrinsic pathway). Apoptosis plays an important role in immune responses by preventing the proliferation of intracellular pathogens, limiting inflammation, and being involved in the activation of certain immune cells, such as neutrophils in vertebrates (Poon et al. 2014; Creagh 2014). On the basis of changes in apoptosis levels in response to a variety of environmental stimuli, apoptosis is thought to play key physiological roles in molluscs, such as maintenance of tissue homeostasis; processing and clearing of environmental pollutants; combating of bacterial, viral, and protistan pathogens; and adjustment to exposure to insecticides, herbicides, and pharmaceuticals (Kiss 2010; Moreau et al. 2015; Romero et al. 2015; Carella et al. 2015; Zhang et al. 2016a). The functional relevance of apoptosis modulation by pathogens and environmental stressors in bivalves, however, is still unclear, since the effect of challenge/exposure on apoptosis levels is not always consistent (Soudant et al. 2013). For example, exposure to *Perkinsus* spp. modulates apoptosis in oyster and clam hemocytes and tissues, but the nature of the modulation depends on the bivalve species and the stage of infection. Advanced stages of *P. marinus* infection in *C. virginica* are generally characterized by suppression of apoptosis, which is, on the other hand, enhanced at early stages of infection (Sunila and LaBanca 2003; Goedken et al. 2005; Hughes et al. 2010; Wang et al. 2017a). Interestingly, the protozoan parasite of eastern oysters *P. marinus* expresses many antiapoptotic genes in response to exposure to oyster pallial fluid, suggesting that this parasite may be able to regulate apoptosis in the host (Pales Espinosa et al. 2014). Basal rates of apoptosis in oysters also differ between the source of hemocytes, ranging from 5–25% in hemocytes in hemolymph to up to 50% in hemocytes within tissues (Sunila and LaBanca 2003; Goedken et al. 2005; Cherkasov et al. 2007; Sokolova 2009)

Main Molecular Players in the Apoptotic Process

Although the major molecules and pathways of apoptosis appear to be conserved between bivalves and other species on the basis of genomic studies (Fig. 17), only a few of them have been characterized functionally. These include the executioner caspase-3 and caspase-1 (caspase-7-like) from *M. gigas,* which appear

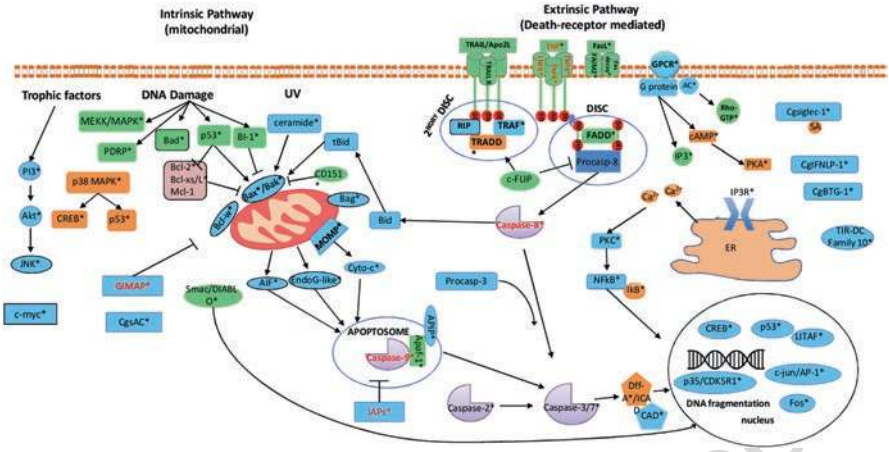


Fig. 17 Apoptosis pathway molecules, with those identified in molluscs indicated with asterisks. Genes identified only in *M. gigas* are prefixed by "Cg" and expanded gene families are shown in red *text*. Molecules that have been only preliminarily identified in molluscs via the eastern oyster genome annotation are denoted with "-like" and genes that been implicated in caspase-independent mechanisms are outlined in black. (Kögel et al. 2013)

to act as intracellular LPS receptors (Xu et al. 2016b; Wang et al. 2017a). Interestingly, bivalves may possess a caspase-independent apoptotic pathway, hypothesized to be involved in apoptosis induced by the protozoan parasite *P. marinus* (Wang et al. 2017a).

Several gene families involved in the apoptotic process have experienced lineage-specific expansions, including tumor necrosis factors (TNF), tumor necrosis factor receptors (TNFRs), caspase 8, inhibitor of apoptosis proteins (IAPs), cysteine-aspartic proteases (caspases), and GTPase of the immune-associated proteins (GIMAPs) (Zhang et al. 2012a; Qu et al. 2015b; McDowell et al. 2016; Li et al. 2016b; Wang et al. 2017a). Enhanced genetic diversity of these apoptosis pathway gene families may allow for more diverse but also pathogen-specific functional responses to disease and therefore increase the ability of apoptosis pathways to aid in stress mitigation and increase survival. For example, while oyster *M. hongkongensis* Chcaspase8s is upregulated with bacterial challenge, *M. gigas* Cgcaspase8–2 responds to viral challenge but not bacterial challenge (Wang et al. 2017a).

Two of these gene families, coding for IAPs and GIMAPs (also known in plants as immune-associated nucleotide-binding genes, or IANs), are of particular interest because of their known critical apoptosis regulatory roles in other organisms, their high level of transcript diversity in bivalves, and their demonstrated differential expression in bivalves after immune challenge. The GIMAP/IAN family has 26 annotated members in *M. gigas,* similar to the predicted 26–28 GIMAPs in the eastern oyster, several of which are downregulated in eastern oyster juveniles after challenge with Roseovarius Oyster Disease (ROD), suggesting an upregulation of apoptosis (McDowell et al. 2016). The functional significance of this expansion in bivalves is unknown, but GIMAPs are known to play key roles in regulation of

lymphocyte survival, T-cell selection and homeostasis, phagolysosomal processing and membrane trafficking in vertebrates, and pathogen resistance in the model plant system *Arabidopsis* (Weiss et al. 2013; Webb et al. 2016).

The CgIAP family represents another expanded apoptosis-related family in oysters, with 48 gene members, likely the result of tandem gene duplications (Qu et al. 2015b; Zhang et al. 2016a; Wang et al. 2017a). IAP proteins have known roles in apoptosis inhibition by interacting with caspases, and direct evidence of this interaction has been shown for CgIAP2, where its characteristic BIR2 domain directly interacts with Cgcaspase-2 (Zhang et al. 2011b; Qu et al. 2015b). Bacterial challenges of the Pacific oyster with the bacterial pathogen *V. anguillarum* have shown increased gene expression over time (Zhang et al. 2011b; Qu et al. 2015b). When two families of Pacific oyster with different susceptibility to ostreid herpesvirus-1 (OsHV-1) were exposed to this virus, CgIAP expression was significantly upregulated in both families though with higher levels of expression in the family most sensitive to OsHV-1 (Zhang et al. 2016a). Another gene family with potential roles in apoptosis worth mentioning here is the TIR-DC family 10, characterized by the presence of two baculovirus inhibitor of apoptosis protein repeat (BIR) domains. This gene family has been found only in bivalves (Gerdol et al. 2017).

Potential Involvement of Autophagy in Immune Response

Not much is known about the role of other forms of programmed cell death in innate immune responses in bivalves. Autophagy, which is involved in innate immunity against intracellular pathogens in vertebrates, is induced in oysters in response to bacterial and viral challenge, as well as environmental stimuli such as changes in salinity, hypoxia, toxins, or lack of nutrition (Carella et al. 2015; Wang et al. 2017a). Genes in the autophagy (ATG) pathway have been described in Pacific oysters, and autophagy is involved in survival after challenge with OsHV-1 and *V. aestuarianus,* two pathogens commonly associated with summer mortality in the Pacific oyster, *M. gigas.* Interestingly, while challenge with OsHV-1 led to induction of autophagy, challenge with *V. aestuarianus* resulted in inhibition of autophagy (Moreau et al. 2015).

Overview of the Immune System of Other Molluscan Classes

We have so far outlined the main molecular and cellular components of the immune system of Bivalvia, the second largest molluscan class. Bivalves have been the subject of extensive immunological research over the past few decades, motivated by the high socioeconomic importance of edible species, their widespread distribution, and their amenability for laboratory research. The largest molluscan class in terms of the number of species, gastropods, has attracted considerable attention for similar reasons. These animals—adapted to the freshwater, marine, and terrestrial environments—present astounding morphological diversification, including snails, slugs, limpets, nudibranchs, and others. This diversity can be correlated with the adaptation of lineage-specific strategies for immune defense, which in some cases has led

to the acquisition of unique traits and advanced mechanisms, such as the somatic diversification of FREPs. The main features of the gastropod immune system are presented in detail in Chap. 12.

Unfortunately, very little information is available concerning several aspects of the basic biology of the other molluscan classes, such as aplacophorans, monoplacophorans, polyplacophorans, and scafopods. Consequently, the immune systems of these animals and the possible peculiar survival strategies that might have been developed in these taxa during their evolution are presently unknown. The few data collected so far concern cellular immunity of chitons, where phagocytic cells located in circulating hemolymph, as well as in connective tissue, seem to bear remarkable immune recognition properties (Crichton et al. 1973; Crichton and Lafferty 1975).

The exception is represented by cephalopods, which have historically attracted major scientific attention, in particular due to their complex nervous system, intelligence, and learning skills. However, immune studies are also emerging, as evidenced by the conspicuous amount of literature produced on this subject over the past few years. The following sections will review the most distinctive peculiarities of the cephalopod immune system of these fascinating animals.

A Short Journey in the Immune System of Cephalopods

Cephalopods (i.e., nautiluses, cuttlefish, squid, and octopuses) comprise over 800 living species (Sweeney and Roper 1998), about 300 belonging to Octopodidae (Jereb and Roper 2016) and including several species complexes (Allcock et al. 2011; Amor et al. 2014; Cheng et al. 2014; Sales et al. 2017). They are considered to rival vertebrates (Packard 1972) for physiological adaptations, complex neural organization, and behavior (Jereb and Roper 2005, 2010; Huffard 2013; Jereb and Roper 2016; Marini et al. 2017). The immune system of cephalopods consists of innate mechanisms and includes cellular and humoral defenses (Ford 1992; Castillo et al. 2015; Pila et al. 2016).

The Highly Complex Circulatory System of Cephalopods

This molluscan taxon is the sole group of animals, other than vertebrates, to enjoy a fully enclosed high-pressure blood system, an example of convergent evolution (Wells 1983). Three hearts (one systemic and two branchial) move blood through an extraordinarily complex network of arteries, veins, and capillaries (Fig. 18), thus representing "a triumph of engineering over design" (Wells and Smith 1987). An overview on the physiology of the circulatory system and its development is available in a number of works (Naef 1928; Boletzky 1968; Wells 1983; Budelmann et al. 1997).

Morphology and Function of Cephalopod Hemocytes

In contrast to bivalves, the circulating blood (hemolymph) in cephalopods turns blue when oxygenated (Wells 1983) because of the presence of hemocyanin. The

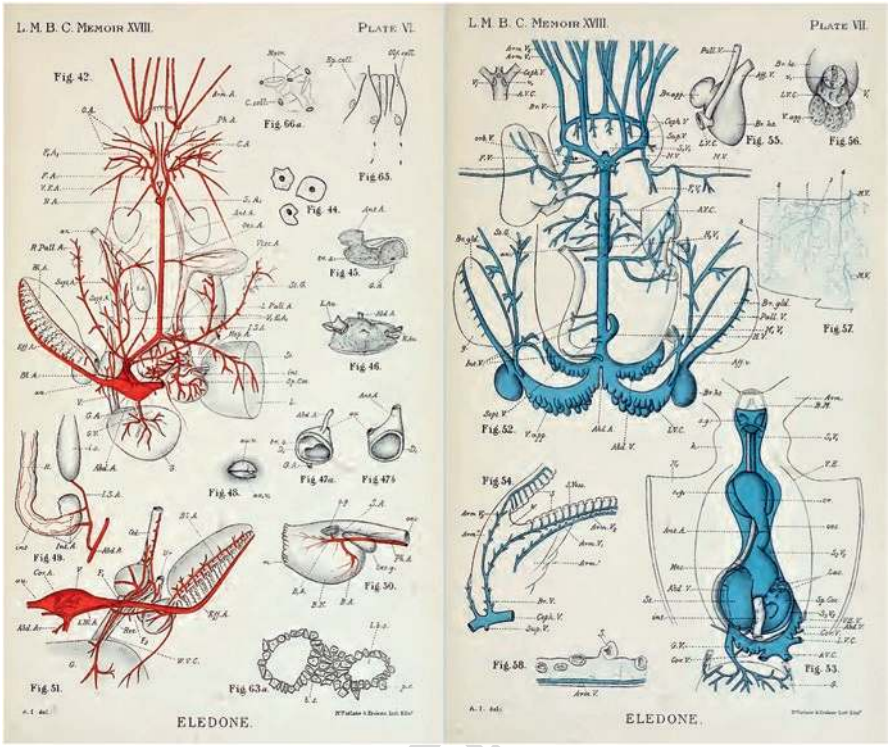


Fig. 18 General outline of the cephalopod circulatory system as exemplified for *Eledone cirrhosa* by Isgrove (1909). In coleoids (cuttlefish, squid, and octopuses), three hearts exist: the systemic heart pumps oxygenated blood (red); the two branchial hearts move blood through the capillaries of the gills (Wells 1983). An extraordinary network of arteries (red), veins, and capillaries exist in cephalopods. The venous system (blue, right) is shown with the principal cephalic vein, pallial veins, three venae cavae, and a large perivisceral blood sinus. In *Nautilus* the circulatory system (not shown) is characterized by large venous spaces, i.e., the pericardium (Owen 1832), differently from what occurs in coleoids

hemocytes—also named leukocytes (Bolognari 1949, 1951), amoebocytes, or granulocytes (Budelmann et al. 1997)—are the "key" cellular components of the immune system of cephalopods. In an analogy to other molluscs, the identification of cellular types in cephalopods and their characterization is often contradictory, since their classification may be biased by the technique that is utilized (Vieira et al. 2017). Furthermore, the variability in observed cells may reflect the physiological status of the animals (Bolognesi and Fenech 2012; Locatello et al. 2013; Castellanos-Martínez et al. 2014b). Attempts to develop a consensus on the nomenclature of hemocytes have been made for some molluscan species (Cheng 1984) but are still lacking for cephalopods. However, we outline their general description on the basis of the few reports available (Fig. 19).

Budelmann et al. (1997) described two types of cells in cephalopod hemolymph. The first type of hemocytes are round or oval cells, with an elongated V-shaped

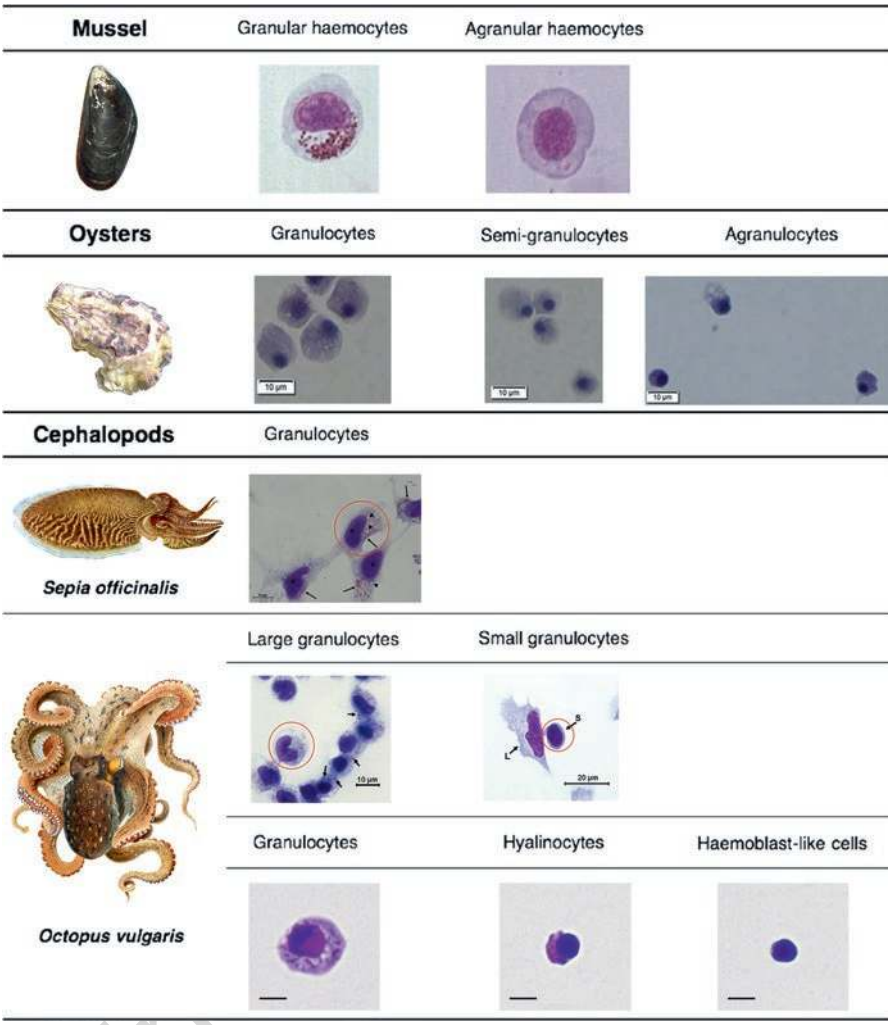


Fig. 19 The different types of hemocytes identified in cephalopod molluscs. Examples from bivalves are provided for comparison. See also Table 2 for further detail. The drawings are based on the original descriptions provided for mussels by Bolognesi and Fenech (2012), for oysters by Wang et al. (2017a), and for the cephalopods *Sepia officinalis* and *Octopus vulgaris* by Le Pabic et al. (2014a) and by Castellanos-Martínez et al. (2014b) and Troncone et al. (2015), respectively

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nucleus, known to extend large pseudopods producing amoeboid locomotion and capable of a phagocytic response and the secretion of pore-forming lysins and cytotoxic oxygen radicals by exocytosis of small granules (Budelmann et al. 1997). The second type include vacuolized round cells, which are relatively sessile (they do not display pseudopods), accumulate into large agglomerates, and are similar in size and shape to hemocytes. Each cell has either numerous small lysosomes or a single large lysosome. They are able to incorporate particles through micropinocytosis.

Vacuolized round cells are thought to correspond to the pore cells of other molluscs and to the monocyte–macrophage system of vertebrates (Budelmann et al. 1997).

Troncone and colleagues (2015) recognized three types of hemocytes in *Octopus vulgaris*: hemoblast-like cells, hyalinocytes, and granulocytes. According to those authors, the hemoblast-like cells are the smallest ones, not motile and without pseudopodia. Hyalinocytes are described as variable in size, with a rounded or oval nucleus, and no or few granules and vacuoles of different diameters in the cytoplasm. The cells are capable of amoeboid movement and can form pseudopodia. Granulocytes are variable in size, highly amoeboid, and able to form many long filopodia. Granulocytes are described as being characterized by an eccentric oval nucleus and numerous cytoplasmic granules of different sizes (endoplasm), while no granules are found in the ectoplasm (Troncone et al. 2015).

In coleoids (cuttlefish, squid, and octopuses) the hemocytes originate from the white body (Bolognari 1949, 1951; Cowden 1972; Cowden and Curtis 1973), a multilobed organ covered by a thin layer of connective tissue surrounding, as cushions, the optic lobes and located in the "orbits" in the head of the animal. White bodies extend between the medial external surfaces of the eyes and the skull, and encapsulate the "central brain." The morphology, structure, and function of this organ were originally described by Bolognari (1949, 1951). A pioneering attempt to isolate the cellular components and to estimate their mitotic activity and culturing in vivo was carried out by Necco and Martin (1963). Further characterization of this organ in the octopus was provided by Cowden (1972), including ultrastructural analysis (Cowden and Curtis 1974). A functional description of the white bodies is also available for *S. officinalis* (Claes 1996) and for sepiolids (see below), while no analogous structures are known in *Nautilus*, to the best of our knowledge.

After histological examination, the white bodies appear as a network of connective fibers, blood vessels, and vascular varosities in which a mass of cellular strings is observed. These are believed to be precursors of the hemocytes (Bolognari 1949, 1951; Cowden 1972). Leukocytes at different stages of "maturity" are identified in the white bodies of *O. vulgaris* (Cowden 1972). According to the classical ultrastructural description, the hemocytoblasts (or reticulum cells of the white bodies) are characterized by an abundant "rough" endoplasmic reticulum, mitochondria, and Golgi, and an irregular large vesicle reported to "contain some internal fibrillar material condensed" in some areas (Cowden and Curtis 1974). These authors also provided a thorough description of other cellular characteristics, and of the transformation of hemocytoblasts to form primary and secondary leukoblasts, and finally mature leukocytes. This last cell type appears to have a folded nucleus containing an abundance of condensed chromatin ... and dense extrachromosomal aggregates. The cytoplasm contains a number of electron-dense, rounded inclusions," possibly derived from the reduction of vesicles characterizing the hemocytoblasts (Cowden and Curtis 1974).

Two main groups of hemocytes are recognized in cephalopods: cells containing many granules (granular hemocytes or granulocytes), and cells with few or no granules (agranular hemocytes, agranulocytes, or hyalinocytes). These correspond to the two types of cells described by Budelmann et al. (1997).

The octopus hemocytes (sensu lato) act as immunocompetent cells in the hemolymph (Ford 1992). They are involved in the recognition and elimination of potential pathogens through phagocytosis, encapsulation, infiltration, and production of reactive agents with oxidizing capacity (i.e., reactive oxygen species (ROS) and reactive nitrogen species (RNS)). Hemocytes are also involved in scar formation, wound healing, and tissue repair by migrating to the site of injury, increasing in number and activity and forming plugs at the wound site to prevent hemolymph loss (Polglase et al. 1983; Féral 1988; Shaw et al. 2016; Imperadore et al. 2017).

The composition and number of hemocytes are highly variable both among species (Le Pabic et al. 2014a) and between individuals (Malham et al. 1998, 2002; Locatello et al. 2013; Roumbedakis et al. 2017) in an analogy to other molluscs (Anisimova et al. 2017). The number of circulating hemocytes appears variable among different individuals following "stressors" such as handling (Malham et al. 1998, 2002), immune challenge (Locatello et al. 2013), or life stages (Roumbedakis et al. 2017). Phagocytosis is known as the primary immune response of hemocytes and has been reported in various species, e.g., *Sepia officinalis* (Le Pabic et al. 2014a), *O. vulgaris* (Novoa et al. 2002; Rodríguez-Domínguez et al. 2006), and *Eledone cirrhosa* (Malham et al. 2002).

Molecular Immunology Studies Are Still at Their Embryonal Stage in Cephalopods

The humoral defense is achieved through soluble molecules (Castillo et al. 2015) such as opsonins, agglutinins, proteolytic enzymes, protease inhibitors, antimicrobials or cytotoxic compounds, phenoloxidase, and its intermediate synthesis products, which are in part similar to those described in detail for bivalve molluscs in the previous sections (Rögener et al. 1985; Lacoue-Labarthe et al. 2009; Alpuche et al. 2010; Le Pabic et al. 2014b; Roumbedakis et al. 2017). However, as evidenced by recent transcriptomic approaches, a relevant fraction of lineage-specific genes with unknown function exists in cephalopods. This observation is particularly relevant considering large high number of unknown mRNAs identified in the transcriptomes obtained from *O. vulgaris* hemocytes (Castellanos-Martínez et al. 2014a) and the white bodies of the sepiolid *Euprymna tasmanica* (Salazar et al. 2015).

Salazar and colleagues (2015) also provided a description of putative *Euprymna* immune-related genes, identifying—for example—NF-κB and components of the Toll signaling pathway, pattern recognition proteins, TNF-receptor-associated factors, and proteins denoting membrane attack complex/perforin domains, which in large part mirror those described in bivalves (see sections "Recognition, Agglutination, and Opsonization," "Signaling and Regulatory Pathways," and "Humoral Immune Effectors").

Although the cellular and "humoral" components of cephalopods have been studied extensively (Castillo et al. 2015), our knowledge of cephalopod immunity is still in its infancy. In brief, evidence exists for (1) a possible role of the white bodies as a hematopoietic and immune organ, and (2) the presence of different types and numbers of circulating cells after challenges. Molecular fingerprints for the immune response have been so far explored only in a limited way (Collins et al.

2012b; Castellanos-Martínez et al. 2014a; Salazar et al. 2015). Preliminary evidence collected over the past few years suggests that cephalopod immunity, like that of other molluscs (see Chap. 12, section "Molluscs Exhibit Immune Priming with Intermediate Degrees of Specificity, and Involving a Plethora of Mechanisms" for a detailed discussion), may show some form of memory. The analysis of the plasticity of innate immune responses in these fascinating organisms is one of the most important future avenues for cephalopod science and, in particular, for immunological studies.

Bobtail Squid as a Model for the Study of Bacterial Symbiosis

The capacity of an animal's immune system to recognize and remove nonself is crucial for its survival and, by tradition, this has been the context in which we have defined immune components, and even how we have designed experiments to understand their roles. This is easy to envision when one considers the detrimental presence of microorganisms to the host, either because of nutrient competition or tissue damage. This kind of association is, by definition, usually considered pathogenic, but this is just one of the three types of symbiotic relationships an animal can establish with another species. The other two types are commensalism (where one species benefits and the other neither benefits nor gets harmed) and mutualism (a type of beneficial relationship between two species, in which both obtain some type of benefit). An animal can establish any one of these associations with the immense variety of microorganisms that share its ecological niche, i.e., bacteria, protozoans, helminths, fungi, or viruses. This section focuses on the major findings resulting from 30 years of study of one of these beneficial interactions, the *Euprymna scolopes–Vibrio fischeri* symbiosis. This model has somewhat challenged our vision on the role of the immune system in metazoans.

The squid–*Vibrio* symbiosis is one of the most studied and better understood binomial associations between an animal and its bacterial symbionts (McFall-Ngai 2008; Castillo et al. 2015; McAnulty and Nyholm 2017; Stabb and Visick 2013; Norsworthy and Visick 2015; Mandel and Dunn 2016). In addition, modern sequencing and proteomic technologies have recently allowed the identification of several molecular players participating in the squid's immune system (Chun et al. 2006; Wier et al. 2010; Collins et al. 2012a, b; Kremer et al. 2013; Salazar et al. 2015). The next paragraphs contain a brief description of this symbiosis, followed by specific information on the molecular players involved, with emphasis on the squid host immune components.

Main Features of the Squid–Vibrio Symbiosis

This mutualistic symbiosis involves the squid *E. scolopes*, also known as the bobtail squid, a relatively small (adult mantle length ~3–4 cm), nocturnal sepiolid species, native to the Hawaiian archipelago (Berry 1912) (Fig. 20, panel b1). The symbionts are Gram-negative marine Proteobacteria members of the Vibrionaceae family, capable of producing bioluminescence by means of luciferase activity under

quorum-sensing conditions. The bacteria reside in the squid in a specialized bilobed structure called the light organ (LO) (McFall-Ngai and Montgomery 1990). The LO is localized on the ventral side of the animal and inside the muscular mantle, just above the funnel or siphon (Fig. 20, panels a1-2, b1-2). In this location, the LO is flushed with ocean water during regular breathing or swimming movements of the mantle. Microorganisms present in the water, including *V. fischeri*, come in direct contact with the LO surface which, in response to bacterial compounds such as lipopolysaccharide (LPS) and peptidoglycan (PG), secretes mucus to which bacteria attach and start aggregating (Nyholm et al. 2000; Foster et al. 2000) (Fig. 20, panel a2). Several studies have found that the mucus contains chemoattractants (N-acetylgalactosamine and N-acetylneurominic acid) (Altura et al. 2011; Mandel et al. 2012), as well as soluble antimicrobials and nitric oxide (Davidson et al. 2004; Kremer et al. 2013). Together, these host-derived products are thought to favor *V. fischeri* attachment while discouraging nonsymbiont organisms from collecting at the site. In addition, the LO of juvenile *E. scolopes* is characterized by having on either side a pair of appendages made from densely ciliated epithelial cells where the mucus is held (Fig. 20, panel b2). The beating cilia help to move aggregated bacteria and particles toward the three open pores that serve as the entrance to the internal part of the LO (Nyholm et al. 2000). As *V. fischeri* cells enter the LO through a pore, they encounter a narrow, ciliated duct that eventually opens into a series of branched and closed-ended spaces known as crypts. Here, the bacteria reach their final place of residence. The lumen of the crypts is covered by epithelial cells with multiple microvilli that secrete mucus and other host molecules, and that, once the squid is colonized, will be in close contact with the bacterial symbionts. Not many *V. fischeri* cells are necessary to seed the LO, as it has been estimated that as few as 3–6 cells can start the colonization of each lobe of this organ (Wollenberg and Ruby 2009). If the bacteria colonizing the LO are capable of producing light, about 12 h after their arrival in the crypts, the combination of light and microbial products is recognized by the host and a developmental signal for a series of programmed morphological changes is initiated. This program includes the following events: (1) apoptosis of the ciliated appendages; (2) fusion of the three pores and ducts into a single one; and (3) an increase in microvilli and swelling of the crypt epithelia (McFall-Ngai and Ruby 1991; Nyholm and McFall-Ngai 2004). The overall result is irreversible loss of the lateral appendages from the LO surface and physiological changes in internal structures over the next 4 weeks that will ensure the maintenance of the newly acquired symbionts (Koch et al. 2014) (Fig. 20, panel b2).

Fig. 20 (continued) on the ciliated appendages. (**b1**) Adult female *E. scolopes* squid side view; the transparent window allows us to see the light organ and accessory nidamental gland locations. (**b2**) Adult light organ with crypts. (**b3**) Host–symbiont interaction zone in adult squid consisting of crypt epithelial cells with microvilli and migrating hemocytes. AE appendage epithelia, ANG accessory nidamental gland, BS blood sinus, CE crypt epithelia, Cr crypts, Hc hemocyte, IS ink sac, Le lens, LO light organ, Mu mucus, NA nidamental gland, P pore, Vf Vibrio fischeri bacteria, YF yellow filters

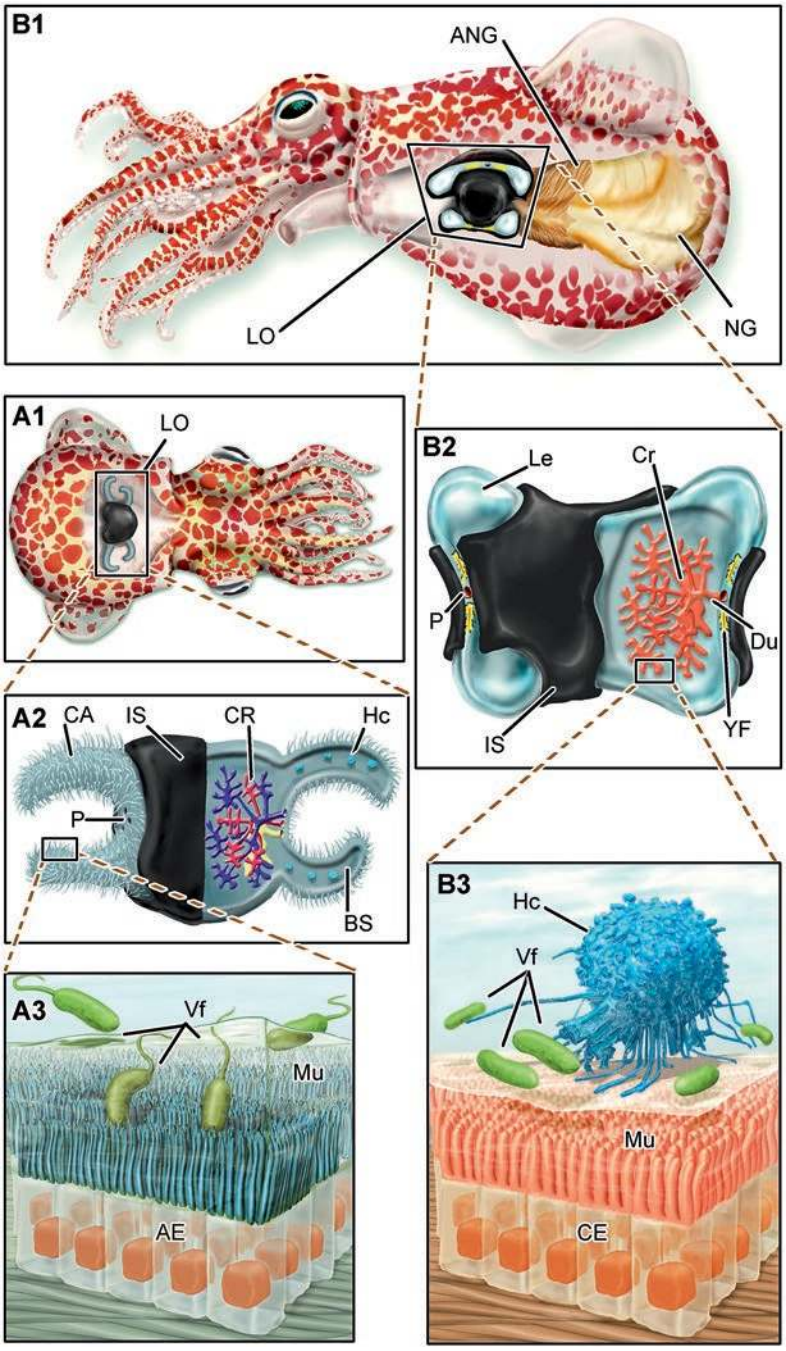

Fig. 20 *Euprymna scolopes* squid and tissues associated with bacterial symbiosis. (**a1**) Juvenile *E. scolopes* squid ventral view. (**a2**) Juvenile light organ with crypts and ciliated appendages. (**a3**) Host–symbiont interaction zone in juvenile squid, consisting of the surface of epithelial cells

Once this association between *E. scolopes* juvenile squid and bacteria is established, the symbiosis will be maintained for the duration of the animal's life (Nyholm and McFall-Ngai 2004). An important characteristic of this symbiosis is the diel rhythm, which consists, among other things, of daily expulsion of the majority (90–95%) of the bacterial population from the LO at dawn (Lee and Ruby 1994; Boettcher et al. 1996; Nyholm and McFall-Ngai 1998). This thick exudate contains live and dead *V. fischeri* cells and also some host hemocytes and epithelial cells (Graf and Ruby 1998; Nyholm and McFall-Ngai 1998). In the 8 h following the emptying of the LO, the remaining population of symbionts quickly grows and divides inside the crypts, until they reach a density high enough to enable quorum sensing, thereby becoming luminescent again at night (Nyholm and McFall-Ngai 1998). It is suggested that the squid uses this light to camouflage itself from potential predators and preys. This is suggested by the presence of several tissues in the LO, including a lens and a reflector, that allow the animal to control the amount of light emitted, with the purpose of replicating down-welling light from the moon and stars. This behavior is known as counterillumination and prevents the production of a shadow during swimming in the water column. (Ruby and McFall-Ngai 1992; Jones and Nishiguchi 2004).

The *Euprymna scolopes–Vibrio fischeri* mutualism offers advantages over other animal model systems for understanding of the physiology and molecular mechanisms of animal–bacterial beneficial associations (Ruby 1999; McFall-Ngai 2008; Lee et al. 2009). This is mainly because this it is a binary association (Ruby and Lee 1998; Mandel 2010), where both organisms can be cultured separately, thereby allowing manipulation of the bacterial introduction, and because the bacterial symbiont is genetically tractable and introductions of mutations and markers are modifications relatively easy to achieve (Ruby 1999; McFall-Ngai 2008; Lee et al. 2009). Moreover, the direct contact and interaction between the two players (host and bacteria) in this symbiosis occur extracellularly, meaning that the bacteria never breach the epithelial integrity of the host tissues. Thus, their interaction occurs via secreted molecules and by means of cell surface molecules both at the level of juvenile squid ciliated appendages (Fig. 20, panel a3) and inside the juvenile and adult LO crypt epithelia (Fig. 20, panel b3).

The Fundamental Role of Hemocytes in the Establishment of Symbiosis

Hemocytes play a major role in the establishment and maintenance of this interaction. As detailed in the previous section, these are motile cells that circulate through the squid vasculature and can reach sites where the bacteria are, and interact with them. For a review on the role of hemocytes on the squid–*Vibrio* symbiosis, the reader is directed to a recent publication by McAnulty and Nyholm (2017). The squid hemocytes play a pivotal role right from the initial stages of colonization. First, the presence of the symbiont causes the proliferation of hemocytes, the number of which peaks about 36 h postcolonization (Koropatnick et al. 2007). Furthermore, these cells play an active role during the apoptotic regression of the LO epithelia, a behavior that is accredited to the presence of *V. fischeri* products

released in the LO crypts. Specifically, and in response to *V. fischeri* outer membrane vesicles (OMV) (Aschtgen et al. 2016) and PGN-tracheal cytotoxin (TCT) (Koropatnick et al. 2004), squid hemocytes move from the circulation and migrate to the sinus space in the ciliated appendages. This migration is also accompanied by upregulation of transcripts involved in protein degradation, suggesting that these cells are involved in facilitating the apoptosis and restructuring of epithelial cells during the LO metamorphosis (Koropatnick et al. 2007). This process is aided by the activity of a matrix metalloproteinase (Koropatnick et al. 2014), as suggested by the upregulation of this enzyme in hemocytes and the LO tissues of symbiotic squids (Chun et al. 2006; Collins et al. 2012b; Schleicher et al. 2014).

In vitro studies have also shown that *E. scolopes* hemocytes can selectively recognize, bind, and engulf bacteria, while showing a degree of tolerance of *V. fischeri* in comparison with other marine bacteria (Nyholm and McFall-Ngai 1998; Nyholm et al. 2009). This recognition is modulated by unknown factors secreted by the symbionts (Nyholm et al. 2009). In addition, to discriminate between bacterial species, hemocytes of adult squid also appear to be "trained" to tolerate the symbiont, as hemocytes from antibiotic-treated squids lose their symbiont recognition capacity and bind *V. fischeri* cells more readily (Nyholm et al. 2009).

Several transcriptome and proteomic studies comparing hemocytes from colonized and noncolonized animals have been performed, which enabled the sequence identification of a number of soluble immune factors (Collins et al. 2012b). Among these, a matrix metalloprotein, a cephalotoxin, a galectin, and a soluble peptidoglycan recognition protein (EsPGRP5) were found to be downregulated in cured hemocytes, while EsC3 transcripts could not be detected in symbiotic animals. These results suggested that the presence of the symbiont modulates the host immune system to avoid its removal (Collins et al. 2012b). The complement component C3 and other complement-like molecules—including CD109 antigen (Yazzie et al. 2015), other thioester-containing proteins, and alpha-2-macroglobulin (Collins et al. 2012b, personal observations)—have also been identified in hemocytes, but their specific role in symbiosis have not been described yet. Like C3, some of these transcripts appear to be modulated in symbiotic squid compared with those not exposed to bacteria, as was the case for CD109 antigen (Yazzie et al. 2015). Furthermore, several transcripts with homology to known PRRs have been identified in hemocytes, including PGRPs and TLRs (Collins et al. 2012b). Hemocyte–proteomics studies have also revealed at least 37 differentially expressed proteins in the adult symbiotic animals compared with cured squid. Some of these are known to be involved in immune-related functions, most notably cathepsins, lysosomal proteins, and various proteases (see section "Proteases and Protease Inhibitors") (Schleicher et al. 2014). It is also worth noting that—as mentioned in section "A Short Journey in the 'Immune System' of Cephalopods,"—like all other cephalopods, squid appear to possess a well conserved immune signaling machinery. It is, however, still unclear how these immune sensors and effector molecules modulate or are modulated by the presence of the bacterial symbiont.

Hemocytes are not only important during the squid colonization process; they are also central to the homeostatic maintenance of the symbiosis. Recent studies

have found that hemocytes have cytoplasmic vesicles that contain chitin (Heath-Heckman and McFall-Ngai 2011). Chitin is an abundant carbohydrate polymer in marine environments and a food source for many planktonic organisms, including bacteria. It has been suggested that hemocytes deliver this nutrient into the LO crypts during the evening and night hours, when the bacteria population is at its higher densities, to provide nutrients to the symbionts. In return, the symbionts utilize this resource via fermentation and, as a consequence, acidify the crypt spaces to a pH of about 5.5 (Kremer et al. 2014). Hemocyanin, the squid's blood pigment and oxygen carrier (Markl 2013), releases oxygen under acidic conditions. Since bacteria need oxygen to produce light, as in the luciferase reaction, the hemocytes are providing a source of food to the bacteria that will in turn promote the formation of the proper environment for light production, which the host uses for its nocturnal activities (Kremer et al. 2014).

The large number of putative immune molecules identified in the aforementioned sequencing studies confirm the involvement of hemocytes in the host response to *V. fischeri* colonization. It is also interesting to note that multiple genes associated with cytoskeletal and lysosomal activities are modulated, reflecting the developmental and morphological changes the host undergoes in response to its association with its bacterial partner. For more information, the reader is directed to the primary study sources (Goodson et al. 2005; Collins et al. 2012b; Schleicher et al. 2014; Salazar et al. 2015).

The Immune Role of the Light Organ

In addition to hemocytes, other squid tissues express immune-related molecules. Many of these were originally discovered during an extensive analysis of expressed sequence tags (ESTs) from the juvenile LO at different times after colonization (Chun et al. 2006), in the transcriptomes of adult LOs at different times during the diel rhythm (Wier et al. 2010), or in a data set of LO transcripts differentially expressed in animals for 3 h to the symbiont (Kremer et al. 2013). The following paragraphs will describe these molecules and their suggested role in the symbiosis.

Receptors and Sensor Molecules

Several receptors were identified in the juvenile LO, including four PGRPs (PGRP1–4) (Chun et al. 2006), whose general role in invertebrate immunity is summarized in section "Other Membrane-Bound Immune Receptors." PGRP1 was found to be localized in the cytoplasm of surface epithelial cells and translocated to the nucleus, a change associated with the apoptosis of the LO appendages (Troll et al. 2009). PGRP2 was secreted in mucus and found to have PGN-catalytic activity, suggesting an antimicrobial purpose (Troll et al. 2010). Furthermore, PGRP2 was also secreted inside the LO crypts but only after colonization, possibly to aid in removal of PGN products released by the symbionts. Finally, PRGP3 had a glycophosphatidylinositol (GPI)-anchoring site, and PRGP4 was a true transmembrane receptor (McFall-Ngai et al. 2010). Additional PRRs identified in *E. scolopes* are members of the LBP/BPIs family of proteins (see section "Lysozymes, BPIs and Other Pore-Forming Molecules"). Not much is known about the function of these

sensor/effector molecules in squid, other than the fact that a BPI transcript was upregulated during LO apoptosis in symbiotic squid. Because of its localization in the LO crypts, this BPI might play a similar antimicrobial role to the PRGPs (Krasity et al. 2011).

Complement System

As mentioned earlier, bivalve molluscs possess a prototypical complement system (see section 4.4). Furthermore, C3-like transcripts have been found in squid hemocytes (Collins et al. 2012b; Schleicher et al. 2014). Transcripts for this and other complement-like molecules were first identified in ESTs from juvenile LOs (Castillo et al. 2009; McFall-Ngai et al. 2010). Immunocytochemical analysis detected the expression of C3 in epithelial cells of several tissues of juvenile squid, including the LO, gills, and skin (Castillo et al. 2009). Other complement homologs have also been identified in *E. scolopes* and its sister species *E. tasmanica* ([name], [year], unpublished data), including C1qDC proteins, C1qBP, and an MBL-like transcript (McFall-Ngai et al. 2010). Preliminary data also point toward the presence of several serine proteases with similarity to MASPs and Factor C ([name], [year], unpublished data), although biological activity for these and the other complement-like proteins remains to be confirmed. Furthermore, TEPs similar to C3 have been identified in *E. scolopes*. Initially thought to be a representative of the insect TEPs (iTEPs) subgroup, Es-CD109 was found to be expressed in several squid tissues, and its transcript was downregulated in the LO of juveniles harboring *V. fischeri* (Collins et al. 2012b; Yazzie et al. 2015). This suggested that, similarly to C3, this microbial sensor is modulated in order to avoid the removal of symbiont cells (Collins et al. 2012b; Yazzie et al. 2015).

Soluble Effector Molecules

One of the first immune-related molecules identified in *E. scolopes* was a halide peroxidase (Tomarev et al. 1993). This enzyme, localized to vesicles in the epithelial cells, was secreted on the ciliated appendages of symbiotic juveniles, possibly as an antimicrobial factor (Weis et al. 1996). Transcripts of enzymes such as chitinase and lysozyme have also been described as upregulated in the first hours of exposure to *V. fischeri*, suggesting a possible involvement in the symbiont selection process (Kremer et al. 2013). The finding of NOS in the squid LO represented another possible antimicrobial source (Davidson et al. 2004). Immunocytochemical studies found NOS and NO in vesicles localized to the mucus on ciliated epithelial cells, where the bacteria aggregate and symbiont selection starts. In addition, NOS was expressed in the crypt ducts and antechambers (Davidson et al. 2004). Furthermore, it was shown that the presence of the symbiont or its products (LPS and TCT) downregulated the expression of NOS and the production of NO (Davidson et al. 2004; Altura et al. 2011). The authors proposed that in this case, the attenuation of NO production was a response by the host, enacted to modify the crypt environment to ease colonization upon symbiont recognition (Altura et al. 2011).

Although hemocyanin is mainly expressed in gills and the branchial heart, it was also detected in the symbiotic LO crypts, where it was suggested to release oxygen,

thereby promoting bacterial growth and bioluminescence (Kremer et al. 2014). Moreover, the detection of a hemocyanin isomer in the mucus secretions of the juvenile LO suggests that this molecule may have a dual role and serve in the symbiont selection process as an antimicrobial agent against nonsymbiotic marine bacteria (Kremer et al. 2014). An additional antimicrobial and bacteriostatic molecule recently reported in *E. scolopes* is galaxin, one of the most highly upregulated transcripts in colonized LOs (Chun et al. 2008; Wier et al. 2010), whose encoded protein is localized to the epithelial cells and mucus secretions of the LO (Heath-Heckman et al. 2014). In vitro assays showed that a peptide fragment of galaxin had inhibitory effects mainly against Gram-positive bacteria, although the growth of *V. fischeri* was also affected (Heath-Heckman et al. 2014). As mentioned earlier, the sensor molecule PGRP2, which binds and degrades bacterial peptidoglycan, is localized to epithelial surfaces exposed to the environment and secreted into the LO mucus, suggesting a role during the initial stages of colonization and selection of the symbiont (Troll et al. 2010). This protein is also detected in the crypt lumen, suggesting that it also assists in modulating host–bacteria interactions once the symbiosis is established (Troll et al. 2010). Another soluble protein with antimicrobial properties found in this squid species is alkaline phosphatase (ALP) (Rader et al. 2012), whose enzymatic activity was upregulated in symbiotic hosts possibly in response to bacterial MAMPs. Indeed, the addition bacterial lipid A and TCT induced the enzymatic activity, while the addition of an inhibitor reduced bacterial colonization by more than 80%. Overall, it was suggested that esap1 has a supporting role in the colonization and maintenance of symbiosis (Rader et al. 2012).

Signaling Molecules

Following the preliminary annotation of the LO-EST database, several molecules pertaining to the canonical TLR signaling (see section "Canonical TLR Signaling") were identified (Goodson et al. 2005). In a related study, three p-63-like (a member of the p-53 family of tumor suppressor proteins) transcripts were identified and localized to the nuclei of LO cells in symbiotic animals, suggesting a role in the apoptosis of appendages (Goodson et al. 2006).

This is a topic that warrants further study, as the capacity of the host to recognize the correct bacterial symbiont from the multitude of bacterial cells in the water may reside in the signaling cascades triggered by *V. fischeri*. One interesting aspect that has been learned since the early studies of this symbiosis is that at first glance, *V. fischeri* bacteria do not seem to contain any evident "symbiont marker" that could help the host to discern the symbionts from other bacteria. Surprisingly, the same molecules present in nonsymbiotic bacteria, including pathogens, are used to communicate with the animal host. These MAMPs, such as LPS and PGN, should be readily recognized by the innate immune system as foreign and usually elicit a response resulting in microbial removal (see section "Phagocytosis"). Similarly, the host interacts with the symbionts using PRRs and signaling pathways known to be usually activated by pathogens. Nonetheless, there is still the potential of discovering novel markers on the symbionts and receptors on the host, especially considering the scarce genomic resources currently available and the unknown

function of most cephalopod genes (see section "A Short Journey in the 'Immune System' of Cephalopods"). The described studies suggest that attention needs to be paid to the context, timing, and very possibly the effector mechanisms elicited in response to the bacterial signals that can make the difference between removal and accommodation.

Accessory Nidamental Gland

E. scolopes is also used to study another very interesting case of symbiosis, in this case involving a consortium of symbionts that may be acquired in different ways. This particular interaction occurs in the accessory nidamental gland (ANG) (Collins et al. 2012a). The ANG is part of the reproductive organs in female squid. This structure is formed by a series of epithelial tubules containing a mixture of bacterial species dominated by Rhodobacteriaceae (Barbieri et al. 2001; Collins and Nyholm 2011; Collins et al. 2012a, 2015). It is thought that some of the components of the ANG bacterial community are added to the jelly coat of eggs during their formation, and that the function of these microorganisms is to protect the developing embryos from environmental infections (Barbieri et al. 1997; Collins et al. 2012a, 2015). In a recent publication, Gromek and colleagues (2016) isolated one of the ANG bacteria (*Leisingera* sp.) from the jelly coat of *E. scolopes* eggs, and in in vitro studies demonstrated that it had antimicrobial activity, producing a pigment that selectively inhibited the growth of several marine bacteria, including *Vibrio* species.

Altogether, the knowledge obtained from the study of these two types of symbiosis has the potential to provide an improved understanding of the complex bacterial associations between animals and microbes. In particular, this might bring new elements to interpret the mechanisms of regulation of bacterial symbiosis in various organs, such as the digestive, respiratory, and urogenital tracts of mammals, further serving as a productive research field for deciphering the multifaceted roles of the immune system in metazoans, which are still not well understood.

Conclusions

The application of -omic tools to the study of bivalve and cephalopod immunology has recently led to exciting discoveries about the extent of the diversity of immune genes in these groups of diverse species. Comparative functional studies using natural and selectively bred disease-resistant strains of bivalves, and in-depth analysis of the powerful model system of the bobtail squid–*Vibrio* symbiosis, as well as the application of gene-editing technologies, have the potential to provide exciting insights into the functional relevance of immune gene family expansion in molluscs and the potential role of this diversity in the specificity and plasticity of immune responses. Other areas of molluscan immunity that have not been understudied until now, because of the lack of tools and resources, include the elucidation of the process of hematopoiesis, the molecular characterization of hemocyte subpopulations, and a thorough characterization of mechanisms underlying maternal immunity and immune priming.

Molluscan immunobiology is gaining renewed importance from the growing challenges posed by human activities, which have a significant impact in particular on anthropized coastal regions (for a detailed discussion, see Chap. 12, section "Challenges for Molluscs in the Anthropocene Epoch"). This, together with the current trends of global climate change, is currently leading significant shifts in the structure of benthic communities due to the introduction of alien species, more resistant to the presence of pollutants and therefore outcompeting native species. Continuous research will be certainly needed to improve our knowledge of the immune system of molluscs, both to preserve endangered endemic populations and to face the challenges posed by emerging diseases targeting commercially and ecologically important species (see Chap. 12, section "Molluscan Conservation Immunology" for a detailed discussion on molluscan conservation immunology).

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Author Queries

Chapter No.: 11 0003596912

Queries	Details Required	Author's Response
AU1	Please confirm the chapter title.	
AU2	Please confirm the affiliation details of all authors.	
AU3	In accordance with our house style, we have used the anglicized city name 'Padua' here.	
AU5	Please ensure that the capitalization and/or italicization of scientific, technical and mathematical terms is correct throughout the chapter or amend them if necessary.	
AU6	Throughout this chapter, please confirm the hierarchy of the section heading levels or amend them if necessary.	
AU7	Please confirm all figures, including their captions and the placement of their citations in the text, or amend them if necessary.	
AU8	Please confirm all section and chapter cross-references throughout this chapter or amend them if necessary.	
AU9	Ben-Horin and Proestou personal communication	
AU10	By 'see below' do you mean 'see the section "Mucosal Immunity: An Important Yet Understudied Topic"? Please confirm this suggested section cross-reference or amend it if necessary.	
AU11	"Gomez-Leon et al. (2008), Kögel et al. (2013), Saito and Vasta (unpublished data), Collins et al. (personal observations)" are cited in the text but details not provided in the reference list. Please provide.	
AU12	Throughout the text, where other text sections are referred to, please cite specific section titles or section numbers (where practicable) rather than using terms such as 'above' or 'below' or 'the previous section(s)' or 'the following section(s)' or 'other sections'.	
AU13	Please confirm if the wording 'Promomers can assemble into trimers' is correct or amend it if necessary.	
AU14	The references Thanasupawat et al (2015), Bohlson et al (2014), Wang et al. (2014c), and Di Prisco and Polese (2015) have been moved to the reference list from the captions of Figs. 8, 9, 11, and 13. Please check and confirm.	
AU15	By 'would allow, in line of principle, additional physiological functions' do you mean 'would, in principle, allow additional physiological functions'?	
AU16	By 'their biological activity of their targets' do you mean 'their biological activity against their targets'?	
AU17	Since there are no tables in this chapter, please confirm if the sentence 'See also Table 2 for further detail.' can be deleted from this figure caption.	
AU18	By 'varosities' do you mean 'varicosities'?	

AU19	The presence of the ellipsis in this sentence and the closing double quotation mark in the next sentence suggests that this wording is part of a quotation, but there is no opening double quotation mark to indicate where the quotation starts. If this is indeed a quotation, please check the relevant text here against the original wording and make any changes necessary to ensure that the original text is quoted accurately. Alternatively, if this is not a quotation, please delete the ellipsis in this sentence and the closing double quotation mark in the next sentence.	
AU20	Please confirm the wording '**b3** Host–symbiont interaction zone' or amend it if necessary.	
AU21	By 'animals for 3 h to the symbiont' do you mean 'animals exposed for 3 h to the symbiont'? Please confirm the correct wording to clarify the intended meaning.	
AU22	Please provide appropriate section cross reference citation here.	
AU23	Here and in the next sentence, please replace the inserted placeholders '[name]' and '[year]' with the name of the source and the year(s) of these unpublished data.	
AU24	Please provide the complete author name details and article title for the reference "Di Prisco and Polese (2015)".	
AU25	Please provide complete reference details for "Jereb and Roper (2005, 2010, 2016)".	
AU26	Please provide the publisher location for "Vasta and Ahmed (2008)".	