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The immune response of cephalopods from head to foot

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ABSTRACT

Cephalopods are a diverse group of marine molluscs that have proven their worth in a vast array of ways, ranging from their importance within ecological settings and increasing commercial value, to their recent use as model organisms in biological research. However, despite their acknowledged importance, our understanding of basic cephalopod biology does not equate their ecological, societal, and scientific significance. Among these undeveloped research areas, cephalopod immunology stands out because it encompasses a wide variety of scientific fields including many within the biological and chemical sciences, and because of its potential biomedical and commercial relevance. This review aims to address the current knowledge on the topic of cephalopod immunity, focusing on components and functions already established as part of the animals' internal defense mechanisms, as well as identifying gaps that would benefit from future research. More specifically, the present review details both cellular and humoral defenses, and organizes them into sensor, signaling, and effector components. Molluscan, and particularly cephalopod immunology has lagged behind many other areas of study, but thanks to the efforts of many dedicated researchers and the assistance of modern technology, this gap is steadily decreasing. A better understanding of cephalopod immunity will have a positive impact on the health and survival of one of the most intriguing and unique animal groups on the planet, and will certainly influence many other areas of human interest such as ecology, evolution, physiology, symbiosis, and aquaculture.

1. Introduction

Cephalopods are marine invertebrates belonging to a class within the phylum Mollusca, the second largest phylum of animals after arthropods [1]. This metazoan group includes a variety of muscular, soft-bodied creatures broadly classified into octopi, cuttlefish, squid, and nautili. A generalized description of their anatomy divides their bodies into three regions: 1) the mantle, a bag-like muscular structure containing the majority of internal organs; 2) the head, which is dominated by two large eyes (one at each side) and enclosing the brain and mouth areas; and 3) the arms and tentacles, usually covered by suckers used for graving and attachment. Locomotion is provided by water propulsion through a funnel-like structure (the syphon) attached to the mantle, along with posterior fins in some species for swimming and directional movement. Inherent diversity within the distinct cephalopod groups allow them to occupy a variety of habitats worldwide; from the frigid waters of the poles, to the temperate and warmer temperatures found in the Mediterranean and tropics. Unlike other molluscs, which are mostly benthic, cephalopods can be found both in benthic and pelagic zones of seas and oceans, as well as close to the water surface, and as deep as 5000 m [2]. With the exception of the nautilus, they also differ from their molluscan relatives by lacking an external shell. They have highly developed visual and nervous systems, and an exquisite capacity to speedily adapt to their environment by means of camouflage, allowing them to be some of the most efficient marine predators [3].

Living members of the class *Cephalopoda* are classified into two subclasses: the *Nautiloidea* containing two genera, *Nautilus* and *Allonautilus* [4]; and the *Coleoidea* with four orders: Sepioidea, Teuthoidea, Octopoda, and Vampyromorpha [2]. Out of an estimated 1000 species of extant cephalopods, at present only 650–700 species have been described and named [5,6]. In the past, the classification of specimens was based on some of the most striking morphological differences among these animals, such as number of arms and tentacles, and the presence/absence of an external shell. Continuous study and recent technological advancements have incorporated new information such as data derived from fossil records, as well as developmental and genetic information that permit more accurate classification and identification [7]. Nonetheless, most people follow

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the original classification, grouping cephalopods into octopi (octopods, eight-armed), squids and cuttlefish (decapods, eight arms plus two tentacles), and the nautili (the only shelled cephalopods).

Cephalopods have always captured human interest and imagination, as evidenced by their depictions in ancient art and the many stories and myths about marine giants and monsters found in the writings of classical authors such as Jules Verne and Victor Hugo [8]. Nowadays, cephalopods maintain their popularity as fantastic creatures, but additionally, they have gained a modern interest for their potential commercial value as food sources [2,3,6], and for their scientific importance as research organisms [e.g. [9–17]. These contemporary interests and their demands have made evident the fact that we know very little about basic cephalopod biology, and that knowledge in this field is lagging behind in comparison to other commercially important species in aquaculture such as bivalves, shrimp, and fish. In recent years, with the aid of modern scientific and technological advances and the efforts from scientists around the world, this gap in knowledge is being bridged, and although a lot more work remains to be done, we are slowly beginning to understand these remarkable animals. A topic of growing interest and significant importance is the basis and workings of the cephalopod immune system. Driving this interest are diverse underlying reasons, which include basic scientific inquiry to improve our understanding of cephalopod biology, phylogeny, and interspecific relationships, as well as other complex and anthropocentric pursuits. Because cephalopods are both predators and prey, changes in their health and population dynamics may have consequences and unknown repercussions on other animal populations, including those that serve as major sources for human consumption. For example, certain species of octopus and squid are important sources of nourishment and income in many countries of Southeast Asia, Europe, and South America [18–24]. Additionally, the popularity and demand of these animals have been increasing steadily worldwide in recent years [25–29]. The Fisheries and Aquaculture Department (FAO) reported an increase of cephalopod catches from approximately one million metric tonnes in 1970 to about 3.6 million metric tonnes in 2010 [30]. This upsurge is, in part, a consequence of changes in capture methods in the fishing industry and efforts trying to overcome the reduced productivity resulting from the overharvesting of popular fish species. As a consequence of these changes, several cephalopod species are being seriously considered as excellent candidates for aquaculture enterprises due to their rapid growth and high protein content [2,31], and for this purpose, new cultivation methods are currently being developed, which aim at improving the health and long-term maintenance of cephalopods in enclosed environments [26,27,32–34]. As researchers study the quality of octopus and squid for human consumption, several species of bacteria, viruses, and parasites have been found to infect cephalopods, some of which are known or have the potential to be transmitted to humans and cause disease [reviewed in [35–38]. To this effect, in order to ensure a responsible utilization of cephalopods as a sustainable human commodity, while at the same time learn from their unique biology, it is imperative to study more about the composition and functioning of their immune system. In the following paragraphs, we present a review containing the current knowledge in the field of immune defenses in cephalopods, focusing on the internal immune components of these animals and their associated functions when this information is available. A summary of this information can be found Table 1 and Figs. 1 and 2 that accompany this review.

2. Invertebrate immunity

Cephalopods, along with all other members of the phylum Mollusca, are invertebrates and thus, lack an adaptive immune system [39,40]. By this we mean that the invertebrate equivalents for B- and T-lymphocytes, as well as their highly diversified antigen-receptors (immunoglobulins and T-cell receptors), have not been found in these organisms [41]. Regardless, invertebrates have an efficient immune system comprised of cellular and humoral components that allow them to interact with microorganisms, discern and remove pathogens, and repair wound and tissue damage [42]. The invertebrate immune system is composed of innate immune cells and molecules which have homologs present in all major metazoan groups. These ancient immune players have regained the appeal and importance they once had when Metchnikoff discovered phagocytosis in starfish and the concept of non-self recognition was conceived [43]. Indeed, the extent and influence the innate immune system has in the development and the efficiency of adaptive responses is now fully recognized [44,45]. In addition, the study of immunity in invertebrate models also experienced a renewed interest when immune-related molecules discovered in these animals, such as the toll receptor in *Drosophila*, were found to have homologs in vertebrates [46]. As a consequence, the study of immunity in lower taxa has been intensified and expanded in the last few decades, not only because of the implications these findings may have in the understanding of the evolution of immune mechanisms, but also because of the potential applications that these comparative studies can have on vertebrate research.

Innate immune functions are phylogenetically ancient and based on the recognition of non-self, missing-self, and the presence of danger signals [47,48]. This recognition is carried out by a variety of receptors that immediately induce the activation of defense effectors, which in turn will kill, remove, or neutralize the foreign invader or offensive material. These immune sensors are collectively called pattern recognition receptors (PRRs) and pattern recognition molecules (PRMs), and include proteins like the toll and toll-like receptors, nucleotide-binding and oligomerization domain-like receptors, scavenger receptors, and lectins. These sensor molecules interact with pathogen/microbial associated molecular patterns (PAMPs/MAMPs) [49,50], which are in their majority invariant microbial surface components such as lipopolysaccharide (LPS), peptidoglycan (PGN), flagellin, and molecules containing conserved glycan residues. Upon recognition and binding of PRRs with their appropriate PAMPs/MAMPs ligands, there will often be changes in the receptors' structural conformation that will allow them to interact with other host proteins involved in signal transduction, gene expression, or activation of effector molecules. Immune effectors are the host's factors performing the killing, neutralization, or removal activities that will protect, prevent infection, or repair damage to the host. These effectors are diverse and include molecules involved in phagocytosis and lysis, secretion of reactive oxygen and nitrogen species (ROS, RNS), antimicrobial peptides, proteinase inhibitors. Similarly, as with adaptive immune responses, the components of innate immunity can be classified as cellular or humoral. In most cases, the cells involved in immune reactions are those with higher probabilities to interact with pathogens, including epithelial cells that cover the host's surfaces exposed to the environment, and those found in the circulatory system and therefore able to infiltrate tissues. The latter are motile, actively circulating cells which, in invertebrate animals are commonly called hemocytes, immunocytes, coelomocytes, or phagocytes. Since circulating cells play pivotal roles in immune surveillance as well as in effector functions, they are of utmost importance; this is especially true if they also contain the sensors, the signaling components, and the capacity to express effector molecules.

Immune reactions have been studied in a handful of cephalopods, mainly in commercially-relevant octopus and squid, making it difficult to make generalizations across species. The information from those studies is presented in the following paragraphs and has

Table 1
Immune molecules identified in common cephalopod model systems.

Cephalopod		PRM/PRR	Receptor	Signal transduction	Effector
	*Euprymna scolopes*	PGRP5 [113,133], Galectin1 [113, 136], LBP1 [128,135], PGRP1 [128, 130], PGRP2 [128,131, 136], LBP2 & 3 [243], Galectin2*, MBL*	C1qBP [113], (PGRP4, TLR) [128], PGRP3 [128, 136], CD109 [227]	(CD63, MyD88, TRAF4 & 6) [113], (ECSIT, IκBα, IKKα-γ, IRAK4, iNF-κB, NF-κB1, MEKK, p50, p65, REL, TOLLIP) [128]	(A2M, Vitronectin) [113], C3 [113,136, 222], TEPs [113,136], Cathepsin D & L2 [133], (PRDX, SOD) [136] ALP [141], Lysozymes [170], NOS [206,207], Galaxin1 [243,244,239], Hemocyanin [238], MASP*
	*Euprymna tasmanica*	(LBP3, PGRP5) [132]	(CD109, FGFR2, LecRK) [132]	(DMBT1, EFTUD2, MAP2K5, NFκB1, TRAF5) [132]	(Ferritin, HSP70, MACPF) [132], C3*, TEPs*
	*Sepiella maindroni*				SOD [213], HSP70*
	*Sepia officinalis*	(LBP2 & 3, PGRP2 & 4, BPI) [58], Lectin [151]	GR [212]		(HSP70, Serpin) [58], Lysozymes [58,169], PO [169], A2M [174], (GPX, SOD) [212], Hemocyanin [231], [236]
	*Eledone cirrhosa*				Lysozyme, A2M [68]
	*Octopus ocellatus*				Serpin [177]
	*Octopus maya*	Lectin [152]			
	*Octopus vulgaris*	(A2M, BMP, BPI, C1q, Ficolin, Galectin, MBL, PGRP) [134], Lectin [153]	(C3R, C5R, FGFR2, LRR, TLR2-4 & 6) [134]	(AIF1, AKT, AP1, FADD, IKKα, IKKβ, IKKε, IRAK4, IRF3, JNK, KLKB1, LITAF, MEKK1, MKK3/6, MKK4, MTOR, MyD88, NF-κB1, Pelino, PIM1, PI3K, RAC1, RIP, Stat1, SOCS2/5, TAB1, TAK1, TBK1, TIRAP, TOLLIP, TRAF2-3 & 5–6) [134], HSBP*	Lysozymes [101, 205], (HSP, PRDX, C5a, SOD, Cathepsin K, VWF, FGF1, Serpin, NOSTRIN, VEGF, TGFβ, C1s, IL17, Granulin) [134], NOS [82,134, 205]

Note: *Direct submission to NCBI-GenBank.

been organized into two main sections: 1) Cellular defenses, which includes mainly hemocyte phagocytic and cytotoxic reactions; and 2) Humoral defenses, which describes the variety of molecules known to be involved in immune responses based on experimental studies, or *in silico* identification through sequence homology with similar components in other organisms. We have not included other forms of defense, such as toxins or venoms found in saliva and ink secretions, nor any form of behavioral defense. Other reports have looked at these aspects of cephalopod defense, and are on their own an interesting topic [e.g. [51–64].

3. Cellular defense

The immune system of cephalopods, akin to that of many other molluscs, includes both humoral and cellular immune components [65]. Many studies performed on the immune system of molluscs have focused on the blood cellular components or hemocytes [e.g. [66–68]. Another major difference between cephalopods and the rest of the molluscan groups is that these animals have a closed circulatory system [69,70] that consists of a central systemic heart, two branchial hearts to assist with blood circulation into the gills [71], and a system of interconnected blood vessels pumping blood throughout the animal's tissues [72]. Previous studies have described the different types of molluscan hemocytes based on morphological and functional characteristics. The most basic classification divides invertebrate circulating blood cells into at least two types, agranular (hyalinocytes) and granular cells [73–79]. In cephalopods, few species have been investigated, and most of the information available has originated from *Octopus vulgaris*, *Eledone cirrhosa, Sepia officinalis* and *Euprymna scolopes*.

Earlier studies originally described only one type of blood cell (granulocyte) in *O. vulgaris* and *E. cirrhosa* [67,80]. More recent studies using electron microscopy and flow cytometry have identified two [81,82] or three [83] types of hemocytes in *O. vulgaris*, and one in *S. officinalis* [84]. Additionally, tissue-adherent hemocytes, or rhogocytes, were described in the branchial heart complex of *S. officinalis* [85], where they were found to share some of the functions of circulating hemocytes such as phagocytosis. The cells described as granulocytes contain a variety of cytoplasmic vacuoles and are the common cell described across cephalopod species.

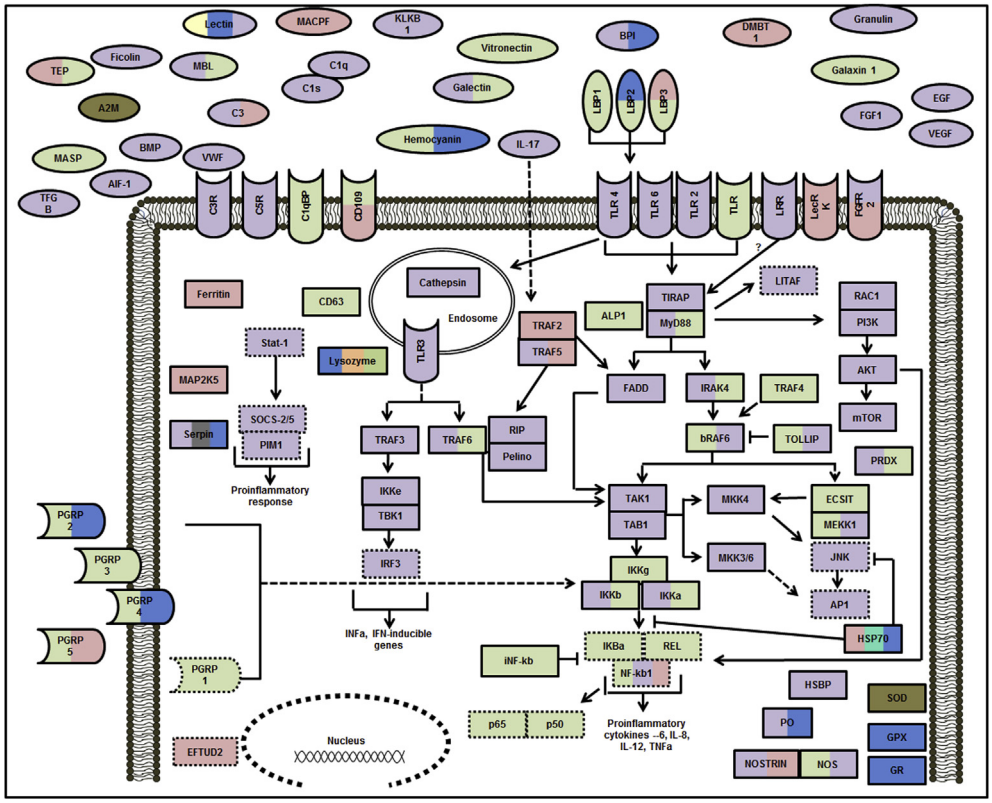

Fig. 1. Schematic diagram representing current knowledge of immune pathways in common cephalopod model systems. Colors indicate in which species each molecule has been reported: green = *Euprymna scolopes*; purple = *Octopus vulgaris*; light pink = *Euprymna tasmanica*; blue = *Sepia officinalis*; yellow = *Octopus maya*; gray = *Octopus occelatus*; orange = *Eledone cirrhosa*; turquoise: *Sepiella maindroni*; brown = identified in more than three organisms. Dashed lines indicate molecules that translocate into the nucleus. Abbreviations are as follows: AP1: activator protein 1; ALP: alkaline phosphatase; BMP: bone morphogenic protein; BPI: bactericidal permeability-increasing protein; C1q: complement component 1 q subcomponent; C1s: complement component 1 s subcomponent; C1qBP: C1q binding protein; C3: complement component 3; C3R: C3 receptor; C5R: C5 receptor; DMBT1: deleted in malignant brain tumor 1; EFTUD2: elongation factor Tu GTP binding domain containing 2; EGF: epidermal growth factor; FGFR2: fibroblast growth factor receptor 2; GPX: glutathione peroxidase; GR: glutathione reductase; H2BFQ: histone h2b type 2-e-like; HSBP: heat shock binding protein; HSP70: heat shock protein 70; IKKγ: Iκβ kinase γ; IL-17: interleukin 17; IRAK4: interleukin-1 receptor-associated kinase 4; KLKB1: kallikrein 1; LecRK: lectin receptor kinase; LBP1: lipopolysaccharide binding protein 1; LBP2: lipopolysaccharide binding protein 2; LBP3: lipopolysaccharide binding protein 3; LRR: leucine rich repeat; MAP2K5: dual specificity mitogen-activated protein kinase kinase 5-like; MACPF: mac/perforin domain containing protein; MASP: mannose binding lectin-associated serine protease; MBL: mannose binding lectin; MyD88: myeloid differentiation primary response 88; NF-κβ1: nuclear factor NF-kappa-β p105 subunit; NOS: nitric oxide synthase; PGRP1: peptidoglycan recognition protein 1; PGRP2: peptidoglycan recognition protein 2; PGRP3: peptidoglycan recognition protein 3; PGRP4: peptidoglycan recognition protein 4; PGRP 5: peptidoglycan recognition 5; PO: phenoloxidase; PI3K: phosphoinositide 3-kinase; RAC1: Ras-related C3 botulimun toxin substrate; REL: proto-oncogene c-Rel; RPS3: Ribosomal protein s3; RPS27a: ubiquitin-40s ribosomal protein s27a; SOD: superoxide dismutase; TGFb: tumor growth factor beta; BTIRAP: Toll-interleukin 1 receptor domain-containing adaptor protein; TLR: toll-like receptor; TRAF4: tumor necrosis factor - receptor-associated factor 4-like; TRAF5: tumor necrosis factor-receptor- associated factor 5-like; TRAF6: tumor necrosis factor - receptor-associated factor 6-like; VEGF: vascular endothelial growth factor; VWF: Von Willebrand Factor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

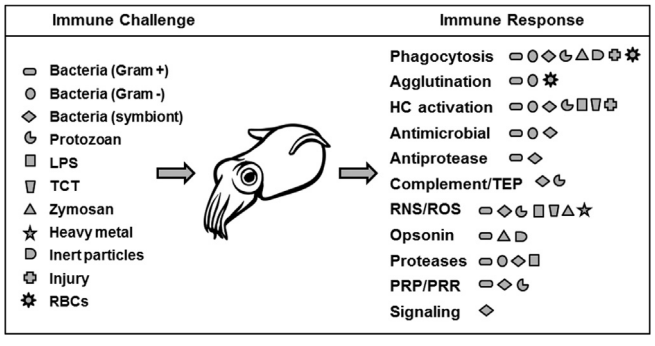

Fig. 2. Summary of known cephalopod immune responses. Diagram depicts a summary of the various immune responses reported in cephalopod studies based on their corresponding immune challenges. LPS: lipopolysaccharide; PRP/PRR: pattern recognition protein/receptor; RNS/ROS: reactive nitrogen/oxygen species; TCT: tracheal cytotoxin; TEP: thioester-containing protein.

However, it is still unclear as to whether these cells, along with the other observed hemocyte types, originate from a common cell-progenitor, as proposed by Hine [77] in bivalves, or if they derive from completely independent cell lines [82,86].

Evidence suggests that cephalopod hemocytes are produced in a specialized tissue called the white body [87–93], a soft and loosely organized tissue found surrounding the optic nerves just behind each eye [65]. It has been proposed that the different cell morphologies found in the white body and in circulation may correspond to different hemocyte lineages, or alternatively, to cells in various stages of development or maturation [82,86].

In invertebrates, hemocytes have been associated with a variety of functions, including ingestion of food particles, phagocytosis and killing, and tissue repair [94]. It is clear that cephalopods contain circulating blood cells that have defense functions, such as phagocytosis and production of immune-active molecules (see below), but at this point it will be premature to make generalizations regarding cell heterogeneity and their corresponding functions until more species are studied, and a better understanding of the hematopoietic process can be defined.

3.1. Phagocytosis

One of the most important immune functions of hemocytes is their capacity to bind, cover, and engulf foreign particles [65,95,96]. Due to their phagocytic and encapsulation activity, and the presence of cytoplasmic granules, hemocytes have been compared to vertebrate granulocytes such as macrophages, neutrophils, and eosinophils [89]. In phagocytosis, microbial invaders or foreign material are detected directly by hemocytes via their surface PRRs or indirectly with the aid of opsonins and their corresponding receptors. Hemocytes are able to bind the foreign material and internalize it into phagosomes, which will later fuse with vacuoles/granules containing digestive enzymes or other bioactive molecules that will help destroy the potential danger [reviewed in [97,98]. Bivalve hemocytes studies in *Crassostrea virginica* and *Mercenaria mercenaria* revealed that granulocytes are the most important cell involved in the phagocytosis of bacteria [99]. Similar results have been observed in cephalopod reports [81,83]. Hemocytes from several cephalopods have been used in *in vivo* and *in vitro* assays to determine their phagocytic activity against bacteria, yeast, and a variety of inert particles. In the octopus *Octopus dolfeini*, Bayne [100] found that the Gram (−) bacteria *Serratia marcescens* was quickly (within 2–4 h after bacterial injection) removed from circulation by hemocytes. Similarly, hemocytes from the octopus *E. cirrhosa* cleared erythrocytes, carbon particles [87], and bacteria (*Vibrio anguillarum*) [68] from the blood after injection, and were observed phagocytosing these same foreign materials in *in vitro* assays [67]. In the common octopus *O. vulgaris*, recent studies have shown that hemocytes migrate to the site of lipopolysaccharide injection [101], and that they are capable of ingesting latex beads [81] and zymosan particles [81,82,102]. Moreover, in the cuttlefish *S. officinalis*, hemocytes were observed phagocytosing fluorescent beads [84].

In some of these phagocytosis studies, the influence of various assay conditions such as time, temperature, ratio of hemocyte-to-foreign particles, and the presence and amount of hemolymph (or plasma) were tested. Results from these studies suggest that sensitivity to these factors may be species specific [84]; for example, in the octopus *O. vulgaris*, low temperature, the ratio of zymosan:hemocyte, and pre-opsonization with hemolymph influenced phagocytosis, but time of incubation had no effect [102]. Meanwhile, studies in *E. cirrhosa* have shown that pre-opsonization of bacteria, higher temperature, and longer incubation time can increase phagocytosis [67]. Finally, in *S. officinalis*, low concentration of plasma and longer incubation time increased phagocytosis, while temperature did not seem to have significant effects. The negative effects of higher concentrations of plasma in phagocytosis observed in *O. vulgaris* and *S. officinalis* experiments were suggested be due to the presence of an agglutinin binding to the material tested, increasing the size of particles, and thus, making them difficult to internalize [84,103].

In addition to being present in the white body and in circulation, cephalopod hemocytes have also been localized in other tissues. This is logical if we think of these cells performing immune surveillance roles. In *S. officinalis*, Buerlein et al. [85] described adhesive cells in the branchial hearts (rhogocytes) that were capable of engulfing bacteria, fluorescent beads, and colloidal gold in a similar fashion to circulating hemocytes. The phagocytic capacity of these tissue-specific hemocytes appears to differ in octopus, where circulating cells displayed lower phagocytic activity when compared to gill hemocytes [71,72].

In the context of beneficial symbiosis, hemocyte phagocytosis has been explored in the *E. scolopes-Vibrio fischeri* model system [104,105]. In this mutualistic animal-microbial symbiosis, *V. fischeri* bacteria live inside a specialized organ called the light organ (LO). The bacteria are acquired horizontally and within a few hours after each juvenile squid hatches. Once this association is established, the symbiosis will be maintained for the duration of the animal's life span [106]. An important characteristic of this symbiosis is the diel rhythm, which consists in the daily removal of the majority of the bacterial population from the LO at dawn, followed by bacterial division and repopulation of the LO-crypts by the remaining symbionts [104,106]. The bacteria is released from the LO in a thick exudate liquid containing mostly bacteria cells, but also some host hemocytes and epithelial cells [104]. Some of those hemocytes were found to contain bacteria inside their cytoplasm, and phagocytosis was suggested as one of their functions [104]. In a study using *in vitro* phagocytosis assays with adult *E. scolopes* hemocytes and various marine bacterial species, the authors found that squid hemocytes were capable of binding and phagocytosing bacteria, but that the symbiont *V. fischeri* cells were phagocytosed at a significantly lower level than those of other bacteria tested [105]. Interestingly, it was also found that hemocytes from antibiotic-treated squid (used to remove bacteria, including the symbiont) bound *V. fischeri* five times more than their non-cured counterparts, and it was suggested that host hemocytes were able to recognize and differentiate symbiont surface molecules and/or their secreted factors from those of non-symbiotic bacteria [105].

3.2. Cytotoxicity

Molluscan hemocytes are known to be able to mediate cytotoxic reactions and cause the lysis of internalized cells by enzymatic digestion or by releasing highly toxic compounds such as ROS and RNS [e.g. [107–112]. In studies with *O. vulgaris*, granulocytes exposed to zymosan particles and *Escherichia coli*-LPS were able to produce nitric oxide (NO) and ROS [81,82]. In addition, hemocyte extracts from this same octopus species were shown to have bactericidal activity against several species (including some pathogenic strains), with stronger antimicrobial activity against Gram (+) than Gram (−) bacteria [83]. In *S. officinalis*, Le Pabic et al. [84] also found evidence of phenoloxidase activity in hemocyte extracts, demonstrating the capacity of hemocytes to be immune effector cells. The capacity to discern pathogens from beneficial partners has been suggested in several studies using *E. scolopes* squid. For example, Collins et al. [113], found that hemocytes in symbiotic animals express lower amounts of NO synthase compared to non-symbiotic (cured) hemocytes, suggesting a down-regulation of this toxic compound in the presence of the symbiont *V. fischeri*.

3.3. Wound healing

An important role of blood cells, in addition to phagocytosis and lysis, is their capacity to assist in tissue repair [65,73]. The effects of tissue damage due to infection and injury were described in the lesser octopus *E. cirrhosa* [114–116]. In these studies it was found that hemocytes respond immediately after wounding by migrating to the site of injury and increasing in number and activity. These cells were observed to form plugs at the wound site to prevent loss of blood, and later, transforming to cover and heal the damaged area [114,115]. Microscopy observations of intestinal tissues from *O. vulgaris* naturally infected with the protozoan *Aggregata octopiana* showed infiltration of hemocytes to the infected areas [117]. In these sites, some of the hemocytes appear to have phagocytosed parasite cells at the sporogonic stage [117]. When bacteria were introduced via injections, the hemocytes that migrated to the affected area had increased number of cytoplasmic granules and, in some cases, cells appeared to have undergone necrosis [116]. The authors interpreted this necrosis as cell death due to exposure to

bacterial toxins, as the bacteria appeared too large to be engulfed. Regardless of the necrotic damage, wounds appear to heal normally after 12 h. In *S. officinalis*, Feral et al. [118], observed migration, accumulation, and spreading of hemocytes after arm amputation, and described the formation of a plug around the wound as described previously in octopi.

3.4. Hemocytes in symbiosis

The surveillance and immune effector activities of hemocytes are not exclusively used for defense against pathogenic microbes. In the beneficial symbiosis model between the sepiolid squid *E. scolopes* and the Gram (−) bacteria *V. fischeri*, hemocytes play important roles during the colonization process and the maintenance of a homeostatic relationship between these two organisms [for reviews see [17,106,119,120]. In the last 20-plus years, studies in this system have revealed that from the moment the squid is exposed to the aquatic environment, there is a constant exchange of chemical signals between the host and the symbiont that modulate both their responses accordingly. Among the responses detected in the host, some include secretion of antimicrobial-containing mucus, beating of epithelial cilia, and production of oxidative molecules and chemoattractants to select and guide bacterial symbionts to their final residence site. The sum of these activities adjusts host tissue conditions to promote *V. fischeri* colonization while at the same time deter other bacteria. Once the LO is colonized, the host tissues that once promoted bacteria accumulation and selection undergo a dramatic transformation. A programed and non-reversible loss of the superficial tissues of the LO is completed in a period of ~96 h post-symbiont exposure, leaving the LO without the structures necessary for the acquisition of bacteria. In early studies, it was recognized that the regression of the LO-epithelia only occurred after the juvenile squid had been exposed to *V. fischeri* for a period of approximately 12 h [121–123]. Later, it was found that bacterial products, specifically *V. fischeri* LPS [124] and PGN-tracheal cytotoxin (TCT) were the molecules responsible for the morphological signal [50]. In addition to signaling the regression of the LO-surface epithelia, the presence of bacterial symbionts also caused the migration of hemocytes to the sinuses of the epithelial appendages [125]. This migration was thought to be mediated by the recognition of *V. fischeri* MAMPs, and the identification of up-regulated genes involved in protein degradation suggested these cells were involved in facilitating the apoptosis of epithelial cells during the regression process [126,127]. In follow-up studies, it was found that *E. scolopes* hemocytes can recognize, bind, and engulf bacteria in a selective manner, binding less to *V. fischeri* cells than to other marine bacteria [105]. In addition to having a capacity to discriminate between bacteria species, the hemocytes of adult squid also appeared to be able to be "trained" to tolerate the symbiont; in experiments where adult squid were "cured" from their symbionts by antibiotic treatment, hemocytes isolated from these animals lost their symbiont-recognition capacity and bound to *V. fischeri* at similar levels as non-symbiont bacteria [105]. The mechanism for this training is still unknown, but the presence of bacterial products may be involved in the modulation of these cells during development and thus, affect their functions. Finally, hemocytes of the squid have been used for several proteomic and transcriptome studies, in which many sequences with homology to immune-related molecules have been identified. In one of these studies, hemocytes from adult animals that were cured from their symbionts, where compared to those from symbiotic animals [113]. Among the immune proteins identified in this study, a homolog of complement component C3 (EsC3), a matrix metalloprotein/vitronectin-like molecule, cephalotoxin, and a soluble form of a peptidoglycan recognition protein (EsPGRP5) were further investigated. Three of these transcripts, the matrix metalloprotein, cephalotoxin and EsPGRP5 were down-regulated in cured hemocytes, while EsC3 was not detected in symbiotic animals. Results suggested that some immune molecules may be down-regulated in symbiotic hemocytes, as a mechanism to avoid killing the beneficial symbiont [113].

3.5. Receptors

Innate immune sensors are molecules that allow organisms to discriminate between self and non-self, playing a crucial role in the initiation of immune responses. These sensors can be divided into soluble proteins, such as lectins and other microbial-binding molecules, and membrane-bound receptors. In this section we will cover host membrane-associated receptors, otherwise known as PRRs. PRRs are molecules that have been conserved through evolution due to their effectiveness in recognizing invariable targets on microbial surfaces [48]. The interaction and association of these sensors with their appropriate ligand triggers a series of intracellular events that conclude with the neutralization or destruction of the recognized target. This destruction can be performed directly by mobilizing the bound-target into intracellular vesicles that will later fuse with lysozomes or other vacuoles containing digestive and toxic compounds. Alternatively, the binding of a PRR to its microbial partner can indirectly initiate and diversify the immune response via cell signaling pathways that can produce effector molecules such as oxidative mediators, antimicrobials, and opsonins [47]. Among the most studied PRRs are the Toll-like receptors and the LPS- and PGN-binding receptors. In cephalopods, some of the first reports on PRRs came from research within the squid-vibrio system [128]. In these studies, several sequences containing microbial binding-domains characteristic of PRRs were identified in an LO-EST dataset; a Toll-like receptor and four PGRPs (EsPGRP 1–4) [128]. In subsequent studies, it was found that PGRP1 was cytoplasmic, PGRP2 was secreted, PGRP3 had a glycophosphatidylinositol (GPI)-anchoring site, and PGRP4 was a true transmembrane receptor [reviewed in [129]. Two PGRP homologs (PGRP2 and PGRP4) were also identified in a recent proteome performed in *S. officinalis* buccal salivary glands [58]. In *E. scolopes*, cytoplasmic PGRP1 was found to be translocated to the nucleus of epithelial cells of the LO-appendages, a localization associated with the apoptosis the LO undergoes as a result of colonization by the symbiont *V. fischeri* [130]. On the other hand, PGRP2 was present in the cytoplasm and mucus secretions of the LO epithelial surface, and was found to have PGN-catalytic activity, suggesting that its secretion onto the mucus had an antimicrobial purpose [131]. More interestingly, PGRP2 was also secreted inside the LO-crypts where the symbiont resides, but only after colonization had occurred, suggesting its secretion was to control the damaging effects that *V. fischeri* PGN may have on host tissues [131]. One additional PGRP was identified in *E. scolopes* hemocytes [113] and in the white body of *Euprymna tasmanica* [132]. Further analysis of PGRP5 revealed the presence of a secretion signal and transcriptional down-regulation in hemocytes from non-symbiotic squid [113,133]. In the octopus, *O. vulgaris* TLR and PGRP transcripts were identified in hemocytes [134]. In this study, the authors found a significant transcript up-regulation for PGRP in the caecum of *A. octopiana* infected animals compared to healthy animals, [134].

Additionally, other microbial-binding proteins, but not necessarily receptors, have been identified in cephalopods; in *E. scolopes* three LPS-binding proteins (LBP1-3) have been identified [reviewed in [135]. Until now, only LBP1 has been further studied and found to be up-regulated during the apoptosis and morphological transformation that occurs in symbiotic squid compared to non-symbiotic juveniles [135]. The protein was also localized to the LO

crypts where it may be serving a similar role as the PGRPs. Cornet et al. [58], found among a variety of secreted proteins in the salivary glands of *S. officinalis*, several bacterial-binding molecules. These proteins were identified as LBP-2, LBP-3, and BP1 (bactericidal/permeability-increasing protein), but functional studies have yet to be reported on these proteins.

Lectins are also known to be present on the membrane of cells, where they serve as receptors for carbohydrate ligands. In hemocytes of *E. scolopes*, a homolog of galectin-4 from the scallop was found to be expressed in higher abundance in hemocytes from symbiotic adult squid compared to cured animals, and the authors suggested that this protein may be serving as a cell-surface receptor involved in the binding and phagocytosis of bacteria [136].

3.6. Signaling

Signal transduction pathways or cascades are commonly involved in the steps following PRR-ligand interactions. Two of the most common signal transduction cascades known to be involved in immune responses are the nuclear factor κB (NFκB) and myeloid differentiation factor 88 (MyD88) pathways. A variety of PRRs are known to initiate these pathways, but the better studied are the Toll and Toll-like receptors (TLRs). TLRs and their associated signaling molecules have been identified and studied in several invertebrate models including arthropods, molluscs, and cnidarians [e.g. [137–139]. In cephalopods, this is probably one of the topics less explored and one that offers great potential in the efforts to understand immune function. In *E. scolopes*, following preliminary identification from an LO-EST database, several signaling molecules were fully sequenced including IRAK4, TRAF6, IKK/NEMO, IB, and RelA/p65 [128]. The authors of this study concluded that squid cells contain all the critical elements (receptors, transducers, and targets) to suggest a functional Toll/NF-kB cascade is present in these animals. In the same squid, a short transcript with homology to MyD88 has been identified, but its full sequence was not confirmed [128]. In another related study, three p-63-like transcripts were sequenced and their expression was investigated in juvenile LOs [140]. P-63 is a member of the p-53 family of tumor suppressor proteins known to be involved, among other functions, in apoptosis. The *E. scolopes* p63 transcripts showed close homology to a previously reported sequence from *Loligo forbesi*. When comparing protein expression and localization in symbiotic *versus* non-symbiotic LOs, western and immunocytochemistry methods revealed that the p-63 isomers were localizing to small spots within nuclei. In a reverse manner, in non-symbiotic LOs, these proteins had a diffuse distribution and were localizing to the cytoplasm of the same cells. The authors concluded that this translocation of p-63 was in response to the presence of the symbiont and involved in the morphological changes associated with colonization [140]. In a recent study, two isoforms of alkaline phosphatase (ALP) were identified in *E. scolopes*, with one of these isoforms (esap1) expressed in the LO and gut [141]. ALP enzymatic activity was found to be influenced by the presence of the bacterial symbiont, resulting in higher enzyme activity in symbiotic animals, especially when the LO had a high bacterial content. Furthermore, the *in vitro* addition of *V. fischeri* MAMPs (lipid A and TCT) induced ALP activity supporting the authors proposed substrate for this enzyme. In addition, juveniles treated with dephosphorylated *V. fischeri*-lipid A (non-reactive) did not exhibited normal apoptosis patterns, suggesting that the activity of ALP plays an important role in the morphogenesis signaling induced by the symbiont. Finally, incubating juvenile squid with levamisole (an ALP-inhibitor) reduced colonization by more than 80%, supporting a role for this enzyme in the colonization and maintenance of the symbiont [141].

4. Humoral defenses

The humoral immune response is comprised of a highly diverse group of soluble molecules that are secreted and present in the blood or hemolymph of animals, and have the potential to be distributed systemically. In cephalopods, most of the information on humoral responses has been obtained from the analysis of hemolymph and, more specifically, from searching for the presence of opsonins and agglutinins, proteolytic enzymes, protease inhibitors, antimicrobials, or cytotoxic compounds such as reactive oxygen/nitrogen species. In the following paragraphs a summary of such studies is presented. Many more putative humoral components have been identified by proteomic and transcriptome studies and are included in a latter section referring publications using these techniques.

4.1. Opsonins and agglutinins

One of the most effective ways to detect and mark non-self material for neutralization, destruction, or killing is by tagging them with host molecules. The molecules responsible for this tagging activity are broadly grouped into opsonins and agglutinins. Many times these molecules are lectins or proteins with lectin-like properties that can bind to specific carbohydrate moieties unique to microorganisms and other non-self cells [142–144]. Lectins play essential roles as sensor molecules, detecting and recognizing PAMPs/MAMPs, and initializing immune responses [145–148].

Earlier studies recognized the capacity of the plasma from *O. vulgaris* [149,150] and *E. cirrhosa* octopi [66,67,87] to agglutinate bacteria and erythrocytes. Furthermore, in a detailed study, Fisher and DiNuzzo [151] methodically tested the agglutination activity of hemolymph and hemocytes of *Octopus maya*, the Japanese squid *Sepiotheutis lessoniana*, and the cuttlefish *S. officinalis* against 73 environmental and 21 clinical bacterial isolates and seven types of erythrocytes. Their results showed the diversity of immune reactions in these animals and supported the presence of lectin-like molecules involved in agglutination. Interestingly, they found that animals raised in captivity showed lower levels of immune response compared to those caught in the wild, a potential consequence of inbreeding and constant laboratory rearing. Recently, a calcium-independent lectin was purified from *O. maya* and was found to agglutinate erythrocytes from various vertebrate species, including rat, guinea pig, and rabbit. Interestingly, this protein also showed sequence homology to a hemocyanin from *Octopus dolfleini* [152].

In addition to lectins, uncharacterized glycoproteins have also been associated with bacteria-binding activity in *O. vulgaris* hemolymph [153], and in mucus secretions on the LO-surface of juvenile *E. scolopes* [154]. In the latter study, the secreted mucus was found to assist in harvesting bacteria from the surrounding waters and promote clumping of microbial cells, including *V. fischeri*. This suggests the presence of lectin-like molecules that are involved in the pre-selection of the symbiont from other diverse microorganisms present in the marine environment [154].

4.2. Proteases and protease inhibitors

Lysozymes include a group of diverse proteases capable of hydrolyzing amino-glycosidic bonds such as those found in the peptidoglycan layers on the surface of prokaryotes [155]. Lysozymes have been identified in many organisms including plants, bacteria and phages [156], and in animals, in which they play an important role in digestion and nutrient breakdown [157,158]. In molluscs, including bivalves and gastropods, the role of lysozymes in defense and inflammation has been well described [75,159–163]. These enzymes are localized inside specialized digestive vacuoles

in hemocytes [161,164,165], or secreted into the hemolymph by these and other cells [99,166]. Lysozymes have also been reported to interact with other components of the immune system and assist in the modulation of defense responses [reviewed in 167, 168]. In cephalopods, very few studies have investigated the activity of lysozymes. Nonetheless, their results support previous work in related species by showing that these proteins offer the host protection against pathogenic organisms by increasing their expression and activity upon exposure to bacteria and their products. For instance, in the octopus *E. cirrhosa*, injection with the Gram (−) bacteria *V. anguillarum* increased lysozyme activity in several tissues but mainly in the branchial heart [68], while tissue damage in *O. vulgaris* caused by injections was sufficient to increase lysozyme activity in the serum, which was more pronounced when *E. coli* LPS was added to the treatment [101]. In the cuttlefish *S. officinalis*, a recent study reported lysozyme activity in a variety of tissues, but most prominently in those exposed to the external environment (integument, mantle) and the circulatory system organs (branchial heart and its appendages, systemic heart, white body and gills) [169]. The authors did not detect lysozyme in the plasma, supporting their observations where they had detected the presence of this enzyme in hemocytes [84]. In addition, lysozyme activity was also detected at high levels in the posterior salivary glands, consistent with known microbial protection and digestive activities for these enzymes [169]. However, further studies are necessary to characterize specific lysosomal enzymes and their activities in cephalopods.

A topic approached by studies using the *E. scolopes-V. fischeri* symbiotic model is the identification of molecules that allow these two organisms to recognize each other and permit colonization of host tissues, while simultaneously restrict access to other bacteria. In a recent study using this model system, this topic was approached by analyzing the LO-transcripts expressed during the first 3 h after symbiont exposure, and comparing those to those from untreated animals [170]. The authors found that in the few hours following exposure to *V. fischeri*, a series of host transcripts are modulated, including proteases such as chitinases and lysozyme. It was suggested that the increase secretion of lysozyme, among other potential antimicrobials (*i.e.* NO and PGRP2), creates a selective environment in the LO mucus that favors the attachment of *V. fischeri* and excludes nonsymbiotic bacteria. In another study reporting proteins expressed in hemocytes from symbiotic adult squid, it was found that several lysozyme-like enzymes were identified, including homologs of cathepsin-D and -L2, and a ganglioside GM2 activator. Using immunocytochemical analysis, authors reported that the cathepsin protein was upregulated in hemocytes from symbiotic animals (compared to cured), and it clearly localized to lysosome-looking vesicles, which potentially indicated a role for this protein in the processing of phagocytized material [133].

Proteolytic enzymes are not exclusive of the animal kingdom; in fact, they are present in all organisms including plants, and bacteria. Therefore, animals need to possess mechanisms to defend against the insult of foreign proteases. One ancient and extremely efficient immune response induced by the attack of microbial proteases is provided by protease inhibitors (PIs) or antiproteases. PIs are a heterogeneous and often species-specific group of proteins that prevent microbial proteases from damaging host cells by either neutralizing or destroying these virulence factors. In general, there are two classes of PIs; ones that inactivate proteases by directly binding to their active sites, and alpha-2 macroglobulins (A2Ms), which neutralize proteases by “trapping” and directing them to hydrolysis [171]. PIs are found in high concentrations in blood, constituting 3–5% of total plasma proteins in mammals [172]. In invertebrates, A2Ms have been reported to be highly abundant, for example in the horseshoe crab *Limulus polyphemus* and in the cuttlefish *S. officinalis*, this PI was reported to be the second most abundant protein in hemolymph [173,174]. In other cephalopods, antiprotease activity was detected on the skin of *Loligo vulgaris* and *L. pealii* [175]. Similarly, the presence and activity of A2M was detected in the hemolymph of *E. cirrhosa,* and antiprotease activity was reported to increase in the hemolymph of octopi injected with bacteria, but not in the hemocytes [68]. Furthermore, Thorgensen et al. [176] reported the identification and purification of an A2M form *O. vulgaris*. The immune defense function of A2M was allotted to this protein in a recent proteome study of the salivary glands from *S. officinalis*, where they identified this protein and proposed it plays a surveillance role in the mouth and/or its secretions [58]. Recently, a study reported the identification and *in vitro* expression of a serine protease inhibitor (serpin) homologue from the octopus *Octopus ocellatus* [177]. This serpin transcript was found to be constitutively expressed in several tissues including muscle, renal sac, gills and hemocytes, but showed highest expression levels in the hepatopancreas. Furthermore, serpin expression in hemocytes was up-regulated after bacterial challenge with *Listonella anguillarum* and *Micrococcus luteus* compared to control animals, and reached a peak at 48 and 24 h respectively [177]. In the same study, the purified protein was found to have inhibitory activity against trypsin and chymotrypsin in a dose-dependent manner, and had bacteriostatic activity on *E. coli* cultures. The exact mechanism of action of this protein has not been reported, but the authors concluded that the data obtained supports a potential immune role in this cephalopod.

4.3. Oxidative stress

Organisms can react to immune challenge or other stressful stimuli by producing reactive oxygen and nitrogen compounds. In vertebrate animals, this response is well studied in cells of the innate immune system like neutrophils and macrophages [reviewed in [178,179]. The resulting molecular intermediates are highly reactive agents with oxidizing capacity that can damage a pathogen's structural and functional components, while at the same time play a dual role by activating host signaling pathways that can amplify and diversify defense responses [reviewed in Refs. [180–182]. Among the most common ROS compounds are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydrogen radical (OH^-), and hypochlorous acid (HClO) [183,184], each characterized by different strengths and target selectivity. Concerning RNS, nitric oxide (NO) and peroxynitrite are both extremely reactive but have shown differences in solubility and reaction times [reviewed in [185–187]. In molluscs, the role of ROS intermediates has been studied in bivalve and gastropod models where they have been associated with responses to pathogenic and environmental stress [reviewed in [188,189]. For example, in the fresh water snail *Biomphalaria glabrata*, an intermediate host of the human parasite *Schistosoma mansoni*, it was found that ROS and RNS were produced by snail hemocytes. Furthermore, the strength of the hemocyte respiratory burst was associated with the ability of a specific snail strain to kill the parasite and be resistant to infections [190–192].

As pointed out by Castellanos-Martinez [193], studies on the oxidative response in cephalopods are scarce. One of the earliest studies investigating oxidative responses in cephalopods reported the identification and partial characterization of a halide peroxidase in the sepiolid squid *E. scolopes*, and having high homology to human myeloperoxidase [194,195]. In these studies, the molecule was first identified as part of the expression profile of the epithelial cells on the surface of the symbiont-occupied LO, where it was found expressed at higher levels compared to non-symbiotic tissues [194], and distributed in cytoplasmic compartments similar to

those seen previously in hemocytes of the mussel *Mytilus edulis* [196]. The expression of this enzyme and its relation to the production of antimicrobial compounds lead the authors to speculate that the squid host may be utilizing the oxidative activity of this protein as an antimicrobial to remove potential pathogens or to modulate and control the symbiont population in the LO [194,195]. A similar peroxidase molecule was identified in the ink gland of *S. officinalis*, where its activity was speculated to be involved in antimicrobial activity and the synthesis of melanin [55]. Peroxidase activity has also been investigated in the octopi *E. cirrhosa* and *O. vulgaris*. Malham et al., in 2002 [197], showed that *E. cirrhosa* hemocytes produced superoxide in response to stress (removing the octopus from the water and exposing it to air for 5 min). Moreover, hemocytes of the octopus *O. vulgaris* produced and secreted ROS upon stimulation with zymosan particles and LPS, while NO production was only observed with zymosan exposure [82]. Analyzing the response of hemocytes in *O. vulgaris* infected with the apicomplexan parasite *A. octopiana*, showed that the oxidative response of hemocytes was inhibited, possibly by a virulent mechanism from the parasite [198]. More specifically, hemocytes of *O. vulgaris* showed a lower expression of peroxiredoxin in infected individuals [198]. Interestingly in a transcriptome study by the same group, the levels of peroxiredoxin transcript were up-regulated in the caecum of infected octopi, suggesting localized activity for this protein [134]. Phenoloxidase activity was detected in a variety of tissues from the cuttlefish *S. officinalis*, with the highest levels detected in the circulatory system organs and integument [169]. High levels were also found in the digestive gland where this protein can be functioning in defense against ingested pathogens or in detoxification and hemocyanin metabolism [169].

Nitric oxide (NO) is an important molecule that has been found to have a variety of functions in living systems, including cell signaling in the nervous and cardiovascular system. It is also potentially toxic as it is a highly oxidizing agent [199]. In its toxic capacity, it is used as part of the innate immune system against invading microorganisms in both vertebrates and invertebrate animals [184,200–202]. NO by itself is difficult to detect in tissues, particularly because it is short-lived and easily dissolved. Instead, the presence or activity of nitric oxide synthase (NOS), the enzyme responsible for NO synthesis, is reported as an indication of NO production. The earliest reports of NOS in cephalopods, found this enzyme expressed in the central nervous system and peripheral tissue of *Octopus*, *Sepia*, *Rossia* and *Loligo* species [203], with proposed functions in nerve impulse transmission. Since then, several studies have investigated the production of RNS and their potential roles in cephalopods such as neurotransmission, learning and behavior, and ink production [reviewed in [204]. For the purpose of this review, we will focus only on those reports that have associated an immune function to these compounds. In a study where circulating and white body hemocytes from *O. vulgaris* were exposed to zymosan and LPS, only zymosan particles were able to induce the production of NO [82]. In the same cephalopod, injection with titanium dioxide particles induced a short and dose-dependent secretion of NO into the hemolymph that was significantly different from control animals at 4 h post-treatment [205].

In the beneficial symbiosis model between the squid *E. scolopes* and the bacteria *V. fischeri*, the production of NO has been studied in relation to the molecular dialogue between host and symbiont, and the changes that these two organisms have to make to accommodate to live together. Immunohistochemical studies have detected the presence of NOS in the *E. scolopes*-LO [206]. In this report, both NO and NOS were detected in the mucus secreted by the surface epithelial cells of the LO where the bacterial symbiont aggregates and selection is thought to begin. Furthermore, exposing juvenile squid to symbiotic bacteria resulted in the down-regulation of both NO and NOS in the ducts and crypts of the LO. This effect was not observed when exposing squid to non-symbiotic bacteria, suggesting that NO plays an important role in the initiation and maintenance of the symbiosis [206]. In a subsequent study, it was shown that the presence of both LPS and TCT from *V. fischeri* were necessary to induce the attenuation of NO and NOS [207]. Previously, it was found that exposure of juvenile squid to *V. fischeri* LPS and TCT was associated with the LO-metamorphosis associated with successful colonization, that is, the apoptosis and morphogenesis of the epithelial surface [50,121,124]. In conclusion, it was proposed that, although we normally think of NO being produced to either destroy or counteract the effects of pathogens, in this case, NO is involved in the communication between the host and its bacterial symbiont, and the bacterial release of LPS and TCT allows the host to recognize its symbiont and respond by sending the proper signals to create a welcoming environment suitable for colonization [reviewed in [17].

It is necessary to remember that exposure to oxidative compounds can be as damaging to the host cells as to the microbes they are trying to deter. In order to protect their own tissues, organisms can secrete enzymes with antioxidant activity and neutralize or remove harmful oxygen species. Some examples of these enzymes include superoxide dismutase (SOD), which catalyzes the transformation of superoxide radicals into H_2O_2 and O_2 [208]. H_2O_2 can later be removed by another antioxidant enzyme, catalase, and produce inert H_2O and O_2 molecules. Other antioxidant enzymes are glutathione peroxidase (GPX) and glutathione reductase (GR). These enzymes are considered housekeeping genes, but their modulation and up-regulation can be regarded as a sign of exposure to pathogenic microorganisms or harmful and toxic environments [209–211]. In this context, antioxidant enzyme activity was detected in the Atlantic brief squid *Lolliguncula brevis* and the cuttlefish *S. officinalis* [212]. In this study, enzymatic levels were higher in the gills compared to the mantle of both species, and authors suggested this was due to the tissue location and its exposure to higher oxygen concentrations. In addition, in *S. officinalis*, the activity of SOD and GPX were found to increase with age, while the enzymatic activity of GR remained constant through the life of the animal. Meanwhile in *L. brevis* no catalase or GR activity could be detected in mantle tissues. The authors concluded that the higher levels of antioxidant activity and their protective properties in *S. officinalis* may be associated with the longer life span of this animal compared to *L. brevis*. In the Japanese spineless cuttlefish *Sepiella maindroni*, a copper/zinc SOD (smSOD) was identified and characterized [213]. Real-time PCR analysis indicated that this enzyme's transcript was significantly (70-fold) up-regulated in the hepatopancreas of the cuttlefish after cadmium exposure and injection of *Vibrio harveyi* (a common marine pathogen). The authors considered this enzyme to be part of the mollusc's acute-phase response and suggested it to be a potential biomarker for infection and heavy metal exposure [213].

4.4. Complement and thioester proteins

The vertebrate complement system consists of a group of serum and membrane associated proteins that play essential roles in immune defense. As part of the innate immune system it is involved in the first response during infections and aids in bridging the innate and adaptive responses [214–217]. In vertebrates, there are at least three well described complement–activation pathways which can be initiated by different sensor molecules: 1. Antibodies and C1q for the classical pathway; 2. Lectins, such as mannose-binding lectin (MBL) or ficolin, for the lectin pathway; and 3. Autocatalytic C3, for the alternative pathway. One common convergence point for all

three pathways is the production of C3b by means of enzymatic cleavage of C3 into two main fragments: C3a and C3b. C3b can then associate with other proteins to stimulate the formation of the membrane attack complex (MAC) and lead to target cell lysis. Alternatively, C3b can serve as an efficient opsonin, blocking and neutralizing dangerous cells and molecules and taking them for final removal and processing by phagocytosis. Moreover, C3 can be further catalyzed and these fragments are known to be biologically active as well, like C3a, an anaphylatoxin with strong leukocyte chemotactic properties [reviewed in [218]. When considering invertebrate complement, the current consensus states that some vertebrate homologs exist in lower taxa, but that this branch of innate immunity is much simpler than that of mammals. For example, Cerenius et al. [219], described the invertebrate complement as a "proto-complement system" and, based on comparative studies across different phyla, it was suggested that these organisms have a basic form of the complement system that will most likely consists of three main components: 1) a sensor molecule, most probably a lectin-like molecule; 2) a serine protease, able to recognize the binding of the sensor to the target and serve as an activator for the effector mechanism; and 3) a C3-like protein, serving as the effector molecule binding to the target and helping in neutralization and removal via phagocytosis [220,221]. This hypothesis remains to be tested experimentally, but as more invertebrate homologs of complement components are identified, a better picture continues to form, and testing this hypothesis becomes easier. In most of the invertebrate organisms used to study the complement system, identification of C3 is considered as the "golden standard", and sufficient evidence for the presence of such an immune mechanism. In cephalopods, C3 has been identified and characterized at the molecular level in the sepiolids *E. scolopes* [222] and *E. tasmanica* (unpublished data). In addition, in *E. scolopes*, C3 transcripts were detected in all tissues tested and the protein was found to be expressed in epithelial cells of several tissues including the LO, gills, and skin of juvenile squid [222]. This is not surprising if we consider C3 as an important surveillance component needed throughout the animal, especially in areas exposed to the outside environment. Furthermore, comparative studies performed in adult *E. scolopes* have suggested an up-regulation of C3 in hemocytes from animals where the symbiont *V. fischeri* is absent [113]. Other molecules with homology to complement components have been identified in transcripts of *E. scolopes* [reviewed in [129] and *E. tasmanica* squid (unpublished data). Complete sequences have been obtained in *E. scolopes* for C1q-like, C1qBP, MBL-like, and MASP-like transcripts, although functional data is still missing to confirm their biological activity.

C3 molecules belong to a large family of related proteins called thioester-containing proteins (TEPs). TEP proteins are divided into 3 major groups: C3-like, A2Ms, and insect TEPs (iTEPs) [223]. As part of the TEP family, these proteins are characterized by several conserved domains, the most characteristic being the thioester domain, which is involved in the binding to target molecules. iTEP proteins appear to have evolved from an A2M ancestor but have functional convergence with C3 molecules, due to their opsonin activity [219]. In addition, other less studied iTEP-related proteins have been identified but their function and evolutionary relation are still being investigated. These include CD109 [224], pregnancy zone protein (PZP) [225], and C3/PZP-like/A2M domain containing 8 protein (CPAMD8) [226]. Precise molecular characterization of TEP-containing proteins is difficult, mainly due to the presence of sequence domains common to many of them. This is especially true when full-length sequences are not available. For example, a TEP identified in *E. scolopes* and *E. tasmanica* was originally thought to be an iTEP, but after further analysis, the sequence revealed closer homology to members of the CD109 group [132,227]. *E. scolopes* CD109 was found to be expressed in most tissues tested and its transcript was down-regulated in LO of juveniles harboring *V. fischeri*. The authors suggested that, similarly to C3, the presence of the symbiont modulates the squid's immune system to avoid damage and removal. In a recent transcriptome study in *O. vulgaris*, a homolog of C1q was identified, and its expression was found to be up-regulated in the gills and caecum of animals suffering from a protozoan infection [134]. Taken together, these results point towards the presence of a functional proto-complement system in the cephalopods, but much more remains to be investigated to determine how the complement system in these animals compares to that in other invertebrate systems.

4.5. Other antimicrobials

In recent studies, several other cephalopod proteins have been found to have defense functions. One such molecule is hemocyanin. Hemocyanins are invertebrate metalloproteins found in the hemolymph of molluscs and arthropods, and mainly known for their role in oxygen transport. Structurally, hemocyanins are made of many individual copper-containing subunits of about 75 kDa that can bind oxygen. Depending on the species, hemocyanin subunits can be arranged in dimers or hexamers, which can, on their own, be arranged in large polypeptide chains or clusters of over 1500 kDa in weight [228]. It was not until the late 1990's that hemocyanin's immune function was recognized when phenoloxidase activity was detected in chelicerate hemocytes [229], and reviewed in [230]. Phenoloxidase activity is important for the melanization process, an essential part of the immune response of insects and crustaceans against physical injury and infection by bacteria and parasites [reviewed in [219]. Although melanization has not been studied in molluscs, recent studies implicate hemocyanins with antimicrobial activity in cephalopods. Earlier studies using immunohistochemistry reported the presence and synthesis of hemocyanin by cells of the branchial gland of *S. officinalis*, *L. brevis*, *Eledone moschata*, and *Octopus vulgais* [231–233], and in the midgut gland of *Nautilius pompilius* [234]. Further biochemical and molecular reports described and compared hemocyanin proteins in a variety of cephalopod species including the *S. lessoniana* [235], *Vampyroteuthis infernalis*, *Benthoctopus* sp., *Octopus dofleini*, *N. pompilius*, *S. officinalis* [236,237], and *E. scolopes* [238]. A recent study associates cephalopod hemocyanin with immune-related activity [238]. In the squid *E. scolopes*, two isoforms of hemocyanin were identified and expressed in multiple tissues but primarily in gills and branchial heart. Protein was detected in the symbiotic LO where it is thought to be secreted by circulating hemocytes and to be involved in oxygen release to promote bacterial growth and bioluminescence (a microbial process requiring oxygen). Moreover, hemocyanin was detected in the apical surface of the juvenile-LO epithelia and in the mucus secretions involved in attachment and selection of environmental bacteria. In *in vitro* studies, a peptide fragment from one of the hemocyanin isomers was found to have antimicrobial activity against a variety of marine bacterial species through phenoloxidase activity, and in this manner participate in the symbiont-selection process.

Also in *E. scolopes*, galaxin, a molecule with antimicrobial and bacteriostatic activity has been recently reported [239]. Galaxins were originally described in other symbiotic systems, namely the coral *Galaxea fascicularis* [240,241] and the hydrothermal giant worm *Riftia pachyptila* [242]. In the squid, galaxin was initially identified as one of the transcripts highly up-regulated and modulated in colonized LOs [243,244]. In a follow-up study, the up-regulation in expression of this transcript was correlated with symbiont growth in the LO following the daily venting of bacteria [239]. Immunolocalization showed the presence of this protein in

the epithelial cells and mucus secretions of the LO, and *in vitro* assays showed that a peptide fragment of galaxin had inhibitory effects on the growth of bacteria, which was mostly against Gram positive bacteria, but also included *V. fischeri*. The inhibitory effects against the symbiont bacteria could be rescued when an antibody against galaxin was added to the media, demonstrating the specific effect of this protein. In summary, it was suggested that galaxin is involved in both the selection of bacteria during the initial colonization stage of the LO, and later, in the maintenance of the symbiosis by controlling bacterial growth in symbiotic animals. An additional antimicrobial protein has been described in *E. scolopes*, the sensor molecule PGRP2 [131]. This protein was shown to have the capacity to bind and degrade bacterial peptidoglycan. Using immunohistochemical methods, PGRP2 was localized to epithelial surfaces exposed to the environment and shown to be secreted into the LO-mucus, suggesting that it played a role during the initial stages of colonization and selection of the symbiont. As with other antimicrobials in this squid, the protein was also found to be secreted into the crypts lumen, suggesting that it could also assist in modulating interactions between the squid host and the bacteria population once the symbiosis is established [131].

5. Conclusions

The interest and importance of cephalopod research continues to increase, and fortunately, they are further recognized as important model organisms for the understanding of a variety of topics from development and evolution [e.g. [245–247], physiology [e.g. [248–251], learning and memory [252], and beneficial symbiosis [17,253], to even hydrodynamics [254,255], camouflage [256–258], and robotics [e.g. [259–261]. In the present review we have summarized the current state of knowledge related to internal immune defenses in cephalopods. Although most of the literature focused on a few species, growing interest and technological advances will permit this field to expand and allow more detailed comparative studies with other molluscan and invertebrate organisms. It is now evident that cephalopods rely on innate components common to all animals and include recognizable molecular members at the three levels of the immune response cascade: sensor, signaling, and effector components. Future challenges include the discovery of more immune components, but also a better understanding of the interrelation between them, such as the specific roles these molecules have in immune reactions. The advantages offered by next generation sequencing and contemporary protein analysis have been welcomed by researchers working with cephalopods. They have given the opportunity to obtain an overview of the various molecular components and their biological behavior based on treatments or their temporal and spatial expression characteristics. More importantly, proteomic and transcriptome studies have opened new areas of inquiry by allowing the identification and selection of targets for future investigation. One successful example is the advances in our understanding on the establishment of beneficial microbial symbiosis using the squid-vibrio model system. The first report of this kind was in 2006 [262], where an annotated EST-library from the LO of *E. scolopes* revealed the presence of many immune-related transcripts. This pivotal study has served to guide the investigation of the molecular interactions between host and symbiont during the early stages of colonization, as well as later during the establishment of symbiotic homeostasis. Since then, several other studies were conducted, including microarray analyses performed on juvenile and adult LOs [243,244], two proteomic studies on adult hemocytes and the LO and its ventate [113,136], a transcriptome from the LO [170], adult hemocytes [113], and the white body of *E. tasmanica* [132], a sister species with similar symbiosis. These studies have identified novel immune-related molecules in sepiolid squid. The complete sequences have already been mentioned in suitable sections in the above paragraphs, and those for which there are known partial sequences are included in Table 1. Similar studies are also available from two other major cephalopod models, the octopus *O. vulgaris* and the cuttlefish *S. officinalis* (Table 1, Fig. 1). In *O. vulgaris* a couple of studies have examined immune components in the blood of octopi, including a two-dimensional gel analysis of hemolymph proteins [198] and a transcriptomic comparison of hemocytes in parasite-infected *versus* healthy animals [134]. Finally, in the cuttlefish *S. officinalis*, a transcriptome and proteome study using the posterior salivary glands identified several immune sensors and effector molecules [58]. Another potential source for elucidating cephalopod immune mechanisms lies in the understanding of signaling cascades linking sensor molecules with their appropriate effector molecules, as well as identifying the molecular modulators associated with such pathways. The benefits of this knowledge could be applied to areas of ongoing research, including that looking at pathogens that afflict cephalopods both in nature and in captivity [e.g. [115,263–267]. Future studies should also address how animals are coping with environmental challenges such as global warming and water pollution, and predict and test potential prophylactic and treatment protocols. One obvious void in the field is the lack of any form of genomic data [268]. Genomic data would supplement our understanding of the diversity and variety of immune components in these animals, as well as permit an in depth evolutionary and comparative analyses with other invertebrates. For example, until now, there is no evidence for unique genes or their pre/post transcriptional regulation as seen in other systems such as the fibrinogen-related proteins (FREPs) in snails [269], the C1q family in bivalves [270], the Down syndrome cell adhesion molecule (or DSCAMs) in crustaceans [271], or the still undefined family of variant proteins in sea urchins knows as the Sp185/333 gene family [272]. What these and other studies in invertebrate immunity have taught us is to expect the unexpected, and given the percentage of cephalopod transcripts that remain unknown, the possibilities for surprises do exist. An important challenge in this field is the availability of immortal cell lines and the standardization of protocols to prepare primary cell lines. To date, the only molluscan cell line available is that of the fresh water snail *B. glabrata* [273], where it has been a valuable tool in the study of host–parasite interactions [274]. Access to such tools for cephalopods will surely reduce the need to rely on live animals for experimentation and expedite the development and advanced in the areas of molecular biology including cell signaling and gene transfer. Overall, our knowledge and understanding of cephalopods and their defense systems has made great progress, but there is still much more to learn. The rewards will provide invaluable knowledge and benefit those animals that we find so beautiful, intriguing, and delicious.

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