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# PHILOSOPHICAL TRANSACTIONS B

## Antimicrobial peptides in marine invertebrate health and disease

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## Antimicrobial peptides in marine invertebrate health and disease

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### Summary

Aquaculture contributes more than one-third of the animal protein from marine sources worldwide. A significant proportion of aquaculture products are derived from marine protostomes that are commonly referred to as “marine invertebrates”. Among them, penaeid shrimp (Ecdysozoa, Arthropoda) and bivalve molluscs (Lophotrochozoa, Mollusca) are economically important. Mass rearing of arthropods and molluscs causes problems with pathogens in aquatic ecosystems that are exploited by humans. Remarkably, species of corals (Cnidaria) living in non-exploited ecosystems also suffer from devastating infectious diseases that display intriguing similarities with those affecting farmed animals. Infectious diseases affecting wild and farmed animals that are present in marine environments are predicted to increase in the future. This paper summarizes the role of the main pathogens and their interaction with host immunity, with a specific focus on antimicrobial peptides (AMPs) and pathogen resistance against AMPs. We also provide a detailed review of penaeid shrimp AMPs and their role at the interface between the host and its resident/pathogenic microbiota. We also briefly describe the relevance of marine invertebrate AMPs in an applied context.

### 1. Economic relevance of marine invertebrates

Oceans and seas cover two-thirds of our planet; provide ecosystem services, such as fishing, aquaculture, carbon sequestration, regulation of water quality and nutrient storage; and support numerous recreational activities, all of which significantly contribute to employment and economic activity [1]. In 2012, aquaculture provided an unprecedented total of 66.6 million tons of seafood, including 175 species of “marine invertebrates”, mainly crustaceans (Ecdysozoa, Arthropoda) and molluscs (Lophotrochozoa, Mollusca) [2]. Farmed crustaceans accounted for 9.7% (6.4 million tons) of the seafood production by volume, but 22.4% (US\$30.9 billion) by value. Shrimp is currently the largest single commodity in terms of value, with the main cultivated species being *Litopenaeus vannamei* and *Penaeus monodon*. In 2012, molluscs, such as oysters, mussels, scallops and clams, accounted for 20.5% (13.7 million tons) of seafood production. Molluscs are essentially produced for food, but they also contributed to 22,400 tons of non-food products, such as pearls and seashells for ornamental and decorative uses. However, disease outbreaks have heavily impacted this intensifying production over the past few years, and emerging infectious diseases are predicted to increase in the future in both wild and farmed animals as a result of climate change [3].

### 2. Infectious diseases affecting marine invertebrates

The health status of marine invertebrates is intimately related to the microbial communities that are present in the aquatic environment, which include both commensals and opportunistic pathogens. While microorganisms hosted by invertebrates help maintain homeostasis, under stressful conditions, some can become highly virulent and severely damage their host [4]. In coastal environments and lagoons, marine invertebrates are exposed to multiple abiotic stresses,

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which can be of anthropogenic origin. Thermal stress, high density [5], and nutrient rich environments [6] are factors favouring infectious diseases in the wild as well as in intensive farming. Remarkably, while marine invertebrates are incredibly diverse in terms of phylogeny and ecological niche, disease patterns are repeatedly found across species. These diseases include temperature-dependent vibrioses [7,8] and polymicrobial diseases [9,10]. Differences are observed in the susceptibility of the animals, from larvae to juveniles and adults, and in the diversity of the pathogens (vibriosis, viruses, etc.) that affect each developmental stage. Relevant examples of infectious diseases are presented below within three different phyla of marine invertebrates (Arthropoda, Mollusca and Cnidaria) that are directly/indirectly and intensively/extensively exploited by humans in a diversity of ecosystems.

**Arthropods.** Diseases in marine arthropods are dominated by those described in penaeid shrimp aquaculture, which is characterized by intensive cultural practices favouring disease development. Twenty viruses are known to infect penaeid shrimp. Two types of viruses cause major viral diseases, namely, DNA viruses, such as the monodon baculovirus (MBV) [11], the white-spot syndrome virus (WSSV) [12], the hepatopancreatic parvovirus (HPV) and the infectious hypodermal and hematopoietic virus (IHHNV) [13], and RNA viruses, such as the yellow-head virus (YHV), the Taura syndrome virus (TSV) [14] and the infectious myonecrosis virus (IMNV) [15]. WSSV is the most severe threat for farmed adult shrimp worldwide and is one of the best studied crustacean viruses [16]. Bacterial infections, particularly vibrioses, are a major concern for the production of shrimp larvae and juveniles. *V. harveyi*, and *V. vulnificus* are associated with larvae mortality [17], whereas *V. damsela*, *V. alginolyticus*, *V. parahaemolyticus*, *V. penaeicida* and *V. nishripulchritudo* cause disease outbreaks in shrimp nurseries or grow-out ponds [17]. In 2010, a new shrimp disease that affects postlarvae has emerged from Asia [18]. This acute hepatopancreatic necrosis disease (AHPND) is caused by a highly virulent strain of *V. parahaemolyticus*, which has acquired a virulence plasmid encoding a pore forming bacterial toxin that is as toxic as the insecticidal *Bacillus* Cry toxin [18].

**Molluscs** from the shellfish industry are affected by a variety of infectious diseases whose importance largely depends on the degree of exploitation and ecosystem health. The most significant epizootic events are caused by bacteria from the *Vibrio* genus [19,20], viruses from the Malacoherpesviridae family [21] and protozoans from the *Perkinsus*, *Marteilia*, *Bonamia* and *Haplosporidium* genera [17]. Some of these microorganisms can affect a broad range of mollusc species at all life stages around the world, while others are highly species-specific. The epizootic events that they cause are frequently devastating. Over the past few decades, abnormal mortalities of juvenile *C. gigas* oysters have affected the US, Japan, Australia and Western Europe. Those mortalities of complex aetiology are due to a temperature-dependent polymicrobial disease that involves pathogenic *Vibrio* strains of the Splendidus clade and the ostreid herpes virus OsHV-1  $\mu$ var [9,17,22]. Vibriosis also cause diseases at other oyster developmental stages. *Vibrio aestuarianus* is responsible for mortality of adult oysters [23], whereas *V. tubiashii* causes a necrotic disease in hatcheries [24].

**Cnidarians**, which live in coastal marine systems and lagoons, also suffer from infectious diseases. Among the known pathogens, all of the classical agents (Eubacteria, cyanobacteria, fungi and viruses) have been described [25,26]. Most diseases were observed in corals. One of the most famous coral diseases is type I White Band Disease (WBD). Described for the first time in the 1970s, WBD caused the loss of up to 95% of the Acroporid found throughout the great Caribbean area [27]. However, as with many coral diseases, the causative agents of type I WBD are unknown and do not fully satisfy the Koch postulate or correspond to microbial consortia [25]. Another well-known example is Black Band Disease, which is caused by cyanobacteria assemblages and other unidentified heterotrophic microbes [10,28]. Many of the known coral pathogens belong to the *Vibrio* genus. While *V. harveyi/charcariae* is the causative agent of type II WBD and other "White Syndromes" [29,30], *V. shiloi* and *V. coralliilyticus* are responsible for bacteria-induced bleaching in corals and tissue lysis in several other cnidarians [43–46].

### 3. Antimicrobial peptides (AMPs) in marine invertebrate immunity, a focus on penaeid shrimp.

The economic consequences of infectious diseases affecting farmed bivalve molluscs and arthropods (crustaceans) have motivated a substantial research effort, which has considerably enriched our knowledge of the immune system of protostomes. Similarly, coral bleaching events are a threat for society and have inspired studies on the interaction of the immune system of cnidarians with its resident microbiota and pathogens. To date, AMPs are among the best described immune effectors of marine invertebrates.

#### 3.1. Diversity and specificity of AMPs in the immune response of marine invertebrates

Similar to AMPs from other phyla [36], most of the AMPs characterized in marine arthropods, including molluscs and cnidarians, are cationic and hydrophobic and target essential components of microbial cell walls and membranes, which determines their spectrum of activity [37]. A high diversity of mechanisms of action has been reported for AMPs from marine invertebrates and is described in detail for some families. For example, mollusc defensins, which are essentially active against Gram-positive bacteria, bind to lipid II, the precursor of peptidoglycan [38]. Arthropod anti-lipopolysaccharide factors (ALFs) and mollusc bactericidal/permeability-increasing protein (BPI), which are essentially active against Gram-negative bacteria, bind to lipopolysaccharide (LPS) [39–41]. Finally, crustacean PvHCt, which is strictly antifungal, permeabilizes the fungal plasma membrane [42].

Those AMP families have been generated through different patterns of diversification (gene duplication, gene copy number variation, recombination and allelic polymorphisms) [37] due to multiple evolutionary drivers and have given rise to functional divergence. Some families appear to have evolved within specific phyla or species [43], whereas others are found in a diversity of phyla [44]. Most AMP families from molluscs, including defensins, big-defensins and BPI (for recent review see [45]), are also found in other protostomes (Ecdysozoa) and/or in deuterostomes (Mammalia). In contrast, in cnidarians, taxonomically restricted AMPs have been described, such as arminins, which are among the most highly expressed genes in *Hydra* [46], or damicornin in the coral *Pocillopora damicornis* [47]. In marine arthropods, the pioneering studies in Chelicerata (horseshoe crabs) have identified both taxonomically restricted (tachyplesins, ALFs) and more widely distributed AMPs (big defensins) [40,48,49]. Finally, in decapod crustaceans, highly diverse AMPs were characterized that are specific to penaeid shrimp (penaeidins, stylicins), crustaceans (crustins) or arthropods (ALFs) [50,51]. The best known AMPs from crustaceans were characterized in penaeid shrimp, and many of them are composed of structural domains that have distinct biological functions [50,52]. A description of the major AMP families in shrimp is provided below. Unlike in insects, there is still little knowledge about the molecular regulation of AMPs in marine invertebrates. In shrimp, which is one of the best described organisms, some AMP families are controlled by NF- $\kappa$ B signalling pathways. Alternatively, mature AMPs can be stored in immune cells and are released upon challenge (see below).

### 3.2. Gene-encoded AMPs from penaeid shrimp

#### 3.2.1. Penaeidins

Penaeidins were the first AMPs characterized in shrimp [53]. Those peptides, which are restricted to species of penaeid shrimp, are abundant in the circulating immune cells (the haemocytes) of *L. vannamei* [53]. They are cationic peptides (4.7-7.2 kDa; pI ~9) composed of an unconstrained N-terminal proline/arginine-rich domain followed by a C-terminal domain that contains an amphipathic helix and two coils stabilized by three disulphide bonds (Table 1) [53,54]. Penaeidins can carry post-translational modifications, such as a N-terminal pyroglutamic acid and an amidated C-terminus. AMPs from this diverse family fall into 4 subgroups (PEN1/2, PEN3, PEN4 and PEN5, Table 1), whose specific sequence signature and biochemical features have been used to standardize their nomenclature after the name of the shrimp species and the penaeidin subgroup [55]. Each subgroup is encoded by distinct genomic sequences [55,56]. While the PEN3 gene is widely distributed among species of penaeid shrimp, PEN2, PEN4 and PEN5 genes are restricted to a given species of shrimp [57]. PEN genes are highly and constitutively expressed in haemocytes of healthy individuals [58] [59]. Penaeidins, which are stored in haemocyte granules, are released in response to microbial challenge [59]. Penaeidin sequence diversity translates into diverse biological activities [60]. Most penaeidins from subgroups PEN1/2, PEN3 and PEN4 are active against Gram-positive bacteria and filamentous fungi, but not Gram-negative bacteria. In contrast, a PEN5 member (*Fenchi* PEN5) from *Fenneropenaeus chinensis* is active against Gram-negative bacteria [56]. *Penmon* PEN5 from *P. monodon* also participates in the shrimp antiviral defence against WSSV [61]. Although little is known about the mechanism of action of penaeidins, the function of the proline/arginine-rich domain was investigated in different penaeidins. This domain was devoid of antimicrobial activity in PEN3 [59], but possessed both antifungal and antibacterial activity in PEN4 [62]. Interestingly, this domain was also reported to behave as a cytokine by attracting haemocytes towards sites of injury [63]. In addition, the cysteine-rich domain of PEN3 was proposed to mediate penaeidin antifungal activity by binding to chitin [59]. The ability of penaeidins to bind to the chitin exoskeleton suggests that penaeidins could play a role in wound healing and/or moulting in shrimp [52].

#### 3.2.2. Anti-lipopolysaccharide factors (ALFs)

ALFs form a diverse family of AMPs that are composed of both cationic and anionic polypeptides. First identified in horseshoe crabs and later in penaeid shrimps [64] and other crustaceans [57], ALFs were named “anti-LPS factors” for their immunomodulatory function. ALFs are able to inhibit the LPS-mediated activation of the limulid coagulation system [40,41]. ALFs contain a hydrophobic N-terminal region with two conserved cysteine residues. The disulphide-bond delimits a  $\beta$ -hairpin structure that is referred to as the LPS-binding domain [65]. Most ALFs bind to Lipid A from Gram-negative bacteria, but they can also interact with lipoteichoic acid (LTA) from Gram-positive bacteria [66] and  $\beta$ -glucan from fungi [67]. The known 3D-structures of ALFs consist of three  $\alpha$ -helices (one at the N-terminus and two at the C-terminus) packed against a four-stranded  $\beta$ -sheet (Table 1) [65,68].

In shrimp, ALFs form a large and diverse family of five groups, namely, ALF-A (anionic and cationic polypeptides of 11.4-11.5 kDa), ALF-B (highly cationic polypeptides of 10.6-11.2 kDa), ALF-C (cationic polypeptides of 11-11.3 kDa), ALF-D (highly anionic polypeptides of 10.7-10.8 kDa) and ALF-E (anionic and cationic polypeptides of 11.4-12.5 kDa) (Table 1) [69,70]. ALFs are encoded by separate genes and are transcribed at basal levels in healthy individuals [69]. In *L. vannamei*, ALF genes are differentially expressed in response to a fungal infection. Although ALF-A gene expression remains stable, the other ALF genes are inducible [69]. While ALFs can be detected in diverse shrimp tissues, results for ALFPm3 strongly suggest that the expression of ALF-B is restricted to haemocytes, which infiltrate shrimp tissues [66]. ALFs are potent and broad spectrum AMPs. For example, cationic Group B ALFs are active against Gram-positive, Gram-negative bacteria, yeast, filamentous fungi and some enveloped viruses [50]. In contrast, anionic Group D ALFs have impaired LPS-binding properties and display very low antimicrobial activity *in vitro* [69].

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### 3.2.3. Crustins

Crustins are antimicrobial polypeptides (6-22 kDa; pI 4-8) containing a Whey Acidic Protein (WAP) domain [71]. This WAP domain, which is also found in some mammalian proteins, supports different biological functions, including antiprotease activities [72]. Crustins are composed of four members (Type I to IV) that are widely distributed across crustaceans [71], which differ by their N-terminal sequence. While the sequence of Type I crustins begins with a cysteine-rich domain, Type II crustins (subtypes IIa and IIb) harbour a glycine-rich hydrophobic domain at the N-terminal position ahead of the cysteine-rich domain that is also found in Type I crustins (Table 1). Comparatively, Type III crustins can contain (or not) a short proline/arginine-domain at the N-terminus, whereas Type IV crustins are composed of two WAP domains, but do not harbour any specific N-terminal sequence (Table 1) [71]. Data on crustin *Pm1* strongly suggest that crustins are mainly expressed by haemocytes [73].

The diversity of crustin sequences supports diverse biological functions. While Type I and type II crustins are mainly antimicrobial, their spectrum of activity varies with the crustin type/sub-type. For instance, in *P. monodon*, a Type IIa crustin is essentially active against Gram-positive bacteria, while Type IIb crustins are active against both Gram-positive and Gram-negative bacteria [73,74]. Interestingly, Type III and Type IV crustins show antimicrobial and/or antiprotease activity [71], whereas Type III crustins lacking the N-terminal proline/arginine-domain and Type IV crustins from shrimp [75,76] only have antiprotease activity [71]. Type IV crustins from crabs exhibit both antimicrobial and antiprotease activities [76,77].

### 3.2.4. Stylicins

Stylicins are anionic (pI 5) multi-domain peptides of 8.9 kDa that are composed of a N-terminal proline/arginine-rich domain followed by a C-terminal domain containing 13 cysteine residues (Table 1) [78]. The recombinant *Ls-Stylicin-1* (from *Litopenaeus stylirostris*) is active against the filamentous fungus *Fusarium oxysporum*, but is not antibacterial. As observed for Group B ALFs, *Ls-Stylicin-1* shows potent LPS-binding activity and was also able to agglutinate Gram-negative bacteria *in vitro* [78].

## 3.3. Shrimp AMPs encrypted in multifunctional proteins

### 3.3.1. Hemocyanin-derived fragments

In addition to gene encoded AMPs, diverse AMPs from crustaceans are encrypted in large proteins carrying non-immune functions. Haemocyanins are respiratory proteins found in arthropods. Interestingly, crustacean haemocyanins release histidine-rich antimicrobial peptides in response to microbial challenge [79,80]. In penaeid shrimp, strictly antifungal peptides are released from the C-terminus of haemocyanins [79]. Recently, the 3D-structure of the antifungal peptide PvHCt from *L. vannamei* was determined, and its mechanism of action was investigated. This histidine-rich AMP was shown to selectively bind to the fungal cell wall and permeabilize fungal membranes by adopting an amphipathic alpha-helical structure. Insertion of PvHCt into the plasma membrane disrupts its integrity as a permeability barrier, leading to a disruption of internal homeostasis and the death of the fungal pathogen [42].

### 3.3.2. Histones and derived fragments

Histones are essential protein components of the chromatin architecture. The antimicrobial activity of histones was first described in deuterostomes (mammalians) [81]. Histones and derived fragments are active against Gram-negative and Gram-positive bacteria, fungi and viruses with various modes of action, including the permeabilization of bacterial cell membrane and binding to bacterial DNA and/or RNA [82]. The role of histones in shrimp defence was first determined in *L. vannamei* [83]. The extracellular release of histones is associated with a defence reaction named ETosis, in which phagocytes release histones associated with extracellular traps (ETs) of DNA that entangle and eventually kill microbes [84]. ETs have now been described in deuterostomes [85] and protostomes, including species of Ecdysozoa (insects [86], crustaceans [87]) and Lophotrochozoa (molluscs [88]). This process is triggered by infection and/or tissue damage. ROS production is a signal that triggers ET formation in mammals [89] and lophotrochozoans [88]. In shrimp, haemocytes also release ETs in response to ROS inducers [87]. It will be of great interest to identify the haemocyte types that are involved in ETosis and determine whether the AMPs that are stored in haemocytes, like penaeidins, contribute to the antimicrobial activity of shrimp ETs.

## 4. AMPs in marine invertebrate immune-microbiota interactions

Marine invertebrates host a broad diversity of microorganisms in their tissues and haemolymph, including vibrios [4], which have the potential to become pathogenic and cause severe disease outbreaks (see section 2). Some adopt intracellular stages and are able to survive inside phagocytes [90,91]. As illustrated in section 3, haemolymph, phagocytes and epithelial tissues are rich in AMPs. We are therefore facing a puzzling paradox, that is, microorganisms have evolved the ability to colonize immune cells/tissues that produce high local concentrations of AMPs. Although still incompletely understood, the recent literature sheds some light on the role of AMPs in the control of microbiota (including pathogens) and the mechanisms by which microorganisms avoid the complex chemical defences of their hosts.



4.1. Essential role of AMPs in the control of the microbiota

The application of gene silencing to non-model organisms has opened the way for *in vivo* functional studies that provide a more exhaustive view on the role of AMPs at the interface between hosts and microorganisms (Table 1). RNA interference (RNAi) confirmed the essential role of AMPs in controlling infections, as demonstrated for Type I crustins from *Marsupenaeus japonicus*, which participate in bacterial clearance in shrimp haemolymph [92,93]. It also confirmed the functional divergence of AMP variants. Indeed, *in vivo*, LvALF1 (Group A ALF) protected *L. vannamei* against *V. penaeicida* and *F. oxysporum*, but not WSSV [94], whereas ALFPm6 (Group C ALF) in *P. monodon* protected against both *V. harveyi* and WSSV [95]. However gene silencing also showed a more complex role of AMPs in the immune response of shrimp. Indeed, Type II crustins, which are not active against Gram-negative bacteria *in vitro* [50], participate in the resistance of *L. vannamei* and *M. japonicus* to Gram-negative *V. penaeicida in vivo* [96,97]. In addition, through the use of gene silencing, AMPs were shown to orchestrate a key interface with the resident microbiota in marine invertebrates. In arthropods (shrimp), ALFs controlled shrimp-associated microbial communities [95,98]. Indeed, silencing of ALFPm3 (Group B ALF) in *P. monodon* caused a rapid propagation of bacteria, resulting in the death of the animals [95]. In freshwater cnidarians (*Hydra*), species-specific AMPs called arminins were found to define host-species-specific bacterial associations [99]. With those findings, host-specific AMPs were proposed to have evolved in early branching metazoans because of the need to control the resident beneficial microbes and not because of invasive pathogens [100]. Novel functions (non-antimicrobial) of AMPs were also discovered through gene silencing. Thus, silencing of PEN3 from the shrimp *P. monodon* resulted in a decrease in the  $\beta$ -integrin-dependent adhesion properties of shrimp haemocytes, revealing a previously unknown immunomodulatory function for penaeidins [101]. Gene silencing techniques applied to anionic AMPs, such as Group D ALFs [69] and stylicins [78], which show poor antimicrobial activity *in vitro*, should enable the characterization of their role in shrimp defence.

4.2. Responses of pathogens to AMPs

4.2.1. Immune suppression of AMPs by pathogens

Among strategies to escape the immune response, the suppression of AMP expression was shown in different *phyla* of marine invertebrates, including arthropods and cnidarians. In the shrimp *L. vannamei*, an infection by the fungal pathogen *Fusarium solani* dramatically repressed the expression of penaeidins, stylicins and Type II crustin [102]. Similarly, immune suppression of AMPs was reported in the coral *P. damicornis*, in which *V. coralliilyticus* exhibits temperature-dependent virulence, resulting in coral bleaching above 24 °C and tissue lysis above 25 °C [7]. In its non-virulent state (below 24 °C), *V. coralliilyticus* induces the transcription of several coral immune genes [103] and the release of damicornin in coral mucus [47]. In contrast, in its virulent state, vibrios penetrating into coral tissues [104] induce a strong transient expression followed by a dramatic repression of damicornin transcription [47]. To date, the molecular mechanisms underlying the immune suppression of AMPs remains to be discovered.

4.2.2. Resistance to AMPs in vibrios

The membranes of microorganisms are an important interface with host AMPs (see section 3). Consequently, in vibrios, many of the most important mechanisms of AMP-resistance rely on outer membrane remodelling. Another important mechanism of resistance is the active efflux of AMPs from the bacterial cell. Those mechanisms have been recently reviewed in detail [105]. The most significant are those in which the minimum inhibitory concentrations (MICs) increase by several fold and are summarized below.

**Membrane charge alteration.** As electrostatic interactions often initiate the binding of cationic AMPs to bacterial membranes, bacteria colonizing metazoan hosts have evolved strategies to lower the net negative charge of cell surface molecules; in Gram-negative bacteria, such modifications are often observed on LPS, the major constituent of their outer membrane [106,107]. Vibrios, including human pathogens, live in close association with marine invertebrates [108], which in turn may have selected AMP-resistant phenotypes among vibrios. A structural modification of LPS was recently found to be responsible for the different AMP-resistant phenotypes observed in *V. cholerae*. Indeed, the Lipid A structure (the anionic membrane anchor of LPS) of *V. cholerae* O1 and O139 [109] contains a hydroxylated secondary acyl chain that is also found in the squid symbiont *V. fischeri* [100]. This structure plays an important role in the resistance to AMPs [109,110], as it can be substituted by di-Glycine residues that lower the negative charge of the whole molecule [111].

**Release of outer membrane vesicles.** Upon outer membrane stress, such as that created by AMPs, bacteria can use the alternate  $\sigma$ E factor to promote the expression of factors that help preserve and/or restore cell envelope integrity. The release of outer membrane vesicles (OMVs) is a  $\sigma$ E-dependent mechanism [112], whose role in AMP resistance has been recently shown in vibrios. Vibrios, including the oyster pathogen *V. tasmaniensis* LGP32, were shown to release OMV protection against membrane-active AMPs [113,114]. Interestingly, OMV release was triggered by oyster plasma, suggesting, as shown in *E. coli* [115], that the membrane-active agents that are present in oyster plasma can trigger OMV release in vibrios. While the major protective effect of OMVs against AMPs has now been shown in two vibrio species and in *E. coli* [116], it is still unknown whether this effect results from a membranous shield-like effect in which OMVs surround vibrios and trap membrane-active AMPs, thereby preventing their interaction with the membranes of the bacterial cell, or a membrane renewal mechanism eliminating AMP-damaged membranes to maintain membrane integrity. The  $\sigma$ E-dependent induction of OMV release tends to support the second hypothesis.

**Active efflux of AMPs.** Once AMPs have breached the membrane barriers of bacteria and reached the cytoplasmic space, they can be expelled into the extracellular milieu by diverse efflux pumps. Pumps of the resistance-nodulation-cell division superfamily (RND) contribute to AMP-resistance in vibrios. However, among the 6 RND efflux pumps of *V. cholerae* [117], only VexAB-TolC is required for AMP resistance *in vitro* [118,119]. The VexAB-TolC pump is structurally and functionally similar to the *E. coli* and *Salmonella enterica* AcrAB-TolC pump [120,121] and the *Pseudomonas aeruginosa* MexAB-OprM systems [122]. VexAB is also the main efflux pump that is involved in the resistance to bile acids, detergents, antibiotics, and PmB [119,123]. To date, among vibrios, *V. cholerae* remains the only species in which AMP-resistance was mediated by an RND efflux system.

## 5. Marine invertebrate AMPs in an applied context

### 5.1 AMPs as therapeutic drugs

Since their first discovery in the 1980s, AMPs have been considered to be promising candidates for therapeutic uses in humans, animals and plant health. However, only a few AMPs have reached phases of clinical and preclinical pipelines [124,125]. AMPs with potential interest for biopharmaceutical companies have been isolated from marine invertebrates. Some AMPs, such as mollusc defensins, have very low MICs in the nanomolar range against Gram-positive bacteria [38]. Their fungal homologue, plectasin, is considered to be a major candidate for therapeutic use [126]. Importantly, AMPs are currently being used for drug development due to their activity as immune modulators, which gives them clinical potential beyond the treatment of antibiotic-resistant strains [127]. Among AMPs from marine invertebrates, ALF-derived peptides have been shown to modulate the inflammatory response in murine macrophage cell lines [128] and display antitumor activity against HeLa cells through the alteration of the cell membrane [129]. Those novel activities may open the way to future drug developments.

### 5.2 AMPs in aquaculture

Disease prevention in marine invertebrate aquaculture has been traditionally based on the control of pathogens or the selection of animals that are resistant to diseases. AMPs of marine invertebrates have been considered for both applications.

**AMPs in the control of pathogens in aquaculture.** In the context of intensifying aquaculture, antibiotics have been used for disease prevention and management, which has resulted in increased bacterial resistance in the environment, and favoured the emergence of resistant strains of major human pathogens [130]. If AMPs are proposed as an alternative to the use of antibiotics, which should be reduced or avoided in aquaculture (FAO/OIE/WHO), there is a risk of promoting the emergence of AMP-resistant strains (see section 4), and this resistance can readily be achieved experimentally [131]. However, AMPs present some important advantages over antibiotics, including the following: (i) they are much less stable in the environment; (ii) they often combine multiple mechanisms of action, *e.g.*, they can both disrupt bacterial membranes and inhibit metabolic pathways; and (iii) they do not increase bacterial mutation rates [132]. However, most of all, it is now recognized that the antimicrobial activity of AMPs largely relies on their immune regulatory properties, including the recruitment and differentiation of immune cells [133], against which microorganisms cannot evolve simple mechanisms of resistance. The finding that AMPs are not simple antimicrobials but are complex orchestrators of host defences has opened new perspectives to combat bacterial infections. AMPs from marine invertebrates have appeared to be attractive candidates to reduce the impact of diseases in closed aquaculture systems that have a low impact on the environment [17]. However, only rare applications have been reported. AMPs have been recently applied in the Polynesian pearl industry [134]. For example, tachyplesin was combined with exopolysaccharides as filming agents instead of the antibiotics that are traditionally used in the grafting process to reduce oyster post-operative mortality and increase pearl quality. The result of this alternative process was similar to that of commercial nuclei that were treated with antibiotics [135]. To date, the use of AMPs as immune modulators has not been reported in aquaculture.

**AMPs as markers for survival capacity in shrimp.** AMPs control homeostasis in marine invertebrates and maintain individuals in a healthy state (see section 4). Inspired by studies in humans, which have investigated the associations between the copy number of AMP encoding genes and susceptibility to diseases [136,137], signatures of AMP expression have been studied in marine invertebrates that are susceptible or resistant to infectious diseases. Thus, in the shrimp *L. stylirostris*, the basal expression of AMP-encoding genes (PEN2 and PEN3, ALF-D, Type II crustin and lysozyme) has been correlated with the capacity of the shrimp to circumvent *V. penaeicida* infections. As a consequence, signatures of AMP expression have been proposed as original molecular markers for selection programs that are dedicated to shrimp resistance to bacterial infections [138].

## 6. Conclusion

Based on our current knowledge, there is probably not a general scheme but a diversity of roles for AMPs in the homeostasis of “marine invertebrates”. Some AMP families are highly abundant in host cells and tissues, whereas others are expressed at levels below inhibitory concentrations. Although the antimicrobial activity of AMPs has been the main focus of research, the immune regulatory properties of AMPs in the defence of marine protostomes and cnidarians now deserve a much greater research effort. In addition, some AMPs appear to have evolved within specific phyla or species,

whereas others evolved from a common ancestor and are widespread in the tree of life. The inspiring example of freshwater cnidarians prompts us to investigate how specific AMP repertoires may have shaped the host-specific microbiota formation of stable holobionts and compare those examples to other metazoan species with less specific AMP repertoires and more versatile microbiota. This research effort is now needed to obtain a more integrated view of the role of AMPs in symbiotic interactions that range from mutualistic to pathogenic to develop a better understanding of the role of AMPs in protostome and cnidarian health and disease.

Authors' Contributions

Authors have written and approved the final version of this manuscript.

Competing Interests

We have no competing interests.

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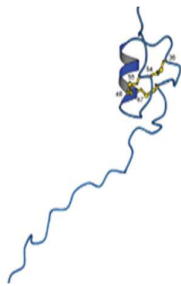













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Table 1: Principal families of AMPs in penaeid shrimp.

AMP	Variant	MW in kDa (charge)	Functions	Structural domains	3D structure
Penaeidins	PEN2	4.71-6.12 (cationic)	anti-Gram-positive activities antifungal activities antitumor activity		 <i>Litvan</i> PEN3 [PDB: 1UE0]
	PEN3	5.58-7.24 (cationic)	anti-Gram-positive activities antifungal activities chitin-binding properties β-integrin-mediated cytokine property		
	PEN4	5.23-5.37 (cationic)	anti-Gram-positive activities antifungal activities		
	PEN5	6.42-6.45 (cationic)	antibacterial and antifungal activities antiviral defense		
ALFs	ALF-A	11.4-11.5 (anionic and cationic)	antibacterial and antifungal defenses		 ALFPm3 [PDB: 2JOB]
	ALF-B	10.6-11.2 (cationic)	antibacterial and antifungal activities antiviral activity against human viruses LPS- and LTA-binding activities inhibition of viral propagation control of hemolymph microbiota		
	ALF-C	11-11.3 (cationic)	antibacterial and antiviral defenses LPS neutralizing activity control of hemolymph microbiota		
	ALF-D	10.7-10.8 (anionic)	not determined		
	ALF-E	11.4-12.5 (anionic and cationic)	bacterial clearance antibacterial defense		
Crustins	Type I	9.34-17.79 (anionic and cationic)	anti-Gram-negative activity bacterial-binding activity bacterial clearance hemocyte phagocytosis antiprotease activity		not determined
	Type II	11.22-22.45 (cationic)	anti-Gram-positive activity LPS- and LTA-binding activities inhibition of viral propagation bacterial agglutination activity antibacterial defense		
	Type III	5.96-7.40 (cationic)	anti-Gram-positive activity bacterial-binding activity antiprotease activity		
	Type IV	10.93-11.14 (cationic)	bacterial-binding activity antiprotease activity		
Stylicins	I	8.9 (anionic)	antifungal activity LPS-binding activity bacterial agglutination activity		not determined
	II		not determined		
<div><div> Glycine-rich domain</div><div> WAP domain</div><div> β-sheet</div><div> PRP-rich domain</div><div> Cysteine-rich domain</div><div> α-helix</div></div>					