

REVIEW

Antimicrobial peptides in crustaceans**RD Rosa, MA Barracco**

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Abstract

Crustaceans are a large and diverse invertebrate animal group that mounts a complex and efficient innate immune response against a variety of microorganisms. The crustacean immune system is primarily related to cellular responses and the production and release of important immune effectors into the hemolymph. Antimicrobial proteins and/or peptides (AMPs) are key components of innate immunity and are widespread in nature, from bacteria to vertebrate animals. In crustaceans, 15 distinct AMP families are currently recognized, although the great majority (14 families) comes from members of the order Decapoda. Crustacean AMPs are generally cationic, gene-encoded molecules that are mainly produced by circulating immune-competent cells (hemocytes) or are derived from unrelated proteins primarily involved in other biological functions. In this review, we tentatively classified the crustacean AMPs into four main groups based on their amino acid composition, structural features and multi-functionality. We also attempted to summarize the current knowledge on their implication both in an efficient response to microbial infections and in crustacean survival.

Key Words: antimicrobial peptides; innate immunity; crustacean defense; crustaceans; decapods

Introduction

Crustaceans compose a large, ancient and diverse animal group that includes many well-known, commercially exploited members, such as shrimp, crab, crayfish and lobster. Together with insects, they are by far the most numerous, diverse and widespread animals on Earth. Crustaceans are primarily marine organisms. They are the most abundant animals inhabiting the world oceans, but there are also freshwater, terrestrial and semi-terrestrial species. During their long-standing existence, crustaceans have confronted a broad variety of challenges to their self-integrity because their natural habitats are typically overloaded with infectious organisms, such as viruses, bacteria, fungi and other parasites. Their evolutionary success confirms the efficient strategies they use for survival in such a potentially hostile and microbe-enriched environment. Part of this coexisting microbial biota is beneficial and can establish advantageous associations with the crustacean in a commensal or mutualistic manner, whereas another

part of this biota is potentially harmful. Indeed, crustaceans can harbor specific microbial communities in their surface and internal epithelia that have important roles in nutrition and defense (Gil-Turnes *et al.*, 1989).

In normal conditions, crustaceans maintain a healthy state and keep infections under control. Externally, they are covered by a hard, rigid exoskeleton that functions as an efficient physico-chemical barrier against mechanical injury and microbe invasion. Their gastrointestinal tract, another important route for pathogen invasion, is also protected almost entirely by chitinous membranes. This cuticular coat, in combination with an acid environment rich in digestive enzymes, is able to inactivate and degrade most viruses and bacteria (Jiravanichpaisal *et al.*, 2006). However, once the cuticle barriers are disrupted, pathogenic and/or opportunist microorganisms can penetrate into the hemocoel and thus activate the internal immune defenses of the crustacean.

Crustaceans lack the complex and highly specific adaptive immune system of vertebrates, which is based on lymphocytes, immunoglobulins and immunological memory. Their internal defenses rely only on innate immune responses that are relatively less specific, but are fast and efficient defenses against microbes. The innate immune system of crustaceans is primarily related to their

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blood or hemolymph and is comprised of cellular and humoral responses. Humoral defenses include pattern-recognition receptors/proteins that recognize pathogen-associated molecular patterns (PAMPs), the production of toxic oxygen and nitrogen metabolites, complex enzymatic cascades leading to melanization, clotting proteins and antimicrobial peptides. Conversely, cellular immune responses are mediated by circulating blood cells or hemocytes (hyaline and granular hemocytes) that are capable of neutralizing and/or eliminating pathogens by phagocytosing microbes and/or trapping them in hemocyte aggregates or nodules or by encapsulating larger microorganisms and parasites (see review of Jiravanichpaisal *et al.*, 2006).

Antimicrobial peptides or proteins (AMPs) are one of the major components of the innate immune defense and are ubiquitously found in all kingdoms from bacteria to mammals, including fungi and plants. AMPs are primarily known as “natural antibiotics” because of their rapid and efficient antimicrobial effects against a broad range of microorganisms, including Gram-positive and Gram-negative bacteria, yeast, filamentous fungi and, to a lesser extent, protozoans and enveloped viruses (Bulet *et al.*, 2004; Yount *et al.*, 2006; Guaní-Guerra *et al.*, 2010). Recent evidence has shown that in addition to their antimicrobial activity, AMPs may encompass a number of other diverse biological roles and are, in fact, multifunctional molecules. It was demonstrated that these peptides have antitumor effects, mitogenic activity and, most importantly, participate in immunoregulatory mechanisms by modulating signal transduction and cytokine production and/or release (Kamysz *et al.*, 2003; Bowdish *et al.*, 2005; Brown and Hancock, 2006; Yount *et al.*, 2006; Easton *et al.*, 2009; Lai and Gallo, 2009; Guaní-Guerra *et al.*, 2010). Thus far, more than 1,500 AMPs sequences have been identified and are accessible on databases (<http://aps.unmc.edu/AP/main.php>) or in journal publications (see for review Thomas *et al.*, 2010).

AMPs are classically described as small cationic, amphipathic, gene-encoded molecules (<10 kDa, 15-100 amino acids) that differ considerably in amino acid sequence and structural conformation. They are commonly found in the blood or epithelial (mucosal) surfaces that are most exposed to microorganisms. More recently, other groups of antimicrobial molecules have been identified that do not fit into this classical definition. These are anionic peptides, proteins larger than 10 kDa and multifunctional proteins that contain antimicrobial sub-domains that are cleaved under certain conditions and generate fragments that resemble classical antimicrobial peptides (Brogden, 2005; Yount *et al.*, 2006).

The AMP mode of action is basically determined by structural conformation, cationic charge and amphipathicity. It is generally agreed that AMPs predominantly act by disrupting the membrane integrity of the cell target. The cationic portion of the peptide is first attracted to the negatively-charged bacterial and fungal cell walls and/or membranes, and following this first electrostatic interaction, the peptide inserts into and

permeabilizes the microbial cell membranes through its hydrophobic portion. The microorganisms are then destroyed via membrane destabilization and/or pore formation (Brogden, 2005; Yount *et al.*, 2006). The detailed mechanism of pore formation (barrel-stave, toroidal and carpet-like models) has been described in detail elsewhere (Brogden, 2005; Salditt *et al.*, 2006). Beyond this direct interaction with microbial membranes, a growing body of evidence suggests that AMPs may have additional mechanisms to inactivate pathogens. Numerous recent studies have documented that AMPs can be translocated into the cytoplasm of the microorganism where they act on specific intracellular targets. Once inside, the peptides interfere with several essential metabolic functions, such as protein, nucleic acid and cell wall syntheses, leading to bacterial cell death (Kamysz *et al.*, 2003; Brogden, 2005; Yount *et al.*, 2006; Hale and Hancock, 2007; Nicolas, 2009). Moreover, it appears that many AMPs may be multifunctional microbicides, acting simultaneously at the cell membrane and internal sites (Yount *et al.*, 2006).

Different families of AMPs have been identified in crustaceans. For economic reasons, the best characterized peptides come from cultivated species, such as marine shrimp. The cultivation of penaeid shrimp is a potent industry worldwide, which generates significant profits for the producing countries. This industry is consistently threatened by devastating disease outbreaks caused by infectious agents that usually lead to massive losses in shrimp production. To overcome this constraint, the study of shrimp immune system, including the antimicrobial peptides, is essential in order to better comprehend their internal defenses and open new perspectives for developing novel strategies to prevent and control infections in aquaculture. Besides marine shrimp, AMPs have been also identified and characterized in a number of other crustaceans, primarily decapods, including marine crabs and lobsters, freshwater crayfishes and prawns and also terrestrial species, such as isopods. The AMPs of penaeid shrimp have been very recently reviewed in great detail (Zhao and Wang, 2008; Tassanakajon *et al.*, 2010).

In this review, we discuss the current information on the different antimicrobial peptide families identified to date in all crustacean groups. Special emphasis is given to their structure, biological properties and involvement in host defense and survival. We also draw attention to the fact that crustaceans possess a significant number of single-gene encoded AMPs that are composed of distinct domains and are larger molecules (>10 kDa) than the classic antimicrobial peptides in other organisms.

Crustacean AMPs: structure, classification and biological properties

The earliest studies on isolation and characterization of AMPs in crustaceans date back to mid-1990s. The first isolated AMPs, including Bac-like, crustins and penaeidins, were purified from the hemolymph of crab and shrimp by biochemical approaches. Since then, novel AMP families have been identified in crustaceans using

Table 1 Summarized characteristics of crustacean AMPs

AMPs	Molecular mass (kDa)	Charge	Crustacean Order	First descriptions
Single-domain linear α-helical AMPs and peptides enriched in certain amino acids				
Bac-like	6.5	cationic	Decapoda (crab)	Schnapp <i>et al.</i> , 1996
Callinectin	3.7	cationic	Decapoda (crab)	Khoo <i>et al.</i> , 1999
Astacidin 2	1.8	cationic	Decapoda (crayfish)	Jiravanichpaisal <i>et al.</i> , 2007a
Armadillidin	5.2	cationic	Isopoda (woodlouse)	Herbinière <i>et al.</i> , 2005
Homarin	4-6	cationic	Decapoda (lobster)	Battison <i>et al.</i> , 2008
Single-domain peptides containing cysteine residues engaged in disulfide bonds				
Defensin	6.7-7.1	cationic	Decapoda (lobster)	Pisuttharachai <i>et al.</i> , 2009b
Anti-LPS factor	7-11	cationic	Decapoda (various)	Gross <i>et al.</i> , 2001 Supungul <i>et al.</i> , 2002
Scygonadin	10.8-11.4	anionic	Decapoda (crab)	Huang <i>et al.</i> , 2006
Multi-domain or chimeric AMPs				
Penaeidin	5.5-6.6	cationic	Decapoda (penaeid shrimp)	Destoumieux <i>et al.</i> , 1997
Crustin	7-14	cationic	Decapoda Amphipoda	Relf <i>et al.</i> , 1999
Hyastatin	11.7	cationic	Decapoda (crab)	Sperstad <i>et al.</i> , 2009b
Arasin	4.3-4.8	cationic	Decapoda (crab)	Stensvåg <i>et al.</i> , 2008
Stylicin	8.9	anionic	Decapoda (penaeid shrimp)	Rolland <i>et al.</i> , 2010
Unconventional AMPs				
Histones and derived fragments	11-15	cationic	Decapoda (penaeid shrimp)	Patat <i>et al.</i> , 2004
Hemocyanin-derived peptides	2.7-8.3 (shrimp)	anionic (shrimp)	Decapoda (shrimp, crayfish)	Destoumieux-Garzón <i>et al.</i> , 2001 Lee <i>et al.</i> , 2003
	1.9 (crayfish)	cationic (crayfish)		

different molecular approaches. At the present time, 15 AMP families or single peptides sharing common molecular features with the currently known AMP families have been recognized in crustaceans. All crustacean AMP families display antimicrobial activities against a number of specific microorganisms (with the exception of two putative β -defensin-like peptides identified in a spiny lobster species by a genomic approach). However, the high diversity of the variants (subgroups and isoforms) found in most crustacean AMP classes can presumably confer a broad spectrum of activity to a single AMP family.

In terms of structure, crustacean AMPs can be primarily defined as small, cationic, amphipathic molecules that are encoded by a single gene, as commonly described in other organisms. However, more recently, this definition was expanded to include less common AMPs, such as anionic peptides, multifunctional proteins that are primarily implicated in other cellular functions and protein-derived fragments that display antimicrobial

activities. Interestingly, an important number of gene-encoded AMPs in crustaceans are composed of different structural domains. Each of these domains possess singular features found in other AMP groups, such as the overrepresentation of specific amino acids or the presence of cysteine residues that form disulfide bonds. It has been proposed that these chimeric peptides could act as multifunctional proteins in different physiological systems in addition to their role in innate immunity (Bachère *et al.*, 2004).

Based on amino acid composition and structure, we tentatively clustered the families of antimicrobial peptides found in crustacean species into four main groups: (1) single-domain linear α -helical AMPs and peptides enriched in certain amino acids, (2) single-domain peptides containing cysteine residues engaged in disulfide bonds, (3) multi-domain or chimeric AMPs and (4) unconventional AMPs including multifunctional proteins and protein-derived fragments that exhibit antimicrobial functions (Table 1). These groups

encompass the major characteristics of crustacean AMPs, although using this classification, some families could be categorized into more than one group.

Single-domain linear α -helical AMPs and peptides enriched in certain amino acids

Linear amphipathic peptides comprise a large group of AMPs that lack cysteine residues engaged in disulfide linkages. These peptides are usually unfolded in solution, but can adopt an amphipathic α -helical structure in the presence of lipid bilayers (Brogden, 2005). They are largely found in many vertebrate and invertebrate species, displaying a broad spectrum of antimicrobial activities against bacteria, fungi and protozoa. Additionally, some linear peptides are enriched with a high proportion of select residues, particularly arginine, proline, glycine, tryptophan or histidine (Tossi and Sandri, 2002). To date in crustaceans, five linear AMPs have been described, including homarin, armadillidin and three proline/arginine-rich peptides (Bac-like, callinectin and astacidin 2).

Proline/arginine-rich peptides

Historically, the first antimicrobial peptide characterized in crustaceans was a 6.5-kDa proline/arginine-rich (or PRP-rich) cationic peptide isolated from the hemocytes of *Carcinus maenas* (Schnapp *et al.*, 1996). Partial sequence analysis of the amino (N)-terminal region revealed that this peptide contains three repetitions of the motif PRP (Proline-Arginine-Proline), which are usually found in other insect antimicrobial peptides (Bulet and Stöcklin, 2005) (Fig. 1A). Moreover, due to significant amino acid similarities to bactenecin-7, a cathelicidin antimicrobial peptide from bovine neutrophils (Frank *et al.*, 1990), this AMP is also known as Bac-like. Similar to the bovine AMP, the 6.5-kDa peptide was active against both Gram-positive (*Micrococcus luteus*) and Gram-negative (*Psychrobacter immobilis*) bacteria in inhibition zone assays. Interestingly, it also shares high sequence similarities with the PRP-rich domain of penaeidins (see section 3.1), an AMP family found exclusively in penaeid shrimp. Unfortunately, this first-identified crustacean AMP was not further characterized, and it remains unclear if its full-length sequence contains only PRP-rich motifs or if it includes additional unidentified molecular domains. New information on its complete amino acid sequence will reveal if Bac-like is related to the penaeidin family or if it is a distinct class of single domain antimicrobial peptides.

A few years later, another PRP-rich peptide, named callinectin, was isolated from the hemocytes of the blue crab *Callinectes sapidus* (Khoo *et al.*, 1999). In contrast to Bac-like, the partially characterized N-terminal sequence of callinectin showed no significant homology with any other known antimicrobial peptide (Fig. 1A). This novel cationic peptide of 3.7 kDa was only tested against *Escherichia coli* D31, and information on its full-length sequence and spectrum of antimicrobial activity are currently lacking. Similar to Bac-like, it is

uncertain if callinectin is a genuine single-domain PRP-rich AMP.

Another peptide belonging to the PRP-rich family was isolated from the hemocytes of the freshwater crayfish *Pacifastacus leniusculus* and was fully characterized (Fig. 1A). This original peptide, named astacidin 2, is composed of 14 amino acids and displays strong antimicrobial activity against both Gram-positive (Minimal Inhibitory Concentration [MIC] of about 5.5-10.3 μ M: *Bacillus* sp., *Staphylococcus aureus* and *M. luteus*) and Gram-negative bacteria (MIC of about 0.5-4.3 μ M: *Shigella flexneri*, *Proteus vulgaris*, *E. coli* and *Pseudomonas aeruginosa*) (Jiravanichpaisal *et al.*, 2007a). Unlike astacidin 1, a crayfish antimicrobial peptide derived from the carboxyl (C)-terminus of hemocyanin (see section 4.2), astacidin 2 is a gene-encoded molecule that is synthesized as a prepeptide and then processed at both N- and C-terminal regions. This highly cationic peptide of 1.8 kDa (isoelectric point or *pI* of 11.8) is constitutively expressed in crayfish hemocytes and shows significant homology to metalnikowin-1 from the insect *Palomena prasina* (Chernysh *et al.*, 1996). Recently, an astacidin 2 homolog was also identified in the crayfish *Procambarus clarkii* (Shi *et al.*, 2009).

Armadillidin: a glycine-rich peptide

Antimicrobial peptides rich in glycine residues have been isolated from a variety of arthropod species, including insects and spiders (Bulet *et al.*, 1991; Lorenzini *et al.*, 2003). In crustaceans, the first glycine-rich antimicrobial peptide, named armadillidin, was purified from the hemocytes of the terrestrial isopod *Armadillidium vulgare* (Herbinière *et al.*, 2005). Armadillidin is a linear cationic peptide (*pI* of 12.1) that is characterized by the presence of a high content of glycine residues (about 47 %). This peptide displays antibacterial activity against the Gram-positive bacteria *Bacillus megaterium* (MIC: 0.5 μ M). The mature peptide (5.2 kDa) is amidated at the C-terminus and encloses a unique five-fold repeated motif GGGFH(R/S) (Fig. 1B). This glycine-rich peptide is the unique crustacean AMP currently characterized in a non-decapod species. Interestingly, armadillidin was found only in the hemocytes of the woodlouse *A. vulgare*, but not in other isopod species such as *Oniscus asellus*, *Porcellio dilatatus petiti* and *Armadillo officinalis* (Herbinière *et al.*, 2005).

Homarin: a linear α -helical peptide

Homarin (also designated as Cationic Antimicrobial Peptide or CAP) is a short linear peptide isolated from the hemocytes of the American lobster *Homarus americanus* and does not contain any specific abundant amino acid residues (Battison *et al.*, 2008). This cationic peptide shares evident amino acid similarities with temporins (Mangoni, 2006) (Fig. 1C). Temporins are small amphipathic α -helical AMPs from amphibian skin that exhibit antimicrobial activity against Gram-positive bacteria (Simmaco *et al.*, 1996). In contrast, two synthetic homarin variants (sCAP-1.1 and sCAP-1.4) were shown to be effective against a *Vibrio* sp. isolated from the lobster intestinal tract and also exhibited protozoacidal activity against an

(A) Proline/arginine-rich peptides

Bac-like* XXV**PRP**E**PRPP**IG**PRPL**P**FP**GGGR**PF**Q**S**

Callinectin* WNSNXFHVGR**RPP**VVGR**RP**GXVXF**RAP**

Astacidin 2 **RPRPNYRPRPIYRP**

(B) Glycine-rich peptide

Amadillidin GHLGRPYIGGGGGFN**RRGGFHRGGGFHRGGGFHSGGGFHRGGGFHS**GGSFGYRG

(C) Linear α -helical peptide

Homarin 1.1 (CAP 1.1) QY**GNLLSLL**NGYR

Fig. 1 Primary structure of crustacean single-domain linear α -helical AMPs and peptides enriched in certain amino acids. (A) The proline and arginine residues are shadowed with a black background (where X is any amino acid residue). (B) The fivefold repeated motif GGGFH(R/S) is shown in bold and underlined. (C) The amino acid sequence of the linear α -helical homarin, which is identical to the amphibian temporins, is represented in bold type. The asterisks indicate the non-yet fully characterized crustacean AMPs.

important ciliate pathogen for lobsters and crabs (Battison *et al.*, 2008).

Single-domain peptides containing cysteine residues engaged in disulfide bonds

This group of single domain AMPs is characterized by the presence of pairs of cysteine residues that are capable of forming intramolecular disulfide bridges. The number of cysteine residues (generally 2 to 12) and their binding result in the formation of cyclic or open-ended cyclic stabilized peptides. Cysteine-containing AMPs are found in many species, from bacteria to eukaryotes, including fungi, plants and both vertebrate and invertebrate animals (Tossi and Sandri, 2002). This AMP group includes thanatin, tachyplesins, gomesin, protegrins and the well-known defensins (plant-, invertebrate-, α -, β - and θ -defensins) (for review, see Bulet *et al.*, 2004). In decapod crustaceans, only three cysteine-containing AMPs have been characterized (defensins, anti-lipopolysaccharide factors and scygonadins), although a significant number of cysteine-containing domains are also present in crustacean multi-domain antimicrobial peptides.

Defensins

The defensin family appears to be the best characterized and the most widespread family of antimicrobial peptides, occurring in most plants and invertebrate and vertebrate animals (Bulet *et al.*, 2004). Curiously, in crustaceans, defensin members

were only very recently identified in the Japanese spiny lobster *Panulirus japonicus* using an expressed sequence tag (EST) approach (Pisuttharachai *et al.*, 2009a). In this species, two different isoforms, PJD1 (7.1 kDa) and PJD2 (6.7 kDa), were detected in various tissues through reverse transcription-polymerase chain reaction (RT-PCR), including hemocytes, heart, gills and hepatopancreas (Pisuttharachai *et al.*, 2009b). Both isoforms possess an N-terminal signal peptide and a C-terminal defensin-like domain. Interestingly, the cysteine pattern found in *P. japonicus* defensins (C-X₄₋₈-C-X₃₋₅-C-X₉₋₁₃-C-X₄₋₇-C-C) is distinct from those present in invertebrate defensins, but identical to that of β -defensins found in vertebrates (Taylor *et al.*, 2008) (Fig. 2A). Unfortunately, to date, defensins are merely considered to be “putative AMPs” in crustaceans because their spectrum of antimicrobial activity has not yet been determined.

Anti-lipopolysaccharide factors

Anti-lipopolysaccharide factors (anti-LPS factors or ALFs) were first purified from the hemolymph cells (amebocytes) of two marine chelicerate arthropods, the horseshoe crabs *Limulus polyphemus* and *Tachypleus tridentatus* (Tanaka *et al.*, 1982). *Limulus* and *Tachypleus* anti-LPS factors (LALF and TALF) have been initially identified as potent inhibitors of LPS-mediated hemolymph coagulation. The coagulation system of horseshoe crabs can be activated by two distinct pathways (triggered by LPS or 1,3,- β -D-glucan) that mediate the activation of the proclotting enzyme,

resulting in the formation of hemolymph clots, a defense reaction against microbial invasion (Kawabata *et al.*, 2009). However, in addition to inhibiting the LPS-mediated coagulation pathway, the horseshoe crab anti-LPS factor also exhibits a strong antibacterial activity against R-types of Gram-negative bacteria, such as *Salmonella minnesota* R595 (Morita *et al.*, 1985), thus acting as a multifunctional protein. Structurally, both LALF and TALF are large cationic peptides of approximately 100 amino acids that contain two conserved cysteine residues and a hydrophobic N-terminal region (Aketagawa *et al.*, 1986; Muta *et al.*, 1987). A cluster of positively-charged residues is comprised between the two cysteine residues that form the disulfide-bond loop, which has been defined as the LPS-binding domain (Hoess *et al.*, 1993). Horseshoe crab anti-LPS factors are stored in the large granules of amebocytes, although no signal sequence has been recognized in the cDNA sequence of TALF (Wang *et al.*, 2002).

In crustaceans, homologues to horseshoe crab ALFs were first identified from the hemocytes of the penaeid shrimp species *Litopenaeus setiferus* (Gross *et al.*, 2001) and *Penaeus monodon* (Supungul *et al.*, 2002) using an EST approach. To date, the genes encoding ALFs have been only identified in decapod crustaceans, such as penaeid shrimps (Tassanakajon *et al.*, 2010), freshwater prawns (Rosa *et al.*, 2008; Lu *et al.*, 2009), crayfish (Liu *et al.*, 2006), lobster (Beale *et al.*, 2008) and crabs (Imjongjirak *et al.*, 2007; Li *et al.*, 2008; Yedery and Reddy, 2009a; Yue *et al.*, 2010). These peptides are part of a very well-characterized family of crustacean AMPs composed of different subgroups and variants that are either encoded by distinct genes or generated by alternative mRNA splicing (Tharntada *et al.*, 2008). In the tiger shrimp *P. monodon*, the most expressed variants, ALFPm2 and ALFPm3, are transcribed by different genes with distinct genomic organization, designated as group A and B genes (Fig. 2B). A genomic structure similar to the shrimp group A gene was also found in the crabs *Scylla paramamosain* and *Eriocheir sinensis* (Imjongjirak *et al.*, 2007; Li *et al.*, 2008). These peptides are constitutively expressed in circulating granular hemocytes, although cis-regulatory elements, which are involved in transcriptional regulation, were also recognized in their gene promoters (Tharntada *et al.*, 2008).

ALF sequences from decapod crustaceans are encoded as precursor molecules. These precursors are composed of a leader sequence followed by a large mature peptide (of about 11 kDa) containing a highly hydrophobic N-terminal region and the two characteristic cysteine residues. Recently, the three-dimensional structure of shrimp ALF heterologously expressed in yeast cells (rALFPm3) was resolved by nuclear magnetic resonance (Yang *et al.*, 2009) (Fig. 2B). The ALF structure consists of three α -helices (one at the N-terminus and two at the C-terminus) packed against a four-stranded β -sheet, as found in limulids. Based on amino acid sequence alignments, the ALF family was tentatively classified into three main groups: ALF Cluster I, II and III (Zhao and Wang, 2008). Later, another classification was proposed, but considered only

penaeid shrimp ALFs: Groups I, II and III ALFs (Tassanakajon *et al.*, 2010). Although an increasing number of studies have focused on the characterization of this AMP family, a consensus nomenclature and classification regarding structural features and phylogenetic relationships among different crustacean species and limulids are not still accessible.

Notably, ALFs exhibit a potent (MIC < 6.25 μ M) and broad spectrum of antimicrobial activity against a large number of both Gram-positive and Gram-negative bacteria, including several opportunistic/pathogenic *Vibrio* species, fungi and human enveloped virus (Somboonwivat *et al.*, 2005; Carriel-Gomes *et al.*, 2007; Yedery and Reddy, 2009a). Interestingly, rALFPm3 showed a bactericidal effect against a large number of bacterial strains, including shrimp pathogens (MIC < 1.56 μ M) (Somboonwivat *et al.*, 2005). Moreover, it was very recently shown that rALFPm3 also has the ability to bind to both negatively-charged lipopolysaccharide (LPS) and lipoteichoic acid (LTA), the major cell wall components of Gram-negative and Gram-positive bacteria, respectively (Somboonwivat *et al.*, 2008). Synthetic peptides corresponding to the ALF putative LPS-binding domain of the kuruma prawn *Marsupenaeus japonicus* displayed an efficient LPS neutralizing activity *in vitro* (Nagoshi *et al.*, 2006), suggesting that crustacean ALFs are also multifunctional proteins.

Interestingly, in their recent review, Smith *et al.* (2010) do not classify ALFs as conventional AMPs, but as *binding proteins*. This classification is due to their ability to bind to LPS (lipid A portion) (Somboonwivat *et al.*, 2008) in addition to their strong antimicrobial property. Moreover, the authors also point out that horseshoe crab ALFs are not segregated into the same granular population (small granules) as the classic antimicrobial peptides (tachyplesin, polyphemusin, tachycitin and tachystatin), but into the large amebocyte granule population together with the components of the coagulation system (Iwanaga and Lee, 2005). This separate compartmentalization apparently suggests that limulid ALFs are primarily implicated in the regulation of the coagulation cascade. However, in crustaceans, the coagulation process is distinct from that of horseshoe crabs and is more similar to the insects (Theopold *et al.*, 2004), and ALFs do not seem to take part in this process.

Scygonadins

In contrast to the cationic hemocyte-produced AMPs, scygonadin is an anionic (pI of 6.09) sex-specific large antimicrobial peptide of 10.8 kDa that was originally purified from the seminal plasma of the mud crab *Scylla serrata* (Huang *et al.*, 2006). The scygonadin gene is composed of three exons and two introns, and its expression is restricted to the ejaculatory duct of adult males (Wang *et al.*, 2007). The purified peptide only showed antibacterial activity against the Gram-positive bacteria *M. luteus* (Huang *et al.*, 2006). Later, a scygonadin homolog of 11.4 kDa, named SSAP (for *S. serrata* antimicrobial protein), was purified from granular hemocytes of the same crab species

(A) Defensins

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PJD1      E P E P S Y F N D C G S N G G S C T R G Y C S Y S N R L P Y T C S L G R T C C R L A Y V
PJD2      E P E P S Y I L D C R T N G G R C V T G Y C S -- N T L P Y S C G G G A I C C R H A Y G
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(B) Anti-lipopolysaccharide factors (ALF)

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ALFPm2    Q G V Q D L L P A L V E K I A G L W H S D E V E F L G H S C R Y S Q R P S F Y R W E L Y F N G R M W C P G W A P F T G R
ALFPm3    Q G W E A V A A A V A S K I V G L W R N E K T E L L G H E C K F T V K P Y L K R F Q V Y Y K G R M W C P G W T A I R G E
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ALFPm2    S R T R S P S G A I E H A T R D F V Q K A L Q S N L I T E E D A R I W L E H
ALFPm3    A S T R S Q S G V A G K T A K D F V R K A F Q K G L I S Q Q E A N Q W L S S
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ALFPm3

(C) Scygonadins

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Scygonadin  G Q A L N K L M P K I V S A I I Y M V G Q P N A G V T F L G H Q C L V E S T R Q P D G F Y T A K M S C A S W T H D N P I
SSAP        G Q A L N K L M P K I V S A I I Y M I G Q P N A G V T F L G H Q C L V E S T R Q P D G F Y T A K M W C T S W T S D N P I
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Scygonadin  V G E G R S R V E L E A L K G S I T N F V Q T A S N Y K K F T I D E V E D W I A S Y
SSAP        V G E G R S R V E L E A L K G S I R N F V Q T A S D Y K K F T I E E V E D W I A S Y
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Fig. 2 Crustacean single-domain peptides containing cysteine residues engaged in disulfide bonds. (A) Amino acid sequence alignment between two defensins isoforms (PJD1 and PJD2) from the Japanese spiny lobster *Panulirus japonicus*. (B) Amino acid sequence comparison between two ALF variants encoded by distinct genes (ALFPm2 and ALFPm3) from the shrimp *Penaeus monodon* and the three-dimensional structure of ALFPm3. (C) Amino acid sequence alignment between the male-specific peptide scygonadin and the non-gender specific *Scylla serrata* antimicrobial protein (SSAP). Identical residues are marked with an asterisk (*) and the cysteine residues are shadowed with a black background.

(Yedery and Reddy, 2009b) (Fig. 2C). Both anionic scygonadin and SSAP (*pI* of 5.7) contain two cysteine residues that are arranged as in ALFs. However, a phylogenetic relationship between both anionic peptides from *S. serrata* and ALFs was not clearly evidenced. In contrast to scygonadin, SSAP is expressed in multiple tissues (as determined by RT-PCR and northern and western blot analyses) of both male and female crabs (Yedery and Reddy, 2009b). SSAP displayed antibacterial activity mainly against Gram-positive bacteria (MIC of about 7.5-30 μ M: *M. luteus*, *Streptococcus pyogenes* and *S. aureus*), but not against yeast and filamentous fungi (Yedery and Reddy, 2009b; Peng *et al.*, 2010).

Multi-domain or chimeric AMPs

Antimicrobial molecules with at least two distinct domains comprise a remarkable group of

crustacean AMPs. Each domain may exhibit particular characteristics of classical single-domain AMPs, such as PRP- or cysteine-rich peptides. Multi-domain AMPs are not common in all living kingdoms, and apart from crustaceans, they have been identified in insects and arachnids (Wicker *et al.*, 1990; Saito *et al.*, 1995). In some cases, chimeric constitution is essential for establishing cationicity and amphipathicity. A cationic domain can be committed in electrostatic interactions with negatively-charged microbial walls, while a hydrophobic domain can be responsible for membrane destabilization. The presence of different structural arrangements in a single molecule can also provide multifunctional and/or synergic properties in addition to its antimicrobial activities. Additional biological functions have been shown for the crustacean multi-domain penaeidins, crustins and hyastatin as discussed hereafter.

Penaeidins

Penaeidins are unquestionably the most well-characterized family of antimicrobial peptides described in crustaceans so far and have been the subject of many review articles (Bachère *et al.*, 2000, 2004; Destoumieux *et al.*, 2000a; Cuthbertson *et al.*, 2008; Tassanakajon *et al.*, 2010). They are chimeric cationic peptides composed of an unstructured N-terminal PRP-rich domain and a C-terminal region containing six cysteine residues that are engaged in three intramolecular disulfide bridges (Destoumieux *et al.*, 1997; Yang *et al.*, 2003; Cuthbertson *et al.*, 2005) (Fig. 3A). Interestingly, the N-terminal domain shares high sequence similarities with PRP-enriched peptides, in particular with crab Bac-like (Schnapp *et al.*, 1996). On the other hand, the C-terminal cysteine-rich domain does not correspond to any other "cysteine motifs" previously described in cysteine-containing AMPs.

Penaeidins were originally isolated from the hemolymph of the Pacific white shrimp *Litopenaeus vannamei* (Destoumieux *et al.*, 1997) and appear to be ubiquitous only in the family Penaeidae. All penaeidin precursors comprise a highly conserved signal peptide followed by a cationic mature peptide (5.48-6.62 kDa) with a calculated isoelectric point above 9 (Bachère *et al.*, 2004). Because the signal peptide is cleaved, the mature peptides can be post-translationally processed by the formation of a pyroglutamic acid in the N-terminus and/or by a C-terminal amidation involving the elimination of a glycine residue (Destoumieux *et al.*, 1997, 2000a). Based on amino acid sequence comparisons and the position of some precise residues in both the N- and C-terminal regions, four distinct subgroups of penaeidins have been classified: PEN2, PEN3, PEN4 and PEN5 (Cuthbertson *et al.*, 2002; Gueguen *et al.*, 2006; Kang *et al.*, 2007) (Fig. 3A). The subgroup PEN1, which was initially purified from shrimp hemolymph, was later classified as a PEN2 variant (Cuthbertson *et al.*, 2002). Each penaeidin subgroup possesses a characteristic amino acid signature and common biochemical features. Gueguen *et al.* (2006) have proposed and developed a specific nomenclature as well as a specialized database for the penaeidin family (PenBase, available at <http://www.penbase.immunaqua.com>).

The genomic structure of the penaeidin genes is variable according to each subgroup. Both PEN2 and PEN4 genes from *L. vannamei* (O'Leary and Gross, 2006), the PEN3 gene from *P. monodon* (Chiou *et al.*, 2007) and the PEN5 gene from the fleshy prawn *Fenneropenaeus chinensis* (Kang *et al.*, 2007) are encoded by two exons interrupted by one intron. In all of these cases, a single intron of variable length divides the PRP- and cysteine-rich domains. Conversely, the PEN3 gene from *L. vannamei* lacks this typical intron sequence (O'Leary and Gross, 2006). Interestingly, in two species of *Litopenaeus*, transcripts of PEN2, PEN3 and PEN4 were all found to be expressed in a single individual (Cuthbertson *et al.*, 2002). In naïve (unchallenged) shrimp, penaeidin precursors are constitutively expressed, processed and addressed to cytoplasmic granules of both granular and semi-

granular hemocytes (Destoumieux *et al.*, 2000b). The production of penaeidins is restricted to granule-containing hemocytes that can be free in the hemolymph or infiltrating shrimp tissues (Bachère *et al.*, 2004). Interestingly, no PEN2 and PEN4 members were described in Asiatic shrimps, while the PEN5 subgroup seems to be absent in "occidental" species (Tassanakajon *et al.*, 2010).

The spectrum of the antimicrobial activity of penaeidins has been studied in detail using native peptides purified from shrimp hemolymph, synthetic peptides and recombinant variants produced in heterologous expression systems (Destoumieux *et al.*, 1997, 1999; Cuthbertson *et al.*, 2004, 2005, 2006; Li *et al.*, 2005; Kang *et al.*, 2007). These peptides have been shown to be particularly effective against Gram-positive bacteria (MIC of about 0.3-2.5 µM: *Aerococcus*, *Micrococcus*, *Bacillus*, *Staphylococcus*) and filamentous fungi (MIC of about 1.25-2.5 µM: *Fusarium*, *Nectria*, *Alternaria*, *Neurospora*, *Botrytis*, *Penicillium*), but poorly or not active against Gram-negative bacteria and marine vibrios (with the exception of *Fenchi* PEN5, which appears to be active against *Klebsiella pneumoniae*). Moreover, recent studies have confirmed that penaeidin subgroups can display distinct antimicrobial activities (for review, see Cuthbertson *et al.*, 2008).

Interestingly, besides their antimicrobial properties, penaeidins are also able to bind to chitin. A conserved chitin-binding motif is recognized in the cysteine-rich domain, whereas the PRP-rich domain is preferentially involved with the antimicrobial activities (Destoumieux *et al.*, 2000a; Cuthbertson *et al.*, 2004). According to Destoumieux *et al.* (2000b), the chitin-binding motif of penaeidins could have additional functions, such as the ability to bind to shrimp carapace upon injury and participate in wound healing and/or molting processes.

Crustins

Crustins are generally defined as multi-domain cationic antibacterial polypeptides (7-14 kDa) containing one whey acidic protein (WAP) domain at the C-terminus (Smith *et al.*, 2008). The first identified crustin member is an 11.5-kDa protein purified from the granular hemocytes of the shore crab *C. maenas* that exhibits specific activity towards Gram-positive marine or salt-tolerant bacteria (Relf *et al.*, 1999). The term *crustin* was later proposed by Bartlett *et al.* (2002) to describe transcripts found in two penaeid shrimp species (*L. vannamei* and *L. setiferus*) with high sequence similarity to the crab 11.5-kDa protein, which was later designated carcinnin (Brockton *et al.*, 2007). Since the first characterization of crustin from *C. maenas*, over 50 crustins and crustin-like sequences have been identified in numerous crustacean species, including crayfish, shrimps, freshwater prawns, crabs and lobsters, and also in non-decapod crustaceans, such as amphipods, (through EST-based approaches) (Smith *et al.*, 2008).

In terms of structure, all known crustin precursors possess a leader sequence at the N-terminus and a C-terminal region containing a WAP domain. "WAP" is a general family of proteins usually found in the whey fraction of mammalian

milk that contains eight conserved cysteine residues in a conserved arrangement, forming a single four disulfide core (4DSC). This molecular motif seems to exert a protease inhibitory activity, in addition to other biological functions (Ranganathan *et al.*, 1999). It is well established that protease inhibitors can play an essential role in crustacean immunity, such as inhibiting microbial proteases or regulating immune-protease cascades (Cerenius and Söderhäll, 2004).

Based on their structural features, Smith *et al.* (2008) categorized crustins into three main types: Types I, II and III (Fig. 3B). Type I crustins comprise the members most related to carcinin and possess a cysteine-rich region of variable length between the leader sequence and the WAP domain. This type of crustins is mainly present in crabs (Relf *et al.*, 1999; Sperstad *et al.*, 2009a; Imjongjirak *et al.*, 2009; Mu *et al.*, 2010; Yue *et al.*, 2010), lobsters (Stoss *et al.*, 2004; Hauton *et al.*, 2006; Christie *et al.*, 2007; Pisuttharachai *et al.*, 2009c), crayfish (Jiravanichpaisal *et al.*, 2007a; Shi *et al.*, 2009), shrimp (Sun *et al.*, 2010) and freshwater prawn (Dai *et al.*, 2009). On the other hand, Type II crustins are characterized by the presence of a hydrophobic region containing an overrepresentation of glycine residues upstream of the cysteine-rich and WAP domains found in Type I crustins. This group is frequently documented in penaeid shrimps (Bartlett *et al.*, 2002; Rattanachai *et al.*, 2004; Supungul *et al.*, 2004; de Lorgeril *et al.*, 2005; Rosa *et al.*, 2007; Zhang *et al.*, 2007; Antony *et al.*, 2010) and crayfish (Jiravanichpaisal *et al.*, 2007a). Conversely, Type III crustins possess a short PRP-rich region between the leader sequence and the single WAP domain, but do not contain the characteristic cysteine-rich domain present in both Type I and II crustins nor the glycine region motif. These peptides are usually known as single-whey domain (SWD) proteins, chelonianin-like proteins or antileukoprotease-like proteins and have been found in shrimp and crayfish species (Jiménez-Vega *et al.*, 2004; Amparyup *et al.*, 2008a; Jia *et al.*, 2008; Du *et al.*, 2010). Interestingly, in penaeids, proteins with two 4DSC motifs have also been recently identified and thus named double WAP domain (DWD)-containing proteins (Jiménez-Vega *et al.*, 2007; Chen *et al.*, 2008; Du *et al.*, 2009). These DWD proteins display protease inhibitory activity against bacterial proteases (Du *et al.*, 2009), but they are not defined as crustins because they have more than one WAP domain in their structure (Smith *et al.*, 2008).

Protease inhibitory and antibacterial activities seem to be related to a particular structure of the WAP domain. Protease inhibitory activity is generally associated with the presence of a methionine residue adjacent to the second cysteine in the 4DSC. This residue is substituted by cationic or hydrophobic amino acids in WAP-containing proteins with antibacterial activity (Hagiwara *et al.*, 2003). Despite the presence of the WAP domain in all crustin groups, antiprotease activities have only been reported for Type III crustins (Amparyup *et al.*, 2008a; Jia *et al.*, 2008). This antiprotease capacity could be important to inactivate microbial proteases during infection and/or regulate endogenous protease cascades, such as the proPO system that

leads to melanization. Thus, type III crustins seem to be multifunctional immune proteins that combine both antimicrobial and antiprotease properties (Amparyup *et al.*, 2008a; Jia *et al.*, 2008).

Some antimicrobial studies have revealed that all crustin groups are mainly active against Gram-positive bacteria (MIC < 8 µM). Susceptible bacteria include the Gram-positive strains of the genera *Micrococcus*, *Aerococcus*, *Planococcus*, *Staphylococcus*, *Streptococcus*, *Corynebacterium* and *Bacillus* (Relf *et al.*, 1999; Zhang *et al.*, 2007; Supungul *et al.*, 2008; Imjongjirak *et al.*, 2009; Sperstad *et al.*, 2009a). Conversely, a Type II crustin from *P. monodon* (crustin-likePm) showed strong antibacterial activity against both Gram-positive and Gram-negative bacteria (MIC < 5 µM), including the crustacean opportunist/pathogen *Vibrio harveyi* (Amparyup *et al.*, 2008b). The antimicrobial activity of crustins appears to be related to the tertiary structure of the 4DSC (tightly constrained by three disulfide bonds and containing a small α-helix). Zhang *et al.* (2007) have shown that the crustin-like CshFc from *F. chinensis*, which lacks the authentic WAP domain, did not affect any bacteria tested, in contrast to the WAP-containing Type II crustin CruFc, which inhibited the growth of Gram-positive bacteria *in vitro*.

Interestingly, another classification for crustins was also proposed by Zhao and Wang (2008). These authors consider that the 'crustin signature' should not be restricted to the sole presence of a WAP domain at the C-terminal region of the molecule. In their classification, the crustin domain comprises the arrangement of the 12 conserved cysteine residues found in Type I and II crustins, which includes the WAP domain (eight cysteines). Accordingly, the SWD (Type III crustins) and DWD-containing proteins, which only contain WAP domains (one for SWD and two for DWD), cannot be considered as authentic crustin molecules. However, as mentioned above, shrimp SWDs combine antiprotease and antimicrobial properties. In addition, regarding the spacing of the cysteine residues within the crustin domain, Zhao and Wang (2008) proposed that shrimp crustins could be recognized as either crustin I or crustin II.

Although crustin antimicrobial peptides have been extensively reviewed and categorized into distinct types, a consensus nomenclature and classification as established for penaeidins (Gueguen *et al.*, 2006) has not yet been proposed. In this review, we adopted the nomenclature used by Smith *et al.* (2008) to facilitate comparisons between the different crustin subgroups already reported in different crustaceans.

The genomic organization of crustins is distinct among the different groups. The genes of two Type I crustins are encoded by four exons interrupted by three introns (Brockton *et al.*, 2007; Imjongjirak *et al.*, 2009), in contrast to the SWDPm2 gene from *P. monodon*, which belongs to the Type III crustin group and contains three exons and two introns (Amparyup *et al.*, 2008a). Furthermore, two crustin variants from the shrimp *P. monodon* are encoded by different genes; crustinPm5 contains four exons separated by three introns, and crustin-likePm contains only two exons and one intron (Amparyup

et al., 2008b; Vatanavicharn *et al.*, 2009). Expression of most crustin-encoding genes has mainly been found in circulating hemocytes, although some reports point to the possibility of expression in other tissues (as determined by RT-PCR), such as heart, ovary and intestines (Smith *et al.*, 2008). Surprisingly, through RT-PCR analysis, transcripts of different crustin types, such as crustin*Pm5*, *Picrustin2*, *Fc-crus 3* and PET-15, were essentially identified in the epipodite, hematopoietic tissue, ovary and olfactory organ, respectively (Stoss *et al.*, 2003; Jiravanichpaisal *et al.*, 2007a; Vatanavicharn *et al.*, 2009; Sun *et al.*, 2010).

Recent findings from our laboratory indicate that the Type II crustin *crusFpau* from the pink shrimp *Farfantepenaeus paulensis* (Rosa *et al.*, 2007) is constitutively produced and stored in the granules of some populations of the shrimp granular hemocytes. Moreover, monospecific polyclonal antibodies raised against the recombinant *rcrusFpau* were able to cross-react with corresponding crustins in the hemocyte granules of other penaeids (*L. vannamei* and *Litopenaeus schmitti*), freshwater prawn (*Macrobrachium potiuna*) and crab (*C. sapidus*), suggesting that these antimicrobial peptides are produced and stored in the hemocyte granules, as described for penaeidins and ALFs (unpublished data).

Hyastatin

Hyastatin is a novel multi-domain antimicrobial peptide that was purified and characterized from the hemocytes of the small spider crab *Hyas araneus* (Sperstad *et al.*, 2009b). The mature cationic molecule (*pI* of 9.84) is a large polypeptide of 11.7 kDa that is composed of a noteworthy glycine-rich N-terminal domain, a short PRP-containing portion and a C-terminal region with six cysteine residues (Fig. 3C). Interestingly, the glycine-rich domain consists of about 27 % of glycine residues and is very similar to that of Type II crustins. On the other hand, both the PRP- and cysteine-containing domains are comparable to those of penaeidins. Interestingly, the arrangement of the six cysteine residues of hyastatin is identical to the cysteine pattern found in all penaeidin groups (Gueguen *et al.*, 2006). Moreover, hyastatin has chitin-binding abilities as described for penaeidins (Destoumieux *et al.*, 2000b). Recently, gene sequences encoding hyastatin peptides were also discovered in EST libraries of other crab species, such as *C. sapidus*, *E. sinensis*, *Cancer magister*, *Portunus trituberculatus* and *Celca pugilator*. To date, information about gene arrangement and organization is not yet available for this peptide.

Hyastatin was assayed against a reduced number of microorganism species but showed a broad spectrum of activity. It is capable to inhibit the growth of yeast (MIC of about 6.3-12.5 μ M: *Saccharomyces cerevisiae* and *Candida albicans*), Gram-positive bacteria (one species: *Corynebacterium glutamicum* - MIC of 0.4 μ M) and Gram-negative bacteria (one species: *E. coli* - MIC of 12.5 μ M), thus differing from penaeidins and crustins that possess a more restricted antimicrobial activity to Gram-positive bacteria. Curiously, the antimicrobial activity of hyastatin appears to be

related to the cysteine-rich domain (Sperstad *et al.*, 2009b) rather than to the PRP-rich domain as presumed for penaeidins (Cuthbertson *et al.*, 2004). Moreover, the chitin-binding property of hyastatin is linked to its N-terminal region (PRP- and glycine-rich domains) instead of to the cysteine-rich domain as in penaeidins (Sperstad *et al.*, 2009b). Therefore, even though hyastatin and penaeidins share sequence and structural similarities, the functional properties of their molecular domains appear to be distinct.

Arasins

Like hyastatin, arasins are cationic chimeric peptides (*pI* ~11) isolated from the hemocytes of *H. araneus* (Stensvåg *et al.*, 2008). These peptides contain a leader sequence of 25 amino acids followed by a linear PRP-rich N-terminal region and a C-terminal portion containing four cysteine residues engaged in two disulfide linkages (Fig. 3D). The N-terminal PRP motif is similar to that of bactenecin-7, metalnikowin-1 and of crustacean PRP-containing AMPs, such as Bac-like and astacidin 2 (Schnapp *et al.*, 1996; Jiravanichpaisal *et al.*, 2007a). Furthermore, the four cysteine residues from the C-terminal region are arranged similarly to vertebrate protegrins (Capone *et al.*, 2010). Like hyastatin, arasin 1 (4.3 kDa) was only tested against a few microorganisms species and exhibited activity against both Gram-positive (*C. glutamicum* - MIC of 0.8 μ M) and Gram-negative (*Listonella anguillarum* and *E. coli* - MIC of 6.3-12.5 μ M) bacteria *in vitro*. Arasin 2 was only identified through sequencing of a hemocyte cDNA library, and its spectrum of antimicrobial activity has not yet been determined. Like most other crustacean antimicrobial peptides, transcripts of both arasin forms were mainly detected in circulating hemocytes (Stensvåg *et al.*, 2008). Unfortunately, information about arasin encoding-genes is not currently available.

Stylicins

Similar to scygonadins, stylicins are anionic peptides with a theoretical *pI* of 5. They are composed of 82 amino acids (8.9 kDa) and are characterized by a proline-rich N-terminal region and a C-terminal portion containing 13 cysteine residues (Rolland *et al.*, 2010) (Fig. 3E). This family of antimicrobial peptides shows homology to mouse cryptdin and was first evidenced in the shrimp *Litopenaeus stylirostris* that was able to survive an infection of the pathogenic *Vibrio penaeicida* (de Lorgeril *et al.*, 2005). These peptides were also identified in EST libraries of two other penaeid species, *L. vannamei* and *P. monodon*. Ls-Stylicin1 from *L. stylirostris* is encoded by two exons interrupted by one intron, and its expression is strictly limited to the hemocytes. Ls-Stylicin1 did not display significant activity against either Gram-positive or Gram-negative bacteria, but exhibited strong antifungal effect on *Fusarium oxysporum*. Interestingly, Ls-Stylicin1 showed a potent LPS-binding activity (comparable to rALFPm3) and was able to agglutinate *V. penaeicida in vitro* in a lectin-like manner (Rolland *et al.*, 2010).

Unconventional AMPs: multifunctional proteins and protein fragments with antimicrobial activities

This last group of crustacean AMPs is composed of multifunctional proteins that primarily serve other functions and protein fragments that display antimicrobial activity and are generated by the processing of larger proteins. These unconventional AMPs, so called by Smith *et al.* (2010), possess important molecular elements found in the structure of classical AMPs, such as charge, hydrophobicity and/or amphipathicity (Brogden, 2005), and have been isolated and characterized from many different invertebrate and vertebrate species. They include whole proteins, such as histones, ribosomal proteins and mammalian milk proteins, and peptide fragments derived from large precursors with no evident antimicrobial properties, such as hemoglobin, lactoferrin and hemocyanin (Bulet *et al.*, 2004). In crustaceans, antimicrobial activities were reported for histones and histone fragments and also for peptides derived from the crustacean oxygen carrier hemocyanin.

Histones and derived fragments

Histones are major protein components of chromatin that are directly implicated in DNA packing and the regulation of gene expression. They are cationic proteins, highly conserved in all eukaryotic cells that might also be involved in antimicrobial defense (Cho *et al.*, 2009). Antimicrobial activity has been reported for different types of histones (H1, H2A, H2B, H3 and H4) in many vertebrate and invertebrate species, confirming their multifunctional properties (Kashima, 1991; Park *et al.*, 1996, 1998; Richards *et al.*, 2001). Their high content of cationic residues and amphipathic secondary structure could be responsible for their antimicrobial property. In fish and amphibians, histones and histone-derived fragments with antimicrobial activity have been found in skin secretions and mucus (Park *et al.*, 1998; Robinette *et al.*, 1998; Fernandes *et al.*, 2002, 2004). The antimicrobial property of histones seems to be related to their capacity of destabilizing bacterial membranes and not to their ability of forming stable pores (Fernandes *et al.*, 2002). However, the mechanisms by which histones are recruited to sites outside the cell nucleus and the regulation of histone production in response to microbial infections is far from being clear.

In crustaceans, histones and histone-derived fragments displaying antimicrobial activities were recently identified in the shrimp *L. vannamei* (Patat *et al.*, 2004). Using biochemical approaches, high levels of the four core histone proteins (H2A, H2B, H3 and H4) were detected in circulating hemocytes. Both full length histone H2A and its N-terminal fragment, which is similar to buforin I and parasin AMPs, were strongly active (MIC < 4.5 μ M) against *M. luteus* and two strains of *Bacillus*. Likewise, a chromatographic fraction containing both H2B/H4 histones inhibited *M. luteus* in liquid growth inhibition assays. Moreover, a shrimp H1-derived

peptide was also found to be active against *M. luteus* (Patat *et al.*, 2004).

Hemocyanin-derived peptides

Hemocyanin is a copper-containing oxygen transport protein in crustaceans that is produced in the hepatopancreas and then released into the plasma. Hemocyanin is the most abundant protein in the crustacean hemolymph, representing more than 95 % of the total protein in the plasma. Arthropod hemocyanins are organized as multiples of hexamers, and each hexamer contains monomers of about 75 kDa (van Holde *et al.*, 2001). In addition to its role as an oxygen carrier, hemocyanin appears as a multifunctional protein since it is also involved in osmoregulation, protein storage and some immune reactions (Decker and Jaenicke, 2004). In chelicerates, the N-terminal region of hemocyanin was suggested to have phenoloxidase activity after proteolytic cleavage (Decker and Rimke, 1998; Nagai and Kawabata, 2000). The prophenoloxidase system (or proPO system) involves a complex cascade in which phenol compounds are oxidized and many toxic molecules (quinones and reactive oxygen intermediates) are generated in response to a microbial infection (Cerenius and Söderhäll, 2004; Cerenius *et al.*, 2008).

In crustaceans, some antimicrobial peptides derived from the C-terminal part of hemocyanin were isolated and characterized from the plasma of the penaeids *L. vannamei* (PvHct) and *L. stylirostris* (PshCt1, PshCt2) (Destoumieux-Garzón *et al.*, 2001) and the crayfish *P. leniusculus* (astacidin 1) (Lee *et al.*, 2003). The three peptides derived from shrimp hemocyanin exhibited strong antifungal activity against many filamentous strains (MIC of 3.15-12.5 μ M) (Destoumieux-Garzón *et al.*, 2001). By contrast, astacidin 1 from crayfish was active against Gram-positive (MIC of 1.9-12.8 μ M: *Bacillus* sp. and *M. luteus*) and Gram-negative bacteria (MIC of 15 μ M: *S. flexneri* and *E. coli*).

All three shrimp hemocyanin-derived peptides are anionic molecules with molecular masses of 2.7 kDa (PvHct), 7.9 kDa (PshCt1) and 8.3 kDa (PshCt2) and are induced or activated in the plasma of animals stimulated by microbial injection (Destoumieux-Garzón *et al.*, 2001). These peptides are generated from the C-terminal region of hemocyanin, which lacks the copper-binding site. In the crayfish *P. leniusculus*, astacidin 1 (about 1.9 kDa) appears to be released from the C-terminus of hemocyanin by a cysteine-like protease, and its production is enhanced by injection of LPS or glucan into the crayfish (Lee *et al.*, 2003).

Involvement of AMPs in crustacean host defense and survival

Evidence of the implication of AMPs in innate host defenses has been reported in multiple species across different taxa, including crustaceans (Hancock and Scott, 2000; Pazgier *et al.*, 2006; de Lorgeril *et al.*, 2008; Conlon, 2010; Smith *et al.*, 2010). In most decapod species, especially in penaeid shrimp, the modulation of gene expression of some AMPs in response to microbial challenge

has been extensively studied (Muñoz *et al.*, 2002, 2004; Sunpugul *et al.*, 2004; Jiravanichpaisal *et al.*, 2007a; Robalino *et al.*, 2007; de la Vega *et al.*, 2008; Sperstad *et al.*, 2010). Interestingly, the expression pattern of the well-characterized crustacean AMPs differs according to the AMP family and/or the crustacean species. ALF transcripts in the circulating hemocytes of *P. monodon* (ALFPm3), *F. chinensis* and *L. stylirostris* increase in abundance in the first hours after a *Vibrio* challenge (Sunpugul *et al.*, 2004; Liu *et al.*, 2005; de Lorgeril *et al.*, 2008). In contrast, the mRNA concentration of shrimp crustins, penaeidins and stylicin significantly decrease at this stage of infection (Muñoz *et al.*, 2004; Sunpugul *et al.*, 2004; de Lorgeril *et al.*, 2008). Furthermore, the expression of these last AMPs returns to basal levels 24-72 h post-infection, followed by a subsequent remarkable increase when compared to unchallenged animals (Muñoz *et al.*, 2004; de Lorgeril *et al.*, 2008).

Conversely, the *in vitro* gene expression of three AMPs (arasin-1, hyastatin and a Type I crustin) in primary hemocyte cultures of the crab *H. araneus* were not affected neither by Gram-positive nor by Gram-negative bacteria stimulation (Sperstad *et al.*, 2010). However, these observations cannot be extended to an *in vivo* situation, in which the conditions are considerably different. Interestingly, in the crayfish *P. leniusculus*, the modulation of crustin expression appears to vary according to the crustin type. The expression of *P*crustin1 (Type I crustin) was increased in circulating hemocytes and hematopoietic tissue after stimulation with both Gram-positive and Gram-negative bacteria, while *P*crustin3 (Type II crustin) expression was only induced after injection with a non-pathogenic Gram-negative bacteria (*Acinetobacter* sp.). In contrast, the expression of *P*crustin2 (another crayfish Type I crustin) and astacin 2 were not modulated by microbial challenge (Jiravanichpaisal *et al.*, 2007a).

According to Bachère *et al.* (2004), two main phases are recognized in shrimp (*L. vannamei*) immune response to a *Vibrio* infection, based on penaeidin expression: Phase I (local reaction) and Phase II (systemic reaction). During the first phase (12 h post-infection), there is a decrease in the mRNA abundance of penaeidins due to the migration of penaeidin-producing hemocytes to the sites of infection. In response to a presumed chemotactic effect, these hemocyte populations release large amounts of penaeidins in the infected tissues. The second phase occurs at about 48 h post-infection and is characterized by the activation of hematopoiesis and the appearance of penaeidin-expressing hemocytes in both hemolymph and shrimp tissues. A similar observation was also observed in ALF kinetics in *P. monodon* after a *V. harveyi* injection (Sombonwivat *et al.*, 2008).

Recently, the *in vivo* role of some crustacean AMPs was investigated in shrimp and crayfish using RNA interference (Liu *et al.*, 2006; de la Vega *et al.*, 2008; Shockey *et al.*, 2009; Tharntada *et al.*, 2009). A significant increase in mortality after silencing crustin gene transcripts was observed in *L. vannamei* infected with the Gram-negative pathogen *V. penaeicida*, but not with the virulent fungus *F.*

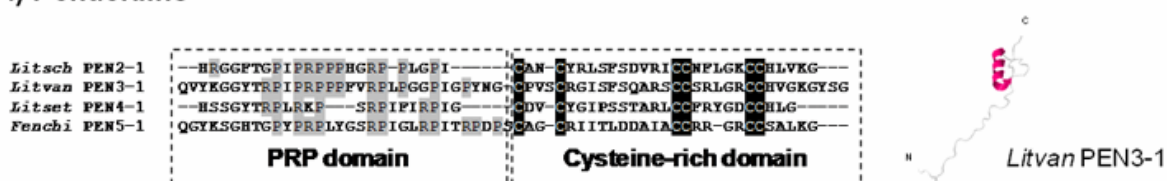
oxysporum, as compared to crustin-expressing animals (Shockey *et al.*, 2009). These results are curious because crustins do not seem to have an effect on marine vibrios, according to *in vitro* assays (see section 3.2). On the other hand, in the same shrimp species, ALF was shown to be involved in both bacterial and fungal infections (de la Vega *et al.*, 2008).

In addition to bacterial infections, crustaceans are also attacked by different classes of viruses. To date, viral infections are the most serious constraint to shrimp farming worldwide, particularly the disease caused by the white spot syndrome virus (WSSV). In the crayfish *P. leniusculus*, injection of ALF-dsRNA resulted in an increased expression of the WSSV envelope protein VP28 in hematopoietic tissue cell cultures, indicating that this molecule is involved in antiviral defense (Liu *et al.*, 2006). Moreover, preincubation of WSSV with rALFPm3 (from *P. monodon*) reduced virus propagation in both crayfish cell cultures (hematopoietic tissue) and infected *P. monodon* shrimp (Tharntada *et al.*, 2009). Conversely, the ALF member LvALF1 does not seem to be responsible for direct virus protection in the shrimp *L. vannamei* (de la Vega *et al.*, 2008). These results, together with differences in ALF antimicrobial activities according to species, suggest that this AMP family could be implicated differentially in the immune responses of each crustacean group.

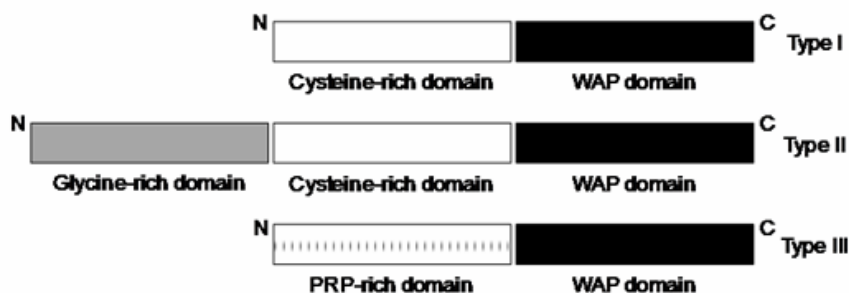
Over the past few years a significant number of large-scale EST sequencing projects have successfully identified many immune genes, such as antimicrobial peptide family genes, in important cultivated shrimp species (Gross *et al.*, 2001; Rojtinnakorn *et al.*, 2002; Sunpugul *et al.*, 2002; Tassanakajon *et al.*, 2006; Dong and Xiang, 2007). Currently, over 170,000 sequences are available in the GenBank database from various tissues of different shrimp species (for review, see Robalino *et al.*, 2009). In addition, the analysis of these EST libraries has confirmed that hemocytes are the main site for AMP synthesis. Sequences encoding AMPs in *L. vannamei* and *L. setiferus* comprise about 20 % of all sequenced transcripts in hemocyte cDNA libraries (Gross *et al.*, 2001). In this context, it has been shown that penaeidins, crustins and ALFs appear to be the most highly expressed AMPs in shrimp hemocytes (Gross *et al.*, 2001; Sunpugul *et al.*, 2002; Dong and Xiang, 2007). Furthermore, in specific EST libraries of *P. monodon*, some AMPs were found to be up-regulated after both WSSV and *V. harveyi* challenge (Tassanakajon *et al.*, 2006).

The developmental expression of the three most well-characterized crustacean AMPs was studied in detail in penaeid shrimp and crayfish. Significant mRNA expression levels of penaeidins, crustins and ALFs were detected in the early developmental stages (nauplius, zoea and mysis) of *L. vannamei*, *P. monodon* and *F. chinensis* (Muñoz *et al.*, 2003; Chiou *et al.*, 2007; Jiravanichpaisal *et al.*, 2007b; Sun *et al.*, 2010; Tassanakajon *et al.*, 2010). Interestingly, two Type II crustins from *F. chinensis* (*Fc*-crus 1 and *Fc*-crus 2) are expressed in several shrimp developmental stages, while *Fc*-crus 3 (the sole Type I crustin member found in penaeid shrimp to date) is found only in the ovaries

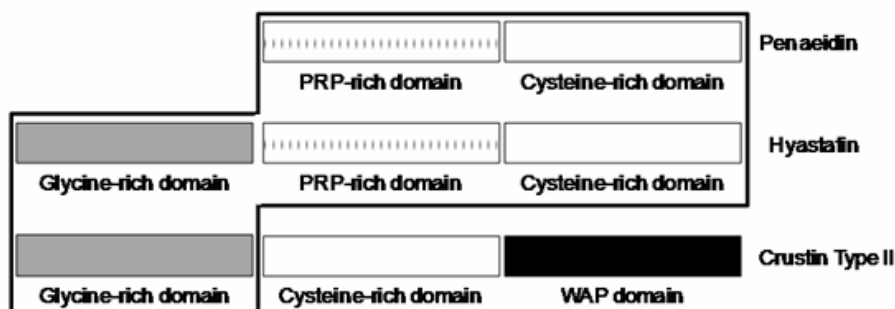
(A) Penaeidins



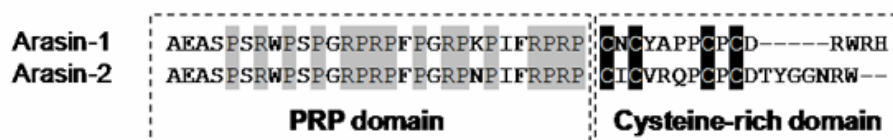
(B) Crustins



(C) Hyastatin



(D) Arasins



(E) Stylicins



Fig. 3 Crustacean multi-domain or chimeric AMP families. (A) Sequence comparison of different penaeidin subgroups (PEN2 to PEN5) and the three-dimensional structure of *Litvan* PEN3-1 from *Litopenaeus vannamei*. (B) A not-to-scale schematic illustration of the three crustins types (Type I to III crustin). (C) Schematic comparison (not-to-scale) of hyastatin, penaeidins and Type II crustins. (D) Amino acid sequence alignment between the two arasins from the spider crab *Hyas araneus*. (E) Schematic representation (not-to-scale) of stylicin from the blue shrimp *Litopenaeus stylirostris*. In the amino acid alignments, the proline/arginine residues and conserved cysteines are highlighted in grey and black boxes, respectively.

(via RT-PCR) of adult animals (Sun *et al.*, 2010). Conversely, in the middle stage of crayfish embryo development, the expression of Type I crustins (*Picrustin1* and *Picrustin2*) is inferior to that of the Type II crustin *Picrustin3* (Zhang *et al.*, 2010). Taken together, these results suggest that some families of AMPs could be preferentially produced during distinct periods of the crustacean life cycle and highlight their importance during ontogenesis.

Recently, genes potentially associated with shrimp survival capacity were identified in *L. stylirostris* using the suppression subtractive hybridization (SSH) strategy (de Lorgeril *et al.*, 2005). Among the genes differentially expressed in the circulating hemocytes of naïve *versus* *Vibrio*-infected shrimp, many were immune-related genes, including some crustacean AMP families. It was shown that the mRNA abundance of penaeidins (*Litsty* PEN2 and *Litsty* PEN3), crustins and stylum (previously called cysteine-rich peptide or cryptdin-like) before a *V. penaeicida* infection could predict shrimp survival. These results evidenced for the first time a relationship between the gene expression profile and the capacity of shrimp to survive an infection (de Lorgeril *et al.*, 2008). Additionally, it was shown that shrimp AMPs could display different expression kinetics after microbial stimulation.

It is noteworthy that one single animal can concomitantly express multiple AMP families, including many different subgroups and isoforms, and that this can confer a broad spectrum of antimicrobial responses to this animal. Unfortunately, at the present moment it is not known if different crustacean AMPs co-localize in the same vesicles of granular hemocytes or if each peptide family is segregated in different granules or cellular subpopulations. It would be also interesting to elucidate if crustacean AMPs co-localize with other immune effectors, such as the proteins of the proPO system and lysosomal degrading enzymes in the hemocyte granules, as shown in detail in limulids (Iwanaga, 2002; Iwanaga and Lee, 2005).

Other considerations on crustacean AMPs

The knowledge acquired over the last two decades on the identification and characterization of antimicrobial peptides in crustaceans has revealed their essential role in the immune response and also in the capacity of these animals to survive infection. However, from the identified AMP families, only a few members have been tested against crustacean pathogens, as marine vibrios that may cause severe infections to these animals (Table 2). From these, solely the ALFs displayed a consistent and potent effect against a variety of *Vibrio* species, including strains that are pathogenic to crustaceans (Somboonwivat *et al.*, 2005). In addition, ALFs are also implicated in crustacean antiviral defense against WSSV (Liu *et al.*, 2006). These properties make the ALFs a particularly interesting anti-infective AMP family within crustaceans. To date, ALF members have been identified in several decapod species, but their occurrence and distribution in other crustacean orders remain unknown. Curiously, the complete genomic sequencing of the water flea *Daphnia pulex* (a basal

crustacean lineage: Class Branchiopoda, Order Cladocera) did not reveal the presence of genes encoding ALFs or any other currently known AMP family identified in crustaceans (McTaggart *et al.*, 2009). These findings are intriguing because gene sequences encoding ALFs are present in ancient chelicerates (horseshoe crabs) as well as in decapods (derived crustacean lineage), which are obviously more phylogenetically related to branchiopods than to chelicerates. Therefore, it remains unclear if ALF sequences were lost in *D. pulex* or if they have never been present.

With respect to crustins, EST sequences containing a putative WAP domain with characteristics of the crustin WAP domain were identified in the marine branchiopod *Artemia franciscana* and the copepod *Calanus finmarchius* (Smith *et al.*, 2008). However, in other molecular aspects, these partial sequences did not share significant similarities with the well-characterized crustin family. Conversely, two EST sequences were identified in the amphipod *Gammarus pulex* (a derived crustacean lineage), including many features of Type II crustins, such as a glycine-rich region similar to those of shrimp and a cysteine-rich region containing the WAP domain (Smith *et al.*, 2008). The authors suggest that although incomplete, these sequences are strong candidates for being true members of the crustin family. Therefore, crustins appear to be the unique AMP family characterized in decapods that is also present in non-decapod species (at least in amphipods) to date. It would be interesting to elucidate if certain AMP families are widely distributed across crustaceans, as it appears to be the case for crustins, while others have a more restricted distribution, such as penaeidins, which are only present in penaeid shrimp.

Another important point concerns the *in vitro* assessment of the activity of the different AMP families of crustaceans (Table 2). In most studies, the physiological conditions of the animals are not taken into account, particularly of marine crustaceans (the most well-studied group in terms of AMPs), which have high salt concentrations in their body fluids. It is well known that AMPs lose their effect at high salinity due to alterations of their charge and structural conformation (Lee *et al.*, 1997; Löfgren *et al.*, 2009). It is therefore essential to confirm whether the AMPs of marine animals are stable under salt conditions to better comprehend their effect *in vivo*. Indeed, it was shown that the penaeidin variant *Litvan* PEN3-1 lost its activity against *M. luteus* at a salinity above 0.9 % (marine salinity is about 3.5 %). Conversely, the antimicrobial property of the ALF *rALFPm3* was not significantly affected by high NaCl concentration (Löfgren *et al.*, 2009), and this is another interesting feature of this molecule. In contrast, carcinin (Type I crustin) requires high salt concentrations for its activity (Relf *et al.*, 1999).

The great majority of AMPs was isolated and/or identified from crustacean hemocytes. It was shown that penaeidins, ALFs and Type II crustins from shrimp are synthesized and stored in the granules of granular hemocytes. However, it was also reported that some AMP families could be also expressed in

Table 2 Antimicrobial properties of crustacean AMPs

AMP families	Bacteria		Fungi	
	Gram-positive	Gram-negative	Filamentous	Yeast
Bac-like	★★★	★★	NT	NT
Callinectin	NT	★★★ ¹	NT	NT
Astacidin 2	★★★	★★★	NT	NT
Armadillidin	★★★ ¹	NA	NT	NT
Homarin	NA	★★	NT	NT
Defensin	NT	NT	NT	NT
ALF	★★★	★★★	★★★	NA
Scygonadin	★★	NA	NA	NA
Penaeidin				
Subgroup 2 (PEN2)	★★★	NA	★★★	NA
Subgroup 3 (PEN3)	★★★	NA	★★★	★★★
Subgroup 4 (PEN4)	★★★	NA	★★★	★★★
Subgroup 5 (PEN5)	★★★	★★	★★★	NT
Crustin ²				
Type I	★★★	NA	NA	★
Type II	★★★	★	★	NT
Type III	★★★	★★★	★★	★★
Hyastatin	★★★ ¹	★★ ¹	NA	★★ ¹
Arasin	★★★ ¹	★★ ¹	NT	NT
Stylicin	NA	★	★★★	NA
Histones and derived fragments	★★★ ¹	NT	NT	NT
Hemocyanin-derived peptides				
Astacidin 1	★★	★★	NT	NT
PvHct/PsHct1/PsHct2	NA	NA	★★★	NT

Minimal inhibitory concentration (MIC) values: ★★★ up to 10 µM; ★★ 10-20 µM; ★ 20-40 µM.

NA: not active (>40 µM).

NT: not tested.

¹AMPs tested against a reduced number of microorganisms.

²Crustin classification proposed by Smith *et al.* (2008).

other crustacean tissues, such as the defensins of the spiny lobster *P. japonicus* (Pisuttharachai *et al.*, 2009b) and certain crustin types (Smith *et al.*, 2008). However, it happens that most of these results are based on RT-PCR and Northern blot analyses whose results can be misleading. Crustaceans possess an open circulatory system, and the circulating blood cells can infiltrate most of their tissues. Consequently, the presence of AMP transcripts in different tissues can actually originate from infiltrated hemocytes. Indeed, the expression of penaeidins was initially identified in a wide range

of tissues in *L. vannamei* using Northern blot analysis (Destoumieux *et al.*, 2000b). However, after *in situ* hybridization assays it was revealed that penaeidins were only expressed in shrimp granular hemocytes (Muñoz *et al.*, 2002). Similar results were also obtained for the ALFs of *F. chinensis* and *P. monodon* (Liu *et al.*, 2005; Soombowiat *et al.*, 2008). Hence, it is of prime importance to determine the gene expression sites of the other crustacean AMP families using more appropriate technical approaches (such as *in situ* hybridization and immunohistochemistry) to avoid misinterpretations.

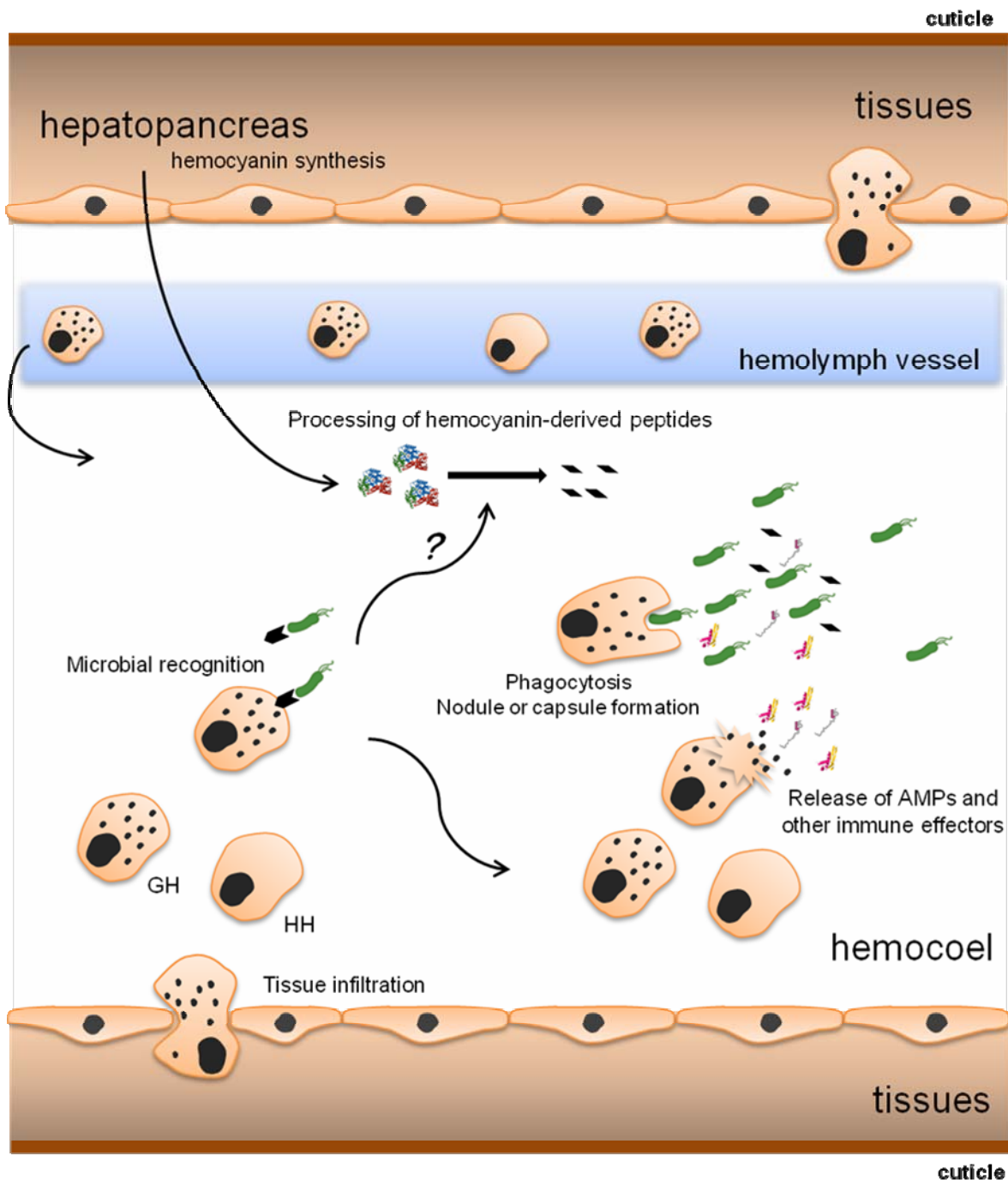


Fig. 4 Schematic illustration of crustacean cellular and humoral immune-reactions after microbial challenge. HH = hyaline hemocytes, GH = granular hemocytes

Another relevant aspect that remains to be clarified concerns the co-localization of the different AMP families within the crustacean hemocytes. To date, it is unclear if the different AMP classes and/or isoforms are selectively or randomly segregated into different granules of distinct granular hemocyte populations. As previously mentioned, this specific subcellular compartmentalization was elegantly

demonstrated in horseshoe crabs, in which the classic AMPs are stored within small granules, while ALF is segregated into large granules in association with the components of the coagulation cascade (Iwanaga and Lee, 2005), suggesting its primary role in horseshoe crab coagulation. In crustaceans, nothing is currently known about the co-localization of the different AMP families within immune cells. A

better elucidation of their storage in the same or different granules could help to predict their behavior during infections. It is generally assumed that crustacean AMPs are promptly released into the hemolymph from the granules upon cell stimulation by invading pathogens (Jiravanichpaisal *et al.*, 2006), as it occurs in limulids (Iwanaga and Lee, 2005). Accordingly, crustacean AMPs should combat and control infections extracellularly in a systemic response. However, these molecules could also exert their activity intracellularly within phagosomes.

Interestingly, a significant number of crustacean AMPs are composed of distinct domains (penaeidins, crustins, hyastatin, arasins and stylicins). This structural feature contrasts with the AMPs commonly described in other organisms, even the phylogenetically related groups, such as limulids (Iwanaga *et al.*, 2005) and insects (Bulet and Stöcklin, 2005), which mainly possess small single-domain peptides. It would be interesting to elucidate when and how the genes encoding these multi-domain proteins have been formed and rearranged during crustacean evolution. The implication of the different domains in different immune or physiological functions in crustaceans is uncertain. Perhaps the biological role of these proteins is primarily a reflex of their overall structural conformation (forming cationic amphipathic structures) and not of their independent intramolecular domains. However, the multifunctional role of some crustacean AMP is better documented. For example, the chitin-binding activity of penaeidin and hyastatin presumably participates in wound healing and molting (Destoumieux *et al.*, 2000a; Sperstad *et al.*, 2009b), the LPS-binding properties of ALFs and stylicins and the protease inhibition activity of Type III crustins block pathogen proteases and/or regulate endogenous protease cascades.

Concerning the unconventional AMPs, the antimicrobial activity of histones and histone-derived fragments was indeed an expected phenomenon in crustaceans because the antimicrobial property of these highly conserved proteins was already reported from fish to mammals. In shrimp, Patat *et al.* (2004) hypothesized that these proteins are stored in hemocyte granules and released into the hemolymph during microbial infection, together with other classical AMPs. However, the presence of histones in granules is unexpected because their transcripts do not possess a signal sequence to direct them to cytoplasmic granules. Expectedly, they contain nuclear localization signals within their N-terminal domain for translocation into the nuclear compartment. Consequently, their storage in hemocyte granules and their presumed release into the hemolymph in response to infection remain to be demonstrated.

Finally, maybe the most important aspect regarding crustacean AMPs concerns the complementary and synergistic antibiotic roles that these molecules probably encompass. It is reasonable to assume that the multiple classes of variants and isoforms of crustacean AMPs, which are concurrently present in the hemocytes of decapods, should act in a cooperative and

complementary manner in order to circumvent infections and ensure host survival (Fig. 4). To date, synergistic experiments have only been reported for *L. vannamei* penaeidins. However, no synergistic effects were observed between Litvan PEN2-1 and Litvan PEN3-1 (Destoumieux *et al.*, 1999). Also, in addition to AMPs, the immune cells of crustaceans produce several other well-known immune effectors, such as lysozyme and other degrading enzymes and the components of the proPO system, to control infections. Smith *et al.* (2010) suggest that lysozyme, a widely distributed antimicrobial enzyme that cleaves the carbohydrate portion of the peptidoglycan from the bacterial cell wall, potential plays a synergistic role with the common antimicrobial peptides. After disrupting the bacterial cell wall, lysozyme could facilitate the access of classic AMPs to permeabilize the bacterial membrane. Therefore, lysozyme could simultaneously serve as a direct effector and synergistic agent for enhancing AMP activity.

Concluding remarks

In conclusion, as illustrated above, both *in vitro* and *in vivo* properties of AMPs confirm that they are indeed essential components of the crustacean immune system. Unfortunately, the vast majority of the current knowledge refers only to the order Decapoda and is far from representing the entire and highly diverse group of crustaceans. This is clearly evidenced by the apparent absence of gene sequences encoding for hitherto described AMPs in the complete genome of the branchiopod *D. pulex*. Perhaps a more refined analysis of its genome could reveal the presence of gene sequences with similarity to some AMP domains that have already been characterized in decapods. Moreover, in view of the exceptional diversity of crustaceans, it is expected that novel unknown AMP families with singular molecular structures and interesting biological properties are waiting to be discovered. On the other hand, concerning the already identified crustacean AMPs, it would be interesting to fill in gaps of missing knowledge, such as their precise mode of action, *in vivo* activity, co-localization with other AMP members and especially their synergistic effect with other AMP families and/or other immunologic effectors also present in the immune cells of crustaceans. This information would certainly significantly contribute to a more comprehensive and integrated vision of the essential role that these molecules exert in crustacean immunity.

References

- Aketagawa J, Miyata T, Ohtsubo S, Nakamura T, Morita T, Hayashida H, *et al.* Primary structure of limulus anticoagulant anti-lipopolysaccharide factor. *J. Biol. Chem.* 261: 7357-7365, 1986.
- Amparyup P, Donpuksa S, Tassanakajon A. Shrimp single WAP domain (SWD)-containing protein exhibits proteinase inhibitory and antimicrobial activities. *Dev. Comp. Immunol.* 32: 1497-1509, 2008a.
- Amparyup P, Kondo H, Hirono I, Aoki T, Tassanakajon A. Molecular cloning, genomic

- organization and recombinant expression of a crustin-like antimicrobial peptide from black tiger shrimp *Penaeus monodon*. Mol. Immunol. 45: 1085-1093, 2008b.
- Antony SP, Bright Singh IS, Philip R. Molecular characterization of a crustin-like, putative antimicrobial peptide, Fi-crustin, from the Indian white shrimp, *Fenneropenaeus indicus*. Fish Shellfish Immunol. 28: 216-220, 2010.
- Bachère E, Destoumieux D, Bulet P. Penaeidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity. Aquaculture 191: 71-88, 2000.
- Bachère E, Gueguen Y, Gonzalez M, de Lorgeril J, Garnier J, Romestand B. Insights into the antimicrobial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. Immunol. Rev. 198: 149-168, 2004.
- Bartlett TC, Cuthbertson BJ, Shepard EF, Chapman RW, Gross PS, Warr GW. Crustins, homologues of an 11.5-kDa antibacterial peptide, from two species of penaeid shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*. Mar. Biotechnol. (NY) 4: 278-293, 2002.
- Battison AL, Summerfield R, Patrzykat A. Isolation and characterization of two antimicrobial peptides from haemocytes of the American lobster *Homarus americanus*. Fish Shellfish Immunol. 25: 181-187, 2008.
- Beale KM, Towle DW, Jayasundara N, Smith CM, Shields JD, Small HJ, et al. Antilipopolysaccharide factors in the American lobster *Homarus americanus*: molecular characterization and transcriptional response to *Vibrio fluvialis* challenge. Comp. Biochem. Physiol. 3D: 263-269, 2008.
- Bowdish DM, Davidson DJ, Hancock RE. A re-evaluation of the role of host defense peptides in mammalian immunity. Curr. Protein Pept. Sci. 6: 35-51, 2005.
- Brockton V, Hammond JA, Smith VJ. Gene characterization, isoforms and recombinant expression of carcinin, an antibacterial protein from the shore crab, *Carcinus maenas*. Mol. Immunol. 44: 943-949, 2007.
- Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3: 238-250, 2005.
- Brown KL, Hancock RE. Cationic host defense (antimicrobial) peptides. Curr. Opin. Immunol. 18: 24-30, 2006.
- Bulet P, Cociancich S, Dimarcq JL, Lambert J, Reichhart JM, Hoffmann D, et al. Insect immunity. Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. J. Biol. Chem. 266: 24520-24525, 1991.
- Bulet P, Stöcklin R. Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept. Lett. 12: 3-11, 2005.
- Bulet P, Stöcklin R, Menin L. Anti-microbial peptides: from invertebrates to vertebrates. Immunol. Rev. 198: 169-184, 2004.
- Capone R, Mustata M, Jang H, Arce FT, Nussinov R, Lal R. Antimicrobial protegrin-1 forms ion channels: molecular dynamic simulation, atomic force microscopy, and electrical conductance studies. Biophys. J. 98: 2644-2652, 2010.
- Carriel-Gomes MC, Kratz JM, Barracco MA, Bachère E, Barardi CR, Simões CM. In vitro antiviral activity of antimicrobial peptides against herpes simplex virus 1, adenovirus, and rotavirus. Mem. Inst. Oswaldo Cruz. 102: 469-472, 2007.
- Cerenius L, Lee BL, Söderhäll K. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29: 263-271, 2008.
- Cerenius L, Söderhäll K. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198: 116-126, 2004.
- Chen D, He N, Xu X. Mj-DWD, a double WAP domain-containing protein with antiviral relevance in *Marsupenaeus japonicus*. Fish Shellfish Immunol. 25: 775-781, 2008.
- Chernysh S, Cociancich S, Briand JP, Hetru C, Bulet P. The inducible antibacterial peptides of the hemipteran insect *Palomena prasina*: Identification of a unique family of prolinerich peptides and of a novel insect defensin. J. Insect Physiol. 42: 81-89, 1996.
- Chiou TT, Lu JK, Wu JL, Chen TT, Ko CF, Chen JC. Expression and characterization of tiger shrimp *Penaeus monodon* penaeidin (mopenaeidin) in various tissues, during early embryonic development and moulting stages. Dev. Comp. Immunol. 31: 132-142, 2007.
- Cho JH, Sung BH, Kim SC. Buforins: histone H2A-derived antimicrobial peptides from toad stomach. Biochim. Biophys. Acta 1788: 1564-1569, 2009.
- Christie AE, Rus S, Goiney CC, Smith CM, Towle DW, Dickinson PS. Identification and characterization of a cDNA encoding a crustin-like, putative antibacterial protein from the American lobster *Homarus americanus*. Mol. Immunol. 44: 3333-3337, 2007.
- Conlon JM. The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell Tissue Res. 2010.
- Cuthbertson BJ, Bullesbach EE, Fievet J, Bachère E, Gross PS. A new class (penaeidin class 4) of antimicrobial peptides from the Atlantic white shrimp (*Litopenaeus setiferus*) exhibits target specificity and an independent proline-rich-domain function. Biochem. J. 381: 79-86, 2004.
- Cuthbertson BJ, Bullesbach EE, Gross PS. Discovery of synthetic penaeidin activity against antibiotic-resistant fungi. Chem. Biol. Drug Des. 68: 120-127, 2006.
- Cuthbertson BJ, Deterding LJ, Williams JG, Tomer KB, Etienne K, Blackshear PJ, et al. Diversity in penaeidin antimicrobial peptide form and function. Dev. Comp. Immunol. 32: 167-181, 2008.
- Cuthbertson BJ, Shepard EF, Chapman RW, Gross PS. Diversity of the penaeidin antimicrobial peptides in two shrimp species. Immunogenetics 54: 442-445, 2002.
- Cuthbertson BJ, Yang Y, Bachère E, Bullesbach EE, Gross PS, Aumelas A. Solution structure of synthetic penaeidin-4 with structural and

- functional comparisons with penaeidin-3. *J. Biol. Chem.* 280: 16009-16018, 2005.
- Dai ZM, Zhu XJ, Yang WJ. Full-length normalization subtractive hybridization: a novel method for generating differentially expressed cDNAs. *Mol. Biotechnol.* 43: 257-263, 2009.
- de la Vega E, O'Leary NA, Shockey JE, Robalino J, Payne C, Browdy CL, *et al.* Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. *Mol. Immunol.* 45: 1916-1925, 2008.
- de Lorgeril J, Gueguen Y, Goarant C, Goyard E, Mugnier C, Fievet J, *et al.* A relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a *Vibrio* infection. *Mol. Immunol.* 45: 3438-3445, 2008.
- de Lorgeril J, Saulnier D, Janech MG, Gueguen Y, Bachère E. Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an infection with *Vibrio penaeicida*. *Physiol. Genomics* 21: 174-183, 2005.
- Decker H, Jaenicke E. Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins. *Dev. Comp. Immunol.* 28: 673-687, 2004.
- Decker H, Rimke T. *Tarantula* hemocyanin shows phenoloxidase activity. *J. Biol. Chem.* 273: 25889-25892, 1998.
- Destoumieux-Garzón D, Saulnier D, Garnier J, Jouffrey C, Bulet P, Bachère E. Crustacean immunity. Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. *J. Biol. Chem.* 276: 47070-47077, 2001.
- Destoumieux D, Bulet P, Loew D, Van Dorsselaer A, Rodriguez J, Bachère E. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *J. Biol. Chem.* 272: 28398-28406, 1997.
- Destoumieux D, Bulet P, Strub JM, Van Dorsselaer A, Bachère E. Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. *Eur. J. Biochem.* 266: 335-346, 1999.
- Destoumieux D, Muñoz M, Bulet P, Bachère E. Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). *Cell. Mol. Life Sci.* 57: 1260-1271, 2000a.
- Destoumieux D, Muñoz M, Cosseau C, Rodriguez J, Bulet P, Comps M, *et al.* Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. *J Cell Sci.* 113 (Pt 3): 461-469, 2000b.
- Dong B, Xiang JH. Discovery of genes involved in defense/immunity functions in a haemocytes cDNA library from *Fenneropenaeus chinensis* by ESTs annotation. *Aquaculture* 272: 208-215, 2007.
- Du ZQ, Li XC, Wang ZH, Zhao XF, Wang JX. A single WAP domain (SWD)-containing protein with antipathogenic relevance in red swamp crayfish, *Procambarus clarkii*. *Fish Shellfish Immunol.* 28: 134-142, 2010.
- Du ZQ, Ren Q, Zhao XF, Wang JX. A double WAP domain (DWD)-containing protein with proteinase inhibitory activity in Chinese white shrimp, *Fenneropenaeus chinensis*. *Comp. Biochem. Physiol.* 154B: 203-210, 2009.
- Easton DM, Nijnik A, Mayer ML, Hancock RE. Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol.* 27: 582-590, 2009.
- Fernandes JM, Kemp GD, Molle MG, Smith VJ. Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Biochem. J.* 368: 611-620, 2002.
- Fernandes JM, Molle G, Kemp GD, Smith VJ. Isolation and characterization of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Dev. Comp. Immunol.* 28: 127-138, 2004.
- Frank RW, Gennaro R, Schneider K, Przybylski M, Romeo D. Amino acid sequences of two proline-rich bactericins. Antimicrobial peptides of bovine neutrophils. *J. Biol. Chem.* 265: 18871-18874, 1990.
- Gil-Turnes MS, Hay ME, Fenical W. Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* 246: 116-118, 1989.
- Gross PS, Bartlett TC, Browdy CL, Chapman RW, Warr GW. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. *Dev. Comp. Immunol.* 25: 565-577, 2001.
- Guani-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM. Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clin. Immunol.* 135: 1-11, 2010.
- Gueguen Y, Garnier J, Robert L, Lefranc MP, Mougnot I, de Lorgeril J, *et al.* PenBase, the shrimp antimicrobial peptide penaeidin database: sequence-based classification and recommended nomenclature. *Dev. Comp. Immunol.* 30: 283-288, 2006.
- Hagiwara K, Kikuchi T, Endo Y, Huqun, Usui K, Takahashi M, *et al.* Mouse SWAM1 and SWAM2 are antibacterial proteins composed of a single whey acidic protein motif. *J. Immunol.* 170: 1973-1979, 2003.
- Hale JD, Hancock RE. Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Rev. Anti. Infect. Ther.* 5: 951-959, 2007.
- Hancock RE, Scott MG. The role of antimicrobial peptides in animal defenses. *Proc. Natl. Acad. Sci. USA* 97: 8856-8861, 2000.
- Hauton C, Brockton V, Smith VJ. Cloning of a crustin-like, single whey-acidic-domain, antibacterial peptide from the haemocytes of the European lobster, *Homarus gammarus*, and its response to infection with bacteria. *Mol. Immunol.* 43: 1490-1496, 2006.

- Herbinière J, Braquart-Varnier C, Greve P, Strub JM, Frere J, Van Dorsselaer A, *et al.* Armadillidin: a novel glycine-rich antibacterial peptide directed against gram-positive bacteria in the woodlouse *Armadillidium vulgare* (Terrestrial Isopod, Crustacean). *Dev. Comp. Immunol.* 29: 489-499, 2005.
- Hoess A, Watson S, Siber GR, Liddington R. Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, *Limulus* anti-LPS factor, at 1.5 Å resolution. *EMBO J.* 12: 3351-3356, 1993.
- Huang WS, Wang KJ, Yang M, Cai JJ, Li SJ, Wang GZ. Purification and part characterization of a novel antibacterial protein Scygonadin, isolated from the seminal plasma of mud crab, *Scylla serrata* (Forskål, 1775). *J. Exp. Mar. Biol. Ecol.* 339: 37-42, 2006.
- Imjongjirak C, Amparyup P, Tassanakajon A, Sittipraneed S. Antilipopolsaccharide factor (ALF) of mud crab *Scylla paramamosain*: molecular cloning, genomic organization and the antimicrobial activity of its synthetic LPS binding domain. *Mol. Immunol.* 44: 3195-3203, 2007.
- Imjongjirak C, Amparyup P, Tassanakajon A, Sittipraneed S. Molecular cloning and characterization of crustin from mud crab *Scylla paramamosain*. *Mol. Biol. Rep.* 36: 841-850, 2009.
- Iwanaga S. The molecular basis of innate immunity in the horseshoe crab. *Curr. Opin. Immunol.* 14: 87-95, 2002.
- Iwanaga S, Lee BL. Recent advances in the innate immunity of invertebrate animals. *J. Biochem. Mol. Biol.* 38: 128-150, 2005.
- Jia YP, Sun YD, Wang ZH, Wang Q, Wang XW, Zhao XF, *et al.* A single whey acidic protein domain (SWD)-containing peptide from fleshy prawn with antimicrobial and proteinase inhibitory activities. *Aquaculture* 284: 246-259, 2008.
- Jiménez-Vega F, Vargas-Albores F. A secretory leukocyte proteinase inhibitor (SLPI)-like protein from *Litopenaeus vannamei* haemocytes. *Fish Shellfish Immunol.* 23: 1119-1126, 2007.
- Jiménez-Vega F, Yepiz-Plascencia G, Söderhäll K, Vargas-Albores F. A single WAP domain-containing protein from *Litopenaeus vannamei* hemocytes. *Biochem. Biophys. Res. Commun.* 314: 681-687, 2004.
- Jiravanichpaisal P, Lee BL, Söderhäll K. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* 211: 213-236, 2006.
- Jiravanichpaisal P, Lee SY, Kim YA, Andren T, Söderhäll I. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern. *Dev. Comp. Immunol.* 31: 441-455, 2007a.
- Jiravanichpaisal P, Puanglarp N, Petkon S, Donnuea S, Söderhäll I, Söderhäll K. Expression of immune-related genes in larval stages of the giant tiger shrimp, *Penaeus monodon*. *Fish Shellfish Immunol.* 23: 815-824, 2007b.
- Kamysz W, Okroj M, Lukasiak J. Novel properties of antimicrobial peptides. *Acta Biochim. Pol.* 50: 461-469, 2003.
- Kang CJ, Xue JF, Liu N, Zhao XF, Wang JX. Characterization and expression of a new subfamily member of penaeidin antimicrobial peptides (penaeidin 5) from *Fenneropenaeus chinensis*. *Mol Immunol.* 44: 1535-1543, 2007.
- Kashima M. H1 histones contribute to candidacidal activities of human epidermal extract. *J. Dermatol.* 18: 695-706, 1991.
- Kawabata S, Koshihara T, Shibata T. The lipopolysaccharide-activated innate immune response network of the horseshoe crab. *Inv. Surv. J.* 6: 59-77, 2009.
- Khoo L, Robinette DW, Noga EJ. Callinectin, an antibacterial peptide from blue crab, *Callinectes sapidus*, hemocytes. *Mar. Biotechnol. (NY)* 1: 44-51, 1999.
- Lai Y, Gallo RL. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol.* 30: 131-141, 2009.
- Lee IH, Cho Y, Lehrer RI. Effects of pH and salinity on the antimicrobial properties of clavanins. *Infect. Immun.* 65: 2898-2903, 1997.
- Lee SY, Lee BL, Söderhäll K. Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus*. *J. Biol. Chem.* 278: 7927-7933, 2003.
- Li C, Zhao J, Song L, Mu C, Zhang H, Gai Y, *et al.* Molecular cloning, genomic organization and functional analysis of an anti-lipopolysaccharide factor from Chinese mitten crab *Eriocheir sinensis*. *Dev. Comp. Immunol.* 32: 784-794, 2008.
- Li L, Wang JX, Zhao XF, Kang CJ, Liu N, Xiang JH, *et al.* High level expression, purification, and characterization of the shrimp antimicrobial peptide, Ch-penaeidin, in *Pichia pastoris*. *Protein Exp. Purif.* 39: 144-151, 2005.
- Liu F, Liu Y, Li F, Dong B, Xiang J. Molecular cloning and expression profile of putative antilipopolsaccharide factor in Chinese shrimp (*Fenneropenaeus chinensis*). *Mar. Biotechnol. (NY)* 7: 600-608, 2005.
- Liu H, Jiravanichpaisal P, Söderhäll I, Cerenius L, Söderhäll K. Antilipopolsaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish *Pacifastacus leniusculus*. *J. Virol.* 80: 10365-10371, 2006.
- Löfgren SE, Smânia A, Smânia EFA, Bachère E, Barracco MA. Comparative activity and stability under salinity conditions of different antimicrobial peptides isolated from aquatic animals. *Aquaculture Res.* 40: 1805-1812, 2009.
- Lorenzini DM, da Silva PIJ, Fogaca AC, Bulet P, Daffre S. Acanthoscurrin: a novel glycine-rich antimicrobial peptide constitutively expressed in the hemocytes of the spider *Acanthoscurria gomesiana*. *Dev. Comp. Immunol.* 27: 781-791, 2003.

- Lu KY, Sung HJ, Liu CL, Sung HH. Differentially enhanced gene expression in hemocytes from *Macrobrachium rosenbergii* challenged in vivo with lipopolysaccharide. *J. Invertebr. Pathol.* 100: 9-15, 2009.
- Mangoni ML. Temporins, anti-infective peptides with expanding properties. *Cell. Mol. Life Sci.* 63: 1060-1069, 2006.
- McTaggart SJ, Conlon C, Colbourne JK, Blaxter ML, Little TJ. The components of the *Daphnia pulex* immune system as revealed by complete genome sequencing. *BMC Genomics* 10: 175, 2009.
- Morita T, Ohtsubo S, Nakamura T, Tanaka S, Iwanaga S, Ohashi K, *et al.* Isolation and biological activities of limulus anticoagulant (anti-LPS factor) which interacts with lipopolysaccharide (LPS). *J. Biochem.* 97: 1611-1620, 1985.
- Mu C, Zheng P, Zhao J, Wang L, Zhang H, Qiu L, *et al.* Molecular characterization and expression of a crustin-like gene from Chinese mitten crab, *Eriocheir sinensis*. *Dev. Comp. Immunol.* 34: 734-740, 2010.
- Muñoz M, Vandenbulcke F, Garnier J, Gueguen Y, Bulet P, Saulnier D, *et al.* Involvement of penaeidins in defense reactions of the shrimp *Litopenaeus stylirostris* to a pathogenic vibrio. *Cell. Mol. Life Sci.* 61: 961-972, 2004.
- Muñoz M, Vandenbulcke F, Gueguen Y, Bachère E. Expression of penaeidin antimicrobial peptides in early larval stages of the shrimp *Penaeus vannamei*. *Dev. Comp. Immunol.* 27: 283-289, 2003.
- Muñoz M, Vandenbulcke F, Saulnier D, Bachère E. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. *Eur. J. Biochem.* 269: 2678-2689, 2002.
- Muta T, Miyata T, Tokunaga F, Nakamura T, Iwanaga S. Primary structure of anti-lipopolysaccharide factor from American horseshoe crab, *Limulus polyphemus*. *J. Biochem.* 101: 1321-1330, 1987.
- Nagai T, Kawabata S. A link between blood coagulation and prophenol oxidase activation in arthropod host defense. *J. Biol. Chem.* 275: 29264-29267, 2000.
- Nagoshi H, Inagawa H, Morii K, Harada H, Kohchi C, Nishizawa T, *et al.* Cloning and characterization of a LPS-regulatory gene having an LPS binding domain in kuruma prawn *Marsupenaeus japonicus*. *Mol. Immunol.* 43: 2061-2069, 2006.
- Nicolas P. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *FEBS J.* 276: 6483-6496, 2009.
- O'Leary NA, Gross PS. Genomic structure and transcriptional regulation of the penaeidin gene family from *Litopenaeus vannamei*. *Gene* 371: 75-83, 2006.
- Park IY, Park CB, Kim MS, Kim SC. Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. *FEBS Lett.* 437: 258-262, 1998.
- Patat SA, Carnegie RB, Kingsbury C, Gross PS, Chapman R, Schey KL. Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. *Eur. J. Biochem.* 271: 4825-4833, 2004.
- Pazgier M, Hoover DM, Yang D, Lu W, Lubkowski J. Human beta-defensins. *Cell. Mol. Life Sci.* 63: 1294-1313, 2006.
- Peng H, Yang M, Huang WS, Ding J, Qu HD, Cai JJ, *et al.* Soluble expression and purification of a crab antimicrobial peptide scygonadin in different expression plasmids and analysis of its antimicrobial activity. *Protein Exp. Purif.* 70: 109-115, 2010.
- Pisuttharachai D, Fagutao FF, Yasuike M, Aono H, Yano Y, Murakami K, *et al.* Characterization of crustin antimicrobial proteins from Japanese spiny lobster *Panulirus japonicus*. *Dev. Comp. Immunol.* 33: 1049-1054, 2009c.
- Pisuttharachai D, Yasuike M, Aono H, Murakami K, Kondo H, Aoki T, *et al.* Expressed sequence tag analysis of phyllosomas and hemocytes of Japanese spiny lobster *Panulirus japonicus*. *Fish Sci.* 75: 195-206, 2009a.
- Pisuttharachai D, Yasuike M, Aono H, Yano Y, Murakami K, Kondo H, *et al.* Characterization of two isoforms of Japanese spiny lobster *Panulirus japonicus* defensin cDNA. *Dev. Comp. Immunol.* 33: 434-438, 2009b.
- Ranganathan S, Simpson KJ, Shaw DC, Nicholas KR. The whey acidic protein family: a new signature motif and three-dimensional structure by comparative modeling. *J. Mol. Graph. Model* 17: 106-113, 134-106, 1999.
- Rattanachai A, Hirono I, Ohira T, Takahashi Y, Aoki T. Cloning of kuruma prawn *Marsupenaeus japonicus* crustin-like peptide cDNA and analysis of its expression. *Fish Sci.* 70: 765-771, 2004.
- Relf JM, Chisholm JR, Kemp GD, Smith VJ. Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from the granular haemocytes of the shore crab, *Carcinus maenas*. *Eur. J. Biochem.* 264: 350-357, 1999.
- Richards RC, O'Neil DB, Thibault P, Ewart KV. Histone H1: an antimicrobial protein of Atlantic salmon (*Salmo salar*). *Biochem. Biophys. Res. Commun.* 284: 549-555, 2001.
- Robalino J, Almeida JS, McKillen D, Colglazier J, Trent HF, 3rd, Chen YA, *et al.* Insights into the immune transcriptome of the shrimp *Litopenaeus vannamei*: tissue-specific expression profiles and transcriptomic responses to immune challenge. *Physiol. Genomics* 29: 44-56, 2007.
- Robalino J, Carnegie RB, O'Leary N, Ouvry-Patat SA, de la Vega E, Prior S, *et al.* Contributions of functional genomics and proteomics to the study of immune responses in the Pacific white leg shrimp *Litopenaeus vannamei*. *Vet. Immunol. Immunopathol.* 128: 110-118, 2009.
- Robinette D, Wada S, Arroll T, Levy MG, Miller WL, Noga EJ. Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like

- antimicrobial proteins. *Cell. Mol. Life Sci.* 54: 467-475, 1998.
- Rojtinnakorn J, Hirono I, Itami T, Takahashi Y, Aoki T. Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. *Fish Shellfish Immunol.* 13: 69-83, 2002.
- Rolland JL, Abdelouahab M, Dupont J, Lefevre F, Bachère E, Romestand B. Stylicins, a new family of antimicrobial peptides from the Pacific blue shrimp *Litopenaeus stylirostris*. *Mol. Immunol.* 47: 1269-1277, 2010.
- Rosa RD, Bandeira PT, Barracco MA. Molecular cloning of crustins from the hemocytes of Brazilian penaeid shrimps. *FEMS Microbiol. Lett.* 274: 287-290, 2007.
- Rosa RD, Stoco PH, Barracco MA. Cloning and characterization of cDNA sequences encoding for anti-lipoplysaccharide factors (ALFs) in Brazilian palaemonid and penaeid shrimps. *Fish Shellfish Immunol.* 25: 693-696, 2008.
- Saito T, Kawabata S, Shigenaga T, Takayenoki Y, Cho J, Nakajima H, *et al.* A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence, and antibacterial activity. *J. Biochem.* 117: 1131-1137, 1995.
- Salditt T, Li C, Spaar A. Structure of antimicrobial peptides and lipid membranes probed by interface-sensitive X-ray scattering. *Biochim. Biophys. Acta* 1758: 1483-1498, 2006.
- Schnapp D, Kemp GD, Smith VJ. Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. *Eur. J. Biochem.* 240: 532-539, 1996.
- Shi XZ, Zhang RR, Jia YP, Zhao XF, Yu XQ, Wang JX. Identification and molecular characterization of a Spatzle-like protein from Chinese shrimp (*Fenneropenaeus chinensis*). *Fish Shellfish Immunol.* 27: 610-617, 2009.
- Shockey JE, O'Leary NA, de la Vega E, Browdy CL, Baatz JE, Gross PS. The role of crustins in *Litopenaeus vannamei* in response to infection with shrimp pathogens: An in vivo approach. *Dev. Comp. Immunol.* 33: 668-673, 2009.
- Simmaco M, Mignogna G, Canofeni S, Miele R, Mangoni ML, Barra D. Temporins, antimicrobial peptides from the European red frog *Rana temporaria*. *Eur. J. Biochem.* 242: 788-792, 1996.
- Smith VJ, Desbois AP, Dyrinda EA. Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. *Mar. Drugs* 8: 1213-1262, 2010.
- Smith VJ, Fernandes JM, Kemp GD, Hauton C. Crustins: enigmatic WAP domain-containing antibacterial proteins from crustaceans. *Dev. Comp. Immunol.* 32: 758-772, 2008.
- Somboonwivat K, Bachère E, Rimphanitchayakit V, Tassanakajon A. Localization of anti-lipoplysaccharide factor (ALFPm3) in tissues of the black tiger shrimp, *Penaeus monodon*, and characterization of its binding properties. *Dev. Comp. Immunol.* 32: 1170-1176, 2008.
- Somboonwivat K, Marcos M, Tassanakajon A, Klinbunga S, Aumelas A, Romestand B, *et al.* Recombinant expression and anti-microbial activity of anti-lipoplysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*. *Dev. Comp. Immunol.* 29: 841-851, 2005.
- Sperstad SV, Haug T, Paulsen V, Rode TM, Strandskog G, Solem ST, *et al.* Characterization of crustins from the hemocytes of the spider crab, *Hyas araneus*, and the red king crab, *Paralithodes camtschaticus*. *Dev. Comp. Immunol.* 33: 583-591, 2009a.
- Sperstad SV, Haug T, Vasskog T, Stensvåg K. Hyastatin, a glycine-rich multi-domain antimicrobial peptide isolated from the spider crab (*Hyas araneus*) hemocytes. *Mol. Immunol.* 46: 2604-2612, 2009b.
- Sperstad SV, Smith VJ, Stensvåg K. Expression of antimicrobial peptides from *Hyas araneus* haemocytes following bacterial challenge in vitro. *Dev. Comp. Immunol.* 34: 618-624, 2010.
- Stensvåg K, Haug T, Sperstad SV, Rekdal O, Indrevoll B, Styrvoid OB. Arasin 1, a proline-arginine-rich antimicrobial peptide isolated from the spider crab, *Hyas araneus*. *Dev. Comp. Immunol.* 32: 275-285, 2008.
- Stoss TD, Nickell MD, Hardin D, Derby CD, McClintock TS. Inducible transcript expressed by reactive epithelial cells at sites of olfactory sensory neuron proliferation. *J. Neurobiol.* 58: 355-368, 2004.
- Sun C, Du XJ, Xu WT, Zhang HW, Zhao XF, Wang JX. Molecular cloning and characterization of three crustins from the Chinese white shrimp, *Fenneropenaeus chinensis*. *Fish Shellfish Immunol.* 28: 517-524, 2010.
- Supungul P, Klinbunga S, Pichyangkura R, Hirono I, Aoki T, Tassanakajon A. Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach. *Dis. Aquat. Organ* 61: 123-135, 2004.
- Supungul P, Klinbunga S, Pichyangkura R, Jitrapakdee S, Hirono I, Aoki T, *et al.* Identification of immune-related genes in hemocytes of black tiger shrimp (*Penaeus monodon*). *Mar. Biotechnol. (NY)* 4: 487-494, 2002.
- Supungul P, Tang S, Maneeruttanarungroj C, Rimphanitchayakit V, Hirono I, Aoki T, *et al.* Cloning, expression and antimicrobial activity of crustinPm1, a major isoform of crustin, from the black tiger shrimp *Penaeus monodon*. *Dev. Comp. Immunol.* 32: 61-70, 2008.
- Tanaka S, Nakamura T, Morita T, Iwanaga S. *Limulus* anti-LPS factor: an anticoagulant which inhibits the endotoxin mediated activation of *Limulus* coagulation system. *Biochem. Biophys. Res. Commun.* 105: 717-723, 1982.
- Tassanakajon A, Amparyup P, Somboonwivat K, Supungul P. Cationic Antimicrobial Peptides in Penaeid Shrimp. *Mar. Biotechnol. (NY)*, 2010 [Epub ahead of print].
- Tassanakajon A, Klinbunga S, Paunglarp N, Rimphanitchayakit V, Udomkit A, Jitrapakdee S, *et al.* *Penaeus monodon* gene discovery project: the generation of an EST collection and

- establishment of a database. *Gene* 384: 104-112, 2006.
- Taylor K, Barran PE, Dorin JR. Structure-activity relationships in beta-defensin peptides. *Biopolymers* 90: 1-7, 2008.
- Tharntada S, Ponprateep S, Somboonwivat K, Liu H, Söderhäll I, Söderhäll K, *et al.* Role of anti-lipopolysaccharide factor from the black tiger shrimp, *Penaeus monodon*, in protection from white spot syndrome virus infection. *J. Gen. Virol.* 90: 1491-1498, 2009.
- Tharntada S, Somboonwivat K, Rimphanitchayakit V, Tassanakajon A. Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci. *Fish Shellfish Immunol.* 24: 46-54, 2008.
- Theopold U, Schmidt O, Söderhäll K, Dushay MS. Coagulation in arthropods: defense, wound closure and healing. *Trends Immunol.* 25: 289-294, 2004.
- Thomas S, Karnik S, Barai RS, Jayaraman VK, Iidicula-Thomas S. CAMP: a useful resource for research on antimicrobial peptides. *Nucleic Acids Res.* 38: D774-780, 2010.
- Tossi A, Sandri L. Molecular diversity in gene-encoded, cationic antimicrobial polypeptides. *Curr. Pharm. Des.* 8: 743-761, 2002.
- van Holde KE, Miller KI, Decker H. Hemocyanins and invertebrate evolution. *J. Biol. Chem.* 276: 15563-15566, 2001.
- Vatanavicharn T, Supungul P, Puanglarp N, Yingvilasprasert W, Tassanakajon A. Genomic structure, expression pattern and functional characterization of crustinPm5, a unique isoform of crustin from *Penaeus monodon*. *Comp. Biochem. Physiol.* 153B: 244-252, 2009.
- Wang DN, Liu JW, Yang GZ, Zhang WJ, Wu XF. Cloning of anti-IPS factor cDNA from *Tachypleus tridentatus*, expression in *Bombyx mori* larvae and its biological activity *in vitro*. *Mol. Biotechnol.* 21: 1-7, 2002.
- Wang KJ, Huang WS, Yang M, Chen HY, Bo J, Li SJ, *et al.* A male-specific expression gene, encodes a novel anionic antimicrobial peptide, scygonadin, in *Scylla serrata*. *Mol. Immunol.* 44: 1961-1968, 2007.
- Wicker C, Reichhart JM, Hoffmann D, Hultmark D, Samakovlis C, Hoffmann JA. Insect immunity. Characterization of a *Drosophila* cDNA encoding a novel member of the dipterocin family of immune peptides. *J. Biol. Chem.* 265: 22493-22498, 1990.
- Yang Y, Boze H, Chemardin P, Padilla A, Moulin G, Tassanakajon A, *et al.* NMR structure of rALF-Pm3, an anti-lipopolysaccharide factor from shrimp: model of the possible lipid A-binding site. *Biopolymers* 91: 207-220, 2009.
- Yang Y, Poncet J, Garnier J, Zatylny C, Bachère E, Aumelas A. Solution structure of the recombinant penaeidin-3, a shrimp antimicrobial peptide. *J. Biol. Chem.* 278: 36859-36867, 2003.
- Yedery RD, Reddy KV. Identification, cloning, characterization and recombinant expression of an anti-lipopolysaccharide factor from the hemocytes of Indian mud crab, *Scylla serrata*. *Fish Shellfish Immunol.* 27: 275-284, 2009a.
- Yedery RD, Reddy KV. Purification and characterization of antibacterial proteins from granular hemocytes of Indian mud crab, *Scylla serrata*. *Acta Biochim. Pol.* 56: 71-82, 2009b.
- Yount NY, Bayer AS, Xiong YQ, Yeaman MR. Advances in antimicrobial peptide immunobiology. *Biopolymers* 84: 435-458, 2006.
- Yue F, Pan L, Miao J, Zhang L, Li J. Molecular cloning, characterization and mRNA expression of two antibacterial peptides: crustin and anti-lipopolysaccharide factor in swimming crab *Portunus trituberculatus*. *Comp. Biochem. Physiol.* 156B: 77-85, 2010.
- Zhang J, Li F, Wang Z, Xiang J. Cloning and recombinant expression of a crustin-like gene from Chinese shrimp, *Fenneropenaeus chinensis*. *J. Biotechnol.* 127: 605-614, 2007.
- Zhang Y, Söderhäll I, Söderhäll K, Jiravanichpaisal P. Expression of immune-related genes in one phase of embryonic development of freshwater crayfish, *Pacifastacus leniusculus*. *Fish Shellfish Immunol.* 28: 649-653, 2010.
- Zhao XF, Wang JX. The antimicrobial peptides of the immune response of shrimp. *Inv. Surv. J.* 5: 162-179, 2008.