Review

Hemolymph proteins: An overview across marine arthropods and molluscs

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A R T I C L E   I N F O

A B S T R A C T

In this compilation we collect information about the main protein components in hemolymph and stress the continued interest in their study. The reasons for such an attention span several areas of biological, veterinarian and medical applications: from the notions for better dealing with the species – belonging to phylum *Arthropoda*, subphylum *Crustacea*, and to phylum *Mollusca* – of economic interest, to the development of 'marine drugs' from the peptides that, in invertebrates, act as antimicrobial, antifungal, antiprotozoal, and/or antiviral agents. Overall, the topic most often on focus is that of innate immunity operated by classes of pattern-recognition proteins.
Significance: The immune response in invertebrates relies on innate rather than on adaptive/acquired effectors. At a difference from the soluble and membrane-bound immunoglobulins and receptors in vertebrates, the antimicrobial, antifungal, antiprotozoal and/or antiviral agents in invertebrates interact with non-self material by targeting some common (rather than some highly specific) structural motifs. Developing this paradigm into (semi) synthetic pharmaceuticals, possibly optimized through the modeling opportunities offered by computational biochemistry, is one of the lessons today's science may learn from the study of marine invertebrates, and specifically of the proteins and peptides in their hemolymph.

1. Introduction – why?

Marine invertebrates are definitely not the organisms that we are more likely to associate with the noun 'animal': we rather do it of *Mammalia* and *Aves*, which share with us – to a larger or smaller extent – the overall layout of body structure; we do it of *Insecta*, which share with us the terrestrial habitat. Closeness, in (current) space and (evolutional) time, seems the criterion our mind follows: what is further ahead hardly matters.

Contrary to common sense, research on marine invertebrates has shown instead that these animals have much to teach: not only in terms of basic knowledge but also in the direction of know-how for practical applications in human and veterinary medicine. To fight viral and bacterial infection these animals cannot count on any form of adaptive/acquired immunity but extensively, and effectively, rely on a number of elements featuring their innate immunity: cells, proteins, peptides whose presence is not stimulated by particular antigens and which interact with non-self material by targeting some common (rather than some highly specific) structural motifs. Knowledge on

this subject is of immediate interest to aquaculture, to manage fishery procedures towards optimal yield of shellfish for human consumption. Knowledge, however, may also be applied to the evaluation of some of the bioactive peptides for possible use in human therapy. Yet another way marine invertebrates are useful to human health has to do with their immunity mechanisms: coagulation of the hemolymph induced by lipopolysaccharide (LPS, a membrane component of gram-negative bacteria) is the biological phenomenon at the basis of the assays for the detection and quantification of bacterial endotoxins (LAL, or TAL, test) [1,2]. Outside the innate defense mechanisms of marine invertebrates, a final item of practical interest is hemocyanin, their oxygen carrier protein. Because of its immunogenicity, hemocyanin has been used as scaffold for the conjugation of small molecules or short peptides, when raising anti-epitope antibodies; because of its peculiar quaternary structure, hemocyanin has also been taken as a possible model for the semi-synthesis of blood substitutes.

From the above, some marine invertebrates may be regarded as farm animals (and eventually as specialty food: *e.g.* shrimps, crayfish, lobsters and crabs), some others as biomedical factories (*Limulus* or *Tachypleus* for

amebocyte lysates, and Megathura crenulata for hemocyanin as biological reagents), all of them as model species for basic investigations. Most components that make these animals worth studying are present in their hemolymph, a fiuid analogous to blood in vertebrates. In an extension to our previous work on the liquid intercellular matrix of blood from a number of *Mammalia* species (Bos taurus [3], Canis familiaris [4], Equus caballus [5], Felis catus [6], Mus musculus [7,8], Rattus norvegicus [9–11], Sus scrofa [12]), we describe in the following the main proteins found in solution in some marine invertebrate hemolymph, and detail their origin, which is often from the hemocytes, the cells suspended in the hemolymph. To one of the proteins, the oxygen carrier hemocyanin, we devote a speciffc section. Because of their interconnection with the above, we also cover the proteome of the hemocytes and mention that of the hepatopancreas. Preliminary to this overview, we deffne the animal groups we mean to review and then briefiy outline their anatomy and physiology, with reference to the role of hemolymph and its components.

While closing this section, we like to refer to a 2012 review dealing with 'hemolymph proteins in marine crustaceans' covering one of the two taxa dealt with in our compilation [13]. All PubMed searches we are referring to were undertaken in the second half of 2020.

1.1. Which animals are we dealing with?

In all our previous scientiffc writing on circulating proteins we have either reported own experimental evidence or reviewed literature data on individual animal species. Only in few cases we had to make any further difference, and it was among breeds [4,14] or strains [15]. In the present survey, on the contrary, the subject of interest spans (Fig. 1) a number of species across two different phyla, *Mollusca* and *Arthropoda*: for the former, a limited number of literature reports deal with species in all the three main classes (*Bivalvia*, *Cephalopoda*, *Gastropoda*); for the latter, a much higher number of reports focus on relatively few species, all belonging to subphylum *Crustacea* and to class *Malacostraca* – except for the horseshoe crabs, belonging to subphylum *Chelicerata*. Both number of reports and number of investigated species seem to correlate – the former positively, the latter negatively – with economic relevance rather than with number of described species: indeed, this figure is almost twice as large for *Mollusca* as for *Crustacea* while the number of reports is approximately equal for the two phyla. Economic relevance also influences the issues actually addressed in each case, and the variety of experimental approaches put to use. Accordingly, the topics covered by the investigations on marine invertebrates' hemolymph are definitely heterogeneous and largely distinct from what we had encountered in our past projects.

1.2. What is the role of hemolymph?

The overall anatomical structure varies extensively not only between *Mollusca* and *Crustacea* but also among *Mollusca*. All the animals share, however, the element that is key to this review, hemolymph as their circulating fluid. Hemolymph fills the central cavity of their bodies, the

hemocoel, and fulfills the functions which *e.g.* in *Mammalia* are split between blood and lymph: it transports oxygen, absorbed through the gills; nutrients, made available by the digestive tract; catabolites, to be disposed of through the excretory systems (nephridia, antennal glands); hormones, and immune cells – the hemocytes. The latter may be classified on a morphological basis, *i.e.* hyaline, semigranular and granular hemocytes in decapod crustaceans, or granulocytes and agranular hyalinocytes in molluscs. The number of circulating cells may significantly vary, reflecting external and internal factors, including an increased demand for new hemocytes in cases of infection or during molting. The location where hematopoiesis takes place varies greatly: *e.g.*, an amebocyte-producing organ (APO) was described in some *Mollusca*, an anterior proliferation center (APC) in some *Crustacea* [16,17]; still, the effector functions of mature hemocytes are known in more detail than the process of their genesis. At a difference from the erythrocytes packed with a medium-size, iron-containing protein – hemoglobin – typical of the blood in *Reptilia*, *Aves* and *Mammalia*, hemolymph carries, in solution (*i.e.*, not membrane-enclosed), a large-size, copper-containing protein – hemocyanin – for oxygen transport.

From the above, the term «hemolymph», applied to invertebrates, identiffes, in strict sense, the combination of hemocytes and of the liquid intercellular substance in which they are suspended, as much as the term «blood» identiffes, for vertebrates, the combination of formed elements and plasma. The correct deffnition for the fiuid resulting from hemolymph coagulation is «hemolymph serum»; we are going to use the complete form in all the clauses in which the meaning might be in question, but we'll shorten it to «hemolymph» when the sense of our writing is clear.

### 1.3. Which omics data do we survey?

In line with our past experience and current research interests, we are going to survey in this review data on hemolymph components as collected with proteomic investigations. While searching literature for relevant scientiffc publications on the subject, we came across a few reports in which other omics approaches were applied instead, addressing surrogate informational macromolecules – the nucleic acids.

The most relevant of these alternative approaches was transcriptomics. The sheer number of reports to be retrieved from PubMed is much higher when searching for transcriptomic than for proteomic investigations (by 50% for *Mollusca* and by 100% for *Crustacea*).

Transcriptome analysis has been the typical way to monitor, for instance, the response of the animals to various challenges (*e.g.* viral infection, or injection of a test substance such as the neuroendocrine-immune factor dopamine) in relevant cells/tissues/organs (*e.g.* Marsupenaeus japonicus hepatopancreas [17], Litopenaeus vannamei hemocytes [18], to address the two compartments from which proteins are secreted into the hemolymph).

In keeping with the trend already commented under 1.1, many such investigations have been carried out in *Crustacea* (including the two reports quoted above) but quite a few examples for the application of

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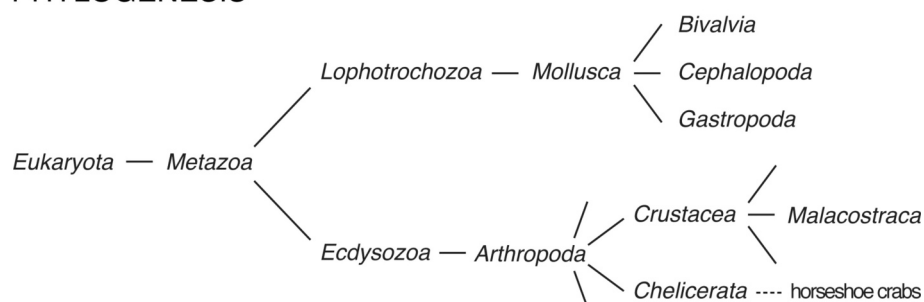
Fig. 1. Phylogenetic relationships among the marine invertebrates dealt with in this review (simpliffed). The ranking of the different terms in the scheme is as follows: *Eukaryota* = superkingdom; *Metazoa* = kingdom; *Lophotrochozoa, Ecdysozoa* = clade; *Mollusca, Arthropoda* = phylum; *Crustacea, Chelicerata* = subphylum; *Bivalvia, Cephalopoda, Gastropoda, Malacostraca* = class (https://www.ncbi.nlm.nih.gov/taxonomy/). The common name «horseshoe crabs» refers to the four species in order *Xiphosura*.

transcriptomics may be found also for *Mollusca* (*e.g.* a very comprehensive investigation on *Saccostrea glomerata* samples – hemolymph, gill, mantle, adductor muscle, gonad and digestive organs – after exposure to different potential stressors – CO_2, salinity, temperature, copper and polycyclic aromatic hydrocarbons [19]).

A subject, namely the long-term antiviral immune priming in Crassostrea gigas by the injection of polyinosinic polycytidylic acid (PolyI:C) in the adductor muscle, after anesthesia with $MgCl_2$, was investigated using both proteomics [20] and transcriptomics [21]. This extensive coverage has to do with the relevance of the procedure as a pseudovaccination against OsHV-1, a major oyster pathogen whose recurrent infection causes huge economic losses to the shellfish farming industry worldwide.

As a further mention, molecular biology procedures other than transcript analysis are applied as well to *Crustacea* and *Mollusca*. Among them, gene knockdown and RNA interference not only score the highest but appear to receive a steadily increasing attention (when comparing the number of reports a few months apart). The main area of interest is also in this case the immune response (just one recent example, the role of Litopenaeus vannamei crustin [22]). The flourishing application of up-to-date technologies to this research field is another indication of its interest, as outlined in the first lines of this writing.

Finally, and moving closer to proteomic issues, we need to draw attention to the limited genomic resources currently available for *Crustacea*. The Genome database (https://www.ncbi.nlm.nih.gov/genome/) contains for them 57 assemblies (410 for organelle DNA), with no nuclear genome close to completeness for its single-copy genes. One of the main challenges is the need to deal with large repetitive genomes, which characterize many decapod crustaceans [23]. As a result, *de novo* sequence data generated through MS/MS procedures may seldom be matched to same-species genomic information; most often researchers have to resort to cross-species identification of homologous proteins *via* similarity searching and use of EST data.

An alternative omics approach to be mentioned here is proteogenomics [24,25]. This strategy includes several steps, some dealing with nucleic acids, some with proteins. The first requires to compile a reference transcriptome for a given species by high-throughput mRNA sequencing (RNA-seq) from its tissues/organs, the second to *in silico* translate, in all possible reading frames, such a nucleotide database into a database of predicted proteins. Identifications of components in a protein sample are made possible by matching to the predicted sequences MS/MS data from a proteomic experiment. Proteogenomics greatly extends standard annotation methods, including the prediction of completely novel genes. As with other omics, the possibilities of identification may be increased through homology searches across cognate species. Some applications of proteogenomics will be presented in the following sections.

In our literature search we retrieved a limited number of omics reports addressing the whole set of circulating low molecular weight molecules; we are going to mention such metabolomics/metabonomics studies all along our compilation.

We mentioned above the Genome database; before moving ahead, we like to quote UniProt (https://www.uniprot.org) as a curated collection of structural and functional information on individual proteins, including for the animal species of interest in this review.

1.4. How is hemolymph collected?

The details of sample collection differ depending on the anatomy and on the size of the sampled species but tend to be similar across all the investigations on a given species. The research articles we quote in this section are thus representative but definitely not exhaustive sources of information on the topic.

For *Crustacea*, different anatomical sites at the heart and legs [26] are specified for hemolymph withdrawal. The collected volume, when mentioned, varies between 0.1 [27] and 1 mL [28]. Some protocols call for hemolymph clotting, some other on the contrary describe the additives (containing EGTA/EDTA [28] or citrate [26]) to be used as anticoagulants.

As for *Mollusca*, the description of hemolymph collection is detailed for instance for *Bivalvia* [29] or for cephalopod Octopus vulgaris (about 1 mL sample obtained). The latter procedure involves anesthesia with $MgCl_2$ [30]. As a rule, hemocytes are removed from plasma by centrifugation; recommended centrifugal force varies between 2000 [20] and 12,000 $\times g$ [30].

2. Arthropoda – Crustacea

For all the reasons mentioned under Introduction, we start our review from *Crustacea*. In our literature search, we found data on over thirty species of crabs, crayfish, lobsters, prawn, shrimps; the two most recurrent genera were *Panaeus*, with seven species, and *Scylla*, with three.

As for the contents, we found no catalogue of hemolymph soluble proteins under baseline conditions, in any species; indeed, proteomic investigations aiming at no more than the description of the protein complement in a given sample have long been discontinued in all fields of biological research. We can still outline the hemolymph composition under baseline conditions if only by compiling information on individual proteins. Conversely, we found a number of comprehensive proteomic investigations devised as differential analyses: in one case, different physiological conditions were compared while in many others contrast was between either absence or presence of (viral) infection or the exposure to toxic compounds. We observed a similar distribution also among data on hemocyte proteins. In addition to proteins, peptides have a crucial importance in the physio-pathological functions of *Crustacea*: we found many reports on these factors and we'll devote a paragraph to this specific topic.

2.1. Studies on individual hemolymph proteins (other than hemocyanin)

The main areas of interest covered by the publications retrieved from PubMed (https://pubmed.ncbi.nlm.nih.gov) on individual crustacean hemolymph proteins mainly address *innate immunity*, featuring both *pattern-recognition proteins (PRPs)* and *enzymes/enzyme inhibitors*; their functional relationships are outlined in Fig. 2, right.

In recent years, five papers have been devoted to *anti-lipopolysaccharide factors (ALFs)*, as expressed by different species (*Portunus trituberculatus* [31], Penaeus monodon [32], *Marsupenaeus japonicas* [33], Fenneropenaeus chinensis [34], *Scylla paramamosain* [35]). The reports explore the site of synthesis of the protein, often expressed in various isoforms in the same species [33–35] and the timing of their presence after challenge with a given noxa [32]. Even more, they detail the organisms that are targets of the biological activity of these molecules: Gram-negative bacteria, as implied by the name, since LPS is the major component of the outer layer of the outer membrane of this type of microorganisms, but also Gram-positive bacteria [35] and virus (white spot syndrome virus (WSSV) [32,34]). All the investigations go further, seeking to identify the molecular counterparts in the target organisms involved in direct interaction with the various ALFs. For instance, Suraprasit et al. use the yeast two-hybrid screening system to identify five potential interacting proteins, then the *in vitro* pull-down assay with these viral proteins in recombinant form as the bait to confirm one of them (WSSV189) as relevant in the binding of the *Penaeus* ALF to WSSV envelope [32]. The effect of mutagenesis on the biological activity of the ALFs is also investigated, by targeting either a conserved amino acid in the LPS-binding domain (LBD) [34] or making reference to single nucleotide polymorphisms (SNPs) in a region outside LBD but close to it [35]. In keeping with our mention (under 1.3) to the widespread use of molecular biology protocols in the study of marine invertebrates, one of the ways to prove the biological function of *M. japonicas* ALF is through knockdown by RNA interference [33]. One paper deals with a likely peptidoglycan-binding protein, tachylectin 5-like immune molecule from Penaeus monodon, characterized by a fibrinogen-related domain; its binding to bacteria cell wall could be at the *N*-acetyl moiety of the GlcNAc-MurNAc repeat unit. The protein is detected in various tissues but upon exposure to bacteria its expression increases mainly in hemocytes and hindgut [36].

Five of the retrieved papers deal with *lectins*, either mannose-binding, from Portunus pelagicus [37], or beta-glucan-binding, from Portunus
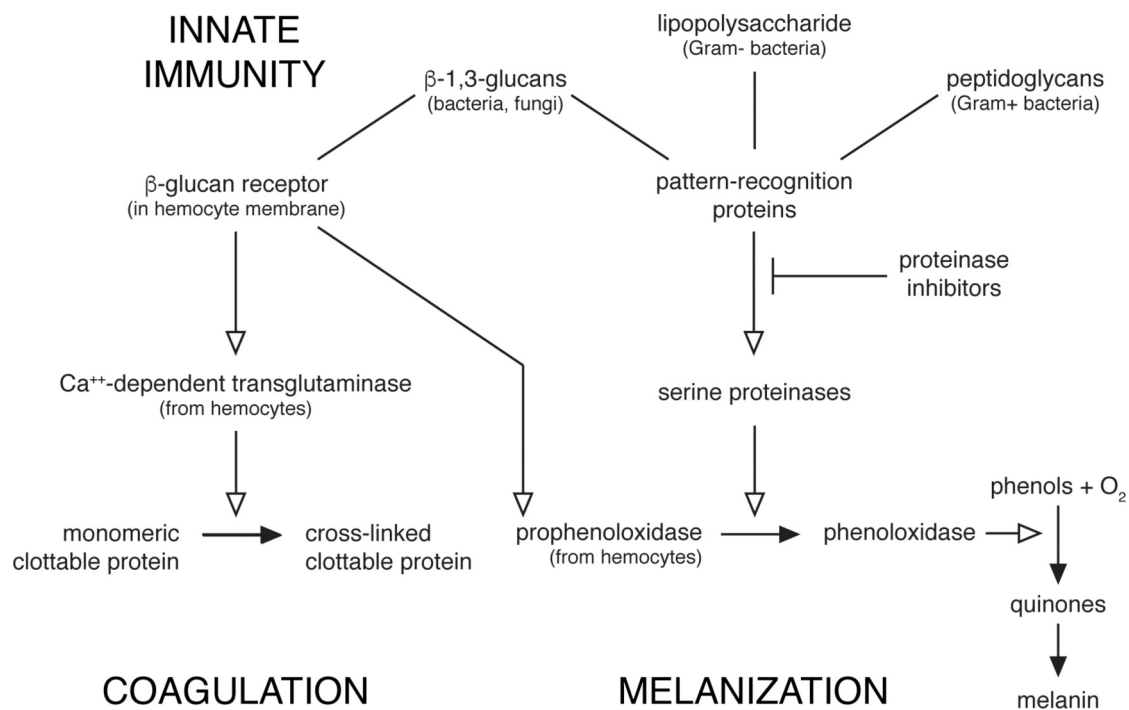
Fig. 2. Outline of the steps of the coagulation and melanization pathways (redrawn from [151,152]). Lines hint to interaction, full arrows to reactions, open arrows to (multistep) processes, —— to inhibition of processes.
The following abbreviations were used in the text: CP, hemolymph clottable protein; LPS, lipopolysaccharide; PO, phenoloxidase; proPO, prophenoloxidase; TG, transglutaminase.

pelagicus [38], *Paratelphusa hydrodromus* [39], from Scylla serrata [40] and from Metapenaeus monoceros [41]. All of them are puriffed by afffnity chromatography, with the relevant sugar moiety as the immobilized ligand. All of them appear to be multifunctional proteins: they promote agglutination, encapsulation and phagocytosis with a broad spectrum of targets spanning Gram-positive (Gram+) and Gram-negative (Gram-) bacteria; they also disrupt the architecture and reduce the thickness of biofflm, a viscous extracellular matrix consisting of carbohydrates that holds the bacteria together and irreversibly binds them to surfaces. Beta-glucan-binding lectins, in addition, promote the activity of phenoloxidase (see next) [39,40] and of serine proteases, and act as radical scavengers, thus cutting their inffammatory potential [39].

Other such proteins have been characterized at the transcriptomic level (ALFs from *Scylla paramamosain* [42] and Exopalaemon carinicauda [43], a galectin from Litopenaeus vannamei [44]).

Phenoloxidase (PO) is a common name of several copper-containing monooxygenases, including tyrosinases, catechol oxidases, and laccases. In *Crustacea*, PO takes part in a complex pathway (in Fig. 2), which involves various cell types, enzymes and enzyme inhibitors, and signaling molecules, and results in the melanization of pathogens and damaged tissues [45]. Upon infection, the starting event of this pathway is the speciffc recognition of bacterial (Gram+ and −), fungal, and viral agents by PRPs. Their interaction triggers a serine proteinase cascade, eventually leading to the cleavage of the inactive proPO to the active PO. Phenoloxidase produces indole groups, which polymerize to melanin; melanotic nodules limit the spread of infecting microorganisms or damaged tissues. The products of the enzymatic reactions, which include reactive oxygen and nitrogen species, are themselves effective against a wide range of pathogens [45]. Different types of PO are either present in hemocytes or synthesized in the hepatopancreas and secreted.

In our literature search, we found information about phenoloxidases from, for instance, Marsupenaeus japonicus [46], Penaeus semisulcatus [47] and Panulirus japonicus [48].

Relevant to melanization are also serpins, a family of serine *protease inhibitors* involved in the regulation of innate immunity pathways, specifically blocking the PO activation cascade. As an example, serpin from Paralithodes camtschaticus was thoroughly characterized both in structure and in function [49]. Another protease inhibitor of interest in connection with immunity is alpha-2-macroglobulin. As described for Penaeus monodon, the protein interacts with the carboxyl-terminus of transglutaminase type II, a key enzyme in the shrimp clotting system, and colocalizes with clottable proteins. This way the protease inhibitor enhances bacterial sequestration by protecting blood clots against ffbrinolysis [50].

This leads us to the topic of *coagulation*, which is closely connected with melanization as an integral part of innate immunity mechanisms (Fig. 2, left). Crucial to the pathway of coagulation are the *hemolymph clottable protein* (CP) and *transglutaminase (TG)*.

Collecting information from proteins in various species, CP is a disulffde-linked homodimeric glycoprotein, over 1600 amino acids in length and over 400 kDa in molecular mass, with a large quantity of associated lipid, primarily the carotenoid pigment astaxanthin. From electron micrographs of the protein, the most prominent feature of its structure is a large cavity spanning the length of the molecule, which is the likely lipid-binding pocket [51–53]. CP bears potential transglutaminase cross-linking sites, *i.e.* Ser-Lys-Thr repeats, Ser- and Gln-rich regions, and polyGln (N = 8–11) [54]; upon cross-linking, CP forms long, branched polymers. Depending on the species, sites of CP synthesis are multiple: central nervous system, gill, lymphoid organ [55] and sub-cuticular epidermis [54], not hepatopancreas. Reduced CP acts as a cysteine type proteinase [56].

TG catalyzes intermolecular or intramolecular epsilon-(gamma-glutamyl) lysine bond formation, resulting in protein polymerization. Two forms of the enzyme are known: TG I is mainly expressed in the hepatopancreas, TG II in the hemocytes [57]; it is mainly the latter to be relevant in the immune resistance processes [58].

The last biological function we have to cover, *lipid transport*, seemingly contrasts with all the above. However, once more we have to mention the propensity of *Crustacea* proteins to be multifunctional and the list of hemolymph lipoproteins starts with two proteins, the high density lipoprotein/beta-glucan binding proteins and the clotting proteins involved, as seen above, in hemostasis and immunity in both sexes. In addition, the former are the main lipid carriers while the latter act as storage proteins in the oocytes matured by females. Vitellogenins (or apolipocrustaceins), conversely, are female-specific lipoproteins, which

supply both proteins and lipids as storage material in the oocyte for later use by the developing embryo. Finally, the existence of large discoidal lipoproteins has been recently reported [59].

2.2. Studies on hemolymph proteome

This section covers both, studies of the hemolymph proteome in the body fluid itself and, if accidentally found, in tissues.

When it comes to comprehensive investigations, we found one report mentioning changes in hemolymph proteome under different physiological conditions. In fact, the research by Head et al. [60] does not actually analyze hemolymph samples, it rather takes note of the variations in the amount of hemolymph proteins in specimens of the Y-organ, an endocrine gland that, together with the X-organ/sinus gland complex, regulates crustacean molting; the source of these proteins in the analyzed samples is of course the hemolymph trapped within the lacunae in the tissue. Across the molting steps analyzed in Gecarcinus lateralis, the authors find – through 2-DE and MS analysis – that, for instance, transglutaminases are more abundant in the intermolt and late premolt stages, whereas the crustacean clotting protein is more abundant in the early, mid, and late premolt stages; for hemocyanin, various molecular species, extensively differing in mass and p*I*, are more represented in different stages of the molting process.

We found as well few reports addressing the changes in hemolymph composition after either infestation with parasites or infection with bacterial or viral agents. One such report deals with the response of Palaemonetes sinensis to the exposure to Tachaea chinensis [61]: the parasitic isopod perforates the host integument with its mandibles and feeds on hemolymph and exudate from the wounds. As in the case above, the proteomic investigation, carried out this time with the iTRAQ approach, uses as sample the tissues of the shrimp; the effects of the parasitic challenge on at least some of the hemolymph proteins, however, are large enough to be detected. None out of 182 up-regulated proteins is a circulating protein; conversely, 4 out of 113 down-regulated proteins – alpha-2-macroglobulin, hemocyanin, a putative clotting protein, and tachylectin – are typical hemolymph components.

Yet another report eventually confronts with the issue connected with actual proteomic analysis of whole hemolymph. In analogy with the setup of *Mammalia* blood, hemolymph contains proteins whose concentrations span several orders of magnitude; the most abundant components thus make it difficult to correctly assess the behavior of the least abundant ones. One of the most logical approaches to improve sensitivity in the analysis of medium to low abundance proteins is to remove as extensively, and as specifically, as possible the most abundant component [62]. For hemolymph this corresponds to hemocyanin (see Section 5). Due to the very high molecular mass of the multimeric protein, much of it may be pelleted by ultracentrifugation; most of the remaining hemocyanin, which includes smaller subunit assemblies, may then be removed by size-exclusion chromatography [63].

All the pathological conditions implied in the above reports may affect the yield of aquacultural activities. Attention has been given to the effects of different production management systems, including the feed supplementation with antibiotics [64]. However, even larger effects, as evaluated by proteomic evidence, appear to result from changes between intensive or extensive rearing conditions, which imply *e.g.* different oxygen and nitrogen concentrations [64].

The report we need to mention last in this section could be as well the first in the following one: indeed, using a proteogenomic approach Leprêtre et al. [65] obtain for Dreissena polymorpha a very comprehensive repertoire of the hemolymph proteome, both soluble circulating proteins and hemocyte components. The authors thoroughly sequence mRNA from whole tissues as well as from five body compartments (total hemolymph, male and female gonads, gills, digestive gland), to obtain almost one million of overlapping coding segments (contigs). Such a transcriptome database is taken as reference for the assignment, in a shotgun setup, of sequences from MS/MS spectra - over 260,000 for hemolymph soluble proteins and over 280,000 for hemocytes. This leads to the discovery of approx. 3000 proteins validated by at least two unique peptide sequences; 49% of them are observed only in hemocytes, 28% only in

plasma and the remaining 23% in both samples. Their putative functions are derived from those of homologous proteins in NCBI and UniProtKB/Swiss-Prot databases (accessed 2017 and 2016, respectively). Over one fourth map to GO term 'immune system process'; the most represented biological process (in terms of normalized spectral abundance factor) is 'response to stress', the most represented molecular function is 'peptidase activity'.

This transcriptome database is again used by the same research group to evaluate the response to two types of biological stress, to which hemolymph samples of Dreissena polymorpha are exposed *ex vivo* [66]. Evaluation of the shotgun peptides is performed by multiple reaction monitoring, using a triple quadrupole MS: identification of a protein is provided by the observation of a specific precursor ion, quantitation by the counts for one or more product ions formed from the precursor in a collision cell. In their experiment Leprêtre et al. feed tryptic peptides to the mass spectrometer through an HPLC device and an electrospray ion source; to correct for variability in chromatographic conditions, elution times are referred to pairs of scout peptides spiked into the peptide samples. The report describes with great detail the setting up of all aspects of the procedure, resulting in the validation of two assays for the relative quantitation of 85 proteins in hemocytes and 89 in plasma. The proteomes of hemocytes and hemolymph plasma are each affected in a distinct way by the biological stress: for the former, the most relevant changes involve pattern recognition receptors, proteases, and protease inhibitors; for the latter, the amount of circulating histones. In addition, exposure to either Cryptosporidium parvum or polyinosinic-polycytidylic acid sometimes brings about concordant effects (*e.g.* increase of C1q containing domain protein in plasma, decrease of clathrin heavy chain and of natterin-like protein in hemocytes) but far more often contrasting effects do occur (*e.g.* in hemocytes, alpha-1-inhibitor 3 increases after protozoan and decreases after nucleic acid challenge).

2.3. Studies on hemocytes

As already shown by the last papers reviewed, contrary to hemolymph soluble proteins, the components of hemocytes have been studied as a whole, with outright proteomics protocols.

The pattern under reference conditions is investigated by de la Ballina et al. [67] for samples from Ostrea edulis. Two cell populations are collected through differential centrifugation: granulocytes (50–70% Percoll interface) and hyalinocytes (10–30% Percoll interface). The patterns of their proteomes after IPG-DALT (1st d on pH 5–8 L IPG, 2nd d on 12.5% SDS-PAGE) are shown in Fig. 3, granulocytes at the top and hyalinocytes at the bottom. The spots specific to either cell types are marked with red dots; those for which a partial sequence could be obtained are numbered 1–20 for granulocytes and 1–14 for hyalinocytes. For 3 granulocyte and 2 hyalinocyte components no matching data were available in existing genomic databases in 2020; identification was thus possible for only 20 plus 14 proteins, most often making reference to *Crassostrea* homologs (12 from C. gigas, 6 from C. virginica, 1 from C. ariakensis; in addition, 3 sequences from Ostrea edulis itself and 1 each from other *Crustacea* or *Mollusca* species). The main functions connected with the identified proteins are not alike for the two cell types: signal transduction, apoptosis, pathogen recognition and intracellular digestion are more represented in granulocytes, while proteins that could be involved in shell production, wound healing and the phenoloxidase system are only found in hyalinocytes. Proteomic data thus support the evidence of granulocytes as more effective in phagocytosis and of hyalinocytes as more involved in the clotting events.

The response to cold stress is instead evaluated by Fan et al. [68] by comparing specimens from Litopenaeus vannamei maintained for 24 h either at 28 or at 13°C. Unfractionated hemocytes are pelleted by centrifugation at 3000 rpm, from citrated hemolymph; the solubilized proteins are fractionated by IPG-DALT (1st d on pH 5–8 L IPG, 2nd d on 12.5% SDS-PAGE) and the differentially represented spots identified by MALDI-TOF/TOF mass spectrometry. The largest number of affected spots corresponds to different molecular species of hemocyanin subunits,

2DE of *Ostrea edulis* hemocyte proteome
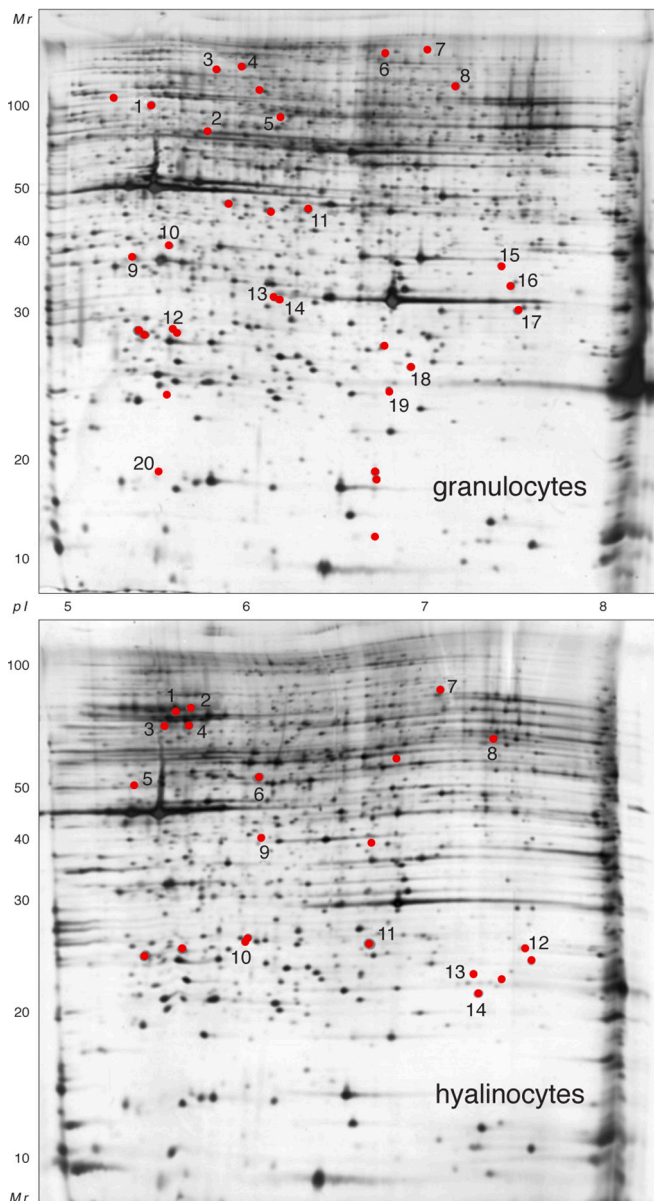
Fig. 3. IPG-DALT of proteins from isolated cell populations of Ostrea edulis hemocytes; 2-DE pattern for granulocytes at the top, for hyalinocytes at the bottom. Protein identiffcations as follows (all details in table 2 of [67]): for granulocytes (top panel), 1 = alpha-mannosidase (lysosomal); 3 = ubiquitin-conjugating enzyme E2W; 4 = actin type I; 6 = pancreatic lipase-related protein 2- like; 7 = RING finger domain and kelch repeat-containing protein DDB_G0271372-like; 8 = NADH dehydrogenase subunit 5; 9 = ankyrin repeat and SOCS box protein 13; 10 = glutathione S transferase 3; 11 = connector enhancer of kinase suppressor of ras 2-like isoform X; 12 = DNA replication complex GINS protein PSF2; 13 = *N*-acetyllactosaminide beta-1,6-*N*-acetylglucosaminyl-transferase; 15 = ribosomal protein S13; 16 = elongation factor 1 alpha; 17 = TD and POZ domain-containing protein 1-like; 18 = DNA replication complex GINS protein PSF2; 19 = mortality factor 4; 20 = heat shock protein HSP90α 1; for hyalinocytes (bottom panel), 1–2 = laccase 1; 3–5 = actin; 7 = transketolase-like protein 2; 8 = flotillin 2a; 9 = methionine adenosyl transferase; 10 = pyridoxine-5′-phosphate oxidase-like isoform X1; 11 = thymosin beta-like isoform X7; 13 = ankyrin repeat domain-containing protein 53; 14 = cement-like protein. Redrawn from figs.1 and 2 in [67].

which are all reduced in abundance after cold stress; other proteins detected at lower levels are transglutaminase and transketolase. Conversely, cystathionase, glyceraldehyde 3-phosphate dehydrogenase, glyoxalase 1 and oncoprotein nm23 are found to increase after cold stress. While oncoprotein nm23 is connected with immunity, the affected enzymes have to do with glycolysis (including glyoxalase, involved in the first step of the pathway that synthesizes (*R*)-lactate from methylglyoxal) or with amino acid metabolism (cystathionase catalyzes the last step in the trans-sulfuration pathway from methionine to cysteine), all belonging to intermediate metabolism. Identifications rely for 3 proteins on Litopenaeus vannamei genomic information, for 2 on homology with proteins from crustaceans (Orconectes cf. virilis, Penaeus monodon), for other 2 from dipteran insects (Aedes aegypti, *Glossina morsitans*), for 1 from a teleost fish (Danio rerio) and for the last 1 from a mammal (Mus musculus).

Spiroplasma eriocheiris, a minimal prokaryotic microorganism and intracellular bacterium lacking cell walls, is the pathogen causing tremor disease in Eriocheir sinensis. In the early stage of the infection, *Spiroplasma* enters the hemocytes and after some cycles of reproduction causes them to lyse. Later on the bacterium enters the thoracic ganglion that controls the pereiopod movement, causing the paroxysmal tremors typical of the disease. The research by Hou et al. [69] addresses both stages of the infection by proteomic means, with the analysis of hemocytes 24 h and of thoracic ganglion 10 days post infection. Proteins extracted with a urea buffer are reduced, alkylated and digested with trypsin, then 6-plex tandem mass tag (TMT) labeled. The peptides are separated by high pH reverse-phase HPLC with an acetonitrile gradient in bicarbonate buffer, resulting in 18 fractions. Identification is through liquid chromatography tandem mass spectrometry (LC-MS/MS). In hemocytes, the sample relevant to this account, 127 proteins increase in abundance while 85 decrease. Many of the differentially abundant proteins involved in the early immune response of the host against *Spiroplasma* infection belong to the pro-phenoloxidase, clotting and hemolytic systems, or have pattern recognition or redox functions.

2.4. Omics approaches to environmental issues

Anthropic impact on environment and biosphere is one of the main concerns for a sustainable economic and social development. On this ground, even if not proteomic in scope, for this short section we singled out a couple of reports addressing the effects on *Crustacea* of chemicals whose increased levels in seawater are heavily connected with human activities.

The ffrst such report deals with the impact on Carcinus maenas of seawater acidiffcation by carbon dioxide, with a concentration- and time-dependence exposure scheme [70]; the perspective is that of metabolomics, with ^{1}H NMR spectroscopy applied to hemolymph as well as to extracts from posterior gills and muscle from one walking leg. The most relevant feature in the hemolymph metabolic ffngerprint of the exposed crabs is the reduced concentration of glycine and proline, which may imply both the supply to body fiuids of proton-buffering ammonia through amino acid catabolism and an adaptation to preserve intracellular isoosmotic regulation.

The second report addresses the responses in Procambarus clarkii to the exposure to nitrite, in a time-dependence setup [71]. The authors single out a number of biological functions and perform transcriptomic evaluation on a number of mRNAs coding for proteins relevant to the selected pathways. In hemocytes, they ffnd up-regulation of cytoplasmic Mn superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferase after 24 h of nitrite exposure, followed by down-regulation of mitochondrial Mn superoxide dismutase, catalase, glutathione peroxidase, and lysozyme after 48 h.

2.5. Studies on peptides

The number of papers reporting on peptides, in various species and with different functions, is relatively high. In their writing some authors refer to peptidomics as a description of either the comprehensive approach [72,73] or

of the analytical strategy [74] they had taken in their research activity. According to their biological function, some of the referred elements may be categorized as neuropeptides, some as antimicrobial peptides.

Neuropeptides, structurally grouped in many families, form the largest class of signaling molecules at the synaptic level in the different districts of the nervous system: the contraction of the heart is controlled by the cardiac ganglion, the ventilatory rhythm of the gills by the thoracic nervous system, the movements of the foregut by the stomatogastric nervous system and the swimmeret system of the tail by the abdominal ganglia. The secretory nerve terminals of two neuroendocrine organs, the X-organ-sinus gland system, in the eyestalk, and the pericardial organ, next to the heart, have direct access to the hemolymph.

In the recent reports we could retrieve, neuropeptides are identified while searching for novel members (27 hits out of 70 Callinectes sapidus resolved neuropeptides, which include orcokinins, orcomyotropin, crustacean hyperglycemic hormone precursor-related peptides, red pigment concentrating hormone, pigment dispersing hormone, proctolin, RFamides, RYamides, and HL/IGSL/IYRamide [75]) or are investigated in connection with physiological responses to environmental factors such as temperature (in Cancer borealis [73]) and pH (in Callinectes sapidus [76]).

Antimicrobial peptides are evolutionarily conserved small molecules consisting of less than 50 amino acid residues with positive charges and strong amphipathy. They possess a broad spectrum of antimicrobial activity against both Gram- and Gram+ bacteria, fungi, protozoan parasites and some enveloped virus. The cationic antimicrobial peptides bind to the negatively charged lipopolysaccharide or lipoteichoic acid of bacteria and cause the perforation of their cell walls. Antimicrobial peptides mainly belong to three classes: beta-defensin-like peptides (*e.g.* from Panulirus argus [77], Homarus americanus [74]) and crustins (*e.g.* from Procambarus clarkii [78], Portunus pelagicus [79], Litopenaeus vannamei [22,80]), anti-lipopolysaccharide factors (*e.g.* from Macrobrachium nipponense [81,82]). In these recent reports they have been characterized as for *e.g.* their structure [77], source [74], and activity [22,79].

Finally, a different biological function from the above is that of Pacifastacus leniusculus astakine. This peptide is involved in hematopoiesis and circulates in hemolymph as a high molecular weight complex, whose formation is reversibly regulated by calcium concentration variations along the molting process [83].

3. *Arthropoda – Chelicerata* … horseshoe crabs

Xiphosura is an order of marine arthropods that first appeared in the Late Ordovician (445.2 ± 1.4 to 443.8 ± 1.5 Mya) and hardly changed since. Currently, only four species persist, which are regarded as living fossils; scientific interest is mainly focused on two of them, Limulus polyphemus and Tachypleus tridentatus, with a different geographical distribution, as made clear by their common names: Atlantic *vs* Japanese horseshoe crab.

Accordingly, the number of recent scientific reports we could retrieve on these animals is much lower than for either *Crustacea* or *Mollusca*, with the exception of two subjects – hemocyanin, dealt with in Section 5, and LAL, which is mentioned in the introduction.

3.1. Studies on individual hemolymph proteins (other than hemocyanin)

Some of the protein names we read in recent PubMed hits correspond to those seen for the corresponding list of *Crustacea* proteins. This is for instance the case for a Tachypleus tridentatus lectin, taking specific molecular interaction with rhamnose in pathogen-associated molecular patterns on the bacterial surface [84]; its targets include both Gram+ (Pseudomonas aeruginosa and Klebsiella pneumoniae) and Gram- bacteria (Streptococcus pneumoniae). Other proteins involved in innate immunity processes are Limulus polyphemus pentraxins: *in vitro* they permeabilize asymmetric planar lipid bilayers that mimic the outer membrane of Gram- bacteria, by forming transmembrane pores, a finding that provides a hint to the mechanism of their *in vivo* activity [85]. At a difference from *Crustacea*, however, a much larger number of UniProt hits have to do with coagulation. The elements of the protease cascade – factor C [86,87], factor B

[88], clotting enzyme [89,90], coagulogen [91,92] – have long been characterized. The final step of the cascade features the cleavage of coagulogen to coagulin. As outlined in Fig. 4, the release of a peptide C, between Arg38 and Arg66, exposes a hydrophobic cove on the head of one coagulin molecule, providing an interaction site for the hydrophobic surface of a second molecule, in a head-to-tail polymerization scheme. The coagulin polymers further aggregate laterally to form thicker fibers, probably through hydrophobic interactions [93]. The gel clot may be further stabilized through crosslinking, by transglutaminase, of coagulin to proxin [94] and of the latter to stablin [95].

3.2. Omic studies

Xiphosura are the topic of comprehensive investigations, including genomic [96,97], transcriptomic [96] as well as proteomic studies. The latter are based on protein capture from Limulus polyphemus hemolymph with combinatorial peptide ligand libraries (CPLL), a procedure that allows to markedly reduce the presence of hemocyanin in the processed specimens; three buffers, at pH 4.0, 7.0 and 9.5, are used for CPLL bead equilibration [98]. Complete lanes from SDS-PAGE separations were sliced and tryptic digests subjected to nano-LC MS/MS analysis. Around 160 unique gene products can be identified *via* the dbEST_limulus as well as *via* comparison with Uniprot_chelicerata and Uniprot_ixodes (animals are other members of the *Chelicerata* subphylum to which *Limulus* belongs); their biological

Coagulin polymerization
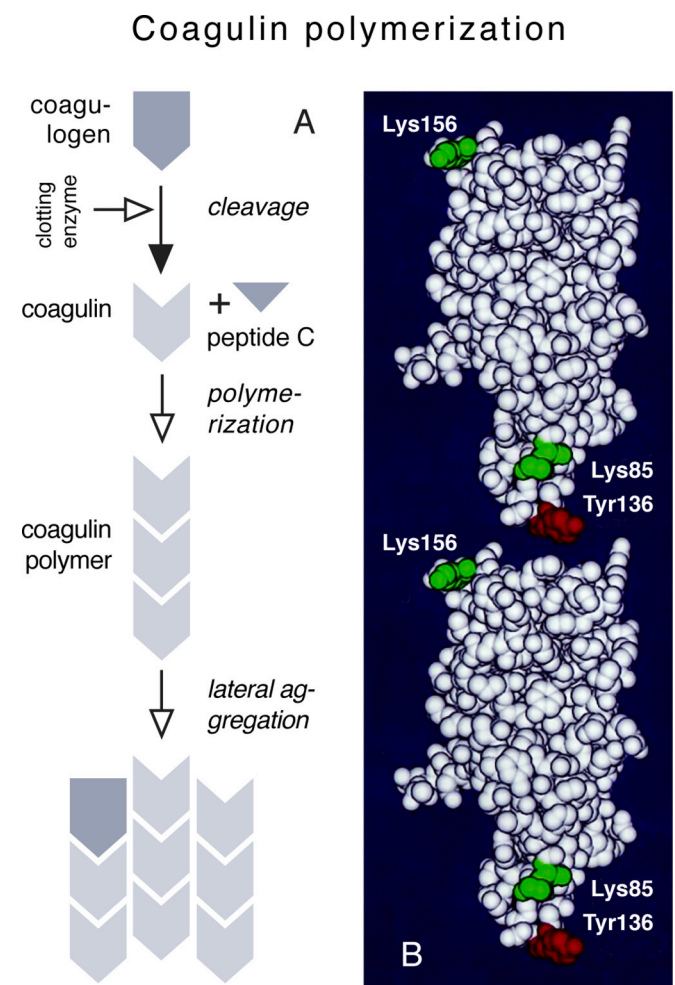
Fig. 4. Coagulin molecule interlinking to form a gel. (A) Outline of the steps. Names of molecules in plain text, names of processes in italics; full arrows hint to reactions, open arrows to (multistep) processes. (B) A molecular model of coagulin-coagulin interaction; neighbor amino acid side chains (from cross-linking experiments) are labeled. Crystal structure of Tachypleus tridentatus protein as resolved at 2.00 Å resolution by X-ray diffraction [153]. Redrawn from figs. 5 and 6 of [93].

functions are outlined in Fig. 5. Approximately ten times more, however, cannot be given a name or be associated with a function as their MS-deduced sequences had no counterparts in the genomic data available at the time of publication, back in 2010.

4. Mollusca

Genomic data are available for 52 *Mollusca* species (https://www.ncbi.nlm.nih.gov/home/genomes/).

In the following, as a rule, we group the evidence we review making reference to the three main *Mollusca* classes: *Bivalvia*, *Cephalopoda*, and *Gastropoda*.

4.1. Studies on individual hemolymph proteins (other than hemocyanin)

It is not surprising that *shell matrix proteins* are among the individual hemolymph components investigated in great depth [99]. The species under consideration in the reference study is Crassostrea virginica (*Bivalvia*), a type of oyster once of major economic relevance but whose survival in the wild has been heavily curtailed by anthropic influence. Two phosphoproteins, of mass 48 and 55 kDa, circulate within the C. virginica hemolymph and interact with the mineral phase during deposition; they are further secreted from hemocytes on induction of shell repair. A comprehensive proteogenomic analysis on the shell matrix of four *Bivalvia* species – *Crassostrea gigas, Mya truncata, Mytilus edulis,* and *Pecten maximus* – is carried out in [100], resulting in the identiffcation of 46–67 proteins per sample, some of which are common to all the test species.

All other proteins for which we retrieve recent publications appear to fulfill multiple functions but share a connection with *immunity*. Galectins are a family of lectins characterized by their binding affinity for beta-galactosides. Their carbohydrate-recognition domain can interact both with pathogens and parasites, promoting their phagocytosis, but also do so with phytoplankton components, participating in uptake and intracellular digestion of microalgae [101]. One more member of this protein family, with strong agglutination activity towards Gram- bacteria, is for instance identified in *Sinonovacula constricta* (*Bivalvia*) from its coding sequence, a 507-bp open reading frame [102]. The same approach is taken

for identifying a transferrin homolog in *Haliotis discus discus* (*Gastropoda*) as the protein corresponding to a 2187-bp open reading frame [103]. Its binding of ferric ions makes transferrin a crucial element in *iron transport* and distribution but also a bacteriostatic agent through the competition with pathogens for a limiting nutritional resource. Challenge with Vibrio parahaemolyticus and Listeria monocytogenes results in increased expression of *Haliotis* transferrin mRNA in keeping with a role as acute-phase protein. Hemoglobin, another iron-containing protein, is present in few *Bivalvia* species: one of them is *Tegillarca granosa* [104]. In addition to *oxygen transport* and peroxidase activities, typical of all hemoglobin variants, the *T. granosa* protein displays various levels of antibacterial activity against Escherichia coli, *Pseudomonas putida, Bacillus subtilis,* and *Bacillus firmus* as a whole protein and against *Vibrio alginolyticus*, Vibrio parahaemolyticus, and *Vibrio harveyi* in the form of peptides resulting from trypsin digestion.

A different type of immune-relevant protein is alpha-2-macroglobulin. In *Pinctada fucata* (*Bivalvia*) its gene can be identified by retrotranscription of the oyster transcripome [105]. Protein concentration is found to be promptly upregulated in hemocytes after challenge with *Vibrio alginolyticus;* knockdown by RNA interference as well as chemical inactivation of the reactive thioester bond significantly reduce the phagocytosis of the parasite.

4.2. Studies on hemolymph proteome

A couple of reports deal with the proteome of the hemolymph from typical *Bivalvia* species under baseline conditions.

The ffrst of them is Mytilus galloprovincialis; the experimental procedure, 2-DE with Coomassie staining, aims at detecting the high-abundance proteins [106]. As shown in Fig. 6, two spot rows are most prominent in the IPG-DALT pattern. The upper corresponds to the metalloprotease astacin, a zinc-containing enzyme reported to catalyze the hydrolysis of peptide bonds in peptides at least ffve amino acids long, preferentially with Ala in P1', and Pro in P2'. The proposed functions of this protease include the processing of extracellular proteins, the degradation of polypeptides, and the activation of growth factors. The lower spot row corresponds instead to extrapallial fluid protein, a carrier of calcium ions that represents the
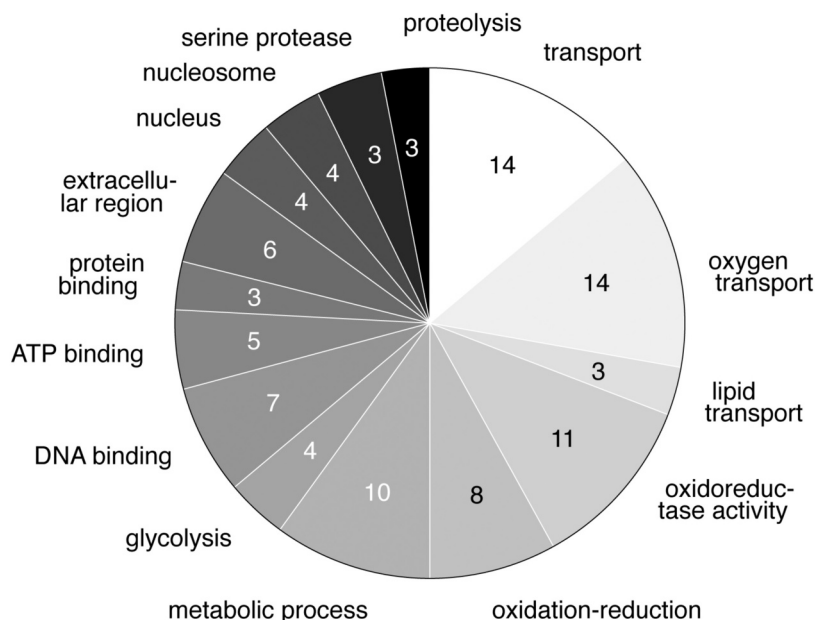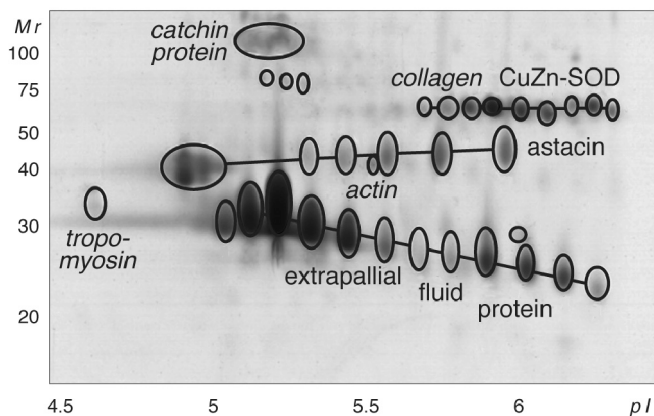
GO terms for *Limulus polyphemus* hemolymph proteins

Fig. 5. Pie chart of the main GO terms associated with the proteins identified in the CPLL eluates of the *Limulus* hemolymph. Redrawn from fig. 4 in [98].

2-DE of *Mytilus galloprovincialis* hemolymph proteome

Fig. 6. IPG-DALT of proteins acetone-extracted from Mytilus galloprovincialis hemolymph (pooled female samples). Protein identiffcations (as in table 2 in [106]) are marked; contaminants from sampling procedures (puncturing the posterior adductor muscle with a 21 G needle and a syringe) are in italics; spots corresponding to different species of a single protein are connected by lines. Approximate p*I* and M*r* scales derived from the molecular parameters of actin and tropomyosin (UniProt) *plus* information about the relevant experimental conditions. Redrawn from fig. 6 in [106].

building block of the soluble organic matrix of the shell. As hinted to by the shape of the spots corresponding to its molecular species, extrapallial fluid protein is a glycoprotein containing a peculiar structure of fucosylated N-glycans. The authors actually go further in the description of the baseline setup, by proving the similarity between the 2-DE pattern for female and male mussels and by meticulously disproving the presence of vitellogenin-like proteins in the hemolymph.

The second species of interest is Mytilus edulis [107]; the investigation this time takes advantage of the proteogenomic approach. Data from nano-LC-MS/MS on tryptic peptides are searched against a database combining all *Mollusca* sequences in UniProt with all sequences derived from the transcriptome of gills of Mytilus galloprovincialis and *Bathymodiolus azoricus* (database access 2014). This way the number of annotated proteins rises from a handful to over 330, spanning a number of biological functions; the main GO terms are plotted in Fig. 7, in three groups, outlined in color: binding proteins, enzymes and transporters. In the same investigation Campos et al. analyze with an identical approach the proteins of M. edulis hemocytes: of the *ca.* 500 proteins annotated for this sample, approximately one-third overlaps the hemolymph proteome.

Other reports compare disease to health conditions. Using the IPG-DALT approach, Castellanos-Martínez et al. [30] investigate both hemolymph and hemocytes of Octopus vulgaris (*Cephalopoda*) infected by the coccidia *Aggregata octopiana*, a parasite responsible for severe gastrointestinal injuries and a malabsorption syndrome. The authors observe no significant differences in hemolymph whereas in hemocytes principal component analysis points to 7 proteins as the major contributors to the overall difference between levels of infection; three of them – filamin, fascin and peroxiredoxin – have a clear connection with immunity.

Hemolymph proteome from oysters (*Bivalvia*) with and without infection is compared by 2-DE in another report. The host species is Ostrea edulis and the parasite is *Bonamia ostreae* (*Cercozoa*) [108]. The comparison between infection-resistant and -susceptible oyster stocks identiffes 7 proteins as candidate markers of resistance to bonamiosis.

4.3. Some more omics

We like to stress here a couple of reports dealing with omics other than proteomics in connection with *Mollusca*.

GO terms for *Mytilus edulis* hemolymph proteins
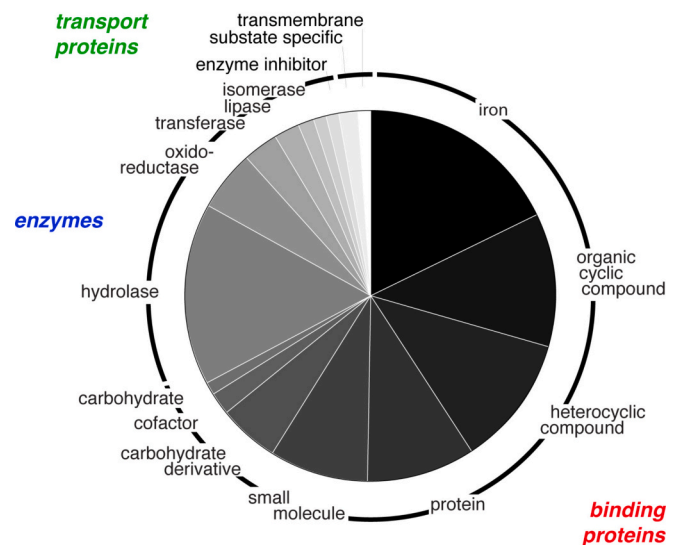
Fig. 7. Pie chart of the main GO terms associated with the proteins identiffed in Mytilus edulis hemolymph. Redrawn from ffg. 3 in [107].

This is the case for an interesting publication isolating and characterizing the genes that are expressed differentially in the hemolymph of *Vibrio alignolyticus*-infected *vs* non-infected *Pinctada martensii* (*Bivalvia*) [109]. A peculiar approach is used in this investigation, *i.e.* suppression subtractive hybridization (SSH), a technology that allows for PCR-based amplification of only cDNA fragments that quali- or quantitatively differ between two transcriptomes. SSH relies on the removal of dsDNA formed by hybridization between control and test sample, thus eliminating cDNAs of similar abundance, and retaining transcripts differentially expressed, or variable in sequence. In the case of infected pearl oysters, significant matches after homologous sequence searches are found for 267 ESTs; the putative biological functions of the inferred genes cover a wide range of cellular processes.

This is also the case for a metabolomic (or metabonomic) report that has to do with Mytilus galloprovincialis (*Bivalvia*) hemolymph [110]. The authors use NMR to assess the changes of the metabolic proffle due to environmental stressors. High temperature (24 °C) and high copper levels (40 µg L^{-1}) turn out to cause a coherent increase of a common set of metabolites (mostly glucose, serine, and lysine). Proteomic investigations dealing with environmental issues are reviewed in the following, under 4.4.

4.4. Environmental issues

In addition to the metabolomics investigation already mentioned under 4.3, a few more reports cover this area of interest.

Two publications, from the same research group, describe the effects on *Saccostrea glomerata* (*Bivalvia*) of the exposure to *heavy metal contamination* [111,112]. In one case, controlled exposure to three environmentally relevant metals (Cu, Pb or Zn) is staged in aquaria; in the other, oysters are tested in the ffeld after being transplanted to sites for which water and sediment content of 10 relevant metals is known (Al, Ca, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn). While all 4-days treatments result in signiffcant changes in hemolymph proteome, there is virtually no overlap between the effects of individual metals as well as in the changes observed in different ffeld sites. Considering the affected biological functions, when individual metals are used, more than 80% of the differential proteins *vs* control map to few GO terms (chief among them, «shell properties»); conversely, upon field exposure, a different assortment and, even more, a different balance of effects is recorded (*e.g.* «shell properties» is not recorded any longer while «cytoskeletal proteins» almost doubles its presence, from 25 to 45% of hits).

While heavy metal toxicity is a long-established concern in environmental surveillance, microplastic (any type of plastic fragment – ffber, bead or pellet – less than 5 mm in length) features an emerging problem. This contaminant heavily impacts on filter feeders such as Mytilus edulis (Bivalvia). The effects on the mussel are investigated over an extended period (52 days) in a mesocosm setting (mesocosm = an outdoor experimental system that examines the natural environment under controlled conditions) in 2019; two types of plastics – polylactic acid (PLA) and high density polyethylene (HDPE) (*PLA: slowly biodegradable, used in packaging as well as in surgical items, and one of the raw materials for fused deposition modeling, or 3D printing; HDPE: not biodegradable but recyclable, used in the production of plastic bottles, corrosion-resistant piping, geomembranes and plastic lumber*) – in fragments tens of micrometers in diameter, at a final concentration of 25 µg L^{-1}, are used as challenge [113]. Fig. 8A shows that the proteome composition allows to clearly differentiate among the three experimental groups, whereas Fig. 8B points out that, among

Effects of microplastics on *Mytilus edulis*

A - *Canonical analysis of principal coordinates*

B - *Changes vs control for concordant proteins*

legend: control ☐   PLA ▨   HDPE ■

Fig. 8. A) Canonical analysis of principal coordinates of the composition of hemolymph proteomes from M. edulis after exposure to 25 µg L^{-1} microplastics, or no microplastics (control). Redrawn from ffg.2 in [113]. B) Fold change *vs* control of proteins in *M.edulis* hemolymph with concordant behavior after exposure to microplastics. Drawn from data of table 1 in [113]. Color-code for both panels in the legend at the bottom.

the over 200 proteins identiffed, a small number change in the same direction and to approximately the same extent upon exposure to both microplastics types; the functions of these proteins are connected with immunity (3/7), detoxiffcation (2/7) and metabolism (1/7); one is instead a predicted (or hypothetical) protein. A recent publication, not dealing with proteomics, complements the information on the effects of exposure of Mytilus galloprovincialis to plastics particles: decrease in phagocytosis, increase in ROS and lysozyme activity, inhibition of NO production, and a change in the associated microbiota, with a shift towards potentially pathogenic bacteria [114].

4.5. Studies on peptides

As we did for *Crustacea*, for *Mollusca* we review some reports dealing with peptides.

An example of investigation in connection with basic physiological processes is the one on the peptidome present during egg-laying of Sepia offcinalis (*Cephalopoda*) [115]. After a transcriptomic survey using an RNAseq approach, the screening in parallel by mass spectrometry of hemolymph and nerve ending contents allows the authors to assign each hit as neuromodulator or neurohormone. Overall, 38 neuropeptide families are identiffed of which 14 are found to be strongly overrepresented in egg-laying females in comparison with mature males.

Two more investigations, instead, deal with defense mechanisms under pathological conditions. One of them refers to (candidate) antimicrobial peptides derived from a speciffc portion, called haliotisin, in E subunit of Haliotis tuberculata (*Gastropoda*) hemocyanin [116]; another refers to the antiviral activity (against OsHV-1) of myticin C peptide from Mytilus galloprovincialis [117].

5. Hemocyanin

Oxygen transport in the body of *Mollusca* and *Arthropoda* is performed by hemocyanins. These metalloproteins contain two copper atoms that can reversibly bind oxygen. Depending on the oxygenation state the Cu (I) deoxygenated form is colorless, whereas the Cu(II) oxygenated form is blue. In contrast to the vertebrate hemoglobin, hemocyanins are suspended directly in the hemolymph.

Oxygen-binding proteins are evolutionarily ancient, likely deriving from enzymes that protected the organism against the toxic oxygen molecule. By developing into multi-subunit, circulating proteins, oxygen could be brought to all different parts of the organism [118]. Hemocyanins in *Mollusca* and *Arthropoda* differ in quaternary structure and sequence, but their active sites are similar. They consist of six highly conserved histidines that bind two copper ions, which together bind one oxygen molecule.

Hemocyanins have diversiffed through multiple gene duplications and functional specializations. Molluscan hemocyanins assemble as large and symmetric multi-domain subunits, forming for instance decameric cylinders in chitons and cephalopods (3500–4500 kDa) or dodecameric cylinders in gastropods and bivalves (8000–10,000 kDa). Each of the 350–450 kDa subunits has 7–8 oxygen-binding units [119].
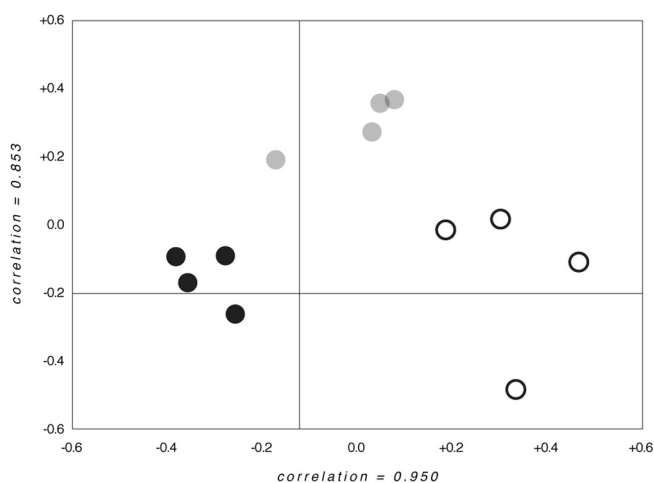
Arthropod hemocyanins are smaller in size and six 75 kDa subunits, each with one oxygen-binding site, assemble into a 450 kDa hexamer. The hexamers assemble further into two to eight multiples of hexamers depending on the class or species. Arthropod hemocyanins are particularly renowned for the number of different kinds of polypeptides within their multi-subunit molecule. The heterogeneity expands the functional properties, as different subunits may have different oxygen affinities or responses to allosteric modifiers, and this creates a large degree of inter- and intra-specific polymorphism [118,120].

Molluscan hemocyanin has phenoloxidase activity, while crustacean hemocyanin shows low-afffnity prophenoloxidase activity only in partially unfolded state. Based on reported differences, a scenario has been proposed for the evolutionary relationships among copper oxygen-binding proteins [121,122]; a comparison among the overall structure of the main oxygen-binding proteins, containing either copper or (heme) iron, is provided by Fig. 9.
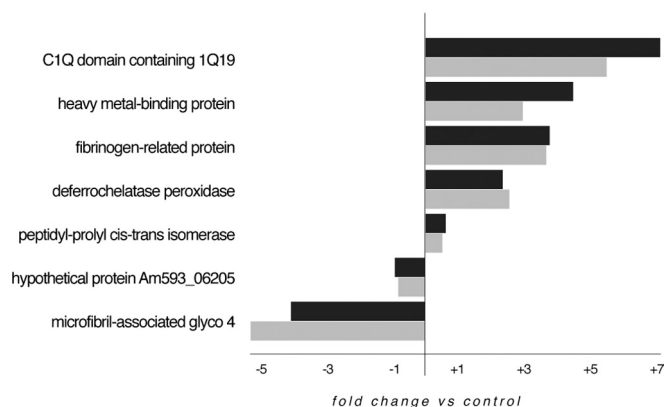
Mass spectrometry played a major role in the characterization of

Oxygen-transport proteins

A - *copper-based*

crustacean hemocyanin

100 Å

molluscan hemocyanin

B - *iron-based*

intracellular hemoglobin (pink blood cells)

extracellular hemoglobin

intracellular hemoglobin (red blood cells)

100 Å

10,000 Å

Fig. 9. An overview on oxygen-transport proteins (derived from ffg. 1 in [118]). Protein structures, from coordinates in the PDB repository (http://www.rcsb.org), are rendered to scale (see the 100 Å rulers at the top right and at the bottom left of the image). An electron micrograph of a mammalian red blood cell is also shown at the bottom right (together with its own 10,000 Å ruler). The protein structures used as references for the different functional classes are, from top to bottom: 6L8S hemocyanin of *Panulirus japonicas*, 4BED hemocyanin of Megathura crenulata, hemerythrin of Phascolopsis gouldii. 1X9F erythrocruorin of *Lumbricus terrestris*, 2HHE hemoglobin of Homo sapiens. Rendering of secondary structure of the proteins as ribbons and of the molecular surface was carried out with MOE software (MOE 2019 Chemical Computing Group, Montreal, Canada). (For interpretation of the references to color in this ffgure legend, the reader is referred to the web version of this article.)

crustacean hemocyanin, especially the - at that time recently developed - mass spectrometry and multi-angle laser light scattering (MALLS) that enabled determination of native as well as subunit masses. It was supplemented by ESI-MS under native and denaturing conditions for estimation of size and post-translational modiffcations [123].

Keyhole limpet hemocyanin (KLH), the respiratory protein of the gastropod mollusc Megathura crenulata, is the most thoroughly studied in this group of proteins. Because of its relative availability, its early characterization and immunological properties, it has found multiple applications in biomedicine. It exists as two isoforms, KLH1 (or KLH-A) and KLH2 (or KLH-B), differing in size of the subunits (449 kDa for KLH1, 392 kDa for KLH2), polymerization/re-association characteristics and O_2-binding constants [124]. KLH activates both the humoral and cellular immune responses, and even in non-immunized individuals KLH-binding lymphocytes and IgG-antibodies cross-reacting with KLH were detected [125]. Its immunologic properties opened new ways for experimental tumor immunotherapy, *via* non-specific or active specific stimulation of the immune system, for instance for the treatment of superficial bladder carcinoma [126,127]. Terminal oligosaccharides of the molecule are key in this function, inducing beneffcial antibody response that is paralleled by increase in natural killer cell activity [128]. As a highly immunogenic T-cell dependent antigen, KLH is also successfully used as a generalized vaccine component, either alone or as a component of the adjuvant cocktail (comprehensive table in [129]). It is also applied in immune competence testing. Conjugated with drugs or other small molecules KLH is a well-liked tool to generate antibodies for use in ELISA or other immunoassays [130,131].

6. Final comments

6.1. Medical relevance of hemolymph protein derivatives

We stated in the Introduction that, in addition to scientiffc grounds, there are practical reasons for studying the hemolymph proteins of selected marine invertebrates. Here we go with the account on some of these.

6.1.1. Hemolymph proteins as *'marine drugs'*

In the previous sections we have repeatedly listed proteins and peptides circulating in the hemolymph; most of them have a role in the defense mechanisms, featuring antimicrobial, antifungal, antiprotozoal, and/or antiviral activities. Some may be considered dedicated anti-infective compounds, whereas others are primarily associated with unrelated biological functions; a number of bioactive peptides are cleavage products from parent proteins with a non-immunological role. Their modes of action in the cognate species (*e.g. Crustacea*) are summarized in Fig. 10: these include proteinase inhibition, trigger to phagocytosis, and enhancement of hematopoiesis [132]. The effects may also be rather indirect: for instance, the (by)products of the catalytic activity of phenoloxidase reduce signiffcantly the number of colony forming units of a number of microorganisms [133]. As a class, the antimicrobial peptides (AMPs) from marine invertebrates are being considered for medical use [134–136]. Indeed, an increasing number of bacterial strains are becoming resistant to one and often to many of the antibiotics in current use, while no new antibiotics are being discovered or developed. AMPs combine a broad-spectrum bactericidal activity and a low potential of inducing resistance. They show a remarkable diversity in structure in comparison with the terrestrial counterparts, and thus offer several possibilities for the design of semisynthetic derivatives, possibly with the assistance of computational biochemistry tools. Most AMPs display a high afffnity to bacteria and to their pathogenicity factors, including LPS of Gram-negative origin, and can prevent endotoxin shock. Accordingly, examples of such applicative studies do focus on endotoxin tolerance [137,138].

We quote here a single example of structure-function studies on a peptide, 24 amino acids in length that corresponds to the lipopolysaccharide-binding domain of a Fenneropenaeus chinensis anti-lipopolysaccharide factor [34,139]. In this appraisal the sequence is modiffed through the substitution of ffve neutral amino acids with lysine, in such a way to shift the net charge from 5 to 10 and the GRAVY index from −0.6 0 to −0.94. The engineered peptide turns out to be associated with higher antimicrobial and bactericidal activities and higher heat stability *vs* the wild type.

Anti-infective activity is not the only possibility for medical use of 'marine drugs': action as antidote is another option. For instance, the plasma protein from Tachypleus tridentatus (*Limulidae*) has a protective effect on acute kidney injury following cyclophosphamide treatment. The effect is

ROLES OF ANTIMICROBIAL PEPTIDES (AMPs) IN IMMUNITY
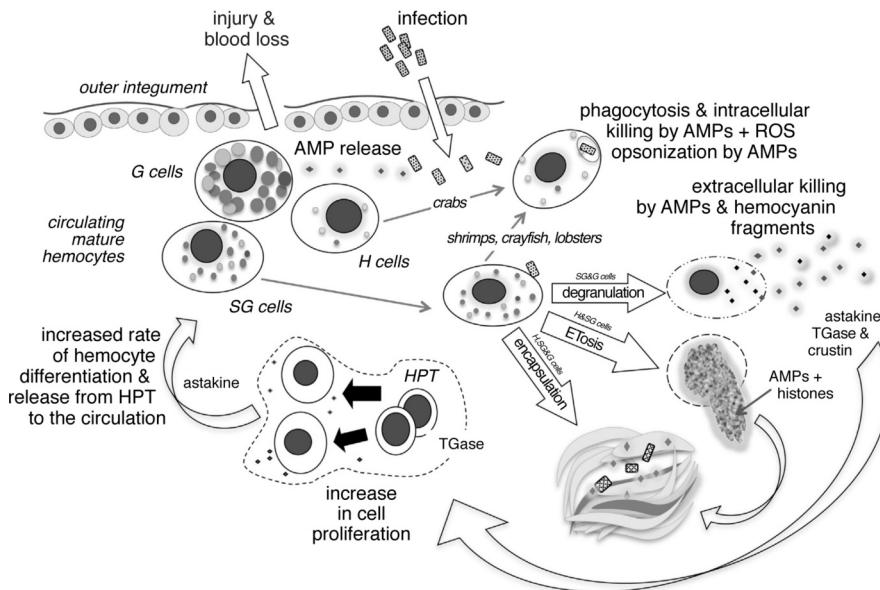
Fig. 10. A summary of the ways by which AMPs contribute to host defense *in vivo* (in *Crustacea*). This graphic depicts the release of AMPs from hemocytes, their delivery to sites of infection, possible role in phagocytosis and involvement in hematopoiesis. Legend: AMPs, antimicrobial peptides; ETosis, formation by neutrophils and mast cells of extracellular traps consisting of a chromatin-DNA backbone with attached antimicrobial peptides and enzymes [154]; G cells, granular hemocytes; H cells, hyaline hemocytes; HTP, hematopoietic tissue; ROS, reactive oxygen species generated by the respiratory burst; SG cell, semi granular hemocytes; TGase, transglutaminase. From ffg. 2 in [132].

mediated by interference in apoptosis and autophagy mechanisms through differential regulation of p38 MAPK and PI3K/Akt signaling pathways [140].

6.1.2. Hemolymph proteins as allergens

While the previous paragraph has dealt with the plus of hemolymph proteins as remedies, this subsection is going to point out their minus as allergens. The prevalence of shellffsh allergy in the population varies from 0% to 10.3%, depending on the methods of diagnosis (most of which relying on self-reported questionnaires); when double-blind, placebo-controlled food challenges are used, the prevalence drops to 0–0.9% [141]. IgE-mediated shellffsh allergy is connected with a number of components of seafood, both *Crustacea* and *Mollusca* [142,143]. Most of them are endocellular proteins, mainly of the muscular tissue: this includes tropomyosin, myosin light chain, sarcoplasmic calcium-binding protein, arginine kinase. However, another is the hemolymph protein hemocyanin. Shrimp hemocyanin behaves as a heat-stable allergen, and cross-reacts with its homolog in house dust mite and probably also snail. Contrary to the above, due to the low amino acid sequence homology between the protein from different shrimp species, belonging to *e.g.* the *Caridae* and the *Penaeidae* family, hemocyanin can lead instead, in some cases, to selective allergy (*i.e.* sensitization to one and tolerance to another species).

6.2. Multifunctional proteins among hemolymph components

We mentioned the notion of multifunctional, or multitasking, proteins along the way noting how often the very same items have to be cited in connection with different biological functions. Last time we did it in the previous paragraph, when pointing out that some peptides relevant in the immune response of marine invertebrates derive from proteins devoid of any such activity. While this feature is not limited to hemolymph proteins, for the present type of proteins their functional diversity is quoted in a number of reports: PubMed retrieves most with the search string « multifunctional protein hemolymph Crustacea », followed by « multifunctional protein hemolymph Mollusca » (*e.g.* [144,145]). Conversely, it is questionable whether any of the hemolymph proteins may be classiffed as moonlighting, *i.e.* as performing multiple autonomous if not unrelated functions without partitioning either between/among domains or between/among proteolytic fragments [146,147]. Search string « moonlighting protein hemolymph » retrieves 3 reports, from a single research group, dealing with lobsters [148], horseshoe

crabs [149], mussels, clams, oysters [150]. All the three studies investigate protein deimination, or citrullination, a post-translational modiffcation caused by calcium-dependent enzymes peptidylarginine deiminases and involving the conversion of arginine to citrulline. The authors remark that the structural and functional protein changes brought about by this PTM "sometimes contribute to protein moonlighting, in health and disease". In such an approach, proteins which Bowden et al. find deiminated in the hemolymph of marine invertebrates, differ between species; overall, the main pathways being involved are relevant in immunity and metabolism.

Whichever the boundaries of their implication, multifunctional proteins are fft to coordinate cellular activities, serving as switches between pathways and helping to respond to changes in the inner milieu, themselves connected with changes in the external environment. While some of the multifunctional proteins alternate between functions in response to speciffc triggers, often mediated by binding with low- or high-molecular weight counterparts, others perform their multiple functions simultaneously. All the examples we have met in our survey seem to comply with the latter paradigm.

Conffict of interest statement

The authors declare no conffict of interest.

Data availability

No data was used for the research described in the article.

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