Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin

Christopher J. Coates1 · Heinz Decker2

Abstract It is now well documented that peptides with enhanced or alternative functionality (termed cryptides) can be liberated from larger, and sometimes inactive, proteins. A primary example of this phenomenon is the oxygen-transport protein hemoglobin. Aside from respiration, hemoglobin and hemoglobin-derived peptides have been associated with immune modulation, hematopoiesis, signal transduction and microbicidal activities in metazoans. Likewise, the functional equivalents to hemoglobin in invertebrates, namely hemocyanin and hemerythrin, act as potent immune effectors under certain physiological conditions. The purpose of this review is to evaluate the true extent of oxygen-transport protein dynamics in innate immunity, and to impress upon the reader the multi-functionality of these ancient proteins on the basis of their structures. In this context, erythrocyte–pathogen antibiosis and the immune competences of various erythroid cells are compared across diverse taxa.

Keywords Antimicrobial peptides · Innate immunity · Myoglobin · Phenoloxidase · Erythrocytes · Enzyme promiscuity · Metabolism · Redox

Abbreviations
- AMP Antimicrobial peptide
- 2-DIGE 2-Dimensional gel electrophoresis
- GE-Hb Giant extracellular hemoglobin
- Hp Haptoglobin
- Hb Hemoglobin
- Hc Hemocyanin
- Hr Hemerythrin
- IFN Interferon
- LPS Lipopolysaccharide
- LTA Lipoteichoic acid
- metHb Methemoglobin
- Mb Myoglobin
- PRR Pathogen recognition receptor
- PAMP Pathogen associated molecular pattern
- PGN Peptidoglycan
- PO Phenoloxidase
- RBCs Red blood cells
- RNS Reactive nitrogen species
- ROS Reactive oxygen species
- SDS Sodium dodecyl sulphate
- SNPs Single nucleotide polymorphisms
- TLR Toll-like receptor
- WBCs White blood cells

Introduction

Maintaining immunity-related proteostasis in metazoans is crucial to survival and recovery from biotic (pathogenic) and abiotic (environmental) traumas. Enlisting oxygen-transport proteins (OTPs: hemoglobin and hemocyanin) to directly combat microbes as well as to supply the O2 necessary to fuel the costly immuno-metabolome is both...
resourceful and economical. The concentration of oxygen within wounds determines a host’s ability to heal and to resist microbial colonisation [1, 2]. Hypoxia and inadequate oxygen tension within tissues can compromise immune cell functionality, e.g. restricting neutrophil respiratory burst [3].

With representatives in almost every known taxon, hemoglobin (Hb), hemocyanin (Hc) and hemerythrin (Hr) are metallated proteins responsible primarily for the sensing, transport and/or storage of O₂ [4]. Hb and Hr are located within the corpuscles (erythrocytes) of vertebrate blood and specialised immune cells (hemerythrocytes) of invertebrate coelomic fluid, respectively [5]. Conversely, Hcs are freely dissolved within the hemolymph plasma of some molluscs and arthropods. Although present in fewer species, it must be noted that two other forms of heme-based OTPs exist, namely chlorocruorin and erythrocruorin from annelids (often referred to as giant extracellular hemoglobins: GE-Hbs) [6–8].

Reports of Hb’s involvement in anti-infective defences have existed since the late 1950s [9], yet only in the last two decades has it become evident that Hb, Hc, and to a lesser extent Hr, contribute to various innate immune mechanisms. Generation of bioactive peptides (cryptides), enzyme promiscuity and pro-inflammatory signalling are but some of the many functions attributed to OTPs, which are expanded upon herein.

The oxygen-transport proteins

Hemoglobins

The presence and diversity of hemoglobins (Hbs) have been confirmed in metazoans, prokaryotes, fungi and flora, with a notable absence in icefish. A common ancestor for globin-related respiratory proteins existed over 1.5 billion years ago [reviewed by 10]. In excess of 250 million Hb molecules are packaged tightly within each human erythrocyte, guaranteeing their stability against proteolysis, a low colloid osmotic pressure, and preventing loss by filtration. Hb and Hr are hematopoietic proteins responsible primarily for the sensing, transport and/or storage of O₂ [4]. Hb and Hr are located within the corpuscles (erythrocytes) of vertebrate blood and specialised immune cells (hemerythrocytes) of invertebrate coelomic fluid, respectively [5]. Conversely, Hcs are freely dissolved within the hemolymph plasma of some molluscs and arthropods. Although present in fewer species, it must be noted that two other forms of heme-based OTPs exist, namely chlorocruorin and erythrocruorin from annelids (often referred to as giant extracellular hemoglobins: GE-Hbs) [6–8].

Reports of Hb’s involvement in anti-infective defences have existed since the late 1950s [9], yet only in the last two decades has it become evident that Hb, Hc, and to a lesser extent Hr, contribute to various innate immune mechanisms. Generation of bioactive peptides (cryptides), enzyme promiscuity and pro-inflammatory signalling are but some of the many functions attributed to OTPs, which are expanded upon herein.

The oxygen-transport proteins

Hemoglobins

The presence and diversity of hemoglobins (Hbs) have been confirmed in metazoans, prokaryotes, fungi and flora, with a notable absence in icefish. A common ancestor for globin-related respiratory proteins existed over 1.5 billion years ago [reviewed by 10]. In excess of 250 million Hb molecules are packaged tightly within each human erythrocyte, guaranteeing their stability against proteolysis, a low colloid osmotic pressure, and preventing loss by filtration. Hb concentration within healthy adults ranges from 120 to 160 mg mL⁻¹ [11].

Vertebrate Hb consists of two identical α-chains and two identical β-chains with molecular masses of ~16 kDa each. Two αβ-dimers assemble in C2 symmetry to form the Hb tetramer (~64 kDa) (Fig. 1). Individual subunits are comparable to monomeric myoglobin (Mb), and in all cases, Hb and Mb fold into a nest of α-helices [12]. Heme prosthetic groups are present in each subunit, consisting of a protoporphyrin ring and a single iron ion in the centre that is coordinated by the proximal histidine of α-helix F. At the other side of the heme, oxygen binds reversibly to the iron in an “end on” coordination (Fig. 1). Binding of oxygen to Hb induces a conformational rotation (15°) of one αβ-dimer against the other, thus switching from a tense (T) deoxygenated state to a relaxed (R) oxygenated state. Cooperative oxygen binding can be modulated by an allosteric effector such as 2,3-diphosphoglycerate in human Hb along the symmetry axis of the tetramer [12]. Vertebrate myoglobins (Mbss) are oxygen storage proteins in red muscle (e.g. cardiac) and other tissues, working to build up a PO₂ gradient from blood vessels to mitochondria for ATP synthesis [13].

Extracellular Hbs (or erythrocruorins) are mostly large, oligomeric proteins with molecular masses up to 3.6 MDa. Vinogradov (1985) classified them into four separate groups: (a) single-domain, single-subunit Hbs (~16 kDa) found in trematodes and some insects, (b) two-domain, multi-subunit Hbs in branchiopod crustaceans such as Daphnia and Triops, (c) multi-domain, multi-subunit Hbs in carapace-free brachiopod crustaceans, the planorbid snails and some clams (~1.7 MDa) and (d) single-domain, multi-subunit Hb aggregates ca. 3.6 MDa in annelids [14]. The first resolved structure of a GE-Hb was from the earthworm, Lumbricus terrestris [6]. This mega-molecule consisted of 144 Hbs and 36 linker subunits assembled to form a core complex with D6 symmetry. Recently, the quaternary structure of Glossoscolex paulistus plasma Hb was presented with a resolution of 3.2 Å, which is the highest resolution reported for a hexagonal bilayer Hb with 12 protomers [15].

Hemocyanins

Although strikingly different in structural appearance, both arthropod and mollusc Hcs contain dicupric (histidine coordinated) groups that reversibly bind molecular oxygen in a side on (μ - η², η²) bridging coordination [16] (Fig. 1). Arthropod Hc is composed of kidney-shaped subunits (~72 kDa, each with an oxygen binding site) arranged into hexamers (Fig. 1) [17]. Hexamers are formed when three subunits assemble back to back and dimerize isologously with a second trimer along the rotational axis (but are twisted against each other by 60°). Individual hexamers or multiples of hexamers have been observed in vitro, the largest of these is an 8 × 6 mer (~3.4 MDa) purified from horseshoe crab genera Limulus and Tachypleus. Vertebrate Hcs and GE-Hbs show strong hierarchies in structural organisation corresponding to hierarchical allosteric interactions (‘nesting’) [18, 19].

Hc concentration in the hemolymph varies greatly depending on the species, ~20–80 mg mL⁻¹. In extreme...
cases, Hc content has been calculated in excess of 140 mg mL\(^{-1}\) in the chelicerate, Limulus polyphemus [16, 20]. Accumulating evidence suggests that Hc is an integral component of biological defence systems within arthropods [reviewed by 21]. Oxygen-carrying Hc can be activated by host (clotting proteins, phospholipids, AMPs, proteases, lipoproteins) and microbial (proteases, membrane ligands) factors to combat infection, parasitism, viremia and physical damages [22–32].

Mollusc Hcs are extremely large protein complexes dissolved in the hemolymph of gastropods and cephalopods [33, 34]. Typically, subunit molecular masses range from 330 to 450 kDa depending on the species. Subunits are composed of 7 or 8 (50 kDa) functional units (FU; Fig. 1), designated FU-a to FU-h. Ten of these subunits form a hollow cylinder with a diameter of \(\sim 310\) Å and a height of \(\sim 160\) Å. Decameric Hcs are found in cephalopods such as Nautilus pompilius [35]. Two of these cylinders can associate along the rotational axis to form di-decamers as observed in marine gastropods [33]. The largest known mollusc Hc is a 13.5 MDa tri-decamer discovered in several species of snail, e.g. Melanoides tuberculata and Terebralia palustris [36]. Renewed interests in Hc structural complexities and the assemblages of associated sugars and lipids aim to exploit the vast therapeutic potential of these megamolecules (Table 1). Especially from molluscs, Hcs are tested for application as bio-adjuvants (viral and bacterial antigens/haptens), immune-stimulants for treatment of cancers such as melanoma, and carrier molecules for vaccines (Table 1) [37–40].

**Hemerythrins**

Hemerythrins (Hr) are relatively rare, non-heme, di-iron, dioxygen-binding proteins present in specialised coelomocytes (hemerythrocytes) of brachiopods, priapulids, and parasitic nematodes [38]. Hemerythrins are structurally distinct from Hc, having non-heme di-iron centers that function as oxygen sensors and signaling molecules. They are characterized by their ability to reversibly bind dioxygen, which is important for their role in oxygen storage and signaling in various biological systems.

**Fig. 1** Three major classes of oxygen-transport proteins. Each of the 4 subunits (\(\alpha_1, \beta_2, \alpha_1, \beta_2\)) making up human Hb (and monomeric myoglobin) contain heme cofactors (Fe\(^{2+}\)–protoporphyrin IX) that bind O\(_2\). Each heme group is indicated by an arrow head. The proximal histidine forms a direct bond with the iron atom, while the distal histidine is suggested to form a hydrogen bond with O\(_2\). The distal His hinders the energetically favoured straight binding of O–O. Arthropod hemocyanin subunits consist of three domains (green, blue, orange) and mollusc hemocyanin FUs consist of two domains (blue, orange). The blue domains possess two copper atoms, each is coordinated by three highly conserved histidine residues. O\(_2\) is bound in a ‘side on’ (\(\mu=\eta^2:\eta^2\)) bridging formation between CuA and CuB.
sipunculids and annelids [50–53]. Most often viewed as an octamer of molecular mass \(108\) kDa (Fig. 1), dimeric, trimeric and tetrameric isoforms of Hr have also been observed. These homo- or hetero-octamers are made up of \(\alpha\)- and \(\beta\)-type subunits, each \(13–14\) kDa in size [54]. Subunits consist of a four-\(\alpha\)-helix motif that houses the two iron ions, one being hexa-coordinated and the other penta-coordinated (bridged by a hydroxyl ion). Between the hydroxyl group and a single iron ion, oxygen is bound reversibly in an ‘end on’ position (Fig. 1)[55]. To date, Hr has not been detected in a deuterostome, whereas many bacteria, fungi and archaea contain Hr-like domains that are seemingly involved in chemotaxis [56]. Muscle-specific hemerythrin (myoHr), which is functionally equivalent to Mb, has been observed in polychaete and sipunculid tissues [57, 58]. Uniquely, the phylum Annelida contains isoforms of all known iron-based OTPs [5, 51].

### Hemoglobin and erythrocytes contribute to mammalian innate immunity

Iron is a precious commodity utilised by microbes for growth and pathogenicity. To colonise and persist in metazoans, microbes must circumvent the many iron-withholding mechanisms of the innate immune response [reviewed by 60]. During infection, hemolytic bacteria (e.g. *Staphylococcus aureus* and *Streptococcus pyogenes*) will lyse erythrocytes to exploit the iron stored within. The extra-erythrocytic Hb is detected and sequestered by the glycoproteins, haptoglobin (Hp) and hemopexin, and the lipid-free apolipoprotein A-I, all of these are freely dissolved in the plasma [60–63]. Binding of Hp to Hb restricts access to the iron centre, thereby neutralising Hb’s pro-oxidative potential and avoiding damage to vasculatures [64]. Macrophages recognise the Hp/Hb complex via the CD163 receptor and internalise the proteins to prevent further inflammation (Fig. 2) [65]. The parasite *Trypanosoma brucei* uses a glycoprotein receptor to consume Hp/Hb complexes for iron removal and recycling. Humans take advantage of this trypanosome receptor by associating lytic molecules (high-density lipoproteins) with Hp-related proteins; tricking the parasite into ingesting the trypanolytic substance [66].

In severe cases of hemolysis (called hemoglobinemia), the excess concentration of Hb overwhelms the scavenging responses and can cause potentially fatal blockages in the
Kidneys [see review 62]. Extracellular Hb is a redox-sensitive molecule with the potential to generate reactive oxygen species (ROS) [67]. The ferrous (Fe$^{2+}$) form can convert hydrogen peroxide (H$_2$O$_2$) into hydroxyl radicals (OH.) and anions (OH$^-$) via Fenton’s reaction. Such ROS disrupt tissue and cellular integrity via the peroxidation of lipids and the oxidation of nucleic and amino acids [68].

Moreover, plasma oxy-Hb can bind to, and react with, nitric oxide (NO) to produce peroxynitrite (ONOO$^-$) and ferric (Fe$^{3+}$) oxidised Hb (methemoglobin) [64, 69, 70] (Fig. 2). NO is an essential antioxidant and plays key roles in immunity, neurotransmission and signalling. In mice and snails (*Biomphalaria glabrata*), resistance to *Schistosoma mansoni* is dependent on the production of NO by macrophages and hemocytes, respectively [71, 72]. NO possesses distinct anti-parasitic properties; therefore, the release of Hb could regulate biological defences targeting *Schistosoma* and *Plasmodium* species [73]. The digestion of blood by these hematophagous parasites yields an insoluble, crystalline Hb-derived product called hemozoin [74]. If hemozoin is not removed from circulation by the spleen and liver, it can be phagocytosed by circulating leukocytes. Accumulation of hemozoin in monocytes is said to interfere with key immune molecules such as protein kinase C and major histocompatibility complex II [75].

Traditionally, pro-inflammatory responses are mediated through the binding of pathogen-associated molecular patterns (PAMPs) by soluble and cell-associated pathogen recognition receptors (PRRs) [76]. PAMPs tend to be extracellular microbial cell wall components (LPS, LTA and β-glucans) and their degenerated membrane fragments. Cell-free Hb is categorised as a damage/danger-associated immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin 297

---

**Fig. 2** Schematic representation of hemoglobin functionality beyond oxygen transport. Hemolysis, whether it is caused by microbes or physical trauma, leads to the uncontrolled release of hemoglobin (Hbs). Extracellular Hb inflicts damage by producing reactive oxygen/nitrogen species (1) and interfering with hepatic, splenic and renal physiologies (2). Inflammation can be avoided/controlled by Hb-scavenging glycoproteins [haptoglobin (Hp), hemopexin (Hx) and apolipoprotein A-I (apoAl)] and soluble receptors (CD163). These proteins intercept Hb, neutralise its oxidant properties, and direct it towards immune cells for degradation and to promote anti-inflammatory responses. Chemical (glutathione) and enzymatic antioxidants (superoxide dismutase, catalase) are recruited also. Anti-inflammatory responses are triggered when Hb binds to PAMPs/DAMPs (3). Microbial ligand (PAMPs)-Hb complexes are recognised by immune cells whereupon pro-inflammatory molecules are released, and Hb is converted into a pseudoperoxidase (POX). If Hb has been enzymatically processed prior to erythrocyte rupture, then hemocidins (antimicrobial peptides) will also be disseminated (4). This scheme was produced by summarising information presented in the following manuscripts: [61, 62, 64, 68, 80].
molecular pattern (DAMP) because it is perceived as an intracellular-derived indicator of pathological traumas such as malaria, sepsis and sickle-cell anaemia [77]. Not only is the Hb oligomer/dimer considered a DAMP, but the heme group (protoporphyrin ring) is recognised independently as an ‘alarmin’ [reviewed by 78]. Proteolytic digestion of extracellular Hb enhances the dissemination of labile heme within the blood, a physiological indicator of cystic fibrosis [68]. The control and removal of naked heme (hemin) from circulation is addressed by the protein hemopexin [62]. Several studies have noted Hb’s ability to interact with microbial ligands (PAMPs), Toll-like receptors (TLRs) and other DAMP molecules (e.g. heat-shock protein HMGB1) [24, 61, 68, 79–81]. Surface plasmon resonance revealed LPS binding sites are present on both the α and β globin chains of Hb [82]. Synthetic peptides representative of these putative ligand-binding regions targeted the lipid A moiety of LPS in vitro, and in doing so, disarmed the endotoxicity. Binding of LPS and/or LTA induces a conformational switch in Hb that causes the structure to loosen somewhat and enable peroxidase activity [24, 82]. Methemoglobin (metHb) alone and in combination with LTA can be recognised by TLR-2 on the neutrophil plasma membrane [81]. Such interactions enhance neutrophil function, initiating an NF-κB signal transduction cascade that culminates in the synthesis of cytokines and other pro-inflammatory agents. Endothelial cells can also detect extracellular Hb via a TLR-4 pathway [83] (Fig. 2).

Many studies (mentioned above) categorised extracellular Hb as harmful to the host and should be removed from circulation before noxious radicals are dispersed. Having said that, plasma Hb is an important warning to white blood cells (WBCs) that homeostasis has been compromised, and recently, Bahl et al. outlined a novel role for Hb in blood coagulation [84]. Macrophages responded to the presence of cell-free Hb by triggering the expression of the vertebrate pro-clotting initiator, tissue factor. Binding of Hb to tissue factor provided it with protection against antioxidants (e.g. glutathione), and reciprocally, the pro-oxidative potential of Hb was suppressed to mitigate collateral damage to the host’s cells. Infection-induced hemolysis and the liberation of Hb promote downstream pro-inflammatory and pro-clotting reactions [84] (Fig. 2). Remarkably, Hb gene expression and protein synthesis were recorded in cytokine (IFNγ) and LPS-stimulated macrophages [85] and surfactant-producing human alveolar type II epithelial cells [86]. The biological function of Hb production outside of erythroid tissues remains unclear. It is postulated, however, Hb may enable these particular cells to cope with nitrosative/oxidative imbalances as macrophages produce excess NO when presented with microbes, and alveolar epithelia are subjected to high levels of CO2 during gas exchange, which may affect cytosolic pH.

Beyond immune cell communication and hemostasis, Hb participates in host defences by releasing AMPs [87], discharging ROS locally [24], and functioning as a microbiostatic molecule (Table 2). The earliest record of Hb’s immune competence was reported over 55 years ago [9]. Hb prepared from human tissue extracts was inhibitory to several enteric bacteria (listed in Table 2), with maximum activity occurring at 37 °C under acidic conditions (pH <5.5) and low salt concentrations (<0.2 M). Bovine, equine, murine and rabbit Hbs were similarly antiseptic. Hb tetramers (~64 kDa) are probably too large to penetrate the bacterial membrane directly. The basic charge of Hb would promote non-specific electrostatic interactions with the acidic moieties of microbial polysaccharides, proceeding to immobilise the cells and prohibit replication. Two consecutive studies by Mak et al. [88] and Parish et al. [89] provided detailed accounts on the conversion of Hbs, Mb and cytochrome c into antimicrobials. Intact Hb and Mb were moderately effective at killing bacteria such as E. coli until the removal of the heme cofactors and partial unfolding of the proteins resulted in a broader spectrum of microbiidal properties and LD50 (μM) values comparable to conventional AMPs. By treating apomyoglobin and apohemoglobin with cyanogen bromide, the globin chains were deconstructed into AMPs ca. 50 amino acids in length (Table 2; Supp. Table 1; Fig. 3). This family of Hb-derived AMPs was referred to as ‘hemocidins’ [88]. Prior to the discovery of hemocidins, human Hb was already known to be a rich source of over 150 regulatory peptides, e.g. hemorphins with opioid-like tendencies [90]. In fact, the first Hb-derived AMP was removed from the gut of the cattle tick Rhipicephalus (Boophilus) microplus [91]. This 3.2 kDa AMP was identical to residues 33–61 (FLSFPTTKTYFPH-FDL SHGSAQVKGHGAK) of bovine α-Hb (Supp. Figure 1), and targeted Gram-positive bacteria, filamentous fungi and yeast [91]. A second tick species, Ornithodoros moubata, contained two anti-Staphylococcal peptides in its midgut after a blood meal [92]. Edman degradation verified the origin of these peptides to be overlapping fragments (residues 1–11; 3–19) from rabbit αHb. When in solution, the bovine peptides Hb33-61 and Hb1-23 do not form comparable to conventional AMPs. By treating apomyoglobin and apohemoglobin with cyanogen bromide, the globin chains were deconstructed into AMPs ca. 50 amino acids in length (Table 2; Supp. Table 1; Fig. 3). This family of Hb-derived AMPs was referred to as ‘hemocidins’ [88]. Prior to the discovery of hemocidins, human Hb was already known to be a rich source of over 150 regulatory peptides, e.g. hemorphins with opioid-like tendencies [90]. In fact, the first Hb-derived AMP was removed from the gut of the cattle tick Rhipicephalus (Boophilus) microplus [91]. This 3.2 kDa AMP was identical to residues 33–61 (FLSFPTTKTYFPH-FDL SHGSAQVKGHGAK) of bovine α-Hb (Supp. Figure 1), and targeted Gram-positive bacteria, filamentous fungi and yeast [91]. A second tick species, Ornithodoros moubata, contained two anti-Staphylococcal peptides in its midgut after a blood meal [92]. Edman degradation verified the origin of these peptides to be overlapping fragments (residues 1–11; 3–19) from rabbit αHb. When in solution, the bovine peptides Hb33-61 and Hb1-23 do not form distinct secondary structures, i.e. they are unfolded. Upon insertion of Hb33-61 into anionic detergent micelles, the cationic peptide establishes an N-terminal β-turn and a C-terminal α-helical arrangement [93]. A flexible region, Pro44-Leu48, forms between the two structural motifs and may act like a hinge to help the peptide penetrate/rupture the lipid bilayers of microbes.

To date, natural sources of human hemocidins include, but may not be limited to, placental tissue, erythrocytes and menstrual vaginal secretions [87]. Liepke et al. observed two AMPs from placental tissue, one from γ-Hb (130–146)
| Organism | Conformational state | Size (kDa) | Charge | Activity range | References |
|----------|----------------------|------------|--------|----------------|------------|
| **Hemoglobin** | | | | | |
| Alligator (*Alligator mississippiensis*) | Hemoglobin tetramer (α₂β₂), α-chain, β-chain | ~68 | Neutral | Antibacterial: Gram— | [89, 133] |
| | | | | Escherichia coli (25–100 μg ml⁻¹) | |
| | | | | Pseudomonas aeruginosa (25–350 μg ml⁻¹) | |
| Blood cockles (clams) (*Scapharca kagoshimensis*) (*Tegillarca granosa*) | Hemoglobin dimer and tetramer | <60 | Neutral | Antibacterial: Gram +/— | [139–141, 144, 145] |
| | Microbial challenge leads to substantial expression of Hb mRNA, peaking at 12 h. | | Neutral | Vibrio parahaemolyticus (11–100 μg ml⁻¹) | |
| | PSVQDAAAQISADVKK | 0.8 | Anionic | Vibrio alginolyticus (12–200 μg ml⁻¹) | |
| | VLASNFGDR | 1.5 | Anionic | Vibrio harveyi (1–200 μg ml⁻¹) | |
| | ISAEEFGK | 2.4 | Anionic | Bacillus subtilis (<180 μg ml⁻¹) | |
| | ISAEGFAINEPMK | 2.5 | Anionic | Bacillus firmus | |
| | GHAIILTYALNNFVDSLDPSR | 3.5 | Neutral | Staphylococcus aureus (>370 μg ml⁻¹) | |
| | MGSSYSDCAAAWAA | | Cationic | Micrococcus tetragenus (<47 μg ml⁻¹) | |
| | LVAVVQAAL | | Cationic | Lipopolysaccharide and peptidoglycan | |
| | LNHGHGLTWYGQNFVDQ | | Cationic | | |
| | LDNADDLEDVARK | | Cationic | | |
| **Catfish (*Ictalurus punctatus*)** | Hb₄P₁: AAKGPSVFTEPVH | 3.7 | Cationic | Anti-parasitic: | [119, 130] |
| | ETWQKFLNVVVAALGKQYH | | pI = 9.22 | Amyloodinium ocellatum (54 μM) | |
| | The Hb-AMP was expressed in skin and gill epithelium when challenged with *I. multifiliis* | | | Ichthyophthirius multifiliis trophont (1.7 μM) | |
| | | | | Tetrahymena pyriformis (6.8 μM) | |
| | | | | Antibacterial: Gram— | |
| | | | | Escherichia coli (3.4 μM) | |
| | | | | Vibrio alginolyticus (13.5 μM) | |
| | | | | Aeromonas hydrophila (3.4 μM) | |
| | | | | Antibacterial: Gram +/— | [9, 91, 93, 100–105, 148–150] |
| | | | | Micrococcus luteus (5–671 μM) | |
| | | | | Staphylococcus epidermidis (21 μM) | |
| | | | | Escherichia coli (IC₅₀ = 0.1 μg ml⁻¹) | |
| | | | | Antibacterial: | |
| | | | | Candida albicans (5 μM) | |
| | | | | Aspergillus nidulans (1.3 μM) | |
| | | | | Saccharomyces cerevisiae (11 μM) | |

* indicates cryptides.
| Organism                      | Conformational state | Size (kDa) | Charge | Activity range                  | References                  |
|-------------------------------|----------------------|------------|--------|---------------------------------|-----------------------------|
| Crocodile* (Crocodylus siamensis) | Hemoglobin tetramer (α₂β₂), α-chain | ~ 68       | Cationic | Antibacterial: Gram +           | [130, 131]                  |
|                               | VLSSDDKCNVKAVW       | 2          | Neutral | Bacillus amyloquefaciens        |                             |
|                               | CKVAG                | 1.2        | Cationic | Bacillus subtilis               |                             |
|                               | KVAGHLEEEYGA         | ~ 1        | Cationic | Bacillus pumilus                |                             |
|                               | WHKVDVAH             | 0.7        | Neutral | Bacillus megaterium             |                             |
|                               | HEAVNH               | 1          | Cationic |                                 |                             |
|                               | ASFGEAVKHLDSIR       | 2.35       | Cationic |                                 |                             |
|                               | VVVAIHHPGSLTPEV      | 2.2        | Cationic |                                 |                             |
|                               | HASLDKF              | 1.1        | Cationic |                                 |                             |
|                               | AIHPGSLTPEVHAS       |            |         |                                 |                             |
|                               | LDKFL                |            |         |                                 |                             |
|                               | AAHPKDFGL            |            |         |                                 |                             |
| Guinea pig (Cavia porcellus)  | Hemoglobin tetramer (α₂β₂) | ~ 68       |         | Antibacterial: Gram –           | [9]                         |
|                               |                      |            |         | Escherichia coli (IC₅₀ = 0.02 µg ml⁻¹) |                             |
|                               |                      |            |         | Antibacterial: Gram +/-         | [9, 80, 94-99]              |
|                               |                      |            |         | Bacillus subtilis, Lactobacillus acidophilus, Salmonella spp. (LD₅₀ = ~5 µM), Staphylococcus aureus (LD₅₀ = ~7 µM), Staphylococcus camosus, Escherichia coli (1–20 µg ml⁻¹), Shigella sonnei, Micrococcus luteus, Enterococcus faecalis (LD₅₀ = 5–8.9 µM), Pseudomonas aeruginosa (LD₅₀ = 4.4–6.7 µM) and Klebsiella spp. (LD₅₀ = 5.8–8.3 µM) |                             |
|                               |                      |            |         | Antifungal: Saccharomyces cerevisiae, Candida albicans (LD₅₀ = 6.25–14 µM), Candida parapsilosis (LD₅₀ = 6.25–12 µM), Candida kruizi (LD₅₀ = 12.5–25 µM) |                             |
|                               |                      |            |         | Ligand binding: lipopolysaccharides |                             |
|                               |                      |            |         |                                 |                             |
| Horse (Equus ferus caballus)  | Hemoglobin tetramer (α₂β₂) | ~ 68       |         | Antibacterial: Gram –           | [9, 89]                     |
|                               |                      |            |         | Escherichia coli (IC₅₀ = 0.4–2 µg ml⁻¹) |                             |
|                               |                      |            |         | Antibacterial: Candida albicans (MIC = 250 µg ml⁻¹) |                             |
|                               |                      |            |         |                                 | [127]                       |
|                               |                      |            |         | Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi and Vibrio cholera |                             |
|                               |                      |            |         |                                 |                             |
| Indian major carp (Cirrhinus mrigala) | Hemoglobin monomers (α & β) | ~ 16       |         | Antibacterial: Gram –           | [124]                       |
|                               |                      |            |         |                                 |                             |
| Japanese eel (Anguilla japonica) | FAHWDLGPSPS         | 2.4        | Cationic | Antibacterial: Gram +/- (10.5–84 µM) |                             |
|                               |                      |            |         |                                 | [124]                       |
|                               |                      |            |         | Edwardsiella tarda, Aeromonas sp., Aeromonas hydrophila, Vibrio alginolyticus, Vibrio parahaemolyticus, Micrococcus luteus and Staphylococcus aureus |                             |
|                               |                      |            |         |                                 |                             |
| Mouse                        | Hemoglobin tetramer (α₂β₂) | ~ 68       |         | Antibacterial: Gram –           | [9]                         |
|                               |                      |            |         | Escherichia coli (IC₅₀ = 0.1 µg ml⁻¹) |                             |
Table 2 continued

| Organism                      | Conformational state                  | Size (kDa) | Charge | Activity range                                      | References |
|-------------------------------|---------------------------------------|------------|--------|----------------------------------------------------|------------|
| Rabbit (Oryctolagus cuniculus) | Hemoglobin tetramer (α₂β₂)            | ~68        | Cationic | Antibacterial: Gram +/−                             | [9, 92]    |
|                               | VLSPADKTNIK                            | 1.2        | pH = 9.53 | Escherichia coli (IC₅₀ = 0.02 µg ml⁻¹)             |            |
|                               | SPADKTNKNTAEKIGS                       | 1.9        | Cationic | Staphylococcus aureus                              |            |
|                               | Peptides were found in stomach of soft tick (Ornithodoros moubata) |            |        |                                                    |            |
| Rat                           | Hemoglobin tetramer (α₂β₂)            | ~68        |        | Antibacterial: Gram −                               | [9]        |
|                               |                                      |            |        | Escherichia coli (IC₅₀ = 0.02 µg ml⁻¹)             |            |
|                               |                                      |            |        |                                                    |            |
| Sea bass (Dicentrarchus labrax)| Hemoglobin-like protein (mRNA expression) |            |        | Antibacterial: Gram −                               | [122]      |
|                               |                                      |            |        |                                                    |            |
| Snake (Thamnophis sirtalis)   | Hemoglobin tetramer (α₂β₂)            | ~68        |        | Antibacterial: Gram +/−                             | [89, 133]  |
|                               |                                      |            |        | Streptococcus faecalis (15 µg ml⁻¹)                |            |
|                               |                                      |            |        |                                                    |            |
| Stingray (Potamotrygon henlei)| β-Globin chain                        | ~16        |        | Antifungal: Candida albicans (≤100 µg ml⁻¹)        | [126]      |
|                               |                                      |            |        |                                                    |            |
| Tuna (Katsuwonus pelamis)     | TQQA-FQKFLAA                           | 2.3        | Cationic | Antibacterial: Gram +/−                             | [125]      |
|                               | VTSALGKQYH                             |            | pH = 9.7 |                                                    |            |
|                               |                                      |            |        |                                                    |            |
| Myoglobin                     | Horse Mb 56–131                        | ~17        |        | Antibacterial: Gram +/−                             | [88, 151]  |
|                               | Whale Apomyoglobin                     |       |        |                                                    |            |
|                               | (Physeter catodon)                     |            |        |                                                    |            |
|                               | Myoglobin fragments: 1–55, 56–131 and 132–153 |            |        |                                                    |            |

* Minimum inhibitory concentration (MIC) or LD₅₀ values were not available for all (poly)peptides listed
a An extended list of hemoglobin/myoglobin-derived peptides is available as supplementary material, Table S1
b Formerly Boophilus microplus
c Cyanogen bromide treatment
and a second from β-Hb (111–146). The latter βHb peptide was present in large amounts, ~360 mg kg\(^{-1}\) tissue, pre-processed from the Hb tetramer within the cytosol of erythrocytes, and could bind to endotoxins [94]. The most convincing evidence for hemocidins functioning in vivo comes from vaginal blood [95–99]. Initial screenings identified 44 hemocidins, most of them originating from the N-terminus of αHb. Two synthetic peptides, Hbα 35–56 and Hbβ115–146, identical to natural peptides purified previously showed preferential activity towards Gram-negative bacteria, less activity toward Gram-positives, and no effect on fungal growth/reproduction [95]. Hbβ115–146 is an acidophilic, halo-tolerant peptide that potentiates the microbicidal effects of common neutrophil-derived immune effectors such as α/β-defensins and lysozyme, which are found in the female urogenital tract [97]. Hemocidins present in the vagina act as bacterial deterrents and assist immune defences during times of intense physiological strain, such as menstruation and childbirth [87, 96]. It remains unclear how hemocidins are formed and deposited into the vagina. The acidic pH may denature the Hb oligomers enough to allow endo/exo-peptidases and neutrophil-derived matrix metalloproteases to separate the peptides in a step-wise manner.

The laboratory of J.L. Ding in Singapore has provided unequivocal evidence to support a role for Hb in innate immunity. Damage to, and lysis of erythrocytes by hemolytic bacteria guides the release of pro-oxidative Hb into the surrounding milieu. The more virulent pathogens continue to secrete proteases for digesting Hb. Microbial ligands and proteases act in synergy and amplify the pseudoperoxidase (POX) activity of Hb. Concurrently, the pathogen is bombarded with a battery of ‘dual-active’ Hb congeners/peptides that bind to exoplasmic membrane structures and inflict localised cytotoxic radicals to weaken/kill the pathogen in situ [24, 80, 82]. Hb-PAMP aggregates are inflammatory agonists (mentioned above), stimulating the expression of cytokines before being recycled by proteasomes and heme-oxygenase within circulating phagocytes (Fig. 2).

Functionally versatile hemocidins have been retrieved from endogenous sources (erythrocytes, ticks, uterine...
secretions) and by in vitro chemical/enzymatic manipulation (e.g. amidation) of commercially sourced globins (listed in Table 2; Supp. Table 1). Synthetic analogues of bovine and human Hb peptides are now screened routinely for therapeutic potential, e.g. anti-HIV-1 [94, 100–106]. Peptide release from larger ‘maternal’ proteins is more widespread than once thought. Indeed, the ‘cryptome’ refers to the entire subset of proteins/peptides released from maternal sources, which have alternative or heightened activities [107–109]. Human lactoferrin [110, 111], lysozyme [112] and cathepsin G [113] are more examples of macromolecules containing encrypted peptides.

**Use of hemoglobin by microbes**

Certain pathogens adapted for intracellular life enlist their own heme containing globins to combat the harsh internal environment of leukocytes. A truncated Hb produced by *Mycobacterium tuberculosis* (HbN) is necessary for infectivity [114]. The microbe-derived HbN decomposes NO produced by macrophages and neutrophils so the bacterium may survive within the cytosol. HbN acts as a NO dioxygenase despite the absence of a true reductase domain [115]. HbN expression intensifies once the bacterium enters the WBC. Subsequently, the protein is glycosylated post-translationally and localised to the cell membrane/wall. The expression of CD80/86 co-stimulatory surface receptors on the phagocyte cell surface is suppressed during *M. tuberculosis* occupancy, linked to an increase in HbN concentration [114]. It appears that HbN not only protects the bacterium from the cytosolic defences of WBCs, but also modulates the expression of host immune factors.

The causative agent of thrush, *Candida albicans*, secretes up to ten acid hydrolases (aspartic peptidases) when attempting to colonise the vagina. These enzymes attack the host’s defences, and are capable of using the existing Hb as a substrate for peptide production. These Hb hydrolysates are effective bactericidals, especially in the presence of *Lactobacillus acidophilus* [99]. Intriguingly, these observations imply that *C. albicans* exploits human Hb to antagonise bacteria and reduce competition within the vagina.

---

**Fig. 4** Hemoglobin-derived antimicrobial peptides from fish. The overlapping encrypted peptides (HbβP-1, 2 and 3) of fish (*Ictalurus punctatus*; GI:318171215) hemoglobin are presented using the crystal structure of rainbow trout hemoglobin (3BOM). The helical structures of each peptide are presented as **ribbons**, and their locations are indicated by **black arrows**. It has not been confirmed whether the peptides retain these structural features upon detachment from the Hb.
Fish hemoglobin and erythrocytes

Functional plasticity of erythrocytes from non-mammalian vertebrates such as trout (Oncorhyncus mykiss) and chickens has been confirmed in vitro [116]. Aside from links to reproductive and endocrine physiologies, these nucleated erythrocytes employ common PRRs (e.g. TLR 3) to detect PAMPs (LPS, PGN) and respond by synthesising a plethora of immune-related mRNAs (chemokine CCL4, IFNγ). These erythrocytes are further capable of communicating the presence of viral mimics (polyninosinic: polycytidylic acid) to macrophages via a type-1 IFN response [116]. Erythrocyte-pathogen antibiosis is frequently encountered across the literature, yet critically, there is now sufficient evidence for erythrocyte-specific roles in fish, reptile and bird innate immunity [reviewed by 117]. First, in 2004, antibacterial proteins were recovered from ruptured erythrocytes of rainbow trout [118]. These cationic proteins targeted Planococcus citreus and E. coli with MIC values in the sub-micromolar range. Although the authors did not purify individual compounds, they speculated (based on size) that histone H2A was involved. It is highly likely some Hb found its way into the final protein extract, regardless of the ‘harsh’ extraction procedure used and the loss of Hb via precipitation [118]. Lately, piscine Hb subunit chains (α & β) and peptides of various sizes have been reported acting in an anti-infective manner (listed in Table 2).

In catfish (Ictalurus punctatus) infected with the ciliate parasite isch (Ichthyophthirius multifiliis), variants of the β-Hb gene were transcribed and translated in epithelial surfaces of the skin and gills, as well as in erythrocytes [119]. In total, three cationic Hb-derived peptides were identified, HbβP-1 to HbβP-3. These peptides emerged from both the C- and N-termini of the β-globin monomer (Fig. 4). The extra-erythrocytic peptide, HbβP-1 (3.7 kDa), was lethal to eukaryotic and prokaryotic fish ectoparasites: Amyloodinium ocellatum, I. multifiliis and Tetrahymena pyriformis [119, 120]. In vivo concentrations of HbβP-1 increased under immune challenge and were selectively toxic to the trophont stage of the parasites. Unlike many of the human hemocidins discussed previously, antimicrobial properties of HbβP-1 were limited to a few Gram-negative bacterial pathogens (Table 2).

Significant increases in Hb mRNAs were detected in several tissues (gill, skin and spleen) of European sea bass, Dicentrarchus labrax, resulting from exposure to acute physical stress (crowding) and pathogenic challenge (Vibrio anguillarum) [121, 122]. This study mirrored patterns of Hc up-regulation in the hepatopancreas of shrimp (L. vannamei) subjected to microbial and thermal stresses [21, 123]. The first fish α-Hb peptide was extracted from the liver of Japanese eel (Anguilla japonica [124]), and following that, a β-Hb peptide was removed from the liver of Tuna (Katsuwonus pelamis [125]). Peptides from each fish were <2.5 kDa in size, positively charged and composed of α-helices. Antimicrobial activity of the tuna Hb peptide (SHβP) was heat stable and pH resistant, but was non-functional in the presence of chymotrypsin and trypsin [125]. C-terminal amidation of SHβP enhanced its activity, perhaps by altering the electrostatic interactions with the anionic bacterial membranes. Generally, post-translational modification of peptides increases their metabolic stability against endogenous peptidases.

The biomolecular composition of stingray (Potamotrygon henlei) mucus was interrogated for the presence of antimicrobials, wherein a β-Hb polypeptide (~16 kDa) was classified [126]. In vitro assays revealed non-specific microbicidal actions of this β-Hb monomer in the presence of bacterial and fungal targets (Table 2). The stingray Hb did not show any adverse cytoxicity upon exposure to mammalian cells, and injection of the protein (10 μM) into the cremaster venules of mice induced an ephemeral response in leukocyte rolling behaviour (visualised using intravital microscopy). These data suggest β-Hb is a putative immune bioactive from stingray mucus and has potential therapeutic applications in humans. The Indian major carp, Cirrhinus mrigala, also contains biologically active α- and β-Hb chains in skin mucus, as well as histones H2A, H3 and H4 [127]. The immune activity of Hbs in fish mucus and skin epithelia serves as a first-line defence against parasites and pathogens.

Reptile hemoglobin

Despite crocodiles spending most of their lives in dirty, microbiologically hazardous waters, they show few signs of severe infection even when seriously wounded [128, 129]. This resistance is due, in part, to the multifunctionality of Hb. Irrespective of the presence or absence of the heme prosthetic group, crocodile (Crocodylus siamensis) Hb tetramers, various degenerated fragments (<21 amino acids), individual globin units (α and β), and synthetic α-Hb monomers were all capable of killing Gram-positive bacteria (four species of Bacilli) but appeared ineffective against Gram-negative bacteria (E. coli) [130, 131]. Electron micrographs taken of B. subtilis [ATCC 6633] cultured in the presence of purified Hb fractions depicted cell membrane irregularities within 2 h. Many of these crocodile hemocidins share typical features of AMPs: net-positive charge, >30 % hydrophobic content and their predicted secondary structural motifs are dominated by α-helices (Table 2; Table S1). The latter is not entirely surprising considering the conserved helical arrangement of all known Hbs (Fig. 3). Crocodile Hb is a potent scavenger of oxygenic radicals in vitro, albeit the
significance of this antioxidant role in vivo has yet to be explored [132].

The antimicrobial features of ectotherm Hbs are not restricted to Gram-positive bacteria, as snake (Thamnophis sirtalis) and alligator (Alligator mississippiensis) Hbs can inhibit the growth of E. coli, Pseudomonas aeruginosa and the pathogenic yeast, C. albicans [89, 133] (Table 2). Alligator Hb failed to suppress Gram-positive bacteria, Streptococcus faecalis and S. aureus, using a disc diffusion approach [89]. The potency of alligator Hb differs between each globin subunit, e.g. up to fivefold less αHb (MIC = 30 μg mL⁻¹) was needed to inhibit yeast compared to βHb (MIC = 150 μg mL⁻¹) [133].

An emerging role for invertebrate (bivalve) hemoglobin in innate immunity

Almost all cephalopods and gastropods utilise Hc to dispense dioxygen to metabolically active tissues; an exception being freshwater snails. Species such as the planorbid snail, Biomphalaria glabrata, use giant extra-cellular Hbs (1.44 MDa) to meet their respiratory needs in what is considered to be an evolutionary abandonment of Hc [134, 135]. In blue-blooded (Cu) and red-blooded (Fe) snails, Hc and Hb are synthesised inside specialist rhogocytes (pore cells) and then released into the plasma [136, 137]. Bivalves lack Hc, instead they utilise cell-bound Hbs which are structurally similar to vertebrate Hbs [5, 138]. Analogous to fish and reptiles, bivalves store their Hb within nucleated erythroid-like cells. Trematode infestation of the Sydney cockle, Anadara trapezia, induced measurable increases in circulating erythrocyte numbers, over double compared to non-parasitised animals [138]. These data add support to earlier findings demonstrating the immune competence of erythrocytes [117].

The cDNA (748 bp) of an intracellular homo-dimeric Hbl (~31 kDa) from the blood clam Tegillarca granosa was cloned and sequenced to reveal ~82 % similarity with Hbs from Scapharca kagoshimensis and Scapharca inaequivalvis [139]. Messenger RNAs of Hbl were expressed constitutively in the hemocytes, adductor muscle, foot, gills, gastrointestinal tract and mantle. When clams were injected with LPS, Vibrio parahaemolyticus and/or PGN, Hbl mRNA transcript numbers increased significantly. The highest levels of expression were detected in the hemocytes, with an 800-fold increase at 12 h post-infection (h.p.i.) compared to the control groups. Differential temporal expression patterns of T. granosa HbII-A and -B genes in hemocytes were also detected in the presence of microbial ligands (LPS and PGN) and intact bacteria [140, 141]. Over 20 individual nucleotide polymorphisms have been identified across all three T. granosa Hb genes [141]. Polymorphic loci at exon2–146 (serine to proline switch) and exon2–23 (alanine to threonine switch) on HbII-A and HbII-B, respectively, were recovered from clams having survived heavy V. parahaemolyticus loads. These amino acid substitutions likely confer alternate functionality to newly synthesised Hbs. Similarly, 13 Hc-associated SNPs have been identified in shrimp (L. vannamei) infected with the same pathogens [142, 143]. These molecular alterations were located in the immunoglobulin-like domain and C-terminal region of the shrimp Hc resulting in improved microbial agglutination properties.

Positive microbialic activities of intact T. granosa HbI and HbII were observed in the presence of E. coli and several Gram-positive bacteria. Seven Hb-derived peptides ranging in size from 0.8 to 3.5 kDa (Table 2) were effective against Gram-negative bacteria only, verified using live/dead cellular staining [144]. These bivalve hemocidins were removed from the Hb protomers via trypsin digestion and purified to homogeneity. The aquatic pathogen, Vibrio harveyi, was particularly sensitive to the neutral Hb peptide 1 (PSVQDAAAQISADVKK), with an MIC value of 1 μg mL⁻¹. Each Hb-derived peptide demonstrated antibacterial potential (Table 2). Additionally, Hb oligomers from S. kagoshimensis proved efficient at killing Gram-positive bacteria, yet had no measurable effect on fungal moulds (Aspergillus niger, Penicillium glaucum) or any Gram-negative bacteria tested [145].

The authors of these studies considered ROS production by peroxidase and PO-like activities of Hb were likely contributing factors to the antimicrobial mechanism. The use of POs to produce ROS and melanins is a conserved defence strategy amongst flora and fauna. PO activities of bivalve hemocytes and proPO within the haemolymph have received much attention [146, 147]. Conversely, studies focussing on Hb-derived PO activities are relatively unheard-of (discussed below).

Inducible phenoloxidase and (pseudo)peroxidase activities of hemocyanins and hemoglobins

Both arthropod and mollusc Hcs can be converted into PO-like enzymes upon physical disruption of the structural motifs in and around the dicopper centres. It is most important to open the entrance to the active site, yet such invasive structural alterations will eventually destroy the PO activity. Either through proteolysis or interactions with endogenous cofactors, placeholder residues (usually with aliphatic or aromatic chemistry) occluding the active sites are dislodged, thus permitting phenolic compounds to be processed into melanin precursors [21, 152]. The POs play vital roles in invertebrate development and contribute to counter-measures targeted towards infectious agents, e.g.
hemocyte encapsulation/nodulation and using toxic quinones to kill pathogens [153]. POs (tyrosinases [EC 1.14.18.] and catecholoxidases [EC 1.10.3.1]) and Hc-d POs catalyse the ortho-hydroxylation of monophenols (β-tyrosine) into diphenols and subsequently oxidise the o-diphenols (L-dihydroxyphenylalanine) into quinones (dopachrome) [154–157]. Arthropod Hc-d PO can convert 5,6-dihydroxyindole directly into melamin, a very resistant polymer net which invaders cannot penetrate [158]. Not all Hcs or POs can carry out the hydroxylation step. Recent data imply that an asparagine residue and a glutamate residue located near the CuB binding site are essential for tyrosinase activity. These residues fix a conserved water molecule and lower its pK value to disrupt the hydrogen from the monophenols (i.e. deprotonate), so the resulting phenolate can bind to CuA to initiate the enzymatic cycle [159–161]. The proton will be bound by this water molecule to form a hydronium ion (H$_3$O$^+$). After release of the final product, namely o-quinone, a hydroxyl group bridges the two copper ions but will be discarded as a water molecule after obtaining a hydrogen back from the hydronium ion. Upon replacing either the asparagine or glutamine residues with different amino acids only catecholoxidase activity is possible [161]. In the absence of a true PO, chelicerates rely on the inducible PO activity of Hc as a substitute [28, 30, 162, 163, 203, 204]. Lately, Hc was found to be a major component of clots formed during hemostasis in the spider, Acanthoscurria geniculata. It is postulated that Hc, like PO, enables protein cross-linking and sclerotisation of the cuticle post-moulting [32]. These findings are supported by earlier studies where Hc was present in abundance throughout the cuticles of shrimp (Penaeus japonicus) and tarantula (Euryepalma californicum) exoskeletons [164, 165].

Both mono-dimeric (31.2 kDa) and hetero-tetrameric (~60 kDa) conformational states of blood clam (S. kagoshimensis) Hb were found to possess PO-like activity. In vitro, Hbs could oxidise diphenols (catechol and L-DOPA; Table 3) to quinones (dopachrome) but were unable to carry out the initial hydroxylation reaction on monophenols [145]. Catalytic turnover was enhanced ~20 % by the polar solvent isopropanol, although exposure to SDS and trypsin led to a 75 % reduction in activity. SDS and ionic liquids are known to induce transient activity in many enzymes, including POs and Hc-d POs [155, 166, 167]. Bivalve Hb may be particularly sensitive to SDS-driven denaturation and tryptic digestion. Thermal and pH ranges of Hb-d PO as well as kinetic parameters such as substrate binding efficiencies ($K_m$) for catechol and L-DOPA are similar to arthropod and mollusc Hc-d POs, notably cuttlefish (Sepia officinalis), snails (Helix pomatia) and crabs (Charybdis japonicus) (Table 3) [144, 145, 168–170]. Hb-d PO activity can be inhibited by known tyrosinase inhibitors (e.g.1-phenyl, 2-thiourea), standard metal chelators (EDTA, DETC) and antioxidants (ascorbic acid) in a similar way to POs [145]. The question remains, however, is Hb a latent PO or simply able to oxidise phenols non-specifically due to the presence of a transient metal ion within the heme cofactor?

In 2007, a seminal paper published by Jiang and co-workers described the pseudoperoxidase activity of human metHb and the PO activity of horseshoe crab Hc in the presence of extracellular proteases released by bacteria and fungi [24]. Hb and Hc were ‘switched on’ upon binding to bacterial ligands, LPS (Gram−) and LTA (Gram+), but were unaffected by laminarin. The generation of superoxide anions (O$_2^−$) by metHb correlated positively with concentrations of microbial stimulants, and equally, was suppressed by the addition of superoxide dismutase. Furthermore, increases in metHb ROS production were recorded inside erythrocytes exposed to different strains of S. aureus, highlighting the significance of this activity in vivo [24]. The peroxidase potential of Hb appears to be conserved amongst metazoans. Clam Hb can oxidise guaiacol (a methylated derivative of catechol) in the presence of H$_2$O$_2$ [144]. The catalytic turnover of phenols into quinones, whether it is by Hc, PO or Hb, generates volatile by-products [21, 24, 144, 145]. These oxidase-related enzymatic by-products boast significant broad-spectrum antimicrobial properties [171] evidenced by mollusc Hb’s inability to kill microbes in the presence of the ion scavenger, glutathione [144]. ROS formation by OTPs arises independently of immune signalling cascades and, therefore, provides an instantaneous assault on pathogens.

**Hemocyanin-derived cryptides**

Hcs are acute phase proteins contributing to host recognition of non-self, pathogen opsonisation and agglutination, hemolysis, melanin biogenesis and virustasis, all of which have been reviewed in detail by Coates and Nairn (2014) [21]. The following section, however, is concerned primarily with the encrypted AMPs of Hc.

Hc-derived peptides were first isolated from hemolymph plasma of commercially relevant shellfish species, one from Litopenaeus vannamei (PvHCt) and two from Penaeus stylirostris (Table 4) [177]. PvHCt failed to inhibit the growth of 17 bacterial species (both Gram +/−), yet revealed its exclusive antifungal activity at concentrations ranging from 3 to 50 μM (Table 4). Most recently, the structure of PvHCt was solved using a combination of $^1$H NMR and circular dichroism (Fig. 5). PvHCt is present in an unordered state in solution, and when incorporated into zwitterionic (DPC) micelles this histidine-rich peptide folds into a linear, amphipathic, α-helical structure with an
Table 3 Inducible o-diphenoloxidase activity in hemoglobin versus hemocyanin

| Substrate kinetics | Dopamine | l-Dopa |
|--------------------|----------|--------|
| Catechol           |          |        |
| HbI—$K_m = 5.7$ mM | $V_{max}$ | $K_m = 2.0$ mM |
| HbII—$K_m = 2.71$ mM | $V_{max}$ | $K_m = 1.22$ mM |
| $K_m = 0.97$ mM    |          |        |
| $K_m = 0.20 \pm 0.03$ mM$^{-1}$ | $V_{max}$ | $K_m = 2.9$ mM |
| $K_m = 1.45 \pm 0.16$ mM | $V_{max}$ | $K_m = 0.59 \pm 0.08$ mM$^{-1}$ |
| $K_m = 3.91 \pm 0.55$ mM$^{-1}$ |          |        |
| $K_m = 2.6$ mM     |          |        |
| $V_{max} = 0.137$ mM min$^{-1}$ |          |        |
| $V_{max} = 0.018$ mM min$^{-1}$ |          |        |
| $K_m = 2.86$ mM     |          |        |
| $K_m = 0.77$ mM     |          |        |
| $K_m = 1.3 \pm 0.1$ mM |          |        |
| $V_{max} = 5.84 \pm 0.24$ μmol min$^{-1}$ | $V_{max} = 2.4 \pm 0.07$ μmol min$^{-1}$ | $K_m = 2.4$ mM |
| $K_m = 9.85 \pm 0.89$ mM | $K_m = 0.431 \pm 0.04$ mM | |
| [4-methylcatechol] |          |        |
| $V_{max} = 0.161 \pm 0.005$ ΔAbs min$^{-1}$ | $V_{max} = 0.143 \pm 0.003$ ΔAbs min$^{-1}$ | $V_{max} = 0.112 \pm 0.002$ ΔAbs min$^{-1}$ |
| $K_m = 7.174 \pm 0.487$ mM | $K_m = 0.181 \pm 0.001$ mM | $K_m = 2.565 \pm 0.115$ mM |
| $K_m = 6.53$ mM |          |        |
| $K_m = 2.4$ mM |          |        |

A arthropod, M mollusc

overall net-negative charge ($pI = 6.16$) (Fig. 5) [178]. Amphipathicity is a key feature of most pore-forming AMPs. Hyphae and spores of *F. oxysporum* were damaged irreversibly within 90 min of PvHCt (20 μM) treatment due to its gross accumulation on the exoplasmic side of the fungal cell wall, but were not dependent on interactions with ergosterol. Cellular pathologies included ‘leakiness’ (4 kDa flux), plasma membrane deterioration, fewer lipid bodies and effete mitochondria. A cationic peptide, termed FCHc-C2, was manufactured recombinantly from the cDNA of shrimp (*Fenneropenaeus chilensis*) Hc [179]. FCHc-C2 and PvHCt share high sequence homology >90%, the only differences being an aspartate is substituted with valine and a glycine is substituted with lysine (Table 4). Amphipathicity does not appear to differ significantly between these two peptides (Supp. Figure 2), yet unlike PvHCt, FCHc-C2 was active against Gram +/− bacteria as well as fungi. The (conventional) basic charge of FCHc-C2 (aided by an additional histidine on the hydrophilic side of the helix) might permit non-selective electrostatic interactions with a broader range of microbes.

Other Hc-derived peptides, namely astacidin 1 and rondonin, were recovered from the hemolymph of immune-stimulated crayfish (*Pacifastacus leniusculus* [180]) and spiders (*Acanthoscurria rondoniae* [181]), respectively. In solution, astacidin 1 forms a β-sheet structure and is active against many bacteria [180] and fungi [182] (Table 4). These peptides are located on the surface of the Hcs and, therefore, exposed to the environment (Fig. 5 [21, 183]). Characterisations of truncated astacidin 1 variants revealed a dependency on the N-terminal residues (FKVQNLHQVVQVFHHH-COOH) for effective microbe killing. Similar to PvHCt, astacidin 1 functions optimally at acidic pH and causes injury to the external membranes of fungi, creating trans-bilayer pores with radii ~2 nm. Rondonin, PvHCt and astacidin 1 all originated from the C-terminal domains (III) of their precursor Hcs, a structurally conserved region organised into a seven-stranded anti-parallel β-barrel (Figs. 1 and 5). Each peptide is likely detached from Hc via directed proteolysis. Aspartyl (pepstatin) and cysteine (E-64) protease inhibitors impeded the production of astacidin 1 in crayfish, indicating the peptide may be cleaved from Hc by cysteine-like proteases released by lysosomes [180]. Concentrations of Hc-derived AMPs in the hemolymph of shrimp and crayfish increased significantly in the presence of microbial ligands, LPS and
| Organism | Conformational state | Size | Charge | Activity (MIC or LD<sub>50</sub>)<sup>+</sup> | References |
|----------|----------------------|------|--------|------------------------------------------|------------|
| Hemerythrin | Medicinal Leech (<em>Hirudo medicinalis</em>) | Octamer | ~ 108 kDa | Antibacterial: Gram+/-<br>Escherichia coli and Micrococcus luteus | [189] |
| | | Up-regulation of Hr confirmed via 2D SDS-PAGE, protein accumulation occurred in tissues of the CNS | | | |
| | Rag (sand) worm (<em>Hediste diversicolor</em>) | MPII, 119 amino acids | 13.7 kDa | Antibacterial: Gram+/-<br>Kocuria kristinae, Micrococcus luteus, Escherichia coli and Vibrio alginolyticus | [192] |
| Hemocyanin | Abalone (<em>Haliotis tuberculata</em>) | YKKFGYRYDSL.ELEGRSISRDELIQQRQEKRFTAGFLKGF (linear α-helix, termed haliotisin) | 5.2 kDa | Cationic: pI = 9.66<br>Bacillus subtilis (0.3–3 lM)<br>Erwinia carotovora (0.8–2.6 μM) | [187] |
| | | | | | |
| | Crayfish (<em>Pacifastacus leniusculus</em>) | FKVQNQHGQVVKIFHH (termed astacidin 1) (β-sheet at pH 4) | 1.9 kDa | Cationic: pI = 10.6<br>Bacillus megaterium (1.9 μM)<br>Bacillus subtilis (15 μM)<br>Staphylococcus aureus (>20 μM)<br>Micrococcus luteus (12.8 μM)<br>Pseudomonas aeruginosa (>20 μM)<br>Escherichia coli (15 μM)<br>Proteus vulgaris (>20 μM)<br>Shigella flexneri (15 μM)<br><br>Antifungal:<br>Candida albicans (6.3 μM)<br>Trichosporon beigelli (6.3 μM)<br>Malassezia furfur (12.5 μM)<br>Trichophyton rubrum (25 μM) | [180, 182] |
| | | | | | |
| | Shrimp (<em>Fenneropenaeus chinensis</em>) | FEVLPNKHQVKPNHGEHHHH (termed FCHc-C2) LVVAVTDGEADAAVEGLHNDIHFHGYSHGKYPDNPQPHGYPLD | 4.9 kDa | Cationic: pI = 7.98<br>Micrococcus luteus (1.3–13 μM)<br>Aeromonas hydrophila (10.6–26 μM)<br>Pseudomonas aeruginosa (13–26 μM)<br>Vibrio anguillarum (13–26 μM)<br><br>Antifungal:<br>Botrytis cinerea (1.3–21.2 μM)<br>Colletotrichum orbiculare (1.1–2.6 μM)<br>Fusarium oxysporum (10.6–26 μM)<br>Pestalotia diospyri (>21.2 μM)<br>Pythium ultimum (>21.2 μM)<br>Sclerotinia sclerotiorum (>21.2 μM) | [179] |
| | | | | | |
| Organism (**Oycypodes** vannamei) | Conformational state | Size | Charge | Activity (MIC or LD50)* | References |
|----------------------------------|----------------------|------|--------|-------------------------|------------|
| Shrimp (**Litopenaeus vannamei**) | FEDLPNGH1QKVFN1GEHIHH (termed PvHCt) (PDB; 2N1C) | 2.76 kDa | Anionic | **Antifungal:** | [177, 178] |

| Organism | Conformational state | Size | Charge | Activity (MIC or LD50)* | References |
|----------|----------------------|------|--------|-------------------------|------------|
| Shrimp (**Penaeus stylirostris**) | LVVARTDGDA5SVNLHENTEHNGSHVY | 8.3 kDa | Anionic | **Antifungal:** | [177] |
|          | VTDGDAVSNLHEHTEYHSNHGYPDK | 7.9 kDa | Anionic | **Antifungal:** | |

| Snail (**Helix aspersa**) | Functional Unit-H | ~60 kDa | **Antibacterial:** | [186] |

| Spider (**Acanthocurria rondoniae**) | IIQYEGKH (termed rondonin) | 1.2 kDa | Cationic | **Antifungal:** | [181] |

| Whelk (**Rapana venosa**) | Functional Units B and E | ~50 kDa | Anionic | **Antibacterial:** | [185] |

|                  | ELVRKNVDHI1SLPVDYELV | ~50 kDa | Anionic | **Antibacterial:** | |

94 % coverage and 72 % identity with **Helix lucorum** Hc
73 % coverage and 70 % identity with **Rapana venosa** Hc2 FU-e;

* Minimum inhibitory concentration (MIC) or LD50 values were not available for all (poly) peptides listed
Therefore, Hc circulating freely in the haemolymph is an immediate source of immune mediators. Although diverse antimicrobial and antiviral properties of mollusc Hc oligomers and several FUs have been noted [21, 184–186], mollusc Hc-derived peptides have received little attention. An interrogation of whelk (*Rapana venosa*) hemolymph led to the identification of a peptide with sequence similarities to a conserved motif at the N-terminus of many Hc FUs [185]. This whelk Hc-derived peptide did not inhibit the growth of *S. aureus* or *Klebsiella pneumoniae*; therefore, its physiological function is a mystery. An in silico study performed on abalone (*Haliotis tuberculata*) Hc indicated the region between the α-helical and β-sandwich domains of FU-e contained encrypted AMPs [187]. A number of synthetic polypeptides resembling this region were antagonistic towards *Erwinia carotovora* and *B. subtilis* in vitro (Table 4). Membrane perturbations visible in electron micrographs of Hc-treated bacteria suggested the amphipathic peptide may act as a pore former. Predictive modelling of the strongest bioactive peptide (termed haliotisin; Table 4) revealed a linear, α-helical structural conformation. Moreover, the peptide is positioned at the surface of the Hc protomer and is flanked by a series of trypsin and chymotrypsin cleavage sites, making it highly accessible during sepsis [187].

So far, no cysteine residues or disulphide bridges were found within any known Hc-derived AMP. This may be advantageous since the peptides can bind more easily to pathogens and be transported more readily through a membrane into the interior, rather than a stiff peptide. Each peptide contains at least three (up to eight) positively charged residues (H, R, K) and differ substantially in their

---

**Fig. 5** Arthropod hemocyanin-derived antimicrobial peptides. The crystal structure of *Panulirus interruptus* hemocyanin (PDB 1HC1) is used to illustrate the location of the encrypted peptides: PvHCT (FEDLPFHIQVKVFNNGHIIH: blue), astacidin I (FKQONQHQQVKKFHH: blue) from crayfish, and PshC2 (LVVAVTDGADAVNLHENTEFHYGSHGQVY: orange) from shrimp on the hemocyanin hexamer (~420 kDa) and corresponding subunit (~70 kDa). Hemocyanin subunit domains I and II are coloured green and purple, respectively. Both peptides are located on the C-terminal subunit (III) of the hemocyanin where they can be liberated through proteolysis. The shrimp peptide (PvHCT) is linear, α-helical and amphipathic, with an overall net-negative charge (theoretical pI = 6.1) as revealed by NMR. The electrostatic surface potential was calculated using UCSF Chimera [202]. PvHCT was isolated from *Litopenaeus vannamei* hemolymph and displays strict fungicidal activity. Both peptides are exposed to the environment even in the hexameric aggregation state. See also [21, 183].
net electrical charges, \( pI = 4-11 \) (Table 4; Fig. 5). The mode of action of Hc-derived AMPs may not be restricted to pore formation, as evidence discussed here hints to a possible role interfering with subcellular organelles.

**Hemerythrin and innate immunity**

Hr functionality is poorly characterised when compared to Hb and Hc. Nevertheless, members of the Hr gene family, including myoHr, participate in respiration, heavy metal detoxification and aspects of innate immunity [188]. Responding to septic shock caused by *E. coli* and *Micrococcus luteus*, Hr expression increased significantly in the leech, *Hirudo medicinalis* [189]. Using a 2-DIGE approach, newly synthesised Hr accumulated within tissues of the central nervous system, referred to as neurohemerythrin. Hr expression was also spatially distributed in peripheral tissues such as muscle, the walls of blood vessels and nephridia (an excretory organ analogous to vertebrate kidneys). The dual functionality of Hr in leech immunity was contemplated; provision of oxygen to fuel the metabolic costs of immune activity, and the sequestration of iron needed by microbes to grow [189].

Metalloprotein II (MPII) is an antibacterial protein found in the coelomic fluid of *Hediste (Nereis) diversicolor* and other polychaetes [190–192]. It is a cadmium binding protein related to the Hr family, ~81 % similar to myoHr [193, 194], and can be produced within specialised coelomocytes (granulocytes type I), somatic muscle cells and the lining of the gut [188, 195]. MPII and myoHr are monomeric isoforms of Hr subunits, displaying almost identical structural architecture (four \( \alpha \)-helix bundle). Upon immune stimulation with intact microbes (*Vibrio alginolyticus, E. coli* and *M. luteus*) or endotoxins, MPII is expelled into the coelomic fluid by granulocytes type I [192]. Concurrently, the enzyme PO is released by granulocytes type II. MPII likely provides the oxygen needed to catalyse phenol hydroxylation/oxidation, thereby facilitating the eventual biogenesis of melanin. This is quite interesting as Hc and Hb are also involved in converting phenols into (semi)-quinone derivatives (see previous sections). The antibacterial properties of MPII are disrupted in the presence of iron or when the coelomic fluid is pre-treated with specific antibodies raised against MPII [192]. Certain sipunculids contain differential coelomocytes (pink blood cells) that express variants of Hr [5, 196]. Depending on their location within the body, cell-specific Hrs bind oxygen with varying affinities: low, moderate and high. Coelomocyte hematopoiesis following severe blood loss (exsanguination) in the peanut worm, *Phascolosoma esculenta*, accompanies the de novo synthesis of Hr [53].

Isoforms of Hr and myoHr have also been found in the salivary complex of the hematophagous leech, *Haementeria depressa*, and during anterior tissue regeneration of the earthworm, *Perionyx excavatus* [197, 198]. These data signify Hr is a multi-functional protein extending beyond its traditional role as a vehicle for molecular oxygen.

In 2011, a 10-kDa polypeptide was extracted from the exoskeleton of a baculovirus-infected crustacean, *Pleuromyconodes planipes* [199]. Protein extracts were capable of inhibiting up to 99.5 % of polyhedrosis nuclear virus replication. An acidic region of this polypeptide, VFYANLDEEHK, shared 100 % coverage and 91–100 % amino acid sequence identity with Hr-like protein subunits from annelids, *Scoloplos armiger* (Accession no. XP_013415662) and *H. medicinalis* (Accession no. Q674M7), and a brachiopod, *Lingula anatina* (Accession no. CAP08294). The authors compared their polypeptide to myoHr, yet in the absence of a known Hr within the Crustacea, caution and further information are required before categorising this protein as an immune effector.

The use of Hr as a defence strategy is not only employed by metazoan hosts. The aquatic pathogen, *Aeromonas hydrophila*, produces a single-domain Hr when inside the cytosol of Japanese eel macrophages to sense O\(_2\) and detoxify ROS [200]. Differential expression of Hr under extreme O\(_2\) conditions (hypoxic, hyperoxic) was recorded in wild-type *A. hydrophila*. The bacterium was also able to cope with high-level exposure to H\(_2\)O\(_2\). Disruption of the Hr gene in *A. hydrophila* mutants (M85) led to a 77 % reduction in survival when incubated with macrophages. Virulence of *A. hydrophila* is dependent on its ability to escape phagosomes using flagellar movements [201]. No differences in motility were found between the wild-type and Hr mutants; therefore, Hr is an important factor in *A. hydrophila* pathogenesis.

**Further considerations**

In many vertebrate and invertebrate systems, the presence of pathogens and parasites in the blood/hemolymph can lead to the de novo synthesis of OTPs (Hb, Hc and Hr). Extracellular proteases that are secreted by microbes target Hb and Hc, leading to the production of ROS/RNS, the conversion of phenolic substrates and release of encrypted immune peptides. The activities of OTP cryptides do not depend on their respective metal prosthetic groups. Hb in the absence of the heme cofactor retains its antimicrobial potency, and likewise, all Hc-derived AMPs originate from the C-terminal domains of arthropod Hc subunits and mollusc Hc functional units where they are not influenced by the distant dicopper centres located within the \( \alpha \)-helical structural domain.
For each OTP there are many bona fide enzymes employing heme, di-iron or dicopper catalytic units: Hb—peroxidase and cytochrome P450; Hc—tyrosinase and ascorbate oxidase; Hr—ribonucleotide reductase and methane mono-oxygenase [4]. Therefore, is enzymatic activity or altered functionality of Hb/Hc/Hr a coincidence of subtle structural rearrangements or interchangeable roles in respiration, detoxification and immunity? A link between OTPs and immunity may be the mitigation or remediation of damage incurred by the host when mounting an immune response. RNS and ROS are equally harmful to the host as they are to microbes, highlighting the essential need for their production to be tightly regulated.

The evolution of OTPs is likely influenced by two co-evolving systems: (1) optimising the precise delivery of dioxygen and storing it within cells (i.e. respiration), and (2) serving as an immediate and efficacious anti-infective agent (i.e. immunity).

Acknowledgments We would like to thank Swansea University (Biosciences) and the Immunology Centre, Mainz for their continued support.

Compliance with ethical standards

Conflict of interest We declare that no conflicts of interest, financial or other, exist.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

1. Jonsson K, Hunt TK, Mathes SJ (1988) Oxygen as an isolated variable influences resistance to infection. Ann Surg 208:783–787
2. Schreml S, Szeimies RM, Prantl L, Landthaler M, Babillas P (2010) Oxygen in acute and chronic wound healing. Brit J Dermatol 163:257–268
3. Allen DB, Maguire JJ, Mahdavian M, Wicke C et al (1997) Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms. Arch Surg 132:991–996
4. Decker H, van-Holde KE (2010) Oxygen and the Evolution of Life. Springer, New York
5. Terwilliger NB (1998) Functional adaptations of oxygen-transport proteins. J Exp Biol 201:1085–1098
6. Royer WE, Strand K, van Heel M, Hendrickson WA (2000) Structural hierarchy in erythrocruorin, the giant respiratory assemblage of annelids. PNAS 97:7107–7111
7. Pallavicini A, Negrisolero E, Barbator R, Dewilde S, Ghiretti-Ma-gald A, Moens L, Lanfranchi G (2001) The primary structure of globin and linker chains from the chlorocruorin of the polychaete Sabella spallanzanii. J Biol Chem 276:26384–26390
8. Riggs AF, Riggs CK (2014) The self-association of the giant hemoglobin from the earthworm, Lumbricus terrestris. Biochem Biophys Acta 1844:1071–1075
9. Hobson D, Hirsch JG (1958) The antibacterial activity of hemoglobins. J Exp Med 107:167–183
10. Burmester T, Hankeln T (2014) Function and evolution of vertebrate globins. Acta Physiol 211:501–514
11. Beutler E, Waalen J (2006) The definition of anemia: what is the lower limit of normal of the blood hemoglobin concentration? Blood 107:1747–1750
12. Yuan Y, Tam MF, Simplaceanu V, Ho C (2015) New look at hemoglobin allostery. Chem Rev 115:1702–1724
13. Wittenberg JB, Wittenberg BA (2003) Myoglobin function reassessed. J Exp Biol 206:2011–2020
14. Vinogradov SN (1985) The structure of invertebrate extracel-lular hemoglobins (erythrocruorins and chlorocruorins). Comp Biochem Physiol Part B 82:1–15
15. Ruggiero Bachega J, Vasconcelos Maluf F, Andi B, D’Muniz Pereira H, Falsarella Carazzolle M, Orville AM, Tabak M, Brandão-Neto J, Garratt RC, Horjales Reboredo E (2015) The structure of the giant haemoglobin from Glossococcox paulistus. Acta Crystallogr D Biol Crystallogr 71:1257–1271
16. Van-Holde KE, Miller KL, Decker H (2001) Hemocyanins and invertebrate evolution. J Biol Chem 276:15563–15566
17. Markl J, Decker H (1992) Molecular structure of the arthropod hemocyanins. In Blood and tissue oxygen carriers (pp. 325–376). Springer Berlin Heidelberg
18. Hellmann N, Weber RE, Decker H (2003) Nested allosteric interactions in extracellular hemoglobin of the leech Macrob-della decora. J Biol Chem 278(45):44355–44360
19. Menze MA, Hellmann N, Decker H, Grieshaber MK (2005) Allosteric models for multimeric proteins: oxygen-linked effector binding in hemocyanin. Biochem 44:10328–10338
20. Coates CJ, Bradford EL, Krome C, Nairn J (2012) Effect of temperature on biochemical and cellular properties of captive Limulus polyphemus. Aquaculture 334–337:30–38
21. Coates CJ, Nairn J (2014) Diverse immune functions of hemo-cyanin. Dev Comp Immunol 45:44–55
22. Nagai T, Osaki T, Kabawata S (2001) Functional conversion of hemocyanin to phenoloxidase by horseshoe crab antimicrobial peptides. J Biol Chem 276:27166–27170
23. Lee SY, Lee BL, Söderhäll K (2004) Processing of crayfish hemocyanin subunits into phenoloxidase. Biochem Biophys Res Comm 322:490–496
24. Jiang N, Tan NS, Ho B, Ding JL (2007) Respiratory protein-mediated reactive oxygen species as an antimicrobial strategy. Nat Immunol 8:1114–1122
25. Hristova R, Dolashki A, Voelter W, Stevanovic S, Dolashka-Angelova P (2008) o-Diphenol oxidase activity of molluscan hemocyanins. Comp Biochem Physiol Part B 149:439–446
26. Dolashka-Angelova P, Lieb B, Velkova L et al (2009) Identification of glycosylated sites in Rapana Hemocyanin by mass spectrometry and gene sequence, and their antiviral effect. Bioconjug Chem 20:1315–1322
27. Guo D, Zhang Y, Zeng D, Wang H, Li X, Li Y, Fan X (2009) Functional properties of hemocyanin from Oncomelania hupensis the intermediate host of Schistosoma japonicum. Exp Parasitol 123:277–281
28. Coates CJ, Kelly SM, Nairn J (2011) Possible role of phosphatidyserine-hemocyanin interaction in the innate immune response of Limulus polyphemus. Dev Comp Immunol 35:155–163
29. Coates CJ, Whalley T, Wyman M, Nairn J (2013) A putative link between phagocytosis-induced apoptosis and hemocyanin-derived phenoloxidase activation. Apoptosis 18:1319–1331
Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin

30. Schenk S, Schmidt J, Hoeger U, Decker H (2015) Lipoprotein-induced phenoloxidase activity in tarantula hemocyanin. Biochim Biophys Acta 1854:939–949
31. Lu X, Lu H, Guo L, Zhang Z et al (2015) Cloning and characterisation of a novel hemocyanin variant LvhMHCV4 from shrimp Litopenaeus vannamei. Fish Shellfish Immunol 46:398–405
32. Sangaard KW, Dyrlund TF, Bechsgaard JS, Scavenius C, Wang T, Bilde T, Enghild JJ (2016) The spider hemolymph clot proteome reveals high concentrations of hemocyanin and von Willebrand factor-like proteins. Biochim Biophys Acta 1864:233–241
33. Markl J (2013) Evolution of molluscan hemocyanin structures. Biochim Biophys Acta 1834:1840–1852
34. Gai Z, Matsuno A, Kato K, Kato S, Khan MRI, Shimizu T et al (2015) Crystal Structure of the 3.8-MDa Respiratory Superoxide Resistant Hemocyanin at 3.0 Å Resolution. Structure 23:2204–2212
35. Gatsogiannis C, Moeller A, Depoix F, Meissner U, Markl J (2007) Nautilus pompilius hemocyanin: 9 Å cryo-EM structure and molecular model reveal the subunit pathway and the interfaces between the 70 functional units. J Mol Biol 374:465–486
36. Gatsogiannis C, Hofnagel O, Markl J, Raunser S (2015) Structure of mega-hemocyanin reveals protein origins in snails. Structure 23:93–103
37. Arancibia S, Campo MD, Nova E, Salazar F, Becker MI (2012)
38. Zheng L, Zhao X, Zhang P, Chen C, Liu S, Huang R et al (2016)
39. Guncheva M, Paunova K, Ossowicz P, Rozwadowski Z, Janus E, Idakieva K et al (2015) Modification of Rapana thomaisiana hemocyanin with choline amino acid salts significantly enhances its antiproliferative activity against MCF-7 human breast cancer cells. RSC Adv 5:63345–63354
40. Guncheva M, Paunova K, Ossowicz P, Rozwadowski Z, Janus E, Idakieva K et al (2016) Rapana thomaisiana hemocyanin modified with ionic liquids with enhanced anti breast cancer activity. Int J Mol Sci 17:2390–2395
41. Zanjani TN, Miranda-Saksena M, Valtchev P, Diefenbach RJ, Lu X, Lu H, Guo L, Zhang Z et al (2015) Cloning and characterisation of a novel hemocyanin variant LvHMCV4 from shrimp Litopenaeus vannamei. Fish Shellfish Immunol 46:398–405
42. Moltedo B, Faunes F, Haussmann D, De Ioannes P, De Ioannes E, Matsunaga T, Zhong TY et al (2014) A Novel Immunomodulatory Hemocyanin from the Limpet Fissurella latimarginata. Eur J Immunol 42:688–699
43. Antonova O, Dolashka P, Toncheva D, Ramensske HG, Fiootenmeyer M, Stenkar RE (1994) Dioxygen and Hemerythrin. Chem Rev 94:715–726
44. Sabbatini PJ, Ragupathi G, Hood C, Aghajanian CA, Juretzka M, Iasonos A et al (2007) Pilot study of a heptavalent vaccine-antigen and molecular model reveal the subunit pathway and the interfaces between the 70 functional units. J Mol Biol 374:465–486
45. Gesheva V, Chausheva S, Mihaylova N, Manolyov I, Doumanova L, Idakieva K, Tchorbanov A (2015) Anti-cancer properties of gastropodan hemocyanins in murine model of colon carcinoma. BMC Immunol 15:34. doi:10.1186/s12864-014-0034-3
46. Gesheva V, Chausheva S, Stefanova N, Mihaylova N, Doumanova L, Idakieva K, Tchorbanov A (2015) Helix pomatia hemocyanin—A novel bio-adjuvant for viral and bacterial antigens. Int Immunopharmacol 26:162–168
47. Stenkamp RE (1994) Dioxygen and Hemerythrin. Chem Rev 114:715–726
48. Bai L, Vanin S, Chabasse C, Mizuguchi K, Vinogradov SN (2008) A phylogenomic profile of hemerythrin, the nonheme diiron binding respiratory proteins. BMC Evol Biol 8:244
49. Meyer A, Lieb B (2010) Respiratory proteins in Sipunculus nudus – Implications for phylogeny and evolution of hemerythrin family. Comp Biochem Physiol B 155:171–177
50. Liu Y, Li C, Su X, Wang M, Li Y, Li Y, Li T (2013) Cloning and characterisation of hemerythrin gene from Sipuncula Phascolosoma esculentum. Genes Genom 35:95–100
51. Antonova O, Dolashka P, Toncheva D, Ramensske HG, Flootenmeyer M, Stefanovic S (2014) In vitro antiproliferative effect of Helix aspersa hemocyanin on multiple malignant cell lines. Z Naturforsch C. 69:325–334
52. Moltedo B, Faunes F, Haussmann D, De Ioannes P, De Ioannes E, Idakieva K et al (2016) Anti-cancer properties of gastropodan hemocyanins in murine model of colon carcinoma. BMC Immunol 15:34. doi:10.1186/s12864-014-0034-3
53. Liu Y, Li C, Su X, Wang M, Li Y, Li Y, Li T (2013) Cloning and characterisation of hemerythrin gene from Sipuncula Phascolosoma esculentum. Genes Genom 35:95–100
54. Okamoto Y, Onoda A, Sugimoto H, Takano Y, Hirota S, Kurtz DM Jr, Shiro Y, Hayashi T (2013) Crystal structure, exogenous ligand binding, and redox properties of an engineered diiron active site in a bacterial hemerythrin. Inorg Chem 52:13014–13020
55. Friesner RA, Baik MH, Gherman BF, Guallar V, Wirstam M, Murphy RB, Lippard SJ (2003) How iron-containing proteins control dioxygen chemistry: a detailed atomic level description via accurate quantum chemical and mixed quantum mechanics/molecular mechanics calculations. Coord Chem Rev 238–239:267–290
56. French CE, Bell JML, Ward FB (2008) Diversity and distribution of hemerythrin-like proteins in prokaryotes. FEMS Microbiol Lett 279:131–145
57. Manwell C, Baker CMA (1988) Magelona haemerythrin: tissue specificity, molecular weights and oxygen equilibria. Comp Biochem Physiol Part B 92:453–463
58. Zanjani TN, Miranda-Saksena M, Valtchev P, Diefenbach RJ, Hueston L, Diefenbach E et al (2016) Abalone hemocyanin blocks the entry of herpes simplex virus 1 into cells. Antimicrob Agents Chemother 60:1003–1012
59. Molteno B, Faunes F, Haussmann D, De Joannes P, De Joannes AE, Puente J, Becker MI (2006) Immuno-therapeutic effect of Concholepas hemocyanin in the murine bladder cancer model: evidence for conserved antitumor properties among hemocyanins. J Urol 176:2690–2695
60. Swartz AM, Li QJ, Sampson JH (2014) Rindopepimut: a promising immuno-therapeutic for the treatment of glioblastoma multiforme. Immunotherapy 6:679–690
61. Sabbatini PJ, Ragupathi G, Hood C, Aghajanian CA, Juretzka M, Iasonos A et al (2007) Pilot study of a heptavalent vaccine-keyhole limpet hemocyanin conjugate plus QS21 in patients with epithelial ovarian, fallopian tube, or peritoneal cancer. Clin Cancer Res 13:4170–4177
62. Miles D, Rocchi H, Martin M, Perren TJ, Cameron DA, Glasy J, Murray JL (2011) Phase III multicenter clinical trial of the siylation-TN (STn)-keyhole limpet hemocyanin (KLH) vaccine for metastatic breast cancer. Oncologist 16:1092–1100
63. Zheng L, Zhao X, Zhang P, Chen C, Liu S, Huang R et al (2016) Hemocyanin from Shrimp Litopenaeus vannamei Has Antiproliferative Effect against HeLa Cell In Vitro. PLoS ONE 11(3):e0151801
64. Antonova O, Dolashka P, Toncheva D, Ramensske HG, Flootenmeyer M, Stefanovic S (2014) In vitro antiproliferative effect of Helix aspersa hemocyanin on multiple malignant cell lines. Z Naturforsch C. 69:325–334
65. Antoinette F, Chausheva S, Mihaylova N, Manolyov I, Doumanova L, Idakieva K, Tchorbanov A (2014) Anti-cancer properties of gastropodan hemocyanins in murine model of colon carcinoma. BMC Immunol 15:34. doi:10.1186/s12864-014-0034-3
66. Atkinson SH, Uyoga SM, Nyatichi E, Macharia AW, Nyutu G, Mwangi K, Ndila et al (2014) Epistasis between the haptoglobin common variant and inflammation genes has a role in the pathogenesis of type 2 diabetes mellitus. PLoS ONE 9(1):e87240
67. Lin T, Sammy F, Yang H, Thundivakkal S, Hellman J, Tracey KJ, Warren HS (2012) Identification of hemopexin as an anti-inflammatory factor that inhibits synergy of hemoglobin with HMGB1 in sterile and infectious inflammation. J Immunol 189:2017–2022
68. Schaer DJ, Buehler PW, Alayash AI, Belcher JD, Vercellotti GM (2013) Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. Blood 121:1276–1284
65. Schaer DJ, Alayash AI, Buehler PW (2007) Gating the radical hemoglobin to macrophages: the anti-inflammatory role of CD163, a scavenger receptor. Antioxid Redox Signal 9:991–999
66. Vanhollebeke B, De Muylder G, Nielsen MJ, Pays A, Tebabi P, Dieu M, Raes M, Moestrup SK, Pays E (2008) A haptoglobin-hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. Science 320:677–681
67. Alayash AI, Patel RP, Cashon RE (2001) Redox reactions of hemoglobin and myoglobin: biological and toxicological implications. Antioxid Redox Signal 3:313–327
68. Lee SK, Goh SY, Wong YQ, Ding L (2015) Response of neutrophilic macrophages to extracellular hemoglobin on the innate immune system. DNA Cell Biol 34:36–40
69. Goj AW, Luchsinger BP, Pawloski JR, Singel DJ, Stamler JS (1999) The oxyhemoglobin reaction of nitric oxide. PNAS 96:9027–9032
70. Auten RL, Davis JM (2009) Oxygen toxicity and reactive oxygen species: the devil is in the details. Pediatric Res 66:121–127
71. James SL, Glaven J (1989) Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. J Immunol 143:4208–4212
72. Hahn UK, Bender RC, Bayne CJ (2001) Involvement of nitric oxide in killing of *Schistosoma mansoni* sporocysts by hemocytes from resistant * Biomphalaria glabrata*. J Parasitol 87:778–785
73. Ascenzi P, Fasano M, Gradoni L (2002) Do hemoglobin and hemocyanin impair *Schistosoma* killing by NO? IUBMB Life 53:287–288
74. Schwarzew, Zurrini F, Ulliers D, Giribaldi G, Ginsburg H, Arese P (1992) Impairment of macrophage functions after ingestion of Plasmodium falciparum-infected erythrocytes or isolated malarial pigment. J Exp Med 176:1033–1041
75. Schwarzew, Zurrini F, Ulliers D, Arese P (1998) Phagocytosis of the malarial pigment, hemoglobin, impairs expression of major histocompatibility complex class II antigen, CD54, and CD11c in human monocytes. Infect Immun 66:1601–1606
76. Akira S, Hemmi H (2003) Recognition of pathogen-associated molecular patterns by TLR family. Immuno Let 85:85–95
77. Scheckter AN (2008) Hemoglobin research and the origins of molecular medicine. Blood 112:3927–3938
78. Soares MP, Bozza MT (2016) Red alert: labile heme is an alarmin. Curr Opin Immunol 38:94–100
79. Yang H, Wang H, Bernik TR, Ivanova S, Uolloa L, Roth J, Eaton JW, Tracey KJ (2002) Globin attenuates the innate immune response to endotoxin. Shock 17:485–490
80. Du R, Ho B, Ding JL (2010) Rapid reprogramming of haemoglobin structure-function exposes multiple dual-antimicrobial potencies. EMBO J 29:632–642
81. Lee SK, Goh SY, Wong YQ, Ding L (2015) Response of neutrophils to extracellular hemoglobin and LTA in human blood system. EBioMedicine 2:225–233
82. Bahl N, Du R, Winarsh I, Ho B, Tucker-Kellogg L, Tidor B, Ding JL (2011) Delineation of lipopolysaccharide (LPS)-binding sites on hemoglobin: from in silico predictions to biophysical characterization. J Biol Chem 286:37793–37803
83. Lisk C, Kominsky D, Ehrentraut S, Bonaventura J, Nuss R, Hassell K et al (2013) Hemoglobin-induced endothelial cell permeability is controlled, in part, via a myeloid differentiation primary response gene-88-dependent signalling mechanism. Am J Respir Cell Mol Biol 49:619–626
84. Bahl N, Winarsh I, Tucker-Kellogg L, Ding JL (2014) Extracellular hemoglobin upregulates and binds to tissue factor on macrophages: implications for coagulation and oxidative stress. Thromb Haemost 111:67–78
85. Liu L, Zeng M, Stamler JS (1999) Hemoglobin induction in mouse macrophages. PNAS 96:6643–6647
86. Newton DA, Rao KMK, Dluhy RA, Baatz JE (2006) Hemoglobin is expressed by alveolar epithelial cells. J Biol Chem 281:5668–5676
87. Mak P (2008) Hemocidins in a functional and structural context of human antimicrobial peptides. Frontiers Biosci 13:6859–6871
88. Mak P, Wojcik K, Silberring J, Dubin A (2000) Antimicrobial peptides derived from heme-containing proteins: Hemocidins. Antonie van Leeuwenhoek 77:197–207
89. Parish CA, Jiang H, Tokiwa Y, Berova N et al (2001) Broad-spectrum antimicrobial activity of hemoglobin. Bioinorg Med Chem 9:377–382
90. Ivanov VT, Karelisin AA, Philippova MM, Nazimov IV, Pletnev VZ (1997) Hemoglobin as a source of endogenous bioactive peptides: The concept of tissue-specific peptide pool. Peptide Sci 43(2):171–188
91. Fogaca AC, da Silva PL, Miranda MT et al (1999) Antimicrobial activity of a bovine hemoglobin fragment in the Tick *Boophilus microplus*. J Biol Chem 274:25330–25334
92. Nakajima Y, Ogihara K, Taylor D, Yamakawa M (2003) Antibacterial hemoglobin fragments from the midgut of the soft tick, *Orophtherodoris moubata* (Acari: Argasidae). J Med Entomol 40:78–81
93. Sforca ML, Machado A, Figueredo RCR, Oyama S Jr, Silva FS, Miranda A, Daffre S, Miranda MT, Spisni A, Pimenta TE (2005) The micelle-bound structure of an antimicrobial peptide derived from the a-chain of bovine hemoglobin isolated from the tick *Boophilus microplus*. Biochem 44:6440–6451
94. Liepke C, Baxmann S, Heine C et al (2003) Human hemoglobin-derived peptides exhibit antimicrobial activity: a class of host defense peptides. J Chromat B 791(1):345–356
95. Mak P, Wojcik K, Worchek J, Sudder P, Dubin A (2004) Antibacterial peptides in human menstrual blood. Peptides 25:1839–1840
96. Mak P, Worchek L, Sudder P, Dubin A, Banas T, Kaim I, Klimk M (2006) Analysis of free hemoglobin level and hemoglobin peptides from human puerperal uterine secretion. J Soc Gynecol Invest 13:285–391
97. Mak P, Siwek M, Pohl J, Dubin A (2007) Menstrual hemocidins Hb115-146 is an acidophilic antibacterial peptide potentiating the activity of human defensins, cathelicidin and lysozyme. Am J Reprod Immunol 57:81–91
98. Deng L, Pan X, Wang Y, Wang L, Zhou X, Li M, Feng Y, Wu Q, Wang B, Huang N (2009) Hemoglobin and its derived peptides may play a role in the antibacterial mechanism of the vagina. Human Repro 24:211–218
99. Bochenska O, Rapala-Kozik M, Wolak N et al (2013) Secreted aspartic peptidases of *Candida albicans* liberate bactericidal hemocidins from human hemoglobin. Peptides 48:49–58
100. Froidevaux R, Krier F, Nedjar-Aroume N et al (2001) Antimicrobial activity of a pepsin-derived bovine hemoglobin fragment. FEBS Letts 491:159–163
101. Daoût D, Dubois V, Bors-Dodita L, Nedjar-Aroume N, Krier F, Chihb N-E, Mary P, Kouach M, Briand G, Guilochon F (2005) New antibacterial peptide derived from bovine hemoglobin. Peptides 26:713–719
102. Marchado A, Sforca ML, Miranda A, Daffre S, Pimenta TE, Spisni A, Miranda MT (2007) Truncation of the amidated fragment 33–61 of bovine a-hemoglobin: effects on the structure
Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin

104. Nedjar-Arroume N, Dubois-Delva V, Adje EY, Traisnel J, Krier F, Mary P, Kouach M, Briand G, Guillochen D (2008) Bovine hemoglobin: An attractive source of antibacterial peptides. Peptides 29:969–977

105. Carvalho LAC, Remuzgo C, Perez KR, Machini MT (2015) Hb40-61a: Novel analogues help expanding the knowledge on chemistry, properties and candidacidal action of this bovine α-hemoglobin-derived peptide. Biochem Biophys Acta 1848:3140–3149

106. Bashir T, Patgaonkar M, Kumar S, Pasi A, Venkata K, Reddy R (2015) Lactoferricin. Cell Mol Life Sci 72:472–479

107. Autelitano DJ, Rajic A, Smith AI, Berndt MC, Ilag LL, Vadas M (2006) The cryptome: a subset of the proteome, comprising cryptic peptides with distinct bioactivities. Drug Disc Today 11:306–314

108. Pimenta DC, Lebrun I (2007) Cryptides: Buried secrets in protein chemistry, properties and candidacidal action of this bovine α-hemoglobin-derived peptide. Biochem Biophys Acta 1726(1):102–114

109. Ner JH, Kotlinska J, Silberring J (2015) HbAHP-25, an in silico designed peptide, inhibits HIV-1 entry by blocking gp120 binding to CD4 receptor. PLoS ONE. doi:10.1371/journal.pone.0124839

110. Preecharram C, Monteiro-dos-Santos J, Seibert CS, Silva PI, Marques EE, Richardson M, Lopes-Ferreira M (2012) Potamotrygon cf. henlei stingray mucus: Biochemical features of a novel antimicrobial protein. Toxicon 60:821–829

111. Singh S, Thakur N, Oliver A, Petruk AA, Hade MD, Sethi D, Jearranaiprepame P, Barnes G, Osborne G, Kawasaki M, Barnes G, Bedin AS, Barnes G (2013) Resistance to hemoglobin: An attractive source of antibacterial peptides. PLoS ONE 8(6):e64268. doi:10.1371/journal.pone.0124839

112. Meloni M, Candusso S, Volpatti D (2015) Preliminary study on expression of antimicrobial peptides in European sea bass (Dicentrarchus labrax) following in vivo infection with Vibrio anguillarum. Fish Shellfish Immunol 43:82–90

113. Fan L, Wang A, Wu Y (2013) Comparative proteomic identification of the hemocyte response to cold stress in white shrimp, Litopenaeus vannamei. J Proteomics 80:196–206

114. Zhang DL, Guan RZ, Huang WS, Xiong J (2013) Isolation and characterization of antibacterial peptide derived from hemoglobin alpha in the liver of Japanese eel, Anguilla japonica. Fish Shellfish Immunol 35:625–631

115. Seo JK, Lee MJ, Jung HG, Go HJ, Kim YJ, Park NG (2014) Antimicrobial function of SHβAP, a novel hemoglobin β chain-related antimicrobial peptide, isolated from the liver of skipjack tuna, Katsuwonus pelamis. Fish Shellfish Immunol 37:173–183

116. Ullal AJ, Litaker RW, Noga EJ (2008) Antimicrobial peptides for the treatment of microbial infections. U.S. Patent No. 6,340,667. Washington, DC: U.S. Patent and Trademark Office

117. Ullal AJ, Noga EJ (2010) Antiparasitic activity of the antimicrobial peptide HbβP-1, a member of the β-hemoglobin peptide family. J Fish Dis 33:657–664

118. Dang C, Cribb TH, Osborne G, Kawasaki M, Bedin AS, Barnes AC (2013) Effect of a hemiuroid trematode on the hemocyte mRNA copy number in several tissues of marine sea bass (Dicentrarchus labrax). BMC Immunol 12(1):1

119. Dang C, Conlan SW, Cribb TH, Osborne G, Kawasaki M, Bedin AS, Barnes AC (2013) Effect of a hemiuroid trematode on the hemocyte mRNA copy number in several tissues of marine sea bass (Dicentrarchus labrax). BMC Immunol 12(1):1

120. Dang C, Cribb TH, Osborne G, Kawasaki M, Bedin AS, Barnes AC (2013) Effect of a hemiuroid trematode on the hemocyte mRNA copy number in several tissues of marine sea bass (Dicentrarchus labrax). BMC Immunol 12(1):1

121. Terova G, Cattaneo AG, Preziosa E, Bernardini G, Saroglia M (2011) Impact of acute stress on antimicrobial polypeptides mRNA copy number in several tissues of marine sea bass (Dicentrarchus labrax). BMC Immunol 12(1):1

122. Meloni C, Candusso S, Galeotti M, Volpatti D (2015) Preliminary study on expression of antimicrobial peptides in European sea bass (Dicentrarchus labrax) following in vivo infection with Vibrio anguillarum. Fish Shellfish Immunol 43:82–90

123. Dang C, Cribb TH, Osborne G, Kawasaki M, Bedin AS, Barnes AC (2013) Effect of a hemiuroid trematode on the hemocyte mRNA copy number in several tissues of marine sea bass (Dicentrarchus labrax). BMC Immunol 12(1):1
immune parameters of the cockle *Anadara trapezia*. Fish Shellfish Immunol 35:951–956

139. Bao Y, Wang Q, Lin Z (2011) Hemoglobin of the bloody clam *Tegillarca granosa* (Tg-Hbl) is involved in the immune response against bacterial infection. Fish Shellfish Immunol 31:517–523

140. Bao Y, Wang Q, Guo XM, Lin ZH (2013) Structure and immune expression analysis of hemoglobin genes from blood clam *Tegillarca granosa*. Gen Mol Res 12:3110–3123

141. Bao Y, Li P, Dong Y et al (2013) Polymorphism of the multiple hemoglobins in blood clam *Tegillarca granosa* and its association with disease resistance to *Vibrio paraahaemolyticus*. Fish Shellfish Immunol 34:1320–1324

142. Zhao X, Guo L, Zhang Y, Liu Y, Zhang X, Lun J, Chen J, Li Y (2012) SNPs of hemocyanin C-terminal fragment in shrimp *Litopenaeus vannamei*. FEBS Lett 586:403–410

143. Guo L, Zhao X, Zhang Y, Wang Z, Zhong M, Guo E, Li S, Lun J (2013) Evidences of SNPs in the variable region of hemocyanin Ig-like domain in shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 35:1532–1538

144. Bao Y, Wang J, Li C, Li P, Wang S, Lin Z (2016) A preliminary study on the antibacterial mechanism of *Tegillarca granosa* hemoglobin by derived peptides and peroxidase activity. Fish Shellfish Immunol 51:9–16

145. Xu B, Zhao J, Zhao J, Zhang Y, Shi Y, Fan T (2015) Role of hemoglobin from blood clam *Scapharca kagoshimensis* beyond oxygen transport. Fish Shellfish Immunol 44:248–256

146. Muñoz P, Meseguer J, Esteban MÁ (2006) Phenoloxidase activity in three commercial bivalve species. Changes due to natural microevolution with *Perkinsus atlanticus*. Fish Shellfish Immunol 20:12–19

147. Hellio C, Bado-Nilles A, Gagnaire B, Renault T, Thomas-Guyon H (2007) Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster. *Crassostrea gigas* (Thunberg) in vitro. Fish Shellfish Immunol 22:433–440

148. Zhang Q, Xu Y, Wang Q, Bang H, Sun Y, Wei X, Hu J (2015) Potential of novel antimicrobial peptide P3 from bovine erythrocytes and its analogs to disrupt bacterial membranes *in vitro* and display activity against drug-resistant bacteria in a mouse model. Antimicrob Agents Chem 59:2835–2841

149. Belmonte R, Cruz CE, Pires JR, Daffre S (2012) Purification and characterization of Hb 98-114: A novel hemoglobin-derived antimicrobial peptide from the midgut of *Rhipicephalus (Boophilus) microplus*. Peptides 37:120–127

150. Hu J, Xu M, Hang B, Wang L, Wang Q, Chen J, Song T, Fu D, Wang Z, Wang S, Liu X (2011) Isolation and characterization of an antimicrobial peptide from bovine hemoglobin α-subunit. World J Microbiol Biotechnol 27:767–771

151. Mak P, Szewczyk A, Mickowska B, Kicinska A, Dubin A (2001) Effect of antimicrobial apomyoglobin 56–131 peptide on liposomes and planar lipid bilayer membrane. *Int J Antimicrob Agents* 17:137–142

152. Cong Y, Zhang Q, Woolford D et al (2009) Structural mechanism of SDS-induced enzyme activity of scorpion hemocyanin revealed by electron cryomicroscopy. Structure 17:749–758

153. Cerdenius L, Lee BL, Söderhäll K (2008) The pro-PO system: pros and cons for its role in invertebrate immunity. Trends Immunol 29:263–271

154. Sánchez-Peñar A, Rodríguez-López JN, García-Cánovas F, García-Carmona F (1995) Tyrosinase: a comprehensive review of its mechanism. Biochim Biophys Acta 1247:1–11

155. Decker H, Nillius Schweikhardt T et al (2007) Similar enzyme activation and catalysis in hemocyanins and tyrosinases. Gene 398:183–191

156. Itoh S, Fukuzumi S (2007) Monoxygenase activity of type 3 copper proteins. *Accounts Chem Res* 40:592–600

157. Rolf M, Schottenheim J, Decker H, Tuczak F (2011) Copper–O2 reactivity of tyrosinase models towards external monophenolic substrates: molecular mechanism and comparison with the enzyme. *Chem Soc Rev* 40:4077–4098

158. Adachi K, Wakamatsu K, Ito S et al (2005) An oxygen transporter hemocyanin can act on the late pathway of melanin synthesis. *Pigm Cell Res* 19:214–219

159. Goldfeder M, Kanteev M, Isaschar-Ovdat S, Adir N, Fishman A (2014) Determination of tyrosinase substrate-binding modes reveals mechanistic differences between type-3 copper proteins. Nat Comm 5

160. Kanteev M, Goldfeder M, Fishman A (2015) Structure-function correlations in tyrosinases. Protein Sci 24:1360–1369

161. Solem E, Tuzcek F, Decker H (2016) Tyrosinase versus Catechol Oxidase: One Asparagine Makes the Difference. *Angew Chem Int Ed* 55:1–6

162. Decker H, Rimke T (1998) Tarantula hemocyanin shows phenoloxidase activity. J Biol Chem 273:25889–25892

163. Decker H, Ryan M, Jaenicke E, Teerwilger N (2001) SDS-induced phenoloxidase activity of hemocyanins from *Limulus polyphemus*, *Eurypelta californicum* and *Cancer magister*. J Biol Chem 276:17796–17799

164. Paul R, Bergner B, Pfeffer-Seidl A, Decker H, Efinger R, Storz H (1994) Gas transport in the haemolymph of arachnids-oxygen transport and the physiological role of haemocyanin. J Exp Biol 188:25–46

165. Adachi K, Endo H, Watanabe T, Nishioka T, Hirata T (2005) Hemocyanin in the exoskeleton of crustaceans: enzymatic properties and immunolocalization. Pigment Cell Res 18:136–143

166. Baird S, Kelly SM, Price NC, Jaenicke E, Meesters C, Nillius D, Decker H, Nairn J (2007) Hemocyanin conformational changes associated with SDS-induced phenol oxidase activation. *Biochim Biophys Acta* 1774:1380–1394

167. Goldfeder M, Egozy M, Ben-Yosef VS, Adir N, Fishman A (2013) Changes in tyrosinase specificity by ionic liquids and sodium dodecyl sulfate. *Appl Microbiol Biotechnol* 97:1953–1961

168. Jaenicke E, Decker H (2008) Kinetic properties of catecholoxidase activity of tarantula hemocyanin. *FEBS J* 275:1518–1528

169. Suzuki K, Shimokawa C, Morioika C, Itoh S (2008) Monoxygenase activity of *Octopus vulgaris* hemocyanin. *Biochemistry* 47:7108–7115

170. Wright J, McCaskill-Clark W, Cain JA, Patterson A, Coates CJ, Nairn J (2012) Effects of known phenoloxidase inhibitors on hemocyanin-derived phenoloxidase from *Limulus polyphemus*. Comp Biochem Physiol Part B 163:303–308

171. Cerenius L, Babu R, Söderhäll K, Jiravanichpaisal P (2010) *In vitro* effects on bacterial growth of phenoloxidase reaction products. J Invert Pathol 103:21–23

172. Fan T, Zhang Y, Yang L, Yang X, Jiang G, Yu M, Cong R (2009) Identification and characterization of a hemocyanin-derived phenoloxidase from the crab *Charybdis japonica*. Comp Biochem Physiol Part B 152:144–149

173. Siddiqui NI, Akosung RF, Gielens C (2006) Location of intrinsic and inducible phenoloxidase activity in molluscan hemocyanin. *Biochem Biophys Res Com* 348:1138–1144

174. Coates CJ, Nairn J (2013) Hemocyanin-derived phenoloxidase activity: A contributing factor to hyperpigmentation in *Nephrops norvegicus*. *Food Chem* 140:361–369

175. Perdomo-Morales R, Montero-Alejo V, Perea E, Pardo-Ruíz Z, Alonso-Jiménez E (2008) Hemocyanin-derived phenoloxidase activity in the spiny lobster *Panulirus argus* (Laurenti, 1804). *Biochim Biophys Acta* 1780:652–658

176. Dolaski A, Voelter W, Dolaskha P (2011) Phenoloxidase activity of intact and chemically modified functional unit RvH1-
Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin

177. Destoumieux-Garzon D, Saulnier D, Garnier J, Jouffrey C, Bulet P, Bachere E (2001) Crustacean immunity-antifungal peptides are generated from the C-terminus of shrimp hemocyanin in response to microbial challenge. J Biol Chem 276:47070–47077

178. Petit VW, Rolland JL, Blond A, Cazevieille C, Djediat C, Peduzzi J, Goulard C, Bachere E, Dupont J, Destoumieux-Garzon D, Rebuffat S (2016) A hemocyanin-derived antimicrobial peptide from the penaeid shrimp adopts an alpha-helical structure that specifically permeabilizes fungal membranes. Biochim Biophys Acta 1860:557–568

179. Qiu C, Sun J, Liu M, Wang B, Jiang K, Sun S et al (2014) Molecular cloning of hemocyanin CDNA from *Fenneropenaeus chinensis* and antimicrobial analysis of two c-terminal fragments. Mar Biotechnol 16:46–53

180. Lee SY, Lee BL, Söderhall K (2003) Processing of an antimicrobial peptide from hemocyanin of the freshwater crayfish *Procambarus clarkii*. J Biol Chem 278:7927–7933

181. Dolashka P, Moshtanska V, Borisova V et al (2011) Antimicrobial peptide from the penaeid shrimp adopts an alpha-helical structure that specifically permeabilizes fungal membranes. Biochim Biophys Acta 1860:557–568

182. Choi H, Lee DG (2014) Antifungal activity and pore-forming mechanism of astacin 1 against *Candida albicans*. Biochimie 105:58–63

183. Decker H, Jaenicke E (2004) Recent findings on phenoloxidase and antimicrobial activity of molluscan hemocyanins. Dev Comp Immunol 30:545–556

184. Dolashka P, Voelter W (2013) Antiviral activity of hemocyanins. Invertebr Surviv J 10:120–127

185. Dolashka P, Moshtanska V, Borisova V et al (2011) Antimicrobial proline-rich peptides from the hemolymph of marine snail *Rapana venosa*. Peptides 32:1477–1483

186. Dolashka P, Dolashki A, Van Bremen J, Fleotenmeyer M, Velkova L, Stefanovic S, Voelter W (2016) Antimicrobial activity of molluscan hemocyanins from *Helix* and *Rapana* snails. Cur Pharmaceut Biotechnol. doi:10.2174/1889201166615007113435

187. Zhuang J, Coates CJ, Zhu H, Zhu P, Wu Z, Xie L (2015) Identification of candidate antimicrobial peptides derived from abalone hemocyanin. Dev Comp Immunol 49:96–102

188. Wang D, Zhou Y, Zhao H, Zhou X, Sun N, Wang B, Yuan X (2012) Molecular cloning, sequencing, and expression analysis of cDNA encoding metalloproteins II (MPII) induced by single and combined metals (Cu(II), Cd(II)) in polychaete *Perinereis aibuhitensis*, Environ Toxicol Pharm 34:841–848

189. Vergote D, Sautiere PE, Vandenbulcke F (2004) Up-regulation of Neuro-hemerythrin expression in the central nervous system of the medicinal leech, *Hirudo medicinalis*, following septic injury. J Biol Chem 279:43828–43837

190. Dhainaut A, Raveillon B, Ahn CH, Park SC (2009) Gene expression profile in the anterior regeneration of the earthworm using expressed sequence tags. Biosci Biotechnol Biochem 73:29–34

191. Galvez-Romero G, Salas-Rojas M, Mendoza Hernandez G, Blanco-Favela F, Aguilar-Setien A (2011) Anti-baculovirus activity in a protein extracted from the exoskeleton of *Pleuroncodes planipes* [Decapoda: Galatheidae]. Fish Shellfish Immunol 31:482–484

192. Zeng WB, Chen WB, Yan QP, Lin GF, Qin YX (2016) Hemerythrin is required for *Aeromonas hydrophila* to survive in the macrophages of *Anguilla japonica*. Gen Mol Res 15; gmr15028074

193. Qin Y, Lin G, Chen W, Huang B, Huang W, Yan Q (2014) Flagellar motility contributes to the invasion and survival of *Aeromonas hydrophila* in Anguilla japonica macrophages. Fish Shellfish Immunol 39:273–279

194. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J Comp Chem 25:1605–1612

195. Bechsgaard J, Vanthournout B, Funch P, Vestbo S, Gibbs RA, Richards S et al (2016) Comparative genomic study of arachnid immune systems indicates loss of beta-1, 3-glucanase-related proteins and the immune deficiency pathway. J Evo Biol 29:277–291

196. Lorenzini DM, da Silva PI, Soares MB, Arruda P, Setubal J, Daffre S (2006) Discovery of immune-related genes expressed in hemocytes of the tarantula spider *Acanthoscurria gomesiana*. Dev Comp Immunol 30:545–556