Fakulta rybářství
a ochrany vod
Faculty of Fisheries
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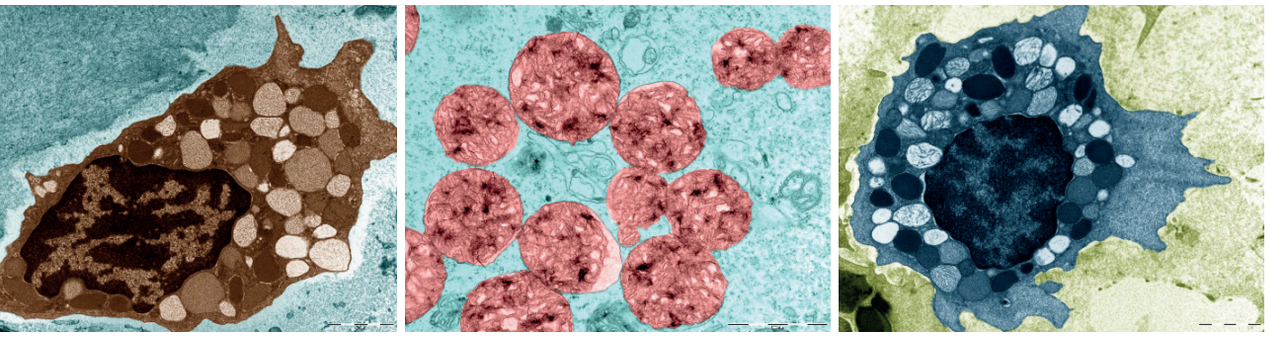
Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

2023



Doctoral thesis

The cellular and molecular characteristics of hemolymph in crayfish

Kifayat Ullah

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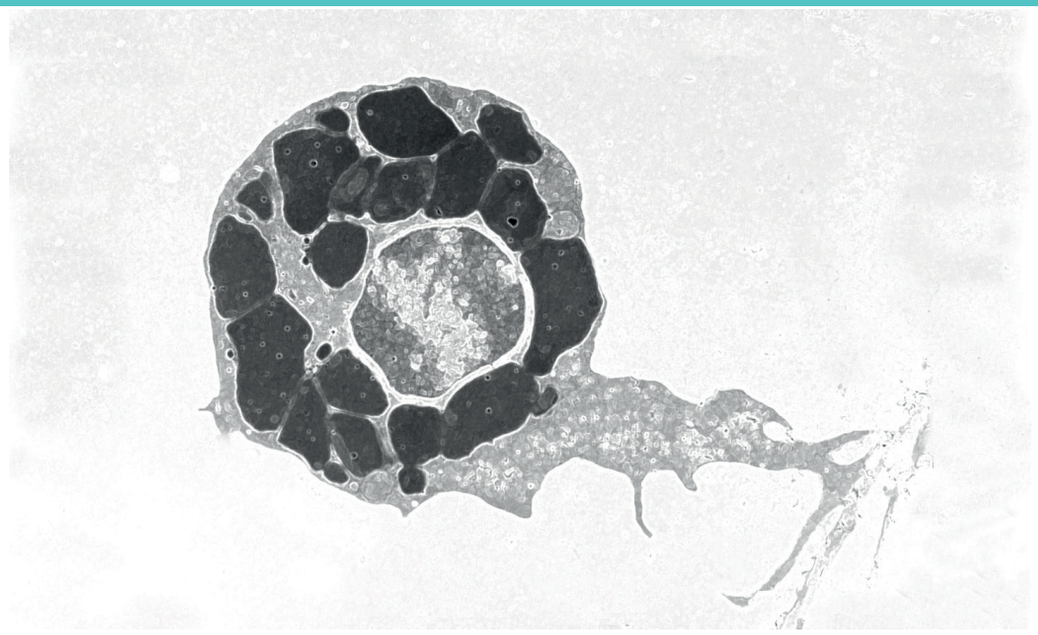
Buněčné a molekulární charakteristiky hemolymfy u raků



Doctoral thesis by
Kifayat Ullah

Czech Republic, Vodňany, 2023

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Title of thesis:
The cellular and molecular characteristics of hemolymph in crayfish
Buněčné a molekulární charakteristiky hemolymfy u raků

Doctoral study program:
Zootechnics / Fishery, full-time

Kifayat Ullah, 2023. The cellular and molecular characteristics of hemolymph in crayfish. Doctoral thesis. University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Czech Republic, 88 pp. ISBN 978-80-7514-188-0 (with the summary in English and Czech)
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Crayfish are decapod crustaceans that play a vital role in aquatic ecosystems, providing valuable ecological services and serving as important links in the food chain (Souty-Grosset et al., 2006). They are also of significant commercial interest, being harvested for human consumption, bait, and the pet trade (FAO, 2019). Moreover, crayfish, in particular marbled crayfish *Procambarus virginalis* have been utilized as model organisms in diverse areas of research, including developmental biology, ecology, regeneration biology, genetics and physiology due to their relatively small size and short generation time, ease of handling, breeding at any time of the year and robustness under laboratory conditions (Vogt, 2008; Hossain et al., 2018). For example, in developmental biology, marbled crayfish have been utilized to investigate the development of transmitter systems in crustaceans by examining the growth of their histamine-immunoreactive neurons in the ventral nervous system (Rieger and Harzsch, 2008). Crayfish have been used to investigate population dynamics, habitat preferences, and the effects of environmental stressors on aquatic communities in the field of ecology (Reynolds et al., 2013). Furthermore, in regeneration and genetic studies, for instance, marbled crayfish have been utilized to examine the gene that encodes for Baboon, a type I TGF-β superfamily receptor involved in the activin pathway and plays an important role in regeneration. The study found that this gene is responsible for controlling the growth, but not patterning, of the new leg during regeneration (Shinji et al., 2016). In physiology, one area of particular interest in crayfish research is the study of their hemolymph. Crayfish have emerged as important models for studying the function and regulation of the crustacean circulatory system, particularly the hemolymph, which serves as the circulatory fluid and plays critical roles in the maintenance of their physiology and immune system (Grubhoffer et al., 2013). This introductory chapter aims to provide an overview to better understand the composition and function of hemolymph in crayfish and discusses the growing body of literature focused on understanding the cellular and molecular characteristics of hemolymph in crustaceans.

1. Hemolymph: the circulatory fluid of crustaceans

Hemolymph is the circulatory fluid found in invertebrates, particularly arthropods and crustaceans. It serves a role analogous to the blood found in vertebrates, but its composition and functions differ in several significant ways that make it a fascinating and important topic of study. Hemolymph is a multifunctional fluid that serves a variety of roles in invertebrates, including the transportation of nutrients, waste products, and hormones, as well as providing hydraulic pressure for locomotion and serving as an immune defense system (Vazquez et al., 2009; Gianazza et al., 2021).

1.1. Differences between hemolymph and mammalian blood

There are several key differences between hemolymph and mammalian blood. One of the most notable differences is the presence of hemocyanin as the primary respiratory protein in many invertebrates, as opposed to hemoglobin in vertebrates (Terwilliger, 2015). Additionally, unlike mammalian blood, hemolymph lacks the variety of cell types found in blood, including erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). Instead, hemolymph contains specialized cells called hemocytes, which play a critical role in the innate immune response (Hartenstein, 2006). Another significant difference between hemolymph and mammalian blood is the presence of an open circulatory system in invertebrates, as opposed to the closed circulatory system found in vertebrates (Grubhoffer

et al., 2013). In an open circulatory system, hemolymph is not confined to vessels but instead bathes tissues directly, providing a less efficient method of nutrient delivery and waste removal (Grubhoffer et al., 2013).

1.2. Hemolymph composition

The composition of hemolymph is highly variable among invertebrates, but it generally consists mainly of water, inorganic ions, small organic molecules, proteins, lipids, and immune cells – the hemocyte (Leta et al., 1996; Hrassnigg et al., 2003; Burmester and Hankeln, 2007; Gianazza et al., 2021). The components of hemolymph are derived in part from the fat body, hemocytes, hepatopancreas, midgut, and epidermis (Gilbert and Chino, 1974). Inorganic ions such as sodium, potassium, calcium, and magnesium are important for maintaining osmotic balance and cellular function (Jungreis et al., 1973). Small organic molecules, including glucose, amino acids, and urea, serve as nutrients and waste products (Edwards, 1982; Lee and Chen, 2003).

One of the primary components of hemolymph is its proteins, which include enzymes, hormones, and transport proteins, which play essential roles in metabolism, homeostasis and immunity (Gianazza et al., 2021). The major proteins found in the hemolymph are hemocyanin, a copper-containing respiratory protein that carries oxygen and serves various immunological roles in many arthropods and crustaceans (Lee et al., 2004; Coates and Nairn, 2014). Unlike hemoglobin, the iron-containing respiratory protein found in vertebrate blood, which imparts a red color to the blood due to oxygenation of hemoglobin via iron within the heme cofactor, the hemocyanin in chelicerate and, to a lesser extent in crustaceans imparts a blue color to hemolymph when oxygenated (Coates and Nairn, 2014). Lipids in hemolymph are primarily found in the form of lipoproteins, which transport lipids between tissues and are involved in lipid metabolism (Yepiz-Plascencia et al., 2000). The cellular components of hemolymph, known as hemocytes, are involved in immune defense and other physiological processes, such as phagocytosis, coagulation and wound healing (Smith and Söderhäll, 1986; Jiravanichpaisal et al., 2006; Matozzo and Marin, 2010).

In addition to its physiological roles, hemolymph plays a crucial role as a component of the invertebrate immune system. The cellular and humoral components of hemolymph work together in the innate immune response under pathogenic challenges and environmental stress (Mengal et al., 2023a). Therefore, the innate immune system of crustaceans heavily relies on hemolymph, which contains various essential components critical for the immune response against pathogens and environmental stressors.

2. The basis of crustacean's innate immunity

The study of immunity in crayfish and other crustaceans has garnered significant attention in recent years due to its importance in understanding the defense mechanisms of aquatic species, potential applications in aquaculture, and broader implications for invertebrate immunology. One of the most important aspects of crustacean biology is their innate immune system, which plays a critical role in protecting them from various pathogens and environmental stressors. Unlike the adaptive immune system found in vertebrates, which relies on the production of specific antibodies and memory cells (Boehm, 2011), the innate immune system of invertebrates is the first line of defense against invading pathogens (Vazquez et al., 2009). It comprises various cellular and molecular mechanisms that work together to recognize and eliminate the pathogen or and ameliorate the harmful effect of various stressors. Crustacean innate immunity is a complex system that involves both cellular and molecular components.

The cellular component of crustacean's innate immunity comprises hemocytes, which are the circulating immune cells in the hemolymph. Hemocytes play a critical role in the recognition and elimination of invading pathogens and in mitigating the deleterious effects of environmental stressors (Pathak, 1993; Jiravanichpaisal et al., 2006). They are involved in various cellular mechanisms, such as hemolymph coagulation, phagocytosis, encapsulation, nodulation, and melanization (Smith and Söderhäll, 1986; Mengal et al., 2023a; Vazquez et al., 2009). The molecular component of crustacean's innate immunity comprises a diverse array of peptides and proteins, such as lectins, antimicrobial peptides, clotting factors, and complement-like proteins. These proteins are involved in various functions, such as pathogen recognition, opsonization, and pathogen elimination (Edwards, 1982; Jiravanichpaisal et al., 2006; Coates and Nairn, 2014; Gianazza et al., 2021).

Hemolymph is the main fluid affected by various environmental stressors in decapods because it is the primary circulatory fluid in direct contact with the external environment, which makes it more susceptible to changes in environmental conditions and exposure to pathogens. Therefore, any changes in the external environment can affect the innate immune status of decapods. Understanding the effects of environmental stressors on hemolymph and the immune system of decapods is essential for developing effective strategies for conservation and disease control.

3. Impact of environmental stressors on the immune system

Crustaceans are constantly exposed to a wide range of environmental stressors, such as changes in temperature, salinity, and pH, as well as exposure to pollutants and pathogens. These stressors can have a profound effect on the immune system of crustaceans, which is responsible for protecting them against pathogens and other harmful agents. Exposure to stressors can lead to changes in the expression of immune-related genes, alterations in the composition and activity of immune cells, and changes in the production of immune-related molecules, such as antimicrobial peptides (Matozzo et al., 2011; Ren et al., 2017; Korkut et al., 2018). In chapter 2, we have reviewed and discussed in detail the effects of environmental factors on the cellular and molecular immune parameters of decapods. Also, we highlighted important knowledge gaps in the immune system of decapods, such as hemocyte classification, phagocytosis, and coagulation, which served as the basis for the experiments conducted in chapters 3 and 4. Therefore, the impact of environmental stressors on the immune system is briefly reviewed here.

Temperature stress is one of the most common environmental stressors affecting crustacean's immune parameters. Exposure to high or low temperatures can have a profound effect on the immune system of crustaceans. For instance, exposure to high temperatures can lead to an increase in the production of heat shock proteins (Mengal et al., 2023b), which are involved in protecting cells from thermal stress. Additionally, it was observed that exposure to high temperatures significantly altered key immune parameters, including total hemocyte count (the immune cells), phenoloxidase, superoxide dismutase and catalase activities in crayfish, *Pacifastacus leniusculus* and crab *Carcinus aestuarii* (Matozzo et al., 2011; Korkut et al., 2018). Thus, high temperatures can lead to a decrease in the activity of hemocytes, which are responsible for phagocytosis and encapsulation of pathogens and can make crustaceans more susceptible to infection by pathogens. Likewise, salinity stress is another common environmental stressor affecting crustacean's immune parameters. Changes in salinity can lead to alterations in the activity and composition of immune cells, as well as changes in the production of immune-related molecules. In the crab *Portunus trituberculatus,* low salinity resulted in a decrease in the hemocyte count and a decrease in

the prophenoloxidase activity (Wang et al., 2018). Changes in water pH also have a significant impact on the immune system of decapods, affecting various immune parameters such as total hemocyte count (THC), differential hemocyte count (DHC), total hemolymph protein, phenoloxidase (PO) activity, reactive oxygen species (ROS) production, and antioxidant responses (Chen et al., 2015). A study on *Penaeus vannamei* shrimp showed that exposure to low (6.5) and high (10.1) pH levels, after being injected with *Vibrio alginolyticus,* resulted in reduced immunity. The study noted a significant reduction in THC, PO activity, respiratory burst, phagocytic activity, superoxide dismutase activity, and clearance efficiency against *V. alginolyticus* (Li and Chen, 2008). In addition, exposure to toxins, such as heavy metals, pesticides, and hydrocarbons, can also have a profound effect on the immune parameters of crustaceans. Toxin exposure can lead to changes in the expression of immune-related genes, alterations in the composition and activity of immune cells, and changes in the production of immune-related molecules (Renault, 2015). Understanding the impact of environmental stressors on crustacean immunity is crucial for developing strategies to mitigate the effects of these stressors, such as improving aquaculture practices and monitoring environmental changes that could affect the health of wild populations.

4. Hemocyte classification and functions in the innate immune system of crustaceans

Hemocytes are a critical component of the innate immune system of crustaceans. They are responsible for recognizing and eliminating invading pathogens, as well as maintaining hemolymph homeostasis (Jiravanichpaisal et al., 2006). Hemocytes are classified into different types based on their morphology, function, and staining properties. However, there is a lack of consensus on the classification of hemocytes, and different classification schemes have been proposed by various researchers (Li et al., 2019; Li et al., 2021; Söderhäll et al., 2022). Despite the lack of consensus on the classification of hemocytes, their importance in the innate immune system of crustaceans cannot be neglected. Hemocytes are involved in various functions, such as pathogen recognition, coagulation, phagocytosis, encapsulation, melanization and opsonization (Smith and Söderhäll, 1986; Jiravanichpaisal et al., 2006; Matozzo and Marin, 2010). They also play a critical role in the regulation of hemolymph homeostasis, such as nutrient transport, waste removal, and osmoregulation (Jiravanichpaisal et al., 2006).

The most commonly used classification scheme for hemocytes is based on their morphological features, such as size, shape, granulation, and nuclear/cytoplasmic ratios. According to this scheme, hemocytes can be classified into three main types: hyalinocytes, semi-granulocytes, and granulocytes (Smith and Söderhäll, 1983; Söderhäll and Smith, 1983). However, the second concept suggests that all morphologically different hemocytes in circulation are actually different developmental stages of a single type of hemocyte (Li et al., 2021). Hyalinocytes are generally the smallest cell type and are characterized by their ovoid shape, high nucleocytoplasmic ratio, and electron-dense deposits in the nucleus. Although largely agranular, hyalinocytes may sometimes contain a few granules (Smith and Söderhäll, 1983). Semi-granulocytes, on the other hand, are relatively larger than hyalinocytes and contain a variable number of smaller granules than those found in granulocytes (Mengal et al., 2023a). Granulocytes are the largest cell type and are characterized by their kidney-shaped nucleus and cytoplasm filled with membrane-bound, electron-dense refractile granules. These granules contain various proteins, such as enzymes and antimicrobial peptides, which are involved in the immune response (Mengal et al., 2023a). Granulocytes play a crucial role in the defense against invading pathogens through various immune functions, including phagocytosis, coagulation, encapsulation, and melanization. Although three types of hemocytes have been characterized based on their morphological features, yet their classification and specific functions, such as phagocytosis and coagulation, remain unclear. Further details on the classification and functions of hemocytes in these processes are discussed in chapters 2 and 3.

5. Phagocytosis in crustaceans

The phagocytic cells, known as hemocytes in invertebrates, recognize and ingest invading pathogens and apoptotic cells through the process of phagocytosis, which involves the engulfment of foreign entities, followed by their degradation and elimination (Giulianini et al., 2007; Flannagan et al., 2012). The process involves several steps, including binding, internalization, formation of the phagosome, fusion with lysosomes, and degradation of the engulfed material (Flannagan et al., 2012, Jiravanichpaisal et al., 2006). Hemocytes use a series of pattern recognition receptors (PRRs) to recognize specific pathogen-associated molecular patterns (PAMPs) to initiate pathogen recognition (Charles A. Janeway and Medzhitov, 2002). They phagocytose pathogenic targets such as bacteria and yeast, as well as apoptotic cells resulting from injury or wounds, thus enabling the animal to defend against pathogens and maintain tissue homeostasis for optimal health.

Although the contributions of hemocytes in the cellular defense reactions in the hemolymph are widely recognized in invertebrates, the types of hemocytes involved in the phagocytic reaction vary among different species and even within the same species. For example, in non-insect invertebrates like decapod crustaceans, different types of hemocytes have been shown to carry out phagocytic activity. The phagocytosis assays of bacteria performed *in vitro* with the hemocytes of *Homarus americanus* and *Panulirus interruptus* have revealed that both large granular hemocytes (LGH) and small granular hemocytes (SGH) are involved in the process, with the strongest response observed in SGH, reaching a maximum of 96% of phagocytizing cells in this hemocyte category (Hose et al., 1990). *In vitro* studies with the hemocytes of *Penaeus monodon, Macrobrachium rosenbergii,* and *M. acanthurus,* have also shown that both LGH and SGH can engulf yeast particles, but apparently not the hyaline hemocytes (HH) (Gargioni and Barracco, 1998). In contrast, a study on *Penaeus monodon* hemocytes revealed that only the hyaline cells, not the LGH or SGH cells, were capable of phagocytosing latex beads (Sung and Sun, 2002). Interestingly, in *Astacus leptodactylus,* it has been suggested that all hemocyte types are involved in the phagocytic activity to some extent, but the SGH are the only ones that respond to all foreign particles, with the highest percentage of phagocytic activity (Giulianini et al., 2007).

Furthermore, in insects like Lepidoptera, many authors have reported that the main hemocyte type that carries out phagocytic activity is the granular hemocyte (Mazet et al., 1994; Ribeiro et al., 1996). In an electron-microscopic study in the silk moth *Bombyx mori,* plasmatocytes were reported to be the main hemocytes responsible for phagocytic reaction (Pathak, 1993). On the other hand, it has also been found that both granular cells and plasmatocytes are involved in phagocytic reactions in the greater wax moth, *Galleria mellonella* (Tojo et al., 2000). These findings highlight the diversity and complexity of the immune responses among different invertebrates species, particularly in decapods.

Besides the contrasting reports on the specific types of phagocytic hemocytes involved in *in vitro* and *in vivo* conditions against abiotic or biotic agents, how phagocytic hemocytes respond to tissue homeostasis and regeneration after an injury or muscle degeneration in natural conditions is not well understood in decapods. To gain a better understanding of hemocyte phagocytic behavior in live animals after muscle injury, we used transmission electron microscopy to study the ultrastructure of hemocytes *in situ.* Chapter 3 provides detailed information regarding the behavior of hemocytes after muscle injury and the types of hemocytes involved in phagocytosis.

6. Morphological characteristics of hemolymph clotting

Coagulation is a crucial defense mechanism in both arthropods and crustaceans with body fluids containing hemolymph. It serves to prevent the loss of blood or hemolymph through breaks in the exoskeleton or wounds, respectively, and to prevent the spread of pathogens throughout the body (Jiravanichpaisal et al., 2006; Cerenius and Söderhäll, 2011). The clotting system, along with the prophenoloxidase-activating system and the production of antimicrobial peptides, is a key component of the innate immune response in invertebrates (Cerenius and Söderhäll, 2011). The ultimate objective of the coagulation process is the formation of a stable clot, which is achieved through the involvement of hemocytes, their granular products and plasma proteins.

In earlier studies, three different types of coagulation mechanisms in crustaceans were proposed based on morphological characteristics: A, B, and C (Perdomo-Morales et al., 2019). Type A coagulation involves the rapid agglutination of hemocytes without the involvement of plasma. During this type of coagulation, a dense network of hemocytes is enough to seal the wound, with clotted hemocytes connected through polymerized fibers. This mechanism is observed in crabs such as *Maja squinado, Loxorhynchus grandis,* and *Cancer pagurus* (Perdomo-Morales et al., 2019). Type B coagulation is characterized by cell aggregation followed by limited clotting of the plasma, as observed in the *lobster Homarus americanus* and the crabs *Carcinus maenas* and *Macropipus puber* (Perdomo-Morales et al., 2019). Finally, Type C coagulation involves the rapid lysis of hemocytes, termed "explosive corpuscles," and immediate clotting of the plasma, resulting in low cell aggregation. This type of coagulation is observed in shrimps and spiny lobsters (Martin et al., 1991).

In addition, the identification of the type of hemocytes that initiate coagulation has been complicated by past confusion over hemocyte classification. For instance, the type C coagulation mechanism suggests that "explosive corpuscles" initiate clotting and undergo cytolysis. Another ultrastructural investigation of hemocytes in three decapod crustaceans (*Homarus americanus, Panulirus interruptus,* and *Loxorhynchus grandis*) reported that the hemocytes triggering blood clotting could be easily distinguished by several features characteristic of hyaline cells. At the transmission electron microscopy level, hyaline cells contain small cytoplasmic deposits that undergo lysis, releasing the content suggesting the hyaline cell's involvement in the clotting process (Hose et al., 1990). Furthermore, a few other reports suggested that hyaline cells are the primary cells that initiate hemolymph coagulation in decapod crustaceans and arthropods (Wood et al., 1971; Ravindranath, 1980). The involvement of granular hemocytes in hemolymph coagulation has also been reported. Dumont and colleagues reported observing a series of cytological changes in granular hemocytes during clotting in *Limulus polyphemus.* These changes included cellular swelling, loss of granular refractivity, degranulation, and cytoplasmic vacuolation (Dumont et al., 1966). However, Ravindranath (1980) pointed out that it is noteworthy that neither Dumont et al. (1966) nor other researchers who had reported similar findings on granular hemocytes observed any changes in the plasma's consistency when the granular contents were released into it, which contradicts the involvement of granular hemocytes in hemolymph coagulation (Ravindranath, 1980).

Hemocyte granules play a vital role in clotting, but there are contrasting reports regarding their ultrastructural forms and types in past studies. Hemocyte granules have been described in several invertebrates; in migratory locust *Locusta migratoria,* two types of granules were reported: ovoid electron-dense granules occupying the marginal area of the cell and granules containing diverse microfibrillar structures covered by a unit membrane (Hoffmann and Stoeckel, 1968). On the other hand, cystocyte granules in mealworm *Tenebrio molitor*

contained only microtubular structures instead of being electron-dense (Stang-Voss, 1970). Furthermore, the granular hemocytes in *Limulus polyphemus* contained not only electron-dense granules but also low-density spherical granules containing microtubules and granules of low density containing particulate matter without microtubular structures when fixed a few minutes after sample collection (Dumont et al., 1966).

Uncertainty and ambiguity regarding the type, function, and fate of different types of hemocytes during coagulation arise from the lack of distinct structural characteristics. *In vitro,* experimental conditions used in past studies may have altered hemocyte morphology, leading to the misidentification of different cell types as these cells are highly unstable once removed from the body. To better understand hemocyte fate during coagulation, we used an *in situ* experimental approach and transmission electron microscopy as a tool to study the ultrastructure of hemocytes and clots during the coagulation process. Chapter 3 provides detailed information regarding hemocyte types and the morphological changes that these cells undergo during coagulation.

7. Molecular characteristics of hemolymph clotting

The open circulation system of crustaceans makes them more vulnerable to hemolymph loss during injury and more susceptible to infections compared to vertebrates (Grubhoffer et al., 2013). However, crustaceans are less likely to develop thrombosis and associated fatal consequences due to their open circulation system. To cope with the challenges of the open circulation system, crustaceans have developed a fast coagulation mechanism that not only promotes wound healing but also serves as a critical part of their immune system (Ravindranath, 1980; Cerenius and Söderhäll, 2011).

Hemolymph clotting is a complex process that involves both cellular and humoral components. The cellular components are mainly the hemocytes (the equivalent of blood cells), which are involved in phagocytosis, encapsulation, nodulation and melanization of foreign materials (Smith and Söderhäll, 1986; Mengal et al., 2023a; Vazquez et al., 2009). The humoral components are mainly the soluble proteins and peptides that are present in the plasma (the liquid part of the hemolymph), which are involved in the recognition, activation, aggregation and cross-linking of clotting factors (Edwards, 1982; Jiravanichpaisal et al., 2006; Coates and Nairn, 2014; Gianazza et al., 2021). Hemolymph clotting not only serves as a physical barrier against pathogens and injuries but also as a source of antimicrobial agents that can kill or inhibit the growth of microorganisms (Jiravanichpaisal et al., 2006).

Arthropods and crustaceans have developed complex mechanisms to utilize the coagulation and entrapment of microorganisms as an effective part of their innate immune system. Among arthropods, the coagulation mechanism is well-described in chelicerate, particularly in horseshoe crabs (Limulidae). The horseshoe crab's coagulation system involves a proteolytic cascade that converts a soluble protein (coagulogen) into an insoluble aggregate (coagulin), which is stored intracellularly until degranulation is initiated on contact with pathogens (Iwanaga, 2002; Kawabata and Muta, 2010). In decapod crustaceans, the clotting protein is found in the hemolymph (plasma), while the coagulation factors are stored intracellularly (Hall et al., 1999; Yeh et al., 1999). Unlike in horseshoe crabs, the coagulation reaction in decapod crustaceans does not involve a proteolytic cascade. Instead, the release of transglutaminase from hemocytes into the hemolymph is sufficient to initiate protein polymerization and clot formation (Hall et al., 1999; Yeh et al., 1999).

Despite extensive characterization of the crayfish coagulation system, no comparative analysis of the entire clot proteome with fresh hemolymph has been conducted for crayfish or any other decapods. In contrast, clotting has been extensively studied in Drosophila

(Karlsson et al., 2004; Scherfer et al., 2004; Lindgren et al., 2008), and more recently, the entire hemolymph clot proteome was characterized without comparison to fresh hemolymph in the Brazilian whiteknee tarantula spider, *Acanthoscurria geniculata* (Sanggaard et al., 2016). Conducting a comparative proteome study of non-clot vs. clot hemolymph in decapod crustaceans would be a valuable step toward gaining a deeper understanding of the molecular mechanisms involved in coagulation and other physiological processes.

Quantitative proteomics has emerged as a powerful tool for studying proteins in biological systems. It enables the identification and quantification of peptides and proteins. By conducting a comparative proteome study of non-clot vs. clot hemolymph in decapod crustaceans, we can identify changes in protein abundances that occur during clot formation, which can contribute to a better understanding of the molecular mechanisms involved in coagulation and other physiological processes. Therefore, we performed the first comparative proteome analysis of non-clot and clot hemolymph in marbled crayfish to gain insights into the molecular mechanisms of hemolymph clotting in crustaceans. Chapter 4 provides detailed information on the molecular characteristics of hemolymph clotting in crustaceans, which can further enrich our knowledge in this field. Overall, proteomics is a valuable tool for advancing our understanding of the molecular mechanisms of hemolymph clotting in crustaceans.

Thesis aim and specific objectives

To investigate the innate immune system of decapod crustaceans, with a focus on hemocyte types, phagocytosis, and coagulation, the study aims to understand the cellular and molecular characteristics of hemolymph under various environmental stress conditions and to investigate the *in situ* ultrastructural and morphological characteristics of hemolymph clotting components, as well as the *in vitro* molecular characteristics of hemolymph coagulation in crayfish.

Thesis objectives:

1. To conduct a comprehensive review to assess the important knowledge gaps in the innate immune system of decapods, including hemocyte types, phagocytosis, and coagulation. And also to review and understand the cellular and molecular characteristics of hemolymph under various environmental stress conditions, with a special focus on immune parameters and abiotic stress mechanisms in decapod crustaceans.

2. To examine the *in situ* ultrastructural and morphological characteristics of hemolymph and its components, with a particular focus on the behavior of hemocytes during coagulation and phagocytosis in marbled crayfish.

3. To investigate the *in vitro* molecular characteristics of hemolymph in relation to coagulation in crayfish using quantitative proteomic profiling techniques.

References

Boehm, T., 2011. Design principles of adaptive immune systems. Nat. Rev. Immunol. 11, 307–317.

Burmester, T., Hankeln, T., 2007. The respiratory proteins of insects. J. Insect Physiol. 53, 285–294.

Cerenius, L., Söderhäll, K., 2011. Coagulation in Invertebrates. J. Innate Immun. 3, 3–8.

Charles A. Janeway, J., Medzhitov, R., 2002. Innate Immune Recognition. Annu. Rev. Immunol. 20, 197–216.

Chen, Y.-Y., Chen, J.-C., Tseng, K.-C., Lin, Y.-C., Huang, C.-L., 2015. Activation of immunity, immune response, antioxidant ability, and resistance against Vibrio alginolyticus in white shrimp *Litopenaeus vannamei* decrease under long-term culture at low pH. Fish Shellfish Immunol. 46, 192–199.

Coates, C.J., Nairn, J., 2014. Diverse immune functions of hemocyanins. Dev. Comp. Immunol. 45, 43–55.

Dumont, J.N., Anderson, E., Winner, G., 1966. Some cytologic characteristics of the hemocytes of Limulus during clotting. J. Morphol. 119, 181–207.

Edwards, H.A., 1982. Free Amino Acids as Regulators of Osmotic Pressure in Aquatic Insect Larvae. J. Exp. Biol. 101, 153–160.

FAO, 2019. Fishery and Aquaculture Statistics 2017. Food and Agriculture Organization of the United Nations Rome.

Flannagan, R.S., Jaumouillé, V., Grinstein, S., 2012. The Cell Biology of Phagocytosis. Annu. Rev. Pathol.-Mech. Dis. 7, 61–98.

Gargioni, R., Barracco, M.A., 1998. Hemocytes of the palaemonids *Macrobrachium rosenbergii* and *M. acanthurus,* and of the *Penaeid Penaeus paulensis.* J. Morphol. 236, 209–221.

Gianazza, E., Eberini, I., Palazzolo, L., Miller, I., 2021. Hemolymph proteins: An overview across marine arthropods and molluscs. J. Proteom. 245, 104294.

Gilbert, L.I., Chino, H., 1974. Transport of lipids in insects. J. Lipid Res. 15, 439–456.

Giulianini, P.G., Bierti, M., Lorenzon, S., Battistella, S., Ferrero, E.A., 2007. Ultrastructural and functional characterization of circulating hemocytes from the freshwater crayfish *Astacus leptodactylus*: Cell types and their role after *in vivo* artificial non-self challenge. Micron. 38, 49–57.

Grubhoffer, L., Rudenko, N., Vancova, M., Golovchenko, M., Sterba, J., 2013. Circulatory system and hemolymph. Biology of Ticks, 2nd ed.; Sonenshine, DE, Roe, RM, Eds, 258–286.

Hall, M., Wang, R., van Antwerpen, R., Sottrup-Jensen, L., Söderhäll, K., 1999. The crayfish plasma clotting protein: A vitellogenin-related protein responsible for clot formation in crustacean blood. Proceedings of the National Academy of Sciences 96, 1965–1970.

Hartenstein, V., 2006. Blood Cells and Blood Cell Development in the Animal Kingdom. Annu. Rev. Cell. Dev. Biol. 22, 677–712.

Hoffmann, J., Stoeckel, M., 1968. Sur les modifications ultrastructurales des coagulocytes au cours de la coagulation de l'hémolymphe chez Locusta migratoria. CR Soc. Biol. (Paris) 162, 2257–2259.

Hose, J.E., Martin, G.G., Gerard, A.S., 1990. A Decapod Hemocyte Classification Scheme Integrating Morphology, Cytochemistry, and Function. Biological Bulletin 178, 33–45.

Hossain, M.S., Patoka, J., Kouba, A., Buřič, M., 2018. Clonal crayfish as biological model: a review on marbled crayfish. Biologia 73, 841–855.

Hrassnigg, N., Leonhard, B., Crailsheim, K., 2003. Free amino acids in the haemolymph of honey bee queens (*Apis mellifera* L.). Amino Acids 24, 205–212.

Iwanaga, S., 2002. The molecular basis of innate immunity in the horseshoe crab. Curr. Opin. Immunol. 14, 87–95.

Jiravanichpaisal, P., Lee, B.L., Söderhäll, K., 2006. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. Immunobiology. 211, 213–236.

Jungreis, A.M., Jatlow, P., Wyatt, G.R., 1973. Inorganic ion composition of haemolymph of the cecropia silkmoth: Changes with diet and ontogeny. J. Insect Physiol. 19, 225–233.

Karlsson, C., Korayem, A.M., Scherfer, C., Loseva, O., Dushay, M.S., Theopold, U., 2004. Proteomic Analysis of the *Drosophila* Larval Hemolymph Clot. J. Biol. Chem. 279, 52033–52041.

Kawabata, S.-i., Muta, T., 2010. Sadaaki Iwanaga: discovery of the lipopolysaccharide- and β1,3-d-glucan-mediated proteolytic cascade and unique proteins in invertebrate immunity. J. Biochem. 147, 611–618.

Korkut, G.G., Söderhäll, I., Söderhäll, K., Noonin, C., 2018. The effect of temperature on bacteria-host interactions in the freshwater crayfish, *Pacifastacus leniusculus*. J. Invertebr. Pathol. 157, 67–73.

Lee, S.Y., Lee, B.L., Söderhäll, K., 2004. Processing of crayfish hemocyanin subunits into phenoloxidase. Biochem. Biophys. Res. Commun. 322, 490–496.

Lee, W.-C., Chen, J.-C., 2003. Hemolymph ammonia, urea and uric acid levels and nitrogenous excretion of *Marsupenaeus japonicus* at different salinity levels. J. Exp. Mar. Biol. Ecol. 288, 39–49.

Leta, M.A., Gilbert, C., Morse, R.A., 1996. Levels of hemolymph sugars and body glycogen of honeybees (*Apis mellifera L.)* from colonies preparing to swarm. J. Insect Physiol. 42, 239–245.

Li, C.-C., Chen, J.-C., 2008. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under low and high pH stress. Fish Shellfish Immunol. 25, 701–709.

Li, F., Xu, L., Hui, X., Huang, W., Yang, F., 2019. Directed differentiation of granular cells from crayfish hematopoietic tissue cells. Fish Shellfish Immunol. 88, 28–35.

Li, F., Zheng, Z., Li, H., Fu, R., Xu, L., Yang, F., 2021. Crayfish hemocytes develop along the granular cell lineage. Sci. Rep. 11, 13099.

Lindgren, M., Riazi, R., Lesch, C., Wilhelmsson, C., Theopold, U., Dushay, M.S., 2008. Fondue and transglutaminase in the *Drosophila* larval clot. J. Insect Physiol. 54, 586–592.

Martin, G.G., Omori, J.E.H.S., Chong, C., Hoodbhoy, T., McKrell, N., 1991. Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod crustaceans. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 100, 517–522.

Matozzo, V., Gallo, C., Marin, M.G., 2011. Effects of temperature on cellular and biochemical parameters in the crab *Carcinus aestuarii* (Crustacea, Decapoda). Mar. Environ. Res. 71, 351–356.

Matozzo, V., Marin, M.G., 2010. The role of haemocytes from the crab *Carcinus aestuarii* (Crustacea, Decapoda) in immune responses: A first survey. Fish Shellfish Immunol. 28, 534–541.

Mazet, I., Pendland, J., Boucias, D., 1994. Comparative analysis of phagocytosis of fungal cells by insect hemocytes versus horse neutrophils. Dev. Comp. Immunol. 18, 455–466.

Mengal, K., Kor, G., Kozák, P., Niksirat, H., 2023a. Effects of environmental factors on the cellular and molecular parameters of the immune system in decapods. Comp. Biochem. Physiol., A: Mol. Integr. Physiol. 276, 111332.

Mengal, K., Kor, G., Kozák, P., Niksirat, H., 2023b. Heat shock proteins adaptive responses to environmental stressors and implications in health management of decapods. Aquaculture Reports 30, 101564.

Pathak, J.P.N., 1993. Cell-mediated defence reactions in insects. In: Pathak, J.P.N. (Ed.), Insect Immunity. Springer Netherlands, Dordrecht, pp. 47–58. 978-94-011-1618-3.

Perdomo-Morales, R., Montero-Alejo, V., Perera, E., 2019. The clotting system in decapod crustaceans: History, current knowledge and what we need to know beyond the models. Fish Shellfish Immunol. 84, 204–212.

Ravindranath, M.H., 1980. Haemocytes in haemolymph coagulation of arthropods. Biol. Rev. 55, 139–170.

Ren, X., Lv J., Gao, B., Li, J., Liu, P., 2017. Immune response and antioxidant status of *Portunus trituberculatus* inoculated with pathogens. Fish Shellfish Immunol. 63, 322–333.

Renault, T., 2015. Immunotoxicological effects of environmental contaminants on marine bivalves. Fish Shellfish Immunol. 46, 88–93.

Reynolds, J., Souty-Grosset, C., Richardson, A., 2013. Ecological roles of crayfish in freshwater and terrestrial habitats. Freshwat. Cray. 9, 197–218.

Ribeiro, C., Simões, N., Brehélin, M., 1996. Insect immunity: the haemocytes of the armyworm *Mythimna unipuncta* (Lepidoptera: Noctuidae) and their role in defence reactions. *In vivo* and *in vitro* studies. J. Insect Physiol. 42, 815–822.

Rieger, V., Harzsch, S., 2008. Embryonic development of the histaminergic system in the ventral nerve cord of the marbled crayfish (Marmorkrebs). Tissue and Cell 40, 113–126.

Sanggaard, K.W., Dyrlund, T.F., Bechsgaard, J.S., Scavenius, C., Wang, T., Bilde, T., Enghild, J.J., 2016. The spider hemolymph clot proteome reveals high concentrations of hemocyanin and von Willebrand factor-like proteins. Biochim. Biophys. Acta 1864, 233–241.

Scherfer, C., Karlsson, C., Loseva, O., Bidla, G., Goto, A., Havemann, J., Dushay, M.S., Theopold, U., 2004. Isolation and characterization of hemolymph clotting factors in *Drosophila melanogaster* by a Pullout Method. Curr. Biol. 14, 625–629.

Shinji, J., Miyanishi, H., Gotoh, H., Kaneko, T., 2016. Appendage Regeneration after autotomy is mediated by baboon in the crayfish *Procambarus Fallax F. Virginalis* Martin, Dorn, Kawai, Heiden and Scholtz, 2010 (Decapoda: Astacoidea: Cambaridae). J. Crust. Biol. 36, 649–657.

Smith, V., Söderhäll K., 1986. Cellular immune mechanisms in the Crustacea, Symp Zool Soc Lond, pp. 59–79.

Smith, V.J., Söderhäll, K., 1983. Induction of degranulation and lysis of haemocytes in the freshwater crayfish, *Astacus astacus* by components of the prophenoloxidase activating system *in vitro*. Cell Tissue Res. 233, 295–303.

Söderhäll, I., Fasterius, E., Ekblom, C., Söderhäll, K., 2022. Characterization of hemocytes and hematopoietic cells of a freshwater crayfish based on single-cell transcriptome analysis. iScience 25, 104850.

Söderhäll, K., Smith, V.J., 1983. Separation of the haemocyte populations of *Carcinus Maenas* and other marine decapods, and prophenoloxidase distribution. Dev. Comp. Immunol. 7, 229–239.

Stang-Voss, C., 1970. Zur Ultrastruktur der Blutzellen wirbelloser Tiere. I. Uber die Haemocyten der Larve des Mehlkafers *Tenebrio molitor* L. Z Zellforsch Mikrosk Anat.

Sung, H.H., Sun, R., 2002. Use of monoclonal antibodies to classify hemocyte subpopulations of tiger shrimp (*Penaeus Monodon*). J. Crust. Biol. 22, 337–344.

Terwilliger, N.B., 2015. Oxygen transport proteins in Crustacea: hemocyanin and hemoglobin. Physiology 4, 359–390.

Tojo, S., Naganuma, F., Arakawa, K., Yokoo, S., 2000. Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*. J. Insect Physiol. 46, 1129–1135.

Vazquez, L., Alpuche, J., Maldonado, G., Agundis, C., Pereyra-Morales, A., Zenteno, E., 2009. Review: Immunity mechanisms in crustaceans. Innate. Immun. 15, 179–188.

Vogt, G., 2008. The marbled crayfish: a new model organism for research on development, epigenetics and evolutionary biology. Journal of Zoology 276, 1–13.

Wang, L., Pan, L., Ding, Y., Ren, X., 2018. Effects of low salinity stress on immune response and evaluating indicators of the swimming crab *Portunus trituberculatus*. Aquacult. Res. 49, 659–667.

Wood, P.J., Podlewski, J., Shenk, T.E., 1971. Cytochemical observations of hemolymph cells during coagulation in the crayfish, *Orconectes virilis*. J. Morphol. 134, 479–487.

Yeh, M.-S., Huang, C.-J., Leu, J.-H., Lee, Y.C., Tsai, I.-H., 1999. Molecular cloning and characterization of a hemolymph clottable protein from tiger shrimp (*Penaeus monodon*). Eur. J. Biochem. 266, 624–633.

Yepiz-Plascencia, G., Vargas-Albores, F., Higuera-Ciapara, I., 2000. Penaeid shrimp hemolymph lipoproteins. Aquaculture. 191, 177–189.

CHAPTER 2

EFFECTS OF ENVIRONMENTAL FACTORS ON THE CELLULAR AND MOLECULAR PARAMETERS OF THE IMMUNE SYSTEM IN DECAPODS

Mengal, K., Kor, G., Kozák, P., Niksirat, H., 2023. Effects of environmental factors on the cellular and molecular parameters of the immune system in decapods. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 276, 111332.

My share on this work was 60 %.

Comparative Biochemistry and Physiology, Part A 276 (2023) 111332

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Effects of environmental factors on the cellular and molecular parameters of the immune system in decapods

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ARTICLE INFO

Edited by Michael Hedrick

Keywords:
Environmental stressors
Stress response
Innate immunity

ABSTRACT

Crustaceans and in particular decapods (i.e. shrimp, crabs and lobsters) are a diverse, commercially and ecologically important group of organisms. They are exposed to a range of environmental factors whose abiotic and biotic components are prone to fluctuate beyond their optimum ranges and, in doing so, affect crustaceans' immune system and health. Changes in key environmental factors such as temperature, pH, salinity, dissolved oxygen, ammonia concentrations and pathogens can provoke stress and immune responses due to alterations in immune parameters. The mechanisms through which stressors mediate effects on immune parameters are not fully understood in decapods. Improved knowledge of the environmental factors – above all, their abiotic components – that influence the immune parameters of decapods could help mitigate or constrain their harmful effects that adversely affect the production of decapod crustaceans. The first part of this overview examines current knowledge and information gaps regarding the basic components and functions of the innate immune system of decapods. In the second part, we discuss various mechanisms provoked by environmental factors and categorize cellular and molecular immune responses to each environmental factor with special reference to decapods.

1. Introduction

Decapod crustaceans include around 10,000 invertebrate species inhabiting both freshwater and marine environments. They are phylogenetically ancient taxa that appeared on Earth in the late Devonian around 360 million years ago (Schram and Dixon, 2004). Today, decapods are found in various aquatic ecosystems and are intensely harvested as an important food resource for humans. According to the latest FAO report, in 2018 global aquacultural production reached 82 million tons (worth US$ 250 billion), of which crustaceans accounted for 9.4 million tons with a sale value of US$ 69.3 billion (Food and Agriculture Organization of the United Nations, 2020).

However, decapods in natural aquatic ecosystems and farms are constantly exposed to various stresses caused by environmental factors, resulting in immune system suppression and increasing susceptibility to disease, which can lead to economic losses. Examples of biotic and abiotic environmental factors include temperature, pH, salinity, oxygen and ammonia concentrations, and pathogens, all of which are known to have significant effects on immune responses in decapod crustaceans (Vargas-Albores et al., 1998; Le Moullac and Haffner, 2000; Jiang et al., 2004c; Pan et al., 2008; Kathyayani et al., 2019). Temperature is one of the major environmental factors that negatively affects decapod immune systems and, for example, increased water temperature causes a significant reduction in the total hemocyte count (THC), phenoloxidase (PO) activity, respiratory burst (RB) and superoxide dismutase (SOD) activity in the whiteleg shrimp *Litopenaeus vannamei* (Cheng et al., 2005b). Also in L. *vannamei*, fluctuations in salinity and pH can cause significant alternations in immune responses (Lu-Qing et al., 2005) and hypoxic stress can profoundly affect immune responses such as THC, differential hemocytes count (DHC), RB and PO (Le Moullac et al., 1998). High levels of environmental ammonia have been reported to affect immune responses, growth and moulting in decapod crustaceans (Chen and Kou, 1992; Jiang et al., 2004c), and pathogen challenges are known to provoke significant alterations in key immune parameters in a range of decapod species (Sun et al., 2015; Ren et al., 2017; Pang et al., 2019; Bouallegui, 2021).

The impact of these environmental factors can be directly assessed by observation of the immune response of organisms. Like other crustaceans, decapods lack adaptive immunity and rely completely on their innate immune system for protection against stressors and pathogens.

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https://doi.org/10.1016/j.cbpa.2022.111332
Received 4 September 2022; Received in revised form 4 October 2022; Accepted 4 October 2022
Available online 12 October 2022

Table 1
Morphological features and functions of hemocytes in decapod crustaceans and the different terminologies used for them in the literature. Hemocyte classification by Söderhäll et al. was used as a widely accepted reference in decapods (Söderhäll and Smith, 1983; Smith and Soderhall, 1983; Soderhall, 2016). The morphological features of immune cells in the text and/or in the micrographs in each reviewed article were used for assigning similar cells with different terminologies to the classification used by Söderhäll and colleagues.

Hemocyte type based on Söderhäll and colleagues (Smith and Soderhall, 1983; Söderhäll and Smith, 1983; Soderhall, 2016) .	Equivalent terminology	Species	Functions
Hyaline cell (HC) The smallest cell, generally ovoid in shape, high nucleocytoplasmic ratio, and electron-dense deposits in the nucleus, largely agranular, sometimes with few granules	Undifferentiated hemocyte (Tsing et al., 1989a)	*Penaeus japonicus, Penaeus monodon, Macrobrachium rosenbergii, and Palaemon adspersus* (Tsing et al., *1989a*)	Phagocytosis (Smith and Ratcliffe, 1978; Sung and Sun, 2002; Giulianini et al., 2007), and hemolymph coagulation (Sternshein and Burton, 1980; Omori et al., 1989; Clare and Lumb, 1994) (Vazquez et al., 2009; Rowley, 2016)
	Lymphocyte (Dall, 1964)	*Metapenaeus mastersii* (Dall, *1964*)	
	A, spindle shaped hyaline cell B, spherical shaped hyaline cell (Sawyer et al., 1970)	*Callinectes sapidus* (Sawyer et al., *1970*)	
	Hyaline cell (Bodammer, 1978)	*Callinectes sapidus* (Bodammer, *1978*)	
	a, prohyalocyte b, hyalocyte (Cornick and Stewart, 1978)	*Homarus americanus* (Cornick and Stewart, *1978*)	
	Agranular cell (Martin and Graves, 1985)	*Sicyonia ingentis and Penaeus californiensis* (Martin and Graves, *1985*)	
	Deposit cell (Omori et al., 1989)	*Sicyonia ingentis* (Omori et al., *1989*)	
	Hyaline cells (Clare and Lumb, 1994)	*Callinectes sapidus* (Clare and Lumb, *1994*)	
	Coagulocyte (Sternshein and Burton, 1980)	*Procambarus spp., Orconectes* (Sternshein and Burton, *1980*)	
	Hyaline cell (Vogan and Rowley, 2002)	*Cancer pagurus* (Vogan and Rowley, *2002*)	
	Phagocytic cell (Smith and Ratcliffe, 1978)	*Carcinus maenas* (Smith and Ratcliffe, *1978*)	
	Agranular hemocyte (Martin et al., 1987)	*Sicyonia ingentis* (Martin et al., *1987*)	
	Hyaline hemocyte (Giulianini et al., 2007)	*Astacus leptodactylus* (Giulianini et al., *2007*)	
Semi-granular cell (SGC) Relatively larger than HC, contains variable number of granules, relatively	Small granule hemocyte (Tsing et al., 1989a)	*Penaeus japonicus, Penaeus monodon, Macrobrachium rosenbergii, and Palaemon*	Encapsulation, nodule formation and phagocytosis (Giulianini et al., 2007; Li et al., 2018),

Table 1 (*continued*)

Hemocyte type based on Söderhäll and colleagues (Smith and Soderhall, 1983; Söderhäll and Smith, 1983; Soderhall, 2016) .	Equivalent terminology	Species	Functions
small granules than GC	Thigmocyte (Dall, 1964)	*adspersus* (Tsing et al., *1989a*) *Metapenaeus mastersii* (Dall, *1964*)	storage and release of proPO molecules and cytotoxicity (Vazquez et al., 2009; Rowley, 2016)
		Finely granular granulocyte (Sawyer et al., 1970) *Callinectes sapidus* (Sawyer et al., *1970*)	
		Intermediate cells (Bodammer, 1978) *Callinectes sapidus* (Bodammer, *1978*)	
		Refractile and chromophobic granulocyte (Cornick and Stewart, 1978) *Homarus americanus* (Cornick and Stewart, *1978*)	
		Small-granule hemocyte (Martin and Graves, 1985) *Sicyonia ingentis and Penaeus californiensis* (Martin and Graves, *1985*)	
		Small-granule hemocyte (Omori et al., 1989) *Sicyonia ingentis* (Omori et al., *1989*)	
Small-granule hemocyte (Clare and Lumb, 1994)		*Callinectes sapidus* (Clare and Lumb, *1994*)	
Amebocyte (Sternshein and Burton, 1980)	*Procambarus spp., Orconectes* (Sternshein and Burton, *1980*)		
Eosinophilic granular cells (Vogan and Rowley, 2002)	*Cancer pagurus* (Vogan and Rowley, *2002*)		
A, Small granule hemocyte (with cytoplasmic deposits) B, Small granule hemocyte (without cytoplasmic deposits) (Martin et al., 1987)	*Sicyonia ingentis* (Martin et al., *1987*)		
Small granule hemocyte (Giulianini et al., 2007)	*Astacus leptodactylus* (Giulianini et al., *2007*)		

(*continued on next page*)

Their innate immune system consists of cellular and humoral components that perform various functions including phagocytosis, encapsulation, nodule formation, clotting, melanization, the activation of antimicrobial peptides (AMPs) and the prophenoloxidase system (proPO) (Le Moullac and Haffner, 2000; Jiravanichpaisal et al., 2006; Vazquez et al., 2009). These immune parameters can act as biomarkers for assessing the impact of environmental factors on decapod immune status. The most-mentioned immune parameters in the literature are the PO, a key component of the proPO-system. THC and DHC refer to the number of immune cells and are important indicators of the immune status and resistance against disease. RB is the mechanism responsible for producing reactive oxygen species (ROS) that help kill phagocytized pathogens and is a key indicator of oxidative stress regulated by antioxidants such as SODs. Lysozyme is an antimicrobial enzyme and acts as an important indicator of immune status.

A large body of work describes the effects of biotic and abiotic environmental factors on the immune parameters of decapods. The molecular mechanisms by which biotic factors such as pathogens induce immune responses are well established in decapod crustaceans. Nevertheless, further efforts are still needed to acquire better understanding of the molecular pathways induced by abiotic stressors and, in particular, how stressor-specific sensors receive signals from a particular abiotic factor and which specific cellular response is triggered by a particular environmental factor, two questions that are not sufficiently well explained in the literature. Therefore, we discuss here different mechanisms by which abiotic environmental factors induce various metabolic pathways associated with immune responses. In addition, this review critically evaluates and summarizes available information in the literature on the effects of altered abiotic environmental conditions and their impact on the immune parameters that can lead to increased susceptibility (e.g. compromised immune response) in decapod crustaceans. To fully understand how these immune mechanisms operate, the first part of this review provides a comprehensive overview of current knowledge of the innate immune system in decapod crustaceans and highlights important knowledge gaps regarding hemocyte types and functions.

2. An overview of the innate immune system in decapods

Successful production of decapod crustaceans depends on the effective use of disease prevention strategies, which requires a comprehensive understanding of the basic functions of the immune system. The immune system in shrimps and prawns has been well studied, given their economic value (Kumaresan et al., 2017; Kulkarni et al., 2021). Decapod crustaceans lack an acquired immune system but have a highly developed innate immune system. The initial non-specific defence of decapod crustaceans against foreign entities is their tough chitinous integument (Amparyup et al., 2013; Rowley, 2016). However, the pathogenic invaders that do enter the hemocoel then have to face up to multiple innate immune responses. The innate immune system of decapods is divided into cellular and non-cellular (humoral) defence responses, as well as a mixture of both that forms coagulation and proPO systems. This classification considers the nature of the effectors – not only their origin – and the functions that act synergistically to produce a coordinated response against foreign pathogens (Hauton, 2012).

2.1. Immune cells in decapods

Hemocytes are crucial components of cellular immune responses in decapods and have diverse cellular immunity functions. Their most important functions include phagocytosis, nodulation and encapsulation, and the release of immune molecules (Smith and Söderhäll, 1986; Smith, 1991; Söderhäll and Cerenius, 1992; Le Moullac and Haffner, 2000; Jiravanichpaisal et al., 2006; Matozzo and Marin, 2010; Qyli et al., 2020). However, it is important to note that the classification of hemocytes in decapods has always been a challenge due to the lack of any unified classification scheme that could distinguish hemocyte types and their related functions (Bauchau et al., 1981; Johansson et al., 2000). The classification criteria of hemocytes used in studies differ and are based on either morphological aspects and/or cytochemical properties, which often make their nomenclature controversial. Due to a lack of uniform consensus on classification criteria, researchers use a wide variety of terminologies in the literature to refer to various hemocyte types. For example, hyaline cells have also been defined as 'lymphocytes' (Dall, 1964), while semi-granular cells have been termed 'thigmocytes' (Dall, 1964) and granular cells 'granular amoebocytes' (Dall,

Table 1 (*continued*)

Hemocyte type based on Söderhäll and colleagues (Smith and Soderhall, 1983; Söderhäll and Smith, 1983; Soderhall, 2016) .	Equivalent terminology	Species	Functions
Granular cell (GC) The largest cell, generally with kidney-shaped nucleus and cytoplasm filled with membrane-bound, electron-dense refractile granules	Large granule hemocyte (Tsing et al., 1989a)	*Penaeus japonicus, Penaeus monodon, Macrobrachium rosenbergii, and Palaemon adspersus* (Tsing et al., *1989a*)	Limited role in phagocytosis (Giulianini et al., 2007; Li et al., 2018), storage and release of proPO molecules, encapsulation and cytotoxicity (Vazquez et al., 2009; Rowley, 2016)
	Large granule amoebocyte (Dall, 1964)	*Metapenaeus mastersii* (Dall, *1964*)	
	Coarsely granular granulocyte (Sawyer et al., 1970)	*Callinectes sapidus* (Sawyer et al., *1970*)	
	Granulocytes (Bodammer, 1978)	*Callinectes sapidus* (Bodammer, *1978*)	
	Refractile and eosinophilic granulocyte (Cornick and Stewart, 1978)	*Homarus americanus* (Cornick and Stewart, *1978*)	
	Large -granule hemocyte (Martin and Graves, 1985)	*Sicyonia ingentis and Penaeus californiensis* (Martin and Graves, *1985*)	
	Large-granule hemocyte (Omori et al., 1989)	*Sicyonia ingentis* (Omori et al., *1989*)	
	Large-granule hemocyte (Clare and Lumb, 1994)	*Callinectes sapidus* (Clare and Lumb, *1994*)	
	Granulocyte (Sternshein and Burton, 1980)	*Procambarus spp., Orconectes* (Sternshein and Burton, *1980*)	
	basophilic granular cell (BG), basophilic/ eosinophilic cell (BEG) (Vogan and Rowley, 2002)	*Cancer pagurus* (Vogan and Rowley, *2002*)	
	Refractile cell (Smith and Ratcliffe, 1978)	*Carcinus maena* (Smith and Ratcliffe, *1978*)	
	Large granule haemocyte (Martin et al., 1987)	*Sicyonia ingentis* (Martin et al., *1987*)	
	Large granule hemocyte (Giulianini et al., 2007)	*Astacus leptodactylus* (Giulianini et al., *2007*)	

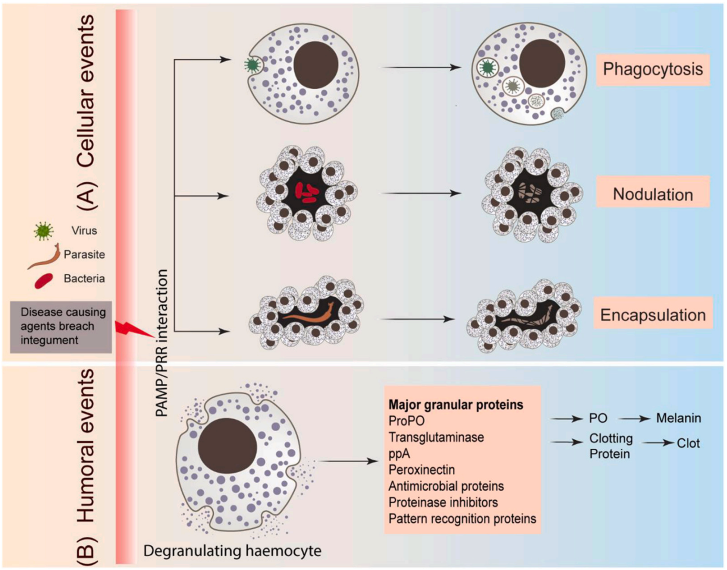

Fig. 1. Scheme of decapod crustaceans' cellular and humoral immune responses and their interaction to pathogens and parasites as biotic environmental factors. (A) The key cellular events are phagocytosis, encapsulation, nodule formation and melanization. (B) The essential responses for the humoral events after activation of granulocytes, prophenoloxidase (proPO), proPO-activating enzyme (ppA), transglutaminase activation, clotting, etc. PAMP/PRR: Pathogen-Associated Molecular Patterns/ Pattern Recognition Receptors.

1964). Despite these contrasting classifications, decapod hemocytes are generally classified into three types, hyaline cells (HC), semi-granular cells (SGC) and granular cells (GC), mainly based on morphological criteria such as cell size, nuclear/cytoplasmic ratios, and the presence/absence and size and number of cytoplasmic granules (Söderhäll and Smith, 1983; Soderhall, 2016). The idea that hemocytes in decapods develop from a single cell lineage and that subpopulations are probably different developmental stages has been mooted in some studies of crustaceans — albeit with little supporting evidence (Tsing et al., 1989b; Jussila et al., 1997; van de Braak et al., 2002). However, a recent study of hemocytes in the crayfish *Cherax quadricarinatus* suggests that the so-called hemocyte subtypes (i.e. SGC and HC) are probably different stages of a single cell type (GC) lineage. Additionally, this study suggests that all hemocytes at different developmental stages are functionally active immune cells that are capable of phagocytosis (Li et al., 2021). A study of the bivalve *Crassostrea rhizophorae* reported that the different subpopulations of hemocytes may in reality be different stages of one type of cell, in which the absence of granules (loss of complexity) could be due to degranulation in the event of an immune response (de Freitas Rebelo et al., 2013). It would be interesting to test the possibility of this hypothesis in future studies of other decapod species using state-of-art morphological and molecular techniques.

Table 1 summarizes different hemocyte types and their synonymous terms in the literature, their related morphological features, and their function in decapod immunity.

2.2. Phagocytosis

Phagocytosis protects the host by engulfing invading pathogens and by helping remove apoptotic cell debris to maintain tissue homeostasis (Li et al., 2018). It starts with the recognition of foreign particles by the hemocytes, which is followed by the engulfing and enclosing of the particle into phagosome. The particle is then shredded into pieces by specialized enzymes, which produce harmless particles that are either recycled for further use by the cell or are discarded (Liu et al., 2020c). Due to the lack of any unified classification scheme of hemocyte types in decapods (Table. 1), contrasting findings regarding the phagocytic role of hemocytes are found in the literature. For example, an in vivo phagocytosis assay in the narrow-clawed crayfish *Astacus leptodactylus* demonstrated phagocytic activity in all three types of hemocytes (Giulianini et al., 2007), whereas a study of the tiger shrimp *Penaeus monodon* reported that phagocytic activity was mainly exhibited by HC rather than SGC or GC (Sung and Sun, 2002). On the other hand, a study of the crayfish *C. quadricarinatus* showed that the 0.2 μm fluorescent microspheres were internalized by SGC and GC cells, thereby suggesting that only two types of hemocytes perform phagocytic activity (Li et al., 2018). Further research is still required in this area to better characterize and classify hemocyte subpopulations and their corresponding phagocytic role in decapods. The schematic model of phagocytosis is shown in Fig. 1 (A).

2.3. Encapsulation and nodulation

Encapsulation and nodulation are two powerful cellular immune reactions that are responsible for the localization and isolation of pathogens and parasites that are too large for phagocytosis by an individual hemocyte (Rowley, 2016).

Encapsulation, whereby hemocytes progressively surround a foreign intruder in multiple layers, efficiently prevents the growth and development of parasites such as cestodes, trematodes and nematodes, as well as pathogenic fungi (Nyhlén and Unestam, 1980; Vranckx and Durliat, 1981; Persson et al., 1987b). It has been shown that the immune cells of the narrow-clawed crayfish are able to encapsulate a parasite *Psorospermium haeckeli* in multiple layers of hemocytes with two different shapes (Vranckx and Durliat, 1981). Furthermore, a study has reported that the crayfish plague *Aphanomyces astaci* that penetrated a wound site immediately encountered encapsulation by hemocytes, with higher and lower levels, respectively, of melanization in resistant signal crayfish

Table 2
Major proteins released from granules of immune cells and their immune functions in decapods.

Proteins from the granules	Species	Function	References
Prophenoloxidase	*Pacifastacus leniusculus*	Component of proPO-system	Aspan and Soderhall, (1991)
proPO-activating enzyme (ppA)	*Pacifastacus leniusculus*	Activation of proPO-system	Aspán et al., (1990)
Peroxinectin	*Pacifastacus leniusculus*	Cell-adhesion activity	Johansson et al., (1995)
Antimicrobial peptides (AMPs)	*Pacifastacus leniusculus*	Antimicrobial activity	Sricharoen et al., (2005; Rosa and Barracco, (2010)
Transglutaminase	*Marsupenaeus japonicus*	Coagulation and regulation of immune genes	Fagutao et al., (2012)
Proteinase inhibitors	*Penaeus monodon*	Proteinase inhibitor and antimicrobial activities	Amparyup et al., (2008)
Pattern recognition proteins (PRPs)	*Penaeus monodon*	Cell-adhesion activity, antimicrobial activity and component of proPO-system	Amparyup et al., (2012a)

Pacifastacus leniusculus and susceptible noble crayfish *Astacus astacus* (Nyhlén and Unestam, 1980).

Nodulation is another effective cellular defence response to large numbers of bacteria that helps the body isolate and neutralize foreign pathogens (Götz, 1986). In vivo studies of the shore crab *Carcinus maenas* injected with two pathogenic bacteria, *Bacillus cereus* and *Moraxella* sp., formed several hemocytic clumps (nodules) that entrapped a large number of microorganisms, thereby effectively constraining the spread of bacteria in the hemocoel (Smith and Ratcliffe, 1980a; Smith and Ratcliffe, 1980b). Similarly, the infection of signal crayfish by *Vibrio areninigrae* caused hemocytes to form nodules against bacterial pathogen in the heart, hepatopancreas and gills (Hernández-Pérez et al., 2021).

Both capsules and nodules are generally accompanied by melanin formation derived from the activation of the prophenoloxidase (proPO) system. Trapped pathogens are killed and dismantled by the cytotoxic quinones in the rigid melanin layer (Vogt, 2008). Encapsulation and nodule formation is illustrated in Fig. 1 (A).

2.4. Recognition of pathogens

Responses to pathogen invaders are very powerful and rapid in decapod crustaceans. They are induced by extracellular signal molecules called pathogen-associated molecular patterns (PAMPs) that are recognized by specialized cell surface receptors called pattern-recognition proteins (PRPs) or pattern-recognition receptors (PRRs) on the surface of immune cells (Janeway Jr and Medzhitov, 2002; Amparyup et al., 2012b; Tassanakajon et al., 2013). When PAMP/PRR interaction occurs, the immune cells are rapidly activated and respond through intracellular signalling cascades, resulting in cellular and humoral immune responses (Tassanakajon et al., 2013) including phagocytosis, encapsulation and nodulation, as well as the activation of proPO and coagulation systems and the release of antimicrobial peptides (Jiravanichpaisal et al., 2006; Amparyup et al., 2012b).

2.5. Prophenoloxidase (proPO) system

The proPO system is an essential part of the humoral immune response and is found in the zymogen form stored in the semi-granular and granular hemocytes (Söderhäll and Smith, 1983). The PO central enzyme is produced after the inactive form proPO is released from the cytoplasm of hemocytes as a result of a stimulation. A variety of PAMPs such as b-1,3-glucans, lipopolysaccharide (LPS) and peptidoglycan released from microbes initiate stimuli by activating serine proteases such as proPO-activating enzyme (ppA), which in turn convert the inactive proPO to an active PO enzyme state (Cerenius et al., 2010). Furthermore, the PO acts upon phenolic compounds and yields melanin and a variety of potentially toxic factors that are important for killing pathogens (Rowley and Powell, 2007; Cerenius et al., 2008). The proPO cascade mechanism has been extensively studied in the signal crayfish (Söderhäll et al., 2009; Cerenius et al., 2010), tiger shrimp (Charoensapsri et al., 2011) and the Chinese mitten crab *Eriocheir sinensis* (Gai et al., 2008). The RNA interference-mediated silencing of proPO gene activity in signal crayfish infected by pathogenic bacterium showed increased bacterial growth and decreased cellular responses such as nodule formation and phagocytosis (Liu et al., 2007). Likewise, the application of the same technique in tiger shrimps challenged with white-spot syndrome virus (WSSV) caused higher mortality (Sutthangkul et al., 2015). PO-mediated melanin synthesis and other humoral events are shown in Fig. 1 A, B.

2.6. Coagulation system

Coagulation of the hemolymph is another crucial humoral defence response. Decapods possess an open circulatory system with a very rapid, powerful coagulation mechanism to prevent the loss of hemolymph and the dissemination of pathogens (Martin et al., 1991). The coagulation system has been extensively studied in signal crayfish as a useful model for clarifying decapod immunity (Cerenius and Söderhäll, 2018; Perdomo-Morales et al., 2019). Transglutaminase (TGase), the central enzyme in hemolymph coagulation, is stored in the granules and released from the cells when hemocytes are activated (Aono and Mori, 1996). TGase is activated by Ca^{2+} contents in plasma, which initiates the polymerization of plasma-clotting protein (CP) molecules into long flexible chains that form the visible clot (Martin et al., 1991; Maningas et al., 2008). CPs are defence molecules that possess multifunctional properties and are essential for recognizing and neutralizing foreign bodies (Iwanaga and Lee, 2005).

2.7. Antimicrobial peptides (AMPs)

As key humoral defence molecules, AMPs are mainly cationic peptides and have been categorized into four groups based on their amino acid composition and structure in decapods: (1) Linear α-helical single-domain AMPs such as homarin and armadillidin; (2) Single-domain peptides AMPs enriched with cysteine residues such as defensin and anti-lipopolysaccharide factor; (3) Multi-domain or chimeric AMPs such as crustins and penaeidin; (4) Non-conventional AMPs that exhibit multifunctional antimicrobial activity such as hemocyanin-derived peptides (Rosa and Barracco, 2010; Becking et al., 2020).

Two key signal transduction pathways including the Toll and immunodeficiency (IMD) pathways are responsible for regulating the expression of AMPs (De Gregorio et al., 2002). The synthesis and release of AMPs from granules of immune cells are rapidly triggered after a microbial challenge via PAMPs/PRPs interaction and the subsequent activation of Toll and IMD signalling pathways (Calderon-Rosete et al., 2018; Tassanakajon et al., 2018; Li et al., 2019). The Toll pathway regulates the expression of AMPs that are mainly active against gram-positive bacteria and fungi (Rutschmann et al., 2002; Valanne et al., 2011), while the IMD pathway mainly regulates AMPs active against gram-negative bacteria and viruses (Lemaitre and Hoffmann, 2007). Table 2 shows the main proteins that are released from immune cells and their functions in decapods.

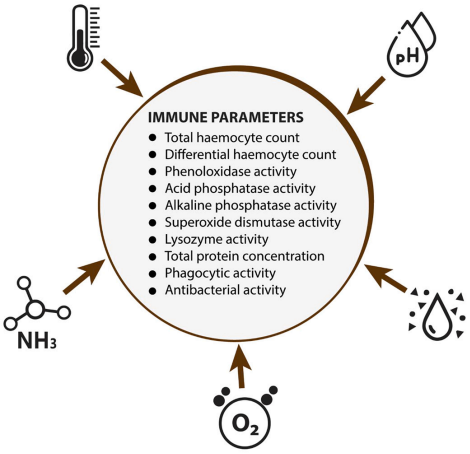

Fig. 2. The illustration shows the key immune parameters that are most commonly affected by abiotic factors such as temperature, pH, salinity, dissolved oxygen and ammonia. These parameters are often used as immune indicators to assess the health status of decapod crustaceans.

3. Interaction of environmental factors and the immune system in decapods

Decapod crustaceans inhabit a diverse range of aquatic environments and are greatly affected by a variety of abiotic and biotic environmental stressors, which have been shown to have profound effects on reactive oxygen species (ROS) production, nucleic acid, lipid and protein stability, and hemocyte counts (Kultz, 2003, 2005; Chang et al., 2009b; Wang et al., 2009; Cheng et al., 2018; Burgos-Aceves et al., 2021; Xu et al., 2022) and so, in turn, can induce significant immunological reactions (Cheng and Chen, 2000; Kumaresan et al., 2017; Burgos-Aceves et al., 2018). Although the mechanisms by which biotic factors and pathogens induce immune responses at cellular and molecular levels in decapods are well studied, the mechanisms that abiotic environmental factors employ to affect specific immune functions at cellular and molecular levels are far less well known. For example, how stressor-specific sensors receive signals from a particular environmental factor and how a specific cellular response is triggered by one of these factors is yet to be investigated in decapods. The overall effects of abiotic factors on the immune parameters of decapods are summarized in Fig. 2.

In the following section, we first elucidate some of the mechanisms through which diverse categories of environmental factors activate the immune system and then classify the resulting immune responses.

3.1. Temperature

3.1.1. Possible mechanism of action of temperature on the immune system

Temperature is probably the most important abiotic environmental factor having an adverse effect on immune responses in decapod crustaceans. Critically high and low temperatures can alter environmental oxygen levels and their demand at cellular and tissue levels. High temperatures are associated with a reduction in the oxygen in the aquatic environment together with a sharp rise in oxygen demand due to the increased energy cost of ventilation and circulation caused by these elevated temperatures (Portner, 2001). On the other hand, critically low temperatures have been shown to reduce mitochondrial aerobic capacity (Portner, 2001). Although water can dissolve more oxygen at lower temperatures, the level of oxygen drops around freezing point because the surface ice layer acts as a barrier against the diffusion of oxygen from the air into water (Pulkkanen and Salonen, 2013). Despite the fact the level of ROS is not high at low temperatures, freeze-tolerant animals can increase their antioxidant levels at freezing point to cope with the oxidative stress that occurs during the thawing and reoxygenation stage (Gorr et al., 2010b). It is estimated that 2–3% of the oxygen consumed by aerobic cells is converted into free oxygen radicals and H_2O_2 (Sohal and Weindruch, 1996). Therefore, higher temperatures outside the optimum tolerance range enhance ROS production and cause oxidative damage to essential cellular biomolecules such as lipids, proteins and DNA in aquatic animals (Kultz, 2005; Malev et al., 2010; Li et al., 2015). To mitigate oxidative stress, the survival of the host cell is supported by molecular protection provided via stress-related proteins and the antioxidative defence system (Feder and Hofmann, 1999; Portner, 2001; Zhou et al., 2010).

However, despite various protective mechanisms, excessive and prolonged thermal stress beyond cells' ability to cope results in the activation of apoptotic mechanisms such as p53–Bax and caspase-dependent apoptotic pathways (Cheng et al., 2015). Consequently, high temperatures may increase apoptosis in hemocytes and reduce THC and immune functions, and increase the vulnerability to pathogens in decapods (Jiravanichpaisal et al., 2004; Cheng et al., 2005b; Wang and Chen, 2006b). The reason for declining phenoloxidase activity after thermal stress could be due to greater hemocyte death followed by a high level of protease inhibitors such as trypsin inhibitor and α- 2-macroglobulin in the hemolymph. Both these protease inhibitors can halt the proPO system by inhibiting ppA as the key enzyme for proPO activation (Sung et al., 1998).

In addition, recent reports suggest that as a response to certain environmental factors, the neuroendocrine-immune system plays a crucial role in maintaining homeostasis and enhancing environmental adaptability (Tong et al., 2022). For example, after exposure to cold stress, production of the hemolymph norepinephrine (NE) increased, which consequently induced the apoptosis of hemocytes via caspase-3 in the whiteleg shrimp (Chang et al., 2009a). As well, thermal stress induced the release of NE and caused significant modulation of the immune responses in the giant freshwater prawn *Macrobrachium rosenbergii* (Chang et al., 2015), indicating thus that the apoptosis of THC might be controlled via the endocrine system. Furthermore, it has been suggested that under cold stress, the decline of THC could be due to weakened production and release of hemocytes from hematopoietic organs, as well as the adherence and immobilization of hemocytes to other tissues such as gills (Johnson, 1980; Victor et al., 1990; Lubawy and Slocinska, 2020).

3.1.2. Immune responses of decapods under thermal stress

The exposure of the narrow-clawed crayfish to high temperatures of up to 30 °C caused a significant increase in the level of DNA damage, THC count, hemolymph glucose and total protein concentrations (Malev et al., 2010). A recent study has reported a correlation between temperature and disease susceptibility in signal crayfish. These crayfish were kept at two temperatures (6 °C and 22 °C) and injected with two pathogenetic gram-negative bacteria strains and LPS. At the lower temperature, the mortality rate was lower, THC decreased and phagocytosis improved, while at the higher temperature the mortality rate and melanization rose. (Korkut et al., 2018). Likewise, Jiravanichpaisal and colleagues studied the effect of water temperature on immune parameters and the infectivity pattern of white-spot disease in both signal and noble crayfish, and demonstrated that temperature greatly altered the immune parameters and mortality rates in infected crayfish (Jiravanichpaisal et al., 2004).

Furthermore, in the tiger shrimp a change in temperature from a control of 26 °C to 34 and 22 °C resulted in lower THC and DHC levels, less phagocytic, phenoloxidase and SOD activity, and greater susceptibility to pathogens (Wang and Chen, 2006b). Significant reductions

Table 3
A summary of the immune responses of decapods to thermal stress.

Species group	Species	Temperature °C	Immune responses	References
Crayfish	*Astacus leptodactylus*	25, 30	↕ Total hemocyte count ↑Total protein concentrations	Malev et al., (2010)
	Pacifastacus leniusculus	6, 22	↓ Total hemocyte count ↑ Melanization ↑ Phagocytic activity	Korkut et al., (2018)
	Pacifastacus leniusculus and Astacus astacus	4, 12, 18, 22	↕ Total hemocyte count ↕ Granular cells – Phenoloxidase activity – prophenoloxidase ↓ Lipopolysacharide glucan binding protein	Jiravanichpaisal et al., (2004)
	Procambarus clarkii	18, 24	↕ Total hemocyte count ↑ Phenoloxidase activity	Dong et al., (2015)
Crabs	*Carcinus aestuarii*	4, 17, 30	↓ Total hemocyte count ↑ Phenoloxidase activity ↓ Superoxide dismutase activity ↓ Catalase activity ↓ Total protein concentrations	Matozzo et al., (2011)
	Carcinus maenas	10, 20	↑ Total hemocyte count	Truscott and White, 1990)
	Carcinus maenas	Water temperatures in February and August	↕ Total hemocyte count ↕ Total protein concentrations ↓Antibacterial activity	Chisholm and Smith, 1994)
Prawns	*Macrobrachium rosenbergii*	22, 28, 34	↕ Total hemocyte count ↕ Differential hemocyte count ↑ Phenoloxidase activity ↑ proPO-system ↓ Superoxide dismutase activity ↓ Phagocytic activity	Chang et al., (2015)
	Macrobrachium rosenbergii	20,21,27,28, 30, 31,33,34	↕ Total hemocyte count ↕ Phenoloxidase activity	Cheng and Chen, 2000)
Shrimps	*Penaeus monodon*	22, 26, 30, 34	↓ Total hemocyte count ↓ Differential hemocyte count ↓ Phenoloxidase activity ↓ respiratory burst ↓ superoxide dismutase activity ↓ phagocytic activity	Wang and Chen, 2006b)
	Litopenaeus vannamei	18, 21, 24, 27, 30	↓ Total hemocyte count ↑ Phenoloxidase activity ↓ Bacteriolytic activity	Lu-Qing et al. (2007)
	Litopenaeus vannamei	17, 20, 23	↓ Total hemocyte count ↑ Reactive oxygen species	Li et al., (2014)
	Litopenaeus vannamei	16, 22, 28	↓ Total hemocyte count ↕ Phenoloxidase activity ↕ Nitric oxide synthase activity ↕ Superoxidase activity ↑ Malondialdehyde content	Jia et al., (2014b)
	Litopenaeus vannamei	20 to 34	↓ Total hemocyte count ↓ Phenoloxidase activity ↓ Respiratory burst ↓ Superoxidase activity ↓ Phagocytic activity	Cheng et al., (2005b)
	Penaeus californiensis	18, 22, 25, 28, 32	↑ Total plasma protein concentration ↓ prophenoloxidase activity	Vargas-Albores et al. (1998)
	Penaeus vannamei	13, 15, 25	↓ Total hemocyte count ↕ Differential hemocyte count ↕ Phenoloxidase activity ↓ prophenoloxidase expression ↕ Lysozyme activity ↕ Gamma-glutamyl transferase activity	Xu et al., (2019)
	Litopenaeus vannamei	18, 21, 24, 27, 30	↓ Total hemocyte count ↓ Differential hemocyte count ↓ Phenoloxidase activity ↓ Serine protease activity	Pan et al., (2008)

↑ = increase.
↓ = decrease.
↕ = increase and decrease.
–= no significant change.

were also observed in phagocytotic responses, antibacterial activity, THC, PO and SOD activity, and RB when whiteleg shrimps were infected with bacteria at a high temperature of 32 °C (Cheng et al., 2005b). In the yellow-leg shrimp *Penaeus californiensis* an increase in temperature from 18 to 32 °C affected both proPO and plasma protein hemolymph parameters, causing a reduction in total proPO level at 32 °C and decrease in plasma total protein at 28 and 32 °C (Vargas-Albores et al., 1998).

In the giant freshwater prawn after exposure to low (22 °C) and high

Table 4
A summary of the immune responses of decapods to pH stress.

Species group	Species	pH	Immune responses	References
Crabs	*Callinectes sapidus*	6.6, 7.0, 7.4, 7.6, 7.8	↓ Phenoloxidase activity	Tanner et al., (2006)
Prawns	*Macrobrachium rosenbergii*	4.6–5.0, 7.5–7.7, 9.0–9.5	↓ total hemocyte count ↓ phenoloxidase activity	Cheng and Chen, 2000)
Shrimps	*Litopenaeus vannamei*	6.8, 8.1	↓ total hemocyte count ↓ Hyaline cells ↓ Granular cells ↓ Respiratory Bursts ↓ Superoxide dismutase activity ↓ Lysozyme activity ↓ phenoloxidase activity ↑ Mortality rate	Chen et al., (2015)
	Litopenaeus vannamei	6.8	↓ Hyaline cells ↓ Granular cells ↓ Semi-granular cells ↓ Total hemocyte count ↓ Phenoloxidase activity ↓ Respiratory burst ↓ Superoxide dismutase activity ↓ Glutathione peroxidase activity ↓ Lysozyme activity	Lin et al., (2010)
	Litopenaeus vannamei	6.5, 8.2, 10.1	↑ Mortality rate ↓ Total Hemocyte count ↓ Phenoloxidase activity ↓ Respiratory burst, ↓ Phagocytic activity ↓ Clearance efficiency ↓ Superoxide dismutase activity	Li and Chen, 2008)
	Fenneropenaeus indicus	6, 5.5	↓ total hemocyte count ↓ phenoloxidase activity ↓ hemolymph protein values	Sharma et al., (2009)
	Litopenaeus vannamei	7.0, 7.5, 9.0, 9.5	↓ Total hemocyte count ↓ Bacteriolytic activity ↓ Antibacterial activity ↑ Phenoloxidase activity	Lu-Qing et al., (2005)

↑ = increase.
↓ = decrease.
↕ = increase and decrease.
–= no significant change.

(34 °C) temperatures immune responses including THC, PO, SOD, RB, and phagocytic activity, and proPO-system-related gene expression were found to have changed significantly (Chang et al., 2015).

When the Mediterranean green crab *Carcinus aestuarii* was exposed to 4, 17, and 30 °C, at temperatures of 4 °C and 30 °C, THC fell significantly, while hemolymph protein concentrations dropped only at 30 °C. In addition, hemocyte proliferation and PO activity increased greatly at both the lowest and highest temperatures, which demonstrates that these crabs can modulate their immune parameters to cope with temperature fluctuations (Matozzo et al., 2011). In the shore crab, a gradual increase in temperature over 12 h from 10 to 20 °C for five days led to a significant rise in hemocyte numbers, whereas a sudden temperature rise initiated no such response (Truscott and White, 1990). The antibacterial activity in shore crabs fell in February and August at the moment of the least and greatest seasonal fluctuation in temperatures (Chisholm and Smith, 1994), which suggests that thermal stress greatly affects the immune parameters in decapod crustaceans. The impact of thermal stress on the immune parameters of decapod crustaceans is summarized in Table 3.

3.2. pH

3.2.1. Possible mechanism of action of pH on the immune system

Decapod crustaceans are sensitive to fluctuations in environmental pH. Its stress probably damages tissues associated with ion exchange such as gills, and causes acid-base imbalances (Mcculloch, 1990). The primary problem associated with acid-induced stress in aquatic animals is excessive secretion and accumulation of mucus on gill filaments leading to respiratory failure, anoxia and death (Daye and Garside, 1975). Greater uptake of hydrogen ions across the gills leads to hemolymph acidosis in crayfish under acidic conditions (Morgan and McMahon, 1982; Wood and Rogano, 1986). Both hemolymph acidosis and alkalosis depend on changes in pH in the surrounding medium and both critically high and low pH increase oxygen consumption in the noble and narrow-clawed crayfish (Cukerzis, 1973). Hemolymph oxygen affinity drops significantly with the decline in hemolymph pH (Mcculloch, 1990) and, in addition, both acidic and alkaline pH stresses have been found to induce RB and DNA damage in the hemocytes and the hepatopancreas of shrimps (Wang et al., 2009). It is important to note that both high and low pH stresses have been reported to lead to substantial immunosuppression and decreased resistance against pathogens in decapods due to a reduction in PO activity and phagocytic activity (Li and Chen, 2008; Chen et al., 2015).

3.2.2. Immune responses of decapods under pH stress

Fluctuations in water pH can greatly alter immune parameters such as THC, DHC, total hemolymph protein, PO activity, ROS production and antioxidant responses in decapods. In the whiteleg shrimp immune parameters and resistance against *Vibrio alginolyticus* decreased in long-term cultures at low pH levels. The immune parameters including THC, HC, GC, RB, PO activity, lysozyme activity (LZM), and SOD activity significantly decreased, and the expression levels of genes including ecCuZnSOD, cytMnSOD, LZM, glutathione peroxidase and penaeidin 3a were also down-regulated at pH 6.8 compared to 8.1. In addition, a separate experiment during the same study showed that during a 24-week challenge with *V. alginolyticus* lower pH can reduce phagocytic index, phagocytic activity and the clearance efficiency of animals (Chen et al., 2015). The Indian white shrimp *Fenneropenaeus indicus* experienced reduced immunity when exposed to low (5.5) and high (9) pH stresses and at lower pH the values of THC, PO activity and hemolymph protein were significantly lower than at a higher pH (Sharma et al., 2009). Exposure of the giant freshwater prawn to the combined effects of pH, temperature and salinity caused a reduction in THC and PO activity in both low (4.6–5.0) and high (9.0–9.5) pH media but not in the middle-ranging pH (7.05–7.7) (Cheng and Chen, 2000).

Furthermore, pH has been shown to influence bacterial virulence in

Table 5
A summary of the immune responses of decapods to the salinity stress.

Species group	Species	Salinity ‰	Immune responses	References
Crabs	*Portunus trituberculatus*	21, 26, 31	↓ Total hemocyte count ↓ prophenoloxidase activity ↑ phenoloxidase activity	Wang et al., (2018)
Prawns	*Macrobrachium rosenbergii*	0, 5, 10, 15	↑ Total hemocyte count ↑ phenoloxidase activity	Cheng and Chen, 2000)
Shrimps	*Fenneropenaeus indicus*	5, 25, 35	↑ Phenoloxidase ↑ Superoxide anion production ↑ Total hemolymph proteins ↑ Acid phosphatase activity ↑ Alkaline phosphatase activity	Selven and Philip, 2013)
	Litopenaeus vannamei	5, 15, 25, 35	↓ Total hemocyte count ↓ Phenoloxidase activity ↓ Respiratory burst ↓ Phagocytic activity ↓ Superoxide dismutase activity ↑ Mortality	Wang and Chen, 2005a)
	Penaeus monodon	0, 15, 35	↓ Total hemocyte count, – Phenoloxidase activity ↓ Nitroblue tetrazolium salt (NBT) reduction – Alkaline phosphatase activity ↓ Acid phosphatase activity	Joseph and Philip, 2007)
	Penaeus monodon	5, 15, 35	↓ Total hemocyte count ↓ Hyaline cell ↓ Phenoloxidase activity ↓ Respiratory burst ↓ Superoxide dismutase activity ↓ Phagocytic activity ↓ Clearance efficiency	Wang and Chen, 2006a)
	Farfantepenaeus paulensis	13, 22, 34	↓ Total hemocyte count – Total serum protein concentration ↓Phenoloxidase activity	Perazzolo et al., (2002)
	Litopenaeus vannamei	5, 30	↓ Total hemocyte count ↓ Bacteriolytic activity ↓ Antibacterial activity ↑ Phenoloxidase activity	Lu-Qing et al., (2005)
	Marsupenaeus japonicus	9,13,17,21,29 and 33 ppt	↓ Total hemocyte count ↑ Phenoloxidase activity	Yu et al., (2003)
	Litopenaeus vannamei	Freshwater and seawater	↑ Total hemocyte count ↓ Respiratory burst ↓ Phenoloxidase activity ↓ Nitric oxide synthase (NOS) ↓ Lysozyme activity	Jia et al., (2014a)
	Litopenaeus vannamei	2.5, 5, 15, 25, 35	↓ Hyaline cells ↓ Granular cells ↓ Phenoloxidase activity ↓ Respiratory bursts ↓ Superoxide dismutase activity ↓ Lysozyme activity	Lin et al., (2012)
	Litopenaeus vannamei	21,26	↓ Total hemocyte count ↓ Differential hemocyte count ↓ Phenoloxidase activity	Pan et al., (2010)
	Litopenaeus vannamei	15, 20, 25, 35	↓ hyaline cell count ↓ Granular cell (semi-granular cell) count ↓ Total hemocyte count ↓ Phenoloxidase activity ↓ Respiratory burst ↓ Superoxide dismutase activity	Li et al., (2010a)

↑ = increase.
↓ = decrease.
↕ = increase and decrease.
–= no significant change.

hosts. For example, the mortality rate is exacerbated in giant freshwater prawns when challenged with *Enterococcus*-like bacterium at high pH (8.8–9.5). However, the exposure of luminous bacteria to low pH (5.5) significantly reduced the pathogenicity of bacteria towards penaeid prawn larvae (Prayitno and Latchford, 1995; Cheng and Chen, 1998). This implies that fluctuations in pH not only influence the health and immune ability of hosts but also affect pathogen virulence in hosts, thereby leading to disease outbreaks. The impact of pH stress on the immune parameters of decapod crustaceans is summarized in Table 4.

3.3. Salinity

3.3.1. Possible mechanisms of action of salinity on the immune system

Acute changes in salinity can disrupt osmoregulatory mechanisms and subsequently suppress physiological and immune mechanisms in decapod crustaceans. As well, both hyper- and hypo-salinities have been shown to have immunosuppressive effects, which reduce resistance to infection (Wang and Chen, 2005b; Wang and Chen, 2006a). The results of studies of terrestrial, freshwater and marine crustaceans suggest that neurohormones like dopamine (DA) perform an important role in ionic and osmotic regulation and are key neuroregulators of the immune

Table 6
A summary of the immune responses of decapods to ammonia stress.

Species group	Species	Range of Ammonia mgL-1	Effect on Immune responses	References
Crabs	*Portunus trituberculatus*	0, 1, 5, 20	↓ Phagocytic activity ↓ Antibacterial and bacteriolytic activity	Yue et al., (2010b)
	Eriocheir sinensis	20, 40, 60, 80	↓ Total hemocyte count ↓ Superoxide dismutase activity	Hong et al., (2007)
	Eriocheir sinensis	1.0, 2.0, 3.0, 4.0,5.0	↓ Total hemocyte count ↓ Lysozyme activity ↓ Phenoloxidase activity ↓ Superoxide dismutase activity	Huang et al., (2006)
Lobsters	*Panulirus homarus*	0, 0.5, 1.5, 3	↓ Total hemocyte count ↓ Phenoloxidase activity	Verghese et al., (2007)
Prawns	*Macrobrachium rosenbergii*	0·55, 1·68, 3·18	– Total hemocyte count – Differential hemocyte count ↓ Phenoloxidase activity ↓ Respiratory burst	Cheng and Chen, 2002)
	Macrobrachium nipponense	0, 5, 10, 15, 20	↕ Superoxide dismutase ↕ catalase ↑ Alkaline phosphatase ↑ Acid phosphatase ↑ Malonaldehyde	Zhang et al., (2015)
	Macrobrachium rosenbergii	1	↓ Total hemocyte count	Hu et al., (2005)
Shrimps	*Penaeus vannamei*	1, 3, 6, 9	↓ Phenoloxidase activity ↓ Superoxide dismutase activity	Kathyayani et al., (2019)
	Litopenaeus vannamei	1.10, 5.24, 11.10, 21.60	↓ Total hemocyte count ↓ Hyaline cells ↓ Granular cells ↓ Phenoloxidase activity ↓ Respiratory bursts ↑ Superoxide dismutase activity ↓ Clearance efficiency to pathogen	Liu and Chen, 2004)
	Penaeus japonicus	5	↓ Total hemocyte count ↓ Plasma protein content ↓ Hemocyte phagocytosis ↓ prophenoloxidase activity ↓ Alkaline phosphatase ↓ Nitric oxide synthase	Jiang et al., (2004b)
	Litopenaeus schmitti	4, 6	↓ Total hemocyte count – Phenoloxidase activity – Hemagglutination activity	Rodríguez-Ramos et al., (2008)
	Litopenaeus vannamei		↓ Total hemocyte count	Qiu et al., (2008)
	Litopenaeus vannamei	0.05,0.15, 0.75, 1.50, 3.0	↓ Prophenoloxidase activity ↓ superoxide dismutase activity ↓ Acid phosphatase	
	Litopenaeus vannamei	0.5, 1.0, 1.5, 2.0, 2.5	↓ Total hemocyte count ↑ Phenoloxidase activity ↓ Bacteriolytic and antibacterial activity	Jiang et al., (2004c)

↑ = increase.
↓ = decrease.
↕ = increase and decrease.
–= no significant change.

system under salinity stress (Morris, 2001; Pan et al., 2010). Experiments have shown that the injection of DA causes significant changes in key immune parameters in decapods including THC, DHC, PO and RB, and a decrease in antibacterial activities (Cheng et al., 2005a; Li et al., 2005; Hu et al., 2007). Similarly, in the whiteleg shrimp under salinity stress, hemolymph concentrations of DA peaked whilst key immune parameters in the hemolymph declined considerably (Pan et al., 2010), suggesting that biogenic amines like DA might have immune-modulatory effects. In addition, high salinity could negatively affect cell viability by activating the proPO system and triggering cell degranulation, which can lead to extreme degranulation and subsequent cell death due to cell lysis (Pan et al., 2011). Therefore, it is assumed that the salinity-induced high DA concentrations of hemolymph could reduce immunity in decapods by reducing THC as a result of cell death. Moreover, the long-term exposure to low salinity stress affects the immunity in seawater decapods via several proteins and biochemical pathways, including the down-regulation of hemocyanins, a group of proteins with respiratory and several immunological functions (Xu et al., 2017).

3.3.2. Immune responses of decapods under salinity stress

Salinity stress has a negative impact on immune parameters and can intensify the virulence of pathogens in hosts leading to higher mortality. According to Selven and Philip, the shrimp *F. indicus* under low (5‰) and high (35‰) salinity stresses showed significant alterations in its immune parameters including PO, intracellular superoxide anion production, total hemolymph proteins, acid phosphatase (ACP) and alkaline phosphatase (ALP) activity when challenged with the marine bacterium *Vibrio harveyi*. However, the virulence of *V. harveyi* was more severe at 35‰ (Selven and Philip, 2013). Likewise, Wang and Chen report the effects of salinity stress at 5, 15, 25, and 35‰ on the immune ability of L. *vannamei* and its susceptibility to *V. alginolyticus*. At low salinities, the immune parameters including THC, PO activity, RB, phagocytic activity and SOD activity, as well as the pathogen clearance efficiency decreased considerably after 12 h, and the mortality of *V. alginolyticus*- injected shrimps reached its peak at the lowest salinity level of 5‰ (Wang and Chen, 2005a). The exposure of the tiger shrimp to 0‰ salinity stress resulted in a substantial depression of immune parameters and increased susceptibility to WSSV infection (Joseph and Philip, 2007). Similarly, the whiteleg shrimp was more susceptible to *V. alginolyticus* at lower (5‰) than higher (35‰) salinity (Wang and Chen, 2005b).

Studies of decapods such as the tiger shrimp, swimming crab *Portunus trituberculatus* and giant freshwater prawn have shown that salinity stress could significantly induce alternations in immune parameters including THC, PO, RB, SOD, ACP, and ALP, and phagocytic activity (Cheng and Chen, 2000; Wang and Chen, 2006a; Joseph and Philip,

Table 7
A summary of the immune responses of decapods to pathogens.

Species group	Species	Pathogens	Tissues	Immune responses	References
Crabs	*Portunus trituberculatus*	WSSV, *Vibrio parahaemolyticus* and *V. alginolyticus*	Hepatopancreas and hemocytes	↓ pro-phenoloxidase-activating system ↓ Lysozyme ↓ Crustin ↑ α2-macroglobulin ↑ NADPH oxidase (NOX) ↑ Nitric oxide synthase	Ren et al., (2017)
	Scylla paramamosain	*(WSSV) or Vibrio alginolyticus*	Hemocytes	↓ Janus kinase ↕ Relish	Zhu et al., (2018)
	Scylla paramamosain	*Beta streptococcus, Vibrio. parahemolyticus, and (rhubarb polysaccharides (immunostimulant))*	Hemocyte, hepatopancreas and intestines	↑ Phenoloxidase ↑ Alkaline phosphatase ↑ Alkaline phosphatasein –Superoxide dismutase –Lysozyme ↑ SpHMC	Cao et al., (2014)
	Eriocheir sinensis	*Vibrio anguillarum*	Hemocytes	↑ Cathepsin C (catC)	Li et al., (2010b)
Crayfishs	*Procambarus clarkii*	*Viral and bacterial- pathogen-associated molecular patterns*	Hepatopancreas	↑ Pc-cathepsin C	Liu et al., (2020b)
	Pacifastacus leniusculus	*Aphanomyces astaci*	Hemolymph	↓ Total hemocyte count	Persson et al., (1987a)
Prawns	*Macrobrachium rosenbergii*	*Spiroplasma* MR-1008	Hepatopancreas	↑ Alkaline phosphatase - Superoxide dismutase ↓ Catalase ↑ β-1,3-glucan-binding protein ↑ Peroxinectin ↑ α2-macroglobulin	Du et al., (2013)
	Macrobrachium rosenbergii	*Aeromonas strains (A. veronii and A. caviae)*		↓ Total hemocyte count ↕ Hyaline cells ↕ Granular cells ↑ Phenoloxidase activity	Sung et al., (2000)
Shrimps	*Litopenaeus vannamei*	WSSV and *Vibrio parahaemolyticus*	Gills	↑ Superoxide dismutase ↑ Peroxidase ↑ Acid phosphatase ↑ Alkaline phosphatase ↑ Acid phosphatase	Pang et al., (2019)
	Litopenaeus vannamei	*Micrococcus lysodeikticus* and WSSV	Hemolymph	↑ Acid phosphatase ↑ Peroxidase ↑ Alkaline phosphatase ↑ Lysozyme phosphatase ↑ Phenoloxidase	Sun et al., (2015)
	Penaeus. vannamei	*Vibrio alginolyticus*	Hemolymph	↓ Total hemocyte count ↓ Phenoloxidase ↓ Superoxide dimutase ↓ Respiratory burst	Hsieh et al., (2008)
	Litopenaeus vannamei	*Vibrio parahaemolyticus*	Hepatopancreas hemolymph and, hemocytes	↓ Total hemocyte counts ↑ Hemocyanin (HEM) ↑ Lysozyme ↕ Acid phosphatase ↕ Alkaline phosphatase ↓ Phenoloxidase	Zhai et al., (2019)
	Fenneropenaeus chinensis	WSSV	Lymphoid organ, gill, hepatopancreas and hematopoietic tissue	– Total hemocyte count ↑ Mitotic index ↑ Phenoloxidase ↑ Superoxide dismutase ↑ Alkaline phosphatase ↑ Acid phosphatase	Zhang et al., (2005)

↑ = increase.
↓ = decrease.
↕ = increase and decrease.
–= no significant change.

2007; Wang et al., 2018). The impact of salinity stress on immune parameters of decapod crustaceans is summarized in Table 5.

3.4. Hypoxia

3.4.1. Possible mechanism of action of hypoxia on the immune system

Dissolved oxygen (DO) is another major environmental factor in aquaculture that can be affected by frost, an abrupt change or death of the phytoplankton in the water body, an increase in the size of the zooplankton population and the decomposition of organic matter such as food leftovers and faeces, which can result in a sudden decrease in DO (Jiang et al., 2005). A prolonged drop in the oxygen supply can induce hypoxia-inducible transcription factor (HIF), a gene known to activate mechanisms against hypoxia that is highly conserved across the animal kingdom. There is also evidence that HIF has an important role in the innate immune response of decapod crustaceans. Therefore, HIF may also trigger immune responses after exposure to hypoxia as an abiotic stressor (Gorr et al., 2010a). In addition, it has been shown that hypoxic

exposure can increase the level of hemocyanin (Hcs), proteins that act as oxygen carriers in the hemolymph of decapod crustaceans (Giomi and Beltramini, 2007). Hcs also play a role in the innate immune response by providing oxygen for phenoloxidase activity or by working as phenoloxidase under certain circumstances (Terwilliger et al., 2006; Jaenicke et al., 2009; Niksirat et al., 2014; Niksirat et al., 2015). Hypoxia can increase the level of antioxidant proteins in the cell and so help cope with the oxidative stress that occurs after a possible reoxygenation of the environment (Gorr et al., 2010a).

There is also evidence to suggest that hypoxic stress can alter immune parameters through the endocrine system. After hypoxia stress in L. *vannamei*, hemolymph DA concentrations increased significantly and subsequently all immune parameters including hemocyte counts, phenoloxidase activity, phagocytic activity of hemocytes, and bacteriolytic and antibacterial activities were found to decline (Hu et al., 2009).

3.4.2. Immune responses of decapods under hypoxic stress

There was a significant decrease in the immune parameters such as THC, bacteriolytic and antibacterial activities, while PO greatly increased in activity when whiteleg shrimps were exposed to hypoxia conditions of 2.0 and 3.5 mg O_2 l^{-1} (Jiang et al., 2005). Likewise, in the blue shrimp *Penaeus stylirostris* acute hypoxia at levels of 1 mg O_2 l^{-1} for 24 h induced significant reductions in THC, DHC and RB, while PO activity greatly increased (Le Moullac et al., 1998). Hypoxic stress greatly affected immune parameters such as PO, SOD and peroxidase (POD), as well as antibacterial and LZM activities in the Chinese shrimp *Fenneropenaeus chinensis* (Li et al., 2006). THC, DHC, RB and PO activity fell considerably and mortality increased at 1.75 compared to 2.75 mg O_2 l^{-1} after 12-h exposure in giant freshwater prawns challenged with *Enterococcus* (Cheng et al., 2002). Furthermore, in juvenile Chinese mitten crabs, hypoxic stress of 1.40 mg O_2 l^{-1} greatly affected the immune parameters THC, DHC and RB (Qiu et al., 2011), thereby suggesting that hypoxic stress has a potentially devastating effect on immune parameters and increases the susceptibility to infections and mortality in decapods.

3.5. Ammonia

3.5.1. Possible mechanism of action of ammonia on immune system

Ammonia is one of the major limiting environmental factors that can have a negative impact on the immune responses of decapod crustaceans and their productivity. In natural waters, total ammonia nitrogen (TAN) is mainly found in two forms, ammonium (NH_4^+) and ammonia (NH_3). The latter is more toxic to aquatic animals since it can diffuse across cell membranes (Frias-Espericueta et al., 2000; Zhao et al., 2020). In intensive aquaculture systems with high-stocking densities, ammonia concentrations rise abruptly, predominantly due to the accumulation of nitrogenous waste from the cultured animals themselves, unconsumed food and other organic matter (Csavas, 1994; Romano and Zeng, 2013). In addition to its role as an important limiting factor in decapod crustacean aquaculture, ammonia is also an important environmental pollutant used as a key water quality parameter for assessing environmental pollution. Exposure of decapod crustaceans to elevated ammonia concentrations can impair many important biological processes such as ionic regulation, cell permeability and immune functions (Young-Lai et al., 1991; Harris et al., 2001; Hong et al., 2007). High ammonia levels can severely damage the gill structure of decapod crustaceans by inducing necrosis and hyperplasia, and harm gill epithelial cells (de Freitas Rebelo et al., 2000; Romano and Zeng, 2007). High ammonia also decreases the expression of Na^+/K^+-ATPase activity and so diminishes the ability of gills to excrete ammonia-N along a gradient, which eventually results in increased hemolymph ammonia-N concentrations (Martin et al., 2011).

Furthermore, recent investigations have suggested that neuroendocrine factors can drive the effects of ammonia on the immune system of decapods (Tong et al., 2022) and, for example, it has been shown that ammonia-N can affect PO and antibacterial and bacteriolytic activities via the eyestalk hormone in L. *vannamei* (Cui et al., 2017a). DA is another neuroendocrine factor that can affect THC, antibacterial and bacteriolytic activities and proPO in *Portunus trituberculatus* as a result of changes in ammonia concentrations (Yue et al., 2010a).

It is widely accepted that hemocytes are the most important component of the decapod immune system (Vazquez et al., 2009). Numerous studies have confirmed that the total hemocyte count decreases significantly under ammonia stress in several decapod crustacean species (Jiang et al., 2004a; Hong et al., 2007; Verghese et al., 2007; Rodríguez-Ramos et al., 2008). A transcriptomic study of the hemocytes of shrimps discovered that high concentrations of hemolymph ammonia could induce the apoptosis of hemocytes. Under ammonia stress, the anti-apoptotic genes such as the inhibitor of apoptosis protein (IAP) and baculoviral IAP repeat-containing protein 2 (Birc2) were greatly down-regulated, whereas the pro-apoptotic genes such as map kinase-interacting serine/threonine (MKNK) and CCAAT/enhancer-binding protein (C/EBP) were significantly up-regulated (Liu et al., 2020a), which suggests that high hemolymph ammonia enhances apoptosis processes and decreases the hemocyte count. Therefore, it is believed that ammonia stress weakens the immune ability by decreasing the number of immune cells, thereby eventually increasing the susceptibility of hosts to pathogens.

Recent gene expression studies have shown that ammonia stress could significantly alter the expression patterns of various important immune genes in decapods. In the swimming crab, the expression levels of immunity-related genes were determined after ammonia exposure. The expression of LZM, antibacterial peptide (crustin) and anti-lipopolysaccharide factor (ALF) genes declined significantly, while the expression of α2-macroglobulin (α2M) gene increased significantly after ammonia-N exposure (Yue et al., 2010b). Furthermore, in the giant tiger shrimp the gene expressions of C-lysozyme, crustin and the anti-lipopolysaccharide factor under high ammonia exposure initially increased for all genes but later decreased for all except crustin (Yang et al., 2015). These genes are mainly expressed and stored as immune molecules in the granules of hemocytes and, when hemocytes are activated, they are released into the hemolymph to perform antibacterial and bacteriolytic functions against pathogens (Yue et al., 2010c).

3.5.2. Immune responses of decapods under ammonia stress

Studies have demonstrated that the exposure of decapod crustaceans to high ammonia concentrations could alter a number of key immune parameters (Hong et al., 2007; Rodríguez-Ramos et al., 2008; Yue et al., 2010c; Romano and Zeng, 2013; Cui et al., 2017b). After seven days of exposure to different concentrations (1.10, 5.24, 11.10, and 21.60 mg l^{-1}) of ammonia, the SOD and PO activity, and clearance efficiency to *V. alginolyticus* in whiteleg shrimps fell significantly. By contrast, superoxide anion increased significantly, and THC, HC and GC showed no significant changes (Liu and Chen, 2004). It has been shown that an increase in pH and temperature can accentuate the negative effects of ammonia stress by converting ammonium to a more toxic un-ionized form of ammonia (Wurts, 2003). Kathyayani and colleagues report the individual and combined effects of ammonia and pH on the Pacific whiteleg shrimp whereby, after exposure to ammonia stress for 14 days, immune parameters such as THC, PO and SOD tended to decline. On the other hand, the combined effect of ammonia and pH 10 provoked 448 times more TAN toxicity than TAN alone, thereby increasing the mortality rate (Kathyayani et al., 2019). Similarly, immune parameters including THC, plasma protein, proPO, nitric oxide synthase (NOS) and ALP showed negative trends in the shrimp *Penaeus japonicus* when exposed to 5 mg l^{-1} ammonia-N (Jiang et al., 2004b).

It has been reported that high concentrations of ammonia could induce an increase in the production of reactive oxygen species (ROS) causing oxidative stress and a reduction in superoxide dismutase activity, thereby weakening the antioxidant ability in decapods (Di Mascio et al., 1991; Pan et al., 2003; Liang et al., 2016). In giant freshwater

prawns exposed to 0.55, 1.68 and 3.18 mg l^{-1} ammonia-N for seven days, RB initially increased significantly at low levels of ammonia but decreased significantly at higher levels of exposure (Cheng and Chen, 2002). Likewise, the exposure of juvenile crabs, *E. sinensis* to 20, 40, 60, and 80 mg of TAN for two days led to significant decreases in THC and SOD activity (Hong et al., 2007). The impact of ammonia stress on the immune parameters of decapod crustaceans is summarized in Table 6. The overall effects of abiotic factors on the immune parameters of decapods are summarized in Fig. 2.

3.6. Biotic factors

3.6.1. Immune responses of decapods to biotic factors

The co-infection of *Vibrio parahaemolyticus* and WSSV in the whiteleg shrimp greatly increased the activity of immune enzymes including SOD, POD, ALP and ACP in the gills; by comparison, the mortality rate in the co-infection group was notably lower than the group infected only by WSSV, which also suggests that the proliferation of WSSV was repressed by *V. parahaemolyticus* (Pang et al., 2019). In addition, when the crab *P. trituberculatus* was challenged with three different pathogens including *V. parahaemolyticus*, *V. alginolyticus,* and WSSV in order to investigate their impact on important immune-related genes, the expression levels of proPO, lysozyme and crustin were downregulated, thereby suggesting the immunosuppressive role of pathogens; however, α2M expression increased over time indicating that pathogens could affect proteinase cascades (Ren et al., 2017). Similarly, when challenged with WSSV and *Micrococcus lysodeikticus*, the whiteleg shrimp showed significantly enhanced immune enzyme activity such as ACP, ALP, PO, POD and LZM in the hemolymph (Sun et al., 2015). However, another study reported that when challenged with *V. alginolyticus*, the whiteleg shrimp had considerably lower immune parameters, namely THC, PO and SOD activities, and RB after 12 h, indicating that the immune responses of shrimps are specific and that immune-associated enzymes act in different ways against each pathogen (Hsieh et al., 2008). The giant freshwater prawn significantly altered its immune enzyme activities when infected by spiroplasma MR-1008 and, for example, AKP, ACP and SOD activity increased, while CAT activity decreased; furthermore, the mRNA levels of seven important immune-related genes including peroxinectin (PE), β-1,3-glucan-binding protein (LGBP), α2M, ALP, ACP, CAT, Cu, and Zn-SOD were all greatly up-regulated after being challenged by spiroplasma MR-1008 in hepatopancreas. On the other hand, the CAT enzyme activity was different from its mRNA transcription level (Du et al., 2013). Sublethal doses of two virulent strains of *Aeromonas* spp. (*A. caviae* and *A. veronii*) greatly decreased the THC in the giant freshwater prawn between 4 and 24 h after injection. As well, an initial increase was observed in HC followed by a sharp decline, while SGC and GC initially decreased slightly and then progressively increased. PO activity increased four-fold immediately after injection and dropped to normal level after 24 h (Sung et al., 2000). In many animals, females possess stronger immune system than males, which give them better resistance to environmental stresses and pathogens (Niksirat et al., 2021). It would be interesting to develop sex-specific treatments to test the sex-based abilities of decapod crustaceans to resist the above-mentioned factors. The impact of disease on changes in immune parameters has been studied in several important decapod species including the crabs *Scylla paramamosain* (Zhu et al., 2018), *Portunus trituberculatus* (Ren et al., 2017) and *Eriocheir sinensis* (Li et al., 2010b), the red swamp crayfish *Procambarus clarkii* (Liu et al., 2020b), signal crayfish (Persson et al., 1987a), American lobster *Homarus americanus* (Clark et al., 2015), the giant freshwater prawn (Du et al., 2013), and the shrimps *Litopenaeus vannamei* (Hsieh et al., 2008; Pang et al., 2019), and *Fenneropenaeus chinensis* (Zhang et al., 2005). The impacts of disease on the immune parameters of decapod crustaceans are summarized in Table 7.

4. Conclusions and future prospects

Hemocytes in circulating hemolymph are the most important component of the innate immune system. Traditionally, hemocytes in crustaceans are classified into three morphological types: HCs, SGCs and GCs. Yet, there is still a lack of consensus regarding the classification of hemocyte types amongst researchers. Recent studies of decapod crustaceans have proposed that the so-called hemocyte sub-populations are probably just different developmental stages of a single cell type. There is an urgent need to explore the process of hemocyte development in decapod crustaceans via the development and application of new observation methods that can directly monitor the whole process of hematopoiesis. A deeper understanding of the immune cell types and their functions will improve management strategies designed to combat the harmful effects of environmental stressors on decapod crustaceans.

Evidence from the reviewed literature suggests that environmental factors influence the immune parameters and health status of decapod crustaceans in a wide variety of ways. The effects of environmental factors on immune responses are often immunosuppressive and therefore these stressors increase the risk of susceptibility to disease. Due to the lack of available data, critical issues like how abiotic stressors operate remain unclear. Understanding the exact mechanisms by which environmental factors and, especially, their abiotic components - influence the immune parameters of decapods could help moderate or constrain the harmful effects of these environmental factors that negatively affect the production of decapod crustaceans. Two excellent starting points would be (i) the use of state-of-art omic techniques to achieve an in-depth understanding of the molecular mechanisms of these environmental factors, and (ii) the localization of biomarkers using in situ hybridization or immunohistochemistry for a better understanding of the cellular and molecular mechanisms underlying each immunological process. Such approaches could foment the development of specific diagnostic morphological and biochemical biomarkers for monitoring the potential impact of environmental factors on target decapod species. Lastly, future research should also target less well-studied abiotic factors such as salinity, hypoxia and, more importantly, the combined effects of abiotic and biotic environmental conditions on disease outbreaks.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data availability

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

Acknowledgements

The study was financially supported by the Grant Agency of the University of South Bohemia, (GAJU) projects No. 055/2022/Z and 012/2022/Z.

References

Amparyup, P., Donpudsa, S., Tassanakajon, A., 2008. Shrimp single WAP domain (SWD)-containing protein exhibits proteinase inhibitory and antimicrobial activities. Dev. Comp. Immunol. 32, 1497–1509.

Amparyup, P., Sutthangkul, J., Charoensapsri, W., Tassanakajon, A., 2012a. Pattern recognition protein binds to lipopolysaccharide and beta-1,3-glucan and activates shrimp prophenoloxidase system. J. Biol. Chem. 287, 10060–10069.

Amparyup, P., Sutthangkul, J., Charoensapsri, W., Tassanakajon, A., 2012b. Pattern recognition protein binds to lipopolysaccharide and β-1, 3-glucan and activates shrimp prophenoloxidase system. J. Biol. Chem. 287, 10060–10069.

Amparyup, P., Charoensapsri, W., Tassanakajon, A., 2013. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. Fish Shellfish Immunol. 34, 990–1001.
Aono, H., Mori, K., 1996. Interaction between hemocytes and plasma is necessary for hemolymph coagulation in the spiny lobster, Panulirus japonicus. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 113, 301–305.
Aspan, A., Soderhall, K., 1991. Purification of prophenoloxidase from crayfish blood-cells, and its activation by an endogenous serine proteinase. Insect Biochem. Mol. Biol. 21, 363–373.
Aspán, A., Sturtevant, J., Smith, V.J., Söderhäll, K., 1990. Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells. Insect Biochem. Mol. Biol. 20, 709–718.
Bauchau, A., Ratcliffe, N., Rowley, A., 1981. Invertebrate Blood Cells. Crustacean. Academic Press, New York.
Becking, T., Delaunay, C., Cordaux, R., Berjeaud, J.M., Braquart-Varnier, C., Verdon, J., 2020. Shedding light on the antimicrobial peptide arsenal of terrestrial isopods: focus on Armadillidins, a New Crustacean AMP Family. Genes (Basel) 11, 93.
Bodammer, J.E., 1978. Cytological observations on the blood and hemopoietic tissue in the crab, Callinectes sapidus. I. The fine structure of hemocytes from intermolt animals. Cell Tissue Res. 187, 79–96.
Bouallegui, Y., 2021. A comprehensive review on Crustaceans' immune system with a focus on freshwater Crayfish in relation to Crayfish Plague Disease. Front. Immunol. 12.
van de Braak, C.B., Botterblom, M.H., Liu, W., Taverne, N., van der Knaap, W.P., Rombout, J.H., 2002. The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (Penaeus monodon). Fish Shellfish Immunol. 12, 253–272.
Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2018. A potential microRNA regulation of immune-related genes in invertebrate haemocytes. Sci. Total Environ. 621, 302–307.
Burgos-Aceves, M.A., Abo-Al-Ela, H.G., Faggio, C., 2021. Impact of phthalates and bisphenols plasticizers on haemocyte immune function of aquatic invertebrates: a review on physiological, biochemical, and genomic aspects. J. Hazard. Mater. 419, 126426.
Calderon-Rosete, G., Gonzalez-Barrios, J.A., Lara-Lozano, M., Pina-Leyva, C., Rodriguez-Sosa, L., 2018. Transcriptional Identification of Related Proteins in the Immune System of the Crayfish Procambarus clarkii. High Throughput 7.
Cao, J., Wang, Z., Zhang, Y., Qu, F., Guo, L., Zhong, M., Li, S., Zou, H., Chen, J., Wang, X., 2014. Identification and characterization of the related immune-enhancing proteins in crab Scylla paramamosain stimulated with rhubarb polysaccharides. Mol. Immunol. 57, 263–273.
Cerenius, L., Söderhäll, K., 2018. Crayfish immunity–Recent findings. Dev. Comp. Immunol. 80, 94–98.
Cerenius, L., Lee, B.L., Soderhall, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29, 263–271.
Cerenius, L., Babu, R., Soderhall, K., Jiravanichpaisal, P., 2010. In vitro effects on bacterial growth of phenoloxidase reaction products. J. Invertebr. Pathol. 103, 21–23.
Chang, C.C., Yeh, M.S., Cheng, W., 2009a. Cold shock-induced norepinephrine triggers apoptosis of haemocytes via caspase-3 in the white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol. 27, 695–700.
Chang, C.C., Jiang, J.R., Cheng, W., 2015. A first insight into temperature stress-induced neuroendocrine and immunological changes in giant freshwater prawn, *Macrobrachium rosenbergii*. Fish Shellfish Immunol. 47, 528–534.
Chang, M., Wang, W.N., Wang, A.L., Tian, T.T., Wang, P., Zheng, Y., Liu, Y., 2009b. Effects of cadmium on respiratory burst, intracellular Ca2+ and DNA damage in the white shrimp Litopenaeus vannamei. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 149, 581–586.
Charoensapsri, W., Amparyup, P., Hirono, I., Aoki, T., Tassanakajon, A., 2011. PmPPAE2, a new class of crustacean prophenoloxidase (proPO)-activating enzyme and its role in PO activation. Dev. Comp. Immunol. 35, 115–124.
Chen, J.C., Kou, Y.Z., 1992. Effects of ammonia on growth and molting of Penaeus-Japonicus Juveniles. Aquaculture. 104, 249–260.
Chen, Y.Y., Chen, J.C., Tseng, K.C., Lin, Y.C., Huang, C.L., 2015. Activation of immunity, immune response, antioxidant ability, and resistance against Vibrio alginolyticus in white shrimp Litopenaeus vannamei decrease under long-term culture at low pH. Fish Shellfish Immunol. 46, 192–199.
Cheng, C.H., Yang, F.F., Liao, S.A., Miao, Y.T., Ye, C.X., Wang, A.L., Tan, J.W., Chen, X. Y., 2015. High temperature induces apoptosis and oxidative stress in pufferfish (Takifugu obscurus) blood cells. J. Therm. Biol. 53, 172–179.
Cheng, C.H., Guo, Z.X., Luo, S.W., Wang, A.L., 2018. Effects of high temperature on biochemical parameters, oxidative stress, DNA damage and apoptosis of pufferfish (Takifugu obscurus). Ecotoxicol. Environ. Saf. 150, 190–198.
Cheng, W., Chen, J.C., 1998. Enterococcus-like infections in Macrobrachium rosenbergii are exacerbated by high pH and temperature but reduced by low salinity. Dis. Aquat. Org. 34, 103–108.
Cheng, W., Chen, J.C., 2000. Effects of pH, temperature and salinity on immune parameters of the freshwater prawn Macrobrachium rosenbergii. Fish Shellfish Immunol. 10, 387–391.
Cheng, W., Chen, J.C., 2002. The virulence of Enterococcus to freshwater prawn Macrobrachium rosenbergii and its immune resistance under ammonia stress. Fish Shellfish Immunol. 12, 97–109.
Cheng, W., Liu, C.H., Hsu, J.P., Chen, J.C., 2002. Effect of hypoxia on the immune response of giant freshwater prawn Macrobrachium rosenbergii and its susceptibility to pathogen enterococcus. Fish Shellfish Immunol. 13, 351–365.
Cheng, W., Chieu, H.T., Tsai, C.H., Chen, J.C., 2005a. Effects of dopamine on the immunity of white shrimp Litopenaeus vannamei. Fish Shellfish Immunol. 19, 375–385.
Cheng, W.T., Wang, L.U., Chen, J.C., 2005b. Effect of water temperature on the immune response of white shrimp *Litopenaeus vannamei* to *Vibrio alginolyticus*. Aquaculture. 250, 592–601.
Chisholm, J.R., Smith, V.J., 1994. Variation of antibacterial activity in the haemocytes of the shore crab, Carcinus maenas, with temperature. J. Mar. Biol. Assoc. UK 74, 979–982.
Clare, A., Lumb, G., 1994. Identification of haemocytes and their role in clotting in the blue crab, Callinectes sapidus. Mar. Biol. 118, 601–610.
Clark, K.F., Acorn, A.R., Wang, H., Greenwood, S.J., 2015. Comparative tissue expression of American lobster (Homarus americanus) immune genes during bacterial and scuticociliate challenge. Fish Shellfish Immunol. 47, 1054–1066.
Cornick, J.W., Stewart, J.E., 1978. Lobster (Homarus-Americanus) hemocytes - classification, differential counts, and associated agglutinin activity. J. Invertebr. Pathol. 31, 194–203.
Csavas, I., 1994. Important factors in the success of shrimp farming. World Aquacult.-Baton Rouge 25, 34.
Cui, Y., Ren, X., Li, J., Zhai, Q., Feng, Y., Xu, Y., Ma, L., 2017a. Effects of ammonia-N stress on metabolic and immune function via the neuroendocrine system in *Litopenaeus vannamei*. Fish Shellfish Immunol. 64, 270–275.
Cui, Y., Ren, X., Li, J., Zhai, Q., Feng, Y., Xu, Y., Ma, L., 2017b. Effects of ammonia-N stress on metabolic and immune function via the neuroendocrine system in *Litopenaeus vannamei*. Fish Shellfish Immunol. 64, 270–275.
Cukerzis, J., 1973. Biologische grundlagen der methode der kunstlichen aufzucht der brut des *Astacus astacus* L. Freshwat. Cray. 1, 187–201.
Dall, W., 1964. Studies on the physiology of a shrimp, Metapenaeus mastersii (Haswell) (Crustacea: Decapoda: Peneidae). I. Blood constituents. Mar. Freshwat. Res. 15, 145–161.
Daye, P.G., Garside, E.T., 1975. Lethal levels of pH for brook trout, *Salvelinus fontinalis* (Mitchill). Can. J. Zool. 53, 639–641.
De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., Lemaitre, B., 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. EMBO J. 21, 2568–2579.
Di Mascio, P., Murphy, M.E., Sies, H., 1991. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. Am J Clin Nutr 53.
Dong, C., Bai, S., Du, L., 2015. Temperature regulates circadian rhythms of immune responses in red swamp crayfish *Procambarus clarkii*. Fish Shellfish Immunol. 45, 641–647.
Du, J., Zhu, H., Liu, P., Chen, J., Xiu, Y., Yao, W., Wu, T., Ren, Q., Meng, Q., Gu, W., Wang, W., 2013. Immune responses and gene expression in hepatopancreas from Macrobrachium rosenbergii challenged by a novel pathogen spiroplasma MR-1008. Fish Shellfish Immunol. 34, 315–323.
Fagutao, F.F., Maningas, M.B., Kondo, H., Aoki, T., Hirono, I., 2012. Transglutaminase regulates immune-related genes in shrimp. Fish Shellfish Immunol. 32, 711–715.
Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61, 243–282.
Food and Agriculture Organization of the United Nations, 2020. The State of World Fisheries and Aquaculture.
de Freitas Rebelo, M., Rodriguez, E.M., Santos, E.A., Ansaldo, M., 2000. Histopathological changes in gills of the estuarine crab *Chasmagnathus granulata* (Crustacea-Decapoda) following acute exposure to ammonia. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 125, 157–164.
de Freitas Rebelo, M., de Souza Figueiredo, E., Mariante, R.M., Nóbrega, A., de Barros, C. M., Allodi, S., 2013. New insights from the oyster Crassostrea rhizophorae on bivalve circulating hemocytes. PLoS One 8, e57384.
Frias-Espericueta, M.G., Harfush-Melendez, M., Paez-Osuna, F., 2000. Effects of ammonia on mortality and feeding of postlarvae shrimp Litopenaeus vannamei. Bull. Environ. Contam. Toxicol. 65, 98–103.
Gai, Y., Zhao, J., Song, L., Li, C., Zheng, P., Qiu, L., Ni, D., 2008. A prophenoloxidase from the Chinese mitten crab Eriocheir sinensis: gene cloning, expression and activity analysis. Fish Shellfish Immunol. 24, 156–167.
Giomi, F., Beltramini, M., 2007. The molecular heterogeneity of hemocyanin: its role in the adaptive plasticity of Crustacea. Gene. 398, 192–201.
Giulianini, P.G., Bierti, M., Lorenzon, S., Battistella, S., Ferrero, E.A., 2007. Ultrastructural and functional characterization of circulating hemocytes from the freshwater crayfish Astacus leptodactylus: Cell types and their role after in vivo artificial non-self challenge. Micron. 38, 49–57.
Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010a. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83, 733–752.
Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010b. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83, 733–752.
Götz, P., 1986. Encapsulation in Arthropods, Immunity in Invertebrates. Springer, pp. 153–170.
Harris, R.R., Coley, S., Collins, S., McCabe, R., 2001. Ammonia uptake and its effects on ionoregulation in the freshwater crayfish Pacifastacus leniusculus (Dana). J. Comp. Physiol. B. 171, 681–693.
Hauton, C., 2012. The scope of the crustacean immune system for disease control. J. Invertebr. Pathol. 110, 251–260.

Hernández-Pérez, A., Söderhäll, K., Sirikharin, R., Jiravanichpaisal, P., Söderhäll, I., 2021. Vibrio areninigrae as a pathogenic bacterium in a crustacean. J. Invertebr. Pathol. 178, 107517.
Hong, M., Chen, L., Sun, X., Gu, S., Zhang, L., Chen, Y., 2007. Metabolic and immune responses in Chinese mitten-handed crab (Eriocheir sinensis) juveniles exposed to elevated ambient ammonia. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 145, 363–369.
Hsieh, S.L., Ruan, Y.H., Li, Y.C., Hsieh, P.S., Hu, C.H., Kuo, C.M., 2008. Immune and physiological responses in Pacific white shrimp (Penaeus vannamei) to *Vibrio alginolyticus*. Aquaculture. 275, 335–341.
Hu, F., Pan, L., Yang, H., 2007. Effects of injecting biogenic amines on immune parameters of white shrimp *Litopenaeus vannamei*. J. Trop. Oceanogr. 26, 64–68.
Hu, F., Pan, L., Jing, F., 2009. Effects of hypoxia on dopamine concentration and the immune response of white shrimp (*Litopenaeus vannamei*). J. Ocean Univ. China 8, 77–82.
Hu, Y.-B., Wang, Y., Jiang, N.-C., 2005. Effects of ammonia-N and nitrite-N on the hemocyte count and ultrastructure of *Macrobrachium rosenbergii*. J. Zhejiang Univ. (Sci.) 32, 691.
Huang, H., Li, Y., Song, X., Wang, Y., Yang, C., 2006. NH~ 4+-N stress on immune function of *Eriocheir sinensis*. Oceanol. Limnol. Sin. 37, 198.
Iwanaga, S., Lee, B.L., 2005. Recent advances in the innate immunity of invertebrate animals. J. Biochem. Mol. Biol. 38, 128–150.
Jaenicke, E., Fraune, S., May, S., Irmak, P., Augustin, R., Meesters, C., Decker, H., Zimmer, M., 2009. Is activated hemocyanin instead of phenoloxidase involved in immune response in woodlice? Dev. Comp. Immunol. 33, 1055–1063.
Janeway Jr., C.A., Medzhitov, R., 2002. Innate immune recognition. Annu. Rev. Immunol. 20, 197–216.
Jia, X., Ding, S., Wang, F., Dong, S., 2014a. A Comparative study on the nonspecific immunity of juvenile *Litopenaeus vannamei* ever inhabiting freshwater and seawater. J. Ocean Univ. China 13, 472–478.
Jia, X.Y., Wang, F., Lu, Y.L., Zhang, D., Dong, S.L., 2014b. Immune responses of *Litopenaeus vannamei* to thermal stress: a comparative study of shrimp in freshwater and seawater conditions. Mar. Freshw. Behav. Physiol. 47, 79–92.
Jiang, G., Yu, R., Zhou, M., 2004a. Modulatory effects of ammonia-N on the immune system of *Penaeus japonicus* to virulence of white spot syndrome virus. Aquaculture. 241, 61–75.
Jiang, G., Yu, R., Zhou, M., 2004b. Modulatory effects of ammonia-N on the immune system of *Penaeus japonicus* to virulence of white spot syndrome virus. Aquaculture. 241, 61–75.
Jiang, L.X., Pan, L.Q., Fang, B., 2005. Effect of dissolved oxygen on immune parameters of the white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol. 18, 185–188.
Jiang, L.-X., Pan, L.-Q., Xiao, G.-Q., 2004c. Effects of ammonia-N on immune parameters of white shrimp *Litopenaeus vannamei*. J. Fish. Sci. China 11, 537–541.
Jiravanichpaisal, P., Soderhall, K., Soderhall, I., 2004. Effect of water temperature on the immune response and infectivity pattern of white spot syndrome virus (WSSV) in freshwater crayfish. Fish Shellfish Immunol. 17, 265–275.
Jiravanichpaisal, P., Lee, B.L., Soderhall, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology. 211, 213–236.
Johansson, M.W., Lind, M.I., Holmblad, T., Thornqvist, P.O., Soderhall, K., 1995. Peroxinectin, a novel cell adhesion protein from crayfish blood. Biochem. Biophys. Res. Commun. 216, 1079–1087.
Johansson, M.W., Keyser, P., Sritunyalucksana, K., Soderhall, K., 2000. Crustacean haemocytes and haematopoiesis. Aquaculture. 191, 45–52.
Johnson, P.T., 1980. Histology of the blue crab, Callinectes sapidus: a model for the Decapoda. Q. Rev. Biol.
Joseph, A., Philip, R., 2007. Acute salinity stress alters the haemolymph metabolic profile of Penaeus monodon and reduces immunocompetence to white spot syndrome virus infection. Aquaculture. 272, 87–97.
Jussila, J., Jago, J., Tsvetnenko, E., Dunstan, B., Evans, L.H., 1997. Total and differential haemocyte counts in western rock lobsters (*Panulirus cygnus* George) under post-harvest stress. Mar. Freshw. Res. 48, 863–867.
Kathyayani, S.A., Poornima, M., Sukumaran, S., Nagavel, A., Muralidhar, M., 2019. Effect of ammonia stress on immune variables of Pacific white shrimp *Penaeus vannamei* under varying levels of pH and susceptibility to white spot syndrome virus. Ecotoxicol. Environ. Saf. 184, 109626.
Korkut, G.G., Soderhall, I., Soderhall, K., Noonin, C., 2018. The effect of temperature on bacteria-host interactions in the freshwater crayfish, Pacifastacus leniusculus. J. Invertebr. Pathol. 157, 67–73.
Kulkarni, A., Krishnan, S., Anand, D., Kokkattunivarthil Uthaman, S., Otta, S.K., Karunasagar, I., Kooloth Valappil, R., 2021. Immune responses and immunoprotection in crustaceans with special reference to shrimp. Rev. Aquac. 13, 431–459.
Kultz, D., 2003. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. J. Exp. Biol. 206, 3119–3124.
Kultz, D., 2005. Molecular and evolutionary basis of the cellular stress response. Annu. Rev. Physiol. 67, 225–257.
Kumaresan, V., Palanisamy, R., Pasupuleti, M., Arockiaraj, J., 2017. Impacts of environmental and biological stressors on immune system of Macrobrachium rosenbergii. Rev. Aquac. 9, 283–307.
Le Moullac, G., Haffner, P., 2000. Environmental factors affecting immune responses in Crustacea. Aquaculture. 191, 121–131.
Le Moullac, G., Soyez, C., Saulnier, D., Ansquer, D., Avarre, J.C., Levy, P., 1998. Effect of hypoxic stress on the immune response and the resistance to vibriosis of the shrimpPenaeus stylirostris. Fish Shellfish Immunol. 8, 621–629.
Lemaitre, B., Hoffmann, J., 2007. The host defense of Drosophila melanogaster. Annu. Rev. Immunol. 25, 697–743.
Li, A.J., Leung, P.T.Y., Bao, V.W.W., Lui, G.C.S., Leung, K.M.Y., 2015. Temperature-dependent physiological and biochemical responses of the marine medaka Oryzias melastigma with consideration of both low and high thermal extremes. J. Therm. Biol. 54, 98–105.
Li, B., Xian, J.A., Guo, H., Wang, A.L., Miao, Y.T., Ye, J.M., Ye, C.X., Liao, S.A., 2014. Effect of temperature decrease on hemocyte apoptosis of the white shrimp Litopenaeus vannamei. Aquac. Int. 22, 761–774.
Li, C., Wang, S., He, J., 2019. The Two NF-kappaB pathways regulating bacterial and WSSV infection of shrimp. Front. Immunol. 10, 1785.
Li, C.C., Chen, J.C., 2008. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under low and high pH stress. Fish Shellfish Immunol. 25, 701–709.
Li, C.C., Yeh, S.T., Chen, J.C., 2010a. Innate immunity of the white shrimp Litopenaeus vannamei weakened by the combination of a Vibrio alginolyticus injection and low-salinity stress. Fish Shellfish Immunol. 28, 121–127.
Li, F., Chang, X., Xu, L., Yang, F., 2018. Different roles of crayfish hemocytes in the uptake of foreign particles. Fish Shellfish Immunol. 77, 112–119.
Li, F., Zheng, Z., Li, H., Fu, R., Xu, L., Yang, F., 2021. Crayfish hemocytes develop along the granular cell lineage. Sci. Rep. 11, 13099.
Li, J.T., Lee, P.P., Chen, O.C., Cheng, W., Kuo, C.M., 2005. Dopamine depresses the immune ability and increases susceptibility to *Lactococcus garvieae* in the freshwater giant prawn, *Macrobrachium rosenbergii*. Fish Shellfish Immunol. 19, 269–280.
Li, W.W., Jin, X.K., He, L., Jiang, H., Xie, Y.N., Wang, Q., 2010b. Molecular cloning, characterization and expression analysis of cathepsin C gene involved in the antibacterial response in Chinese mitten crab, *Eriocheir sinensis*. Dev. Comp. Immunol. 34, 1170–1174.
Li, Y.Q., Li, J., Wang, Q.Y., 2006. The effects of dissolved oxygen concentration and stocking density on growth and non-specific immunity factors in Chinese shrimp, *Fenneropenaeus chinensis*. Aquaculture. 256, 608–616.
Liang, Z., Liu, R., Zhao, D., Wang, L., Sun, M., Wang, M., Song, L., 2016. Ammonia exposure induces oxidative stress, endoplasmic reticulum stress and apoptosis in hepatopancreas of pacific white shrimp (*Litopenaeus vannamei*). Fish Shellfish Immunol. 54, 523–528.
Lin, Y.C., Tayag, C.M., Huang, C.L., Tsui, W.C., Chen, J.C., 2010. White shrimp Litopenaeus vannamei that had received the hot-water extract of Spirulina platensis showed earlier recovery in immunity and up-regulation of gene expressions after pH stress. Fish Shellfish Immunol. 29, 1092–1098.
Lin, Y.C., Chen, J.C., Li, C.C., Morni, W.Z., Suhaili, A.S., Kuo, Y.H., Chang, Y.H., Chen, L. L., Tsui, W.C., Chen, Y.Y., Huang, C.L., 2012. Modulation of the innate immune system in white shrimp *Litopenaeus vannamei* following long-term low salinity exposure. Fish Shellfish Immunol. 33, 324–331.
Liu, C.-H., Chen, J.-C., 2004. Effect of ammonia on the immune response of white shrimpLitopenaeus vannamei and its susceptibility to *Vibrio alginolyticus*. Fish Shellfish Immunol. 16, 321–334.
Liu, F., Li, S., Yu, Y., Sun, M., Xiang, J., Li, F., 2020a. Effects of ammonia stress on the hemocytes of the Pacific white shrimp *Litopenaeus vannamei*. Chemosphere. 239, 124759.
Liu, H., Jiravanichpaisal, P., Cerenius, L., Lee, B.L., Sorderhall, I., Soderhall, K., 2007. Phenoloxidase is an important component of the Defense against Aeromonas hydrophila infection in a crustacean, *Pacifastacus leniusculus*. J. Biol. Chem. 282, 33593–33598.
Liu, Q.N., Kausar, S., Gul, I., Zhou, H.L., Abbas, M.N., Dai, L.S., 2020b. The red swamp crayfish, Procambarus clarkii cathepsin C, participates in the innate immune response to the viral and bacterial pathogens. Fish Shellfish Immunol. 100, 436–444.
Liu, S., Zheng, S.C., Li, Y.L., Li, J., Liu, H.P., 2020c. Hemocyte-mediated phagocytosis in Crustaceans. Front. Immunol. 11, 268.
Lubawy, J., Slocinska, M., 2020. Characterization of Gromphadorhina coquereliana hemolymph under cold stress. Sci. Rep. 10, 12076.
Lu-Qing, P., Ling-Xu, J., Jing-Jing, M., 2005. Effects of salinity and pH on immune parameters of the white shrimp Litopenaeus vannamei. J. Shellfish Res. 24, 1223–1227.
Lu-Qing, P., Bo, F., Ling-Xu, J., Jing, L., 2007. The Effect of Temperature on Selected Immune Parameters of the White Shrimp, *Litopenaeus vannamei*. J. World Aquacult. Soc. 38, 326–332.
Malev, O., Srut, M., Maguire, I., Stambuk, A., Ferrero, E.A., Lorenzon, S., Klobucar, G.I., 2010. Genotoxic, physiological and immunological effects caused by temperature increase, air exposure or food deprivation in freshwater crayfish Astacus leptodactylus. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 152, 433–443.
Maningas, M.B., Kondo, H., Hirono, I., Saito-Taki, T., Aoki, T., 2008. Essential function of transglutaminase and clotting protein in shrimp immunity. Mol. Immunol. 45, 1269–1275.
Martin, G.G., Graves, B.L., 1985. Fine structure and classification of shrimp hemocytes. J. Morphol. 185, 339–348.
Martin, G.G., Hose, J.E., Kim, J.J., 1987. Structure of hematopoietic nodules in the ridgeback prawn, Sicyonia ingentis: light and electron microscopic observations. J. Morphol. 192, 193–204.
Martin, G.G., Hose, J.E., Omori, S., Chong, C., Hoodbhoy, T., Mckrell, N., 1991. Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod Crustaceans. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 100, 517–522.
Martin, M., Fehsenfeld, S., Sourial, M.M., Weihrauch, D., 2011. Effects of high environmental ammonia on branchial ammonia excretion rates and tissue Rh-protein mRNA expression levels in seawater acclimated Dungeness crab

Metacarcinus magister. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 160, 267–277.
Matozzo, V., Marin, M.G., 2010. The role of haemocytes from the crab Carcinus aestuarii (Crustacea, Decapoda) in immune responses: a first survey. Fish Shellfish Immunol. 28, 534–541.
Matozzo, V., Gallo, C., Marin, M.G., 2011. Effects of temperature on cellular and biochemical parameters in the crab *Carcinus aestuarii* (Crustacea, Decapoda). Mar. Environ. Res. 71, 351–356.
Mcculloch, D.L., 1990. Metabolic response of the grass shrimp palaemonetes-Kadiakensis Rathbun, to acute exposure of Sublethal changes in Ph. Aquat. Toxicol. 17, 263–274.
Morgan, D., McMahon, B., 1982. Acid tolerance and effects of sublethal acid exposure on iono-regulation and acid-base status in two crayfish Procambarus clarki and Orconectes rusticus. J. Exp. Biol. 97, 241–252.
Morris, S., 2001. Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. J. Exp. Biol. 204, 979–989.
Niksirat, H., Andersson, L., James, P., Kouba, A., Kozak, P., 2014. Proteomic profiling of the signal crayfish Pacifastacus leniusculus egg and spermatophore. Anim. Reprod. Sci. 149, 335–344.
Niksirat, H., James, P., Andersson, L., Kouba, A., Kozak, P., 2015. Label-free protein quantification in freshly ejaculated versus post-mating spermatophores of the noble crayfish *Astacus astacus*. J. Proteome 123, 70–77.
Niksirat, H., Siino, V., Steinbach, C., Levander, F., 2021. High-resolution proteomic profiling shows sexual dimorphism in zebrafish heart-associated proteins. J. Proteome Res. 20, 4075–4088.
Nyhlén, L., Unestam, T., 1980. Wound reactions and *Aphanomyces astaci* growth in crayfish cuticle. J. Invertebr. Pathol. 36, 187–197.
Omori, S.A., Martin, G.G., Hose, J.E., 1989. Morphology of Hemocyte Lysis and Clotting in the Ridgeback Prawn, Sicyonia-Ingentis. Cell Tissue Res. 255, 117–123.
Pan, C.-H., Chien, Y.-H., Hunter, B., 2003. The resistance to ammonia stress of Penaeus monodon Fabricius juvenile fed diets supplemented with astaxanthin. J. Exp. Mar. Biol. Ecol. 297, 107–118.
Pan, L., Xie, P., Hu, F., 2010. Responses of prophenoloxidase system and related defence parameters of Litopenaeus vannamei to low salinity. J. Ocean Univ. China 9, 273–278.
Pan, L., Hu, F., Zheng, D., 2011. Effect of dopamine injection on the hemocyte count and prophenoloxidase system of the white shrimp *Litopenaeus vannamei*. J. Ocean Univ. China 10, 280.
Pan, L.Q., Hu, F.W., Jing, F.T., Liu, H.J., 2008. The effect of different acclimation temperatures on the prophenoloxidase system and other defence parameters in *Litopenaeus vannamei*. Fish Shellfish Immunol. 25, 137–142.
Pang, H., Wang, G., Zhou, S., Wang, J., Zhao, J., Hoare, R., Monaghan, S.J., Wang, Z., Sun, C., 2019. Survival and immune response of white shrimp Litopenaeus vannamei following single and concurrent infections with WSSV and *Vibrio parahaemolyticus*. Fish Shellfish Immunol. 92, 712–718.
Perazzolo, L.M., Gargioni, R., Ogliari, P., Barracco, M.A.A., 2002. Evaluation of some hemato-immunological parameters in the shrimp *Farfantepenaeus paulensis* submitted to environmental and physiological stress. Aquaculture. 214, 19–33.
Perdomo-Morales, R., Montero-Alejo, V., Perera, E., 2019. The clotting system in decapod crustaceans: history, current knowledge and what we need to know beyond the models. Fish Shellfish Immunol. 84, 204–212.
Persson, M., Cerenius, L., Söderhäll, K., 1987a. The influence of haemocyte number on the resistance of the freshwater crayfish, Pacifastacus leniusculus Dana, to the parasitic fungus *Aphanomyces astaci*. J. Fish Dis. 10, 471–477.
Persson, M., Vey, A., Söderhäll, K., 1987b. Encapsulation of foreign particles in vitro by separated blood cells from crayfish, *Astacus leptodactylus*. Cell Tissue Res. 247, 409–415.
Portner, H.O., 2001. Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. Sci. Nat. 88, 137–146.
Prayitno, S.B., Latchford, J.W., 1995. Experimental Infections of Crustaceans with luminous bacteria related to photobacterium and vibrio - effect of salinity and ph on infectiosity. Aquaculture. 132, 105–112.
Pulkkanen, M., Salonen, K., 2013. Accumulation of low-oxygen water in deep waters of ice-covered lakes cooled below 4 degrees C. Inland. Waters. 3, 15–24.
Qiu, D.-Q., Zhou, X.-J., Qiu, M.-S., 2008. Study on anti-disease ability of *Litopenaeus vannamei* and the biological control of vibrio parahaemolyticus bacteriophage under stresses of ammonia nitrogen. Acta Hydrobiol. Sin. 32, 455–461.
Qiu, R.J., Cheng, Y.X., Huang, X.X., Wu, X.G., Yang, X.Z., Tong, R., 2011. Effect of hypoxia on immunological, physiological response, and hepatopancreatic metabolism of juvenile Chinese mitten crab *Eriocheir sinensis*. Aquac. Int. 19, 283–299.
Qyli, M., Aliko, V., Faggio, C., 2020. Physiological and biochemical responses of mediterranean green crab, *Carcinus aestuarii*, to different environmental stressors: evaluation of hemocyte toxicity and its possible effects on immune response. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 231, 108739.
Ren, X., Lv, J., Gao, B., Li, J., Liu, P., 2017. Immune response and antioxidant status of *Portunus trituberculatus* inoculated with pathogens. Fish Shellfish Immunol. 63, 322–333.
Rodríguez-Ramos, T., Espinosa, G., Hernández-López, J., Gollas-Galván, T., Marrero, J., Borrell, Y., Alonso, M.E., Bécquer, U., Alonso, M., 2008. Effects of Echerichia coli lipopolysaccharides and dissolved ammonia on immune response in southern white shrimp *Litopenaeus schmitti*. Aquaculture. 274, 118–125.
Romano, N., Zeng, C.S., 2007. Ontogenetic changes in tolerance to acute ammonia exposure and associated gill histological alterations during early juvenile development of the blue swimmer crab, *Portunus pelagicus*. Aquaculture. 266, 246–254.
Romano, N., Zeng, C.S., 2013. Toxic effects of ammonia, nitrite, and nitrate to decapod crustaceans: a review on factors influencing their toxicity, physiological consequences, and coping mechanisms. Rev. Fish. Sci. 21, 1–21.
Rosa, R.D., Barracco, M.A., 2010. Antimicrobial peptides in crustaceans. Invertebr. Surviv. J. 7, 262–284.
Rowley, A.F., 2016. The immune system of Crustaceans. In: Ratcliffe, M.J.H. (Ed.), Encyclopedia of Immunobiology. Academic Press, Oxford, pp. 437–453.
Rowley, A.F., Powell, A., 2007. Invertebrate immune systems specific, quasi-specific, or nonspecific? J. Immunol. 179, 7209–7214.
Rutschmann, S., Kilinc, A., Ferrandon, D., 2002. Cutting edge: the toll pathway is required for resistance to Gram-positive bacterial infections in Drosophila. J. Immunol. 168, 1542–1546.
Sawyer, T.K., Cox, R., Higginbottom, M., 1970. Hemocyte values in healthy blue crabs, *Callinectes sapidus*, and crabs infected with the amoeba, *Paramoeba perniciosa*. J. Invertebr. Pathol. 15, 440–446.
Schram, F.R., Dixon, C.J., 2004. Decapod phylogeny: addition of fossil evidence to a robust morphological cladistic data set. Bull. Mizunami Fossil Museum 31, 19.
Selven, S., Philip, R., 2013. Salinity a significant environmental factor for V ibrio harveyi virulence in F enneropenaeus indicus. Aquac. Res. 44, 747–759.
Sharma, S.R.K., Jayaprakash, S., Philipose, K.K., Radhakrishnan, E.V., 2009. Effect of salinity and pH on selected immune functions of the Indian white shrimp, *Fenneropenaeus indicus* (H. Milne Edwards, 1837). Indian J. Fish. 56, 183–187.
Smith, V.J., 1991. Invertebrate immunology: phylogenetic, ecotoxicological and biomedical implications. Comp. Haematol. Int. 1, 61–76.
Smith, V.J., Ratcliffe, N., 1978. Host defence reactions of the shore crab, *Carcinus maenas* (L.), in vitro. J. Mar. Biol. Assoc. UK 58, 367–379.
Smith, V.J., Ratcliffe, N., 1980a. Cellular defense reactions of the shore crab, *Carcinus maenas*: in vivo hemocytic and histopathological responses to injected bacteria. J. Invertebr. Pathol. 35, 65–74.
Smith, V.J., Ratcliffe, N.A., 1980b. Host defense reactions of the shore crab, carcinus-maenas (L) - clearance and distribution of injected test particles. J. Mar. Biol. Assoc. UK 60, 89–102.
Smith, V.J., Soderhall, K., 1983. Induction of degranulation and lysis of haemocytes in the freshwater crayfish, *Astacus astacus* by components of the prophenoloxidase activating system in vitro. Cell Tissue Res. 233, 295–303.
Smith, V.J., Söderhäll, K., 1986. Cellular immune mechanisms in the Crustacea. Symp. Zool. Soc. Lond. 59–79.
Soderhall, I., 2016. Crustacean hematopoiesis. Dev. Comp. Immunol. 58, 129–141.
Söderhäll, I., Wu, C., Novotny, M., Lee, B.L., Söderhäll, K., 2009. A novel protein acts as a negative regulator of prophenoloxidase activation and melanization in the freshwater crayfish *Pacifastacus leniusculus*. J. Biol. Chem. 284, 6301–6310.
Söderhäll, K., Cerenius, L., 1992. Crustacean immunity. Annu. Rev. Fish Dis. 2, 3–23.
Söderhäll, K., Smith, V.J., 1983. Separation of the haemocyte populations of Carcinusmaenas and other marine decapods, and prophenoloxidase distribution. Dev. Comp. Immunol. 7, 229–239.
Sohal, R.S., Weindruch, R., 1996. Oxidative stress, caloric restriction, and aging. Science. 273, 59–63.
Sricharoen, S., Kim, J.J., Tunkijjanukij, S., Soderhall, I., 2005. Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish. Dev. Comp. Immunol. 29, 1017–1031.
Sternshein, D.J., Burton, P.R., 1980. Light and electron microscopic studies of crayfish hemocytes. J. Morphol. 165, 67–83.
Sun, C.B., Wang, G., Chan, S.F., 2015. Effects of artificial infection of *Litopenaeus vannamei* by micrococcus lysodeikticus and WSSV on the activity of immunity related enzymes. Fish Shellfish Immunol. 46, 778–786.
Sung, H.H., Sun, R., 2002. Use of monoclonal antibodies to classify hemocyte subpopulations of tiger shrimp (*Penaeus monodon*). J. Crustac. Biol. 22, 337–344.
Sung, H.H., Hwang, S.F., Tasi, F.M., 2000. Responses of giant freshwater prawn (*Macrobrachium rosenbergii*) to challenge by two strains of *Aeromonas* spp. J. Invertebr. Pathol. 76, 278–284.
Sung, H.-H., Chang, H.-J., Her, C.-H., Chang, J.-C., Song, Y.-L., 1998. Phenoloxidase activity of hemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. J. Invertebr. Pathol. 71, 26–33.
Sutthangkul, J., Amparyup, P., Charoensapsri, W., Senapin, S., Phiwsaiya, K., Tassanakajon, A., 2015. Suppression of shrimp melanization during white spot syndrome virus infection. J. Biol. Chem. 290, 6470–6481.
Tanner, C.A., Burnett, L.E., Burnett, K.G., 2006. The effects of hypoxia and pH on phenoloxidase activity in the Atlantic blue crab, *Callinectes sapidus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 144, 218–223.
Tassanakajon, A., Somboonwiwat, K., Supungul, P., Tang, S., 2013. Discovery of immune molecules and their crucial functions in shrimp immunity. Fish Shellfish Immunol. 34, 954–967.
Tassanakajon, A., Rimphanitchayakit, V., Visetnan, S., Amparyup, P., Somboonwiwat, K., Charoensapsri, W., Tang, S., 2018. Shrimp humoral responses against pathogens: antimicrobial peptides and melanization. Dev. Comp. Immunol. 80, 81–93.
Terwilliger, N.B., Ryan, M., Phillips, M.R., 2006. Crustacean hemocyanin gene family and microarray studies of expression change during eco-physiological stress. Integr. Comp. Biol. 46, 991–999.
Tong, R.X., Pan, L.Q., Zhang, X., Li, Y.F., 2022. Neuroendocrine-immune regulation mechanism in crustaceans: a review. Rev. Aquac. 14, 378–398.
Truscott, R., White, K.N., 1990. The influence of metal and temperature stress on the immune-system of crabs. Funct. Ecol. 4, 455–461.
Tsing, A., Arcier, J.-M., Brehélin, M., 1989a. Hemocytes of penaeid and palaemonid shrimps: morphology, cytochemistry, and hemograms. J. Invertebr. Pathol. 53, 64–77.

Tsing, A., Arcier, J.M., Brehelin, M., 1989b. Hemocytes of penaeid and palaemonid shrimps - morphology, cyto-chemistry, and hemograms. J. Invertebr. Pathol. 53, 64–77.
Valanne, S., Wang, J.H., Ramet, M., 2011. The Drosophila toll signaling pathway. J. Immunol. 186, 649–656.
Vargas-Albores, F., Hinojosa-Baltazar, P., Portillo-Clark, G., Magallon-Barajas, F., 1998. Influence of temperature and salinity on the yellowleg shrimp, *Penaeus californieinsis* Holmes, prophenoloxidase system. Aquac. Res. 29, 549–553.
Vazquez, L., Alpuche, J., Maldonado, G., Agundis, C., Pereyra-Morales, A., Zenteno, E., 2009. Review: Immunity mechanisms in Crustaceans. Innate. Immun. 15, 179–188.
Verghese, B., Radhakrishnan, E.V., Padhi, A., 2007. Effect of environmental parameters on immune response of the Indian spiny lobster, *Panulirus homarus* (Linnaeus, 1758). Fish Shellfish Immunol. 23, 928–936.
Victor, B., Narayanan, M., Jones Nelson, D., 1990. Gill pathology and hemocyte response in mercury exposed Macrobrachium idae(Heller). J. Environ. Biol. 11, 61–65.
Vogan, C.L., Rowley, A.F., 2002. Effects of shell disease syndrome on the haemocytes and humoral defences of the edible crab, *Cancer pagurus*. Aquaculture. 205, 237–252.
Vogt, G., 2008. How to minimize formation and growth of tumours: potential benefits of decapod crustaceans for cancer research. Int. J. Cancer 123, 2727–2734.
Vranckx, R., Durliat, M., 1981. Encapsulation of *Psorospermium haeckeli* by the haemocytes of Astacus leptodactylus. Cell. Mol. Life Sci. 37, 40–42.
Wang, F.I., Chen, J.C., 2006a. Effect of salinity on the immune response of tiger shrimp Penaeus monodon and its susceptibility to Photobacterium damselae subsp. damselae. Fish Shellfish Immunol. 20, 671–681.
Wang, F.I., Chen, J.C., 2006b. The immune response of tiger shrimp *Penaeus monodon* and its susceptibility to *Photobacterium damselae* subsp damselae under temperature stress. Aquaculture. 258, 34–41.
Wang, L., Pan, L.Q., Ding, Y.G., Ren, X.Y., 2018. Effects of low salinity stress on immune response and evaluating indicators of the swimming crab *Portunus trituberculatus*. Aquac. Res. 49, 659–667.
Wang, L.U., Chen, J.C., 2005b. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* at different salinity levels. Fish Shellfish Immunol. 18, 269–278.
Wang, L.-U., Chen, J.-C., 2005a. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* at different salinity levels. Fish Shellfish Immunol. 18, 269–278.
Wang, W.N., Zhou, J., Wang, P., Tian, T.T., Zheng, Y., Liu, Y., Mai, W.J., Wang, A.L., 2009. Oxidative stress, DNA damage and antioxidant enzyme gene expression in the Pacific white shrimp, *Litopenaeus vannamei* when exposed to acute pH stress. Comp. Biochem. Physiol., C: Toxicol. Pharmacol. 150, 428–435.
Wood, C.M., Rogano, M.S., 1986. Physiological responses to acid stress in crayfish (Orconectes): haemolymph ions, acid–base status, and exchanges with the environment. Can. J. Fish. Aquat. Sci. 43, 1017–1026.
Wurts, W.A., 2003. Daily pH cycle and ammonia toxicity. World Aquacult. 34, 20–21.
Xu, C., Li, E., Liu, Y., Wang, X., Qin, J.G., Chen, L., 2017. Comparative proteome analysis of the hepatopancreas from the Pacific white shrimp *Litopenaeus vannamei* under long-term low salinity stress. J. Proteome 162, 1–10.
Xu, H., Bai, X.X., Li, Y., Li, J.J., Meng, Y., Xu, Z.Q., Tang, J.Q., Lu, Y., Huang, Y.H., 2022. Changes in the immunity, histopathology, and metabolism of crayfish (*Procambarus clarkii*) in response to drought. Animals 12.
Xu, Z., Guan, W., Xie, D., Lu, W., Ren, X., Yuan, J., Mao, L., 2019. Evaluation of immunological response in shrimp *Penaeus vannamei* submitted to low temperature and air exposure. Dev. Comp. Immunol. 100, 103413.
Yang, L.S., Yang, Q.B., Jiang, S.G., Li, Y., Zhou, F.L., Li, T., Huang, J.H., 2015. Metabolic, immune responses in prawn (*Penaeus monodon*) exposed to ambient ammonia. Aquac. Int. 23, 1049–1062.
Young-Lai, W., Charmantier-Daures, M., Charmantier, G., 1991. Effect of ammonia on survival and osmoregulation in different life stages of the lobster *Homarus americanus*. Mar. Biol. 110, 293–300.
Yu, Z.M., Li, C.W., Guan, Y.Q., 2003. Effect of salinity on the immune responses and outbreak of white spot syndrome in the shrimp *Marsupenaeus japonicus*. Ophelia. 57, 99–106.
Yue, F., Pan, L., Xie, P., Li, J., 2010a. Effects of ammonia exposure on prophenoloxidase system and immune parameters of swimming crab Portunus trituberculatus. J. Fish. Sci. China/Zhongguo Shuic. Kexue 17.
Yue, F., Pan, L., Xie, P., Zheng, D., Li, J., 2010b. Immune responses and expression of immune-related genes in swimming crab *Portunus trituberculatus* exposed to elevated ambient ammonia-N stress. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 246–251.
Yue, F., Pan, L., Xie, P., Zheng, D., Li, J., 2010c. Immune responses and expression of immune-related genes in swimming crab Portunus trituberculatus exposed to elevated ambient ammonia-N stress. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 246–251.
Zhai, Q., Li, J., Feng, Y., Ge, Q., 2019. Evaluation of combination effects of Astragalus polysaccharides and florfenicol against acute hepatopancreatic necrosis disease-causing strain of *Vibrio parahaemolyticus* in *Litopenaeus vannamei*. Fish Shellfish Immunol. 86, 374–383.
Zhang, W.Y., Jiang, Q.C., Liu, X.Q., Pan, D.M., Yang, Y.H., Yang, J.X., 2015. The effects of acute ammonia exposure on the immune response of juvenile freshwater Prawn, macrobrachium nipponense. J. Crustac. Biol. 35, 76–80.
Zhang, Z.F., Shao, M.Y., Kang, K.H., 2005. Changes of enzyme activity and hematopoiesis in Chinese prawn Fenneropenaeus chinensis (Osbeck) induced by white spot syndrome virus and zymosan A. Aquac. Res. 36, 674–681.
Zhao, M.M., Yao, D.F., Li, S.K., Zhang, Y.L., Aweya, J.J., 2020. Effects of ammonia on shrimp physiology and immunity: a review. Rev. Aquac. 12, 2194–2211.
Zhou, J., Wang, L., Xin, Y., Wang, W.N., He, W.Y., Wang, A.L., Liu, Y.A., 2010. Effect of temperature on antioxidant enzyme gene expression and stress protein response in white shrimp, *Litopenaeus vannamei*. J. Therm. Biol. 35, 284–289.
Zhu, F., Qian, X., Ma, X., 2018. Comparative transcriptomic analysis of crab hemocytes in response to white spot syndrome virus or *Vibrio alginolyticus* infection. Fish Shellfish Immunol. 80, 165–179.

CHAPTER 3

HEMOCYTE COAGULATION AND PHAGOCYTIC BEHAVIOR IN EARLY STAGES OF INJURY IN CRAYFISH (ARTHROPODA: DECAPODA) AFFECT THEIR MORPHOLOGY

Mengal, K., Kor, G., Kouba, A., Kozák, P., Niksirat, H., 2023. Hemocyte coagulation and phagocytic behavior in early stages of injury in crayfish (Arthropoda: Decapoda) affect their morphology. Developmental & Comparative Immunology, 141, 104618.

My share on this work was 60 %.

Developmental and Comparative Immunology 141 (2023) 104618

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Hemocyte coagulation and phagocytic behavior in early stages of injury in crayfish (Arthropoda: Decapoda) affect their morphology

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ARTICLE INFO

Keywords:
Degranulation
Hemolymph coagulation
Muscle degeneration
Phagocytosis

ABSTRACT

Crustacean hemocytes are important mediators of immune functions such as coagulation and phagocytosis. We employed an *in situ* approach to investigate the ultrastructural behavior of hemocytes during coagulation and phagocytosis in the early stages after injury caused by leg amputation, using transmission electron microscopy technique in marbled crayfish *Procambarus virginalis*. Hemocytes underwent drastic morphological changes during coagulation. The morphology of the cytoplasmic granules changed from electron-dense to electron-lucent forms in an expanding manner. The transformed granules containing amorphous electron-lucent material were observed to merge and discharge their contents into extracellular space for coagulation. We also observed that the contents of the nucleus participate in the process of coagulation. In addition, leg amputation induced extensive muscle degeneration and necrotic tissues were avidly taken up by the phagocytic hemocytes containing distinct phagosomes. Interestingly, we observed for the first time how the digested contents of phagocytized necrotic tissues are incorporated into granules and other cellular components that change the cell morphology by increasing the granularity of the hemocytes. Nevertheless, the degranulation of hemocytes during coagulation can also reduce their granularity. Given that morphological traits are important criteria for hemocyte classification, these morphological changes that occur during coagulation and phagocytosis must be taken into account.

1. Introduction

An immediate defense response to injury in decapod crustaceans is the activation of the coagulation system, which leads to hemolymph clotting at the damaged site. Coagulation refers to transformation of hemolymph as an extracellular fluid from a liquid to a gel state. It is well established that coagulation is a complex and dynamic process that involves cell-to-cell and cell-to-extracellular matrix interactions (Majno and Joris, 2004; Sirikharin et al., 2017; Sritunyalucksana and Söderhäll, 2000). Hemolymph coagulation plays a critical role in innate immune responses in decapods (Vazquez et al., 2009) creating a physical barrier around the wound that prevents the loss of hemolymph and maintains hemostasis and, at the same time, restricts the entry of microbes into the hemocoel, thereby hindering their systemic dissemination (Cerenius and Söderhäll, 2013; Kopacek et al., 1993; Theopold et al., 2004). Since decapods possess an open circulatory system, they use clot formation as a defense system much more extensively than vertebrates with no potential danger of thromboses (Loof et al., 2011).

In general, decapod crustacean hemocytes are grouped into three major classes, namely, hyaline cells (HCs), semigranular cells (SGCs), and granular cells (GCs) based on cytoplasmic granularity, density, nuclear size, and staining properties (Mengal et al., 2022; Söderhäll, 2016; Söderhäll and Smith, 1983). The production of hemocytes takes place mainly in the hematopoietic tissue after which they are released into the circulation where they finally mature and perform various biological functions including coagulation and phagocytosis (Junkunlo et al., 2016; Lin et al., 2008; Sirikharin et al., 2018; Sirikharin et al., 2020; Söderhäll, 2013; Söderhäll and Junkunlo, 2019). Initial changes in clot formation resulting from the actions of hemocytes promote hemolymph coagulation and melanization (Vafopoulou et al., 2007). Hemocytes are highly labile cells that undergo rapid morphological transformations during the clotting process (Clare and Lumb, 1994b; Omori et al., 1989a; Sternshein and Burton, 1980). One of the key features of hemocytes is the presence of large, homogeneous-to-irregularly shaped cytoplasmic granules (Burgos-Aceves et al., 2021a,b; Sternshein and Burton, 1980) packed with various immune molecules that participate in coagulation and other immune functions, and are released upon injury (Kumar et al., 2013; Sricharoen et al., 2005).

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https://doi.org/10.1016/j.dci.2022.104618
Received 8 November 2022; Received in revised form 12 December 2022; Accepted 12 December 2022
Available online 13 December 2022

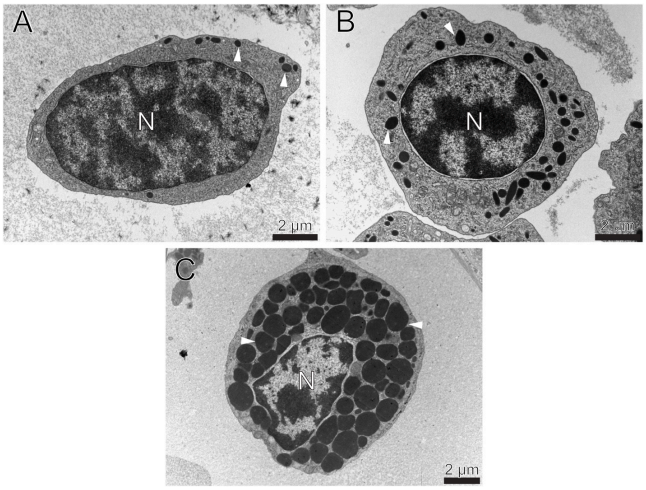

Fig. 1. A–C. Transmission electron micrographs of *P. virginalis* hemocytes. (A) A hyaline cell showing a high nucleus/cytoplasm ratio. (B) A semi-granular cell showing a round centrally located nucleus containing different amounts of small electron-dense cytoplasmic granules (white arrowheads) of different sizes. (C) A granular cell filled with typical, relatively large electron-dense cytoplasmic granules (white arrowheads). M, mitochondrion; N, nucleus.

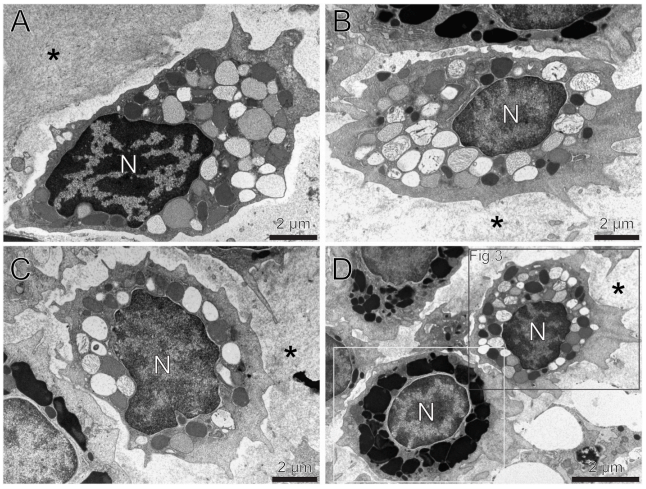

Fig. 2. A–D. Transmission electron micrographs of several activated hemocytes containing expanding and decondensing granules during hemolymph coagulation in *P. virginalis*. The individual activated semigranulocytes are shown in Figs. A–C. Note the large intact granulocyte highlighted by the white square and the activated semigranulocyte highlighted by the black square in Fig. 2D. The details of the activated semigranulocyte in Fig. 2D highlighted by the black square are shown in greater magnification in Fig. 3. Clotted hemolymph is indicated by asterisks in the micrographs. N, nucleus.

Molecular aspects of the role hemocytes play in the clotting system have been studied previously in decapod crustaceans (Cerenius and Söderhäll, 2013; Maningas et al., 2013; Perdomo-Morales et al., 2019; Vazquez et al., 2009). Briefly, coagulation is induced when transglutaminase (TGase) is released from hemocyte granules upon injury. It then becomes activated by the Ca^{2+} in the plasma and, finally, starts crosslinking the plasma-clotting protein (CP) into large aggregates (Hall et al., 1999; Junkunlo et al., 2018; Soderhall and Soderhall, 2022; Wang et al., 2001). Earlier ultrastructural studies have noted certain morphological features of hemocytes during coagulation in decapods such as the crayfish *Procambarus* spp. and *Orconectes* spp. (Sternshein and Burton, 1980), ridgeback prawn *Sicyonia ingentis* (Omori et al., 1989a), and blue crab *Callinectes sapidus* (Clare and Lumb, 1994b). Nevertheless, *in situ* ultrastructural aspects of hemocyte behavior during coagulation remain poorly understood.

Hemocytes also play a major role in cellular immune functions such

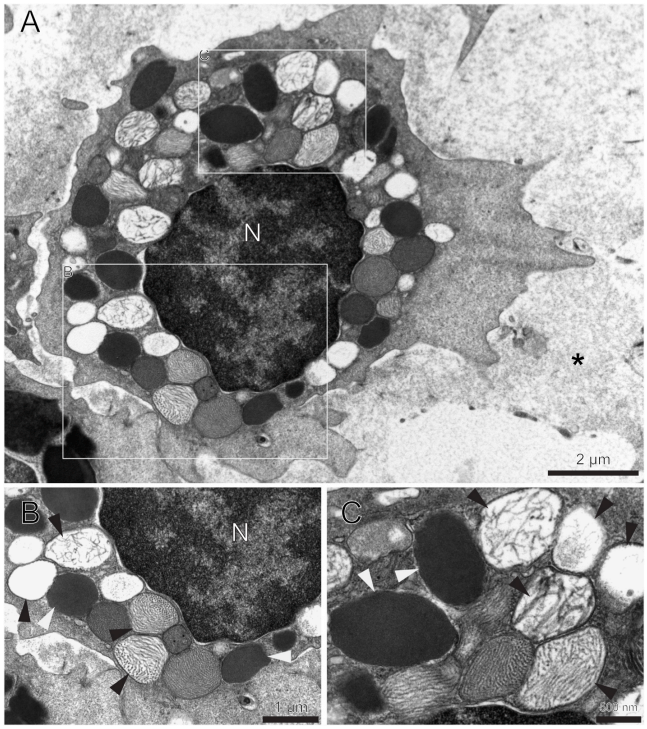

Fig. 3. A–C. Transmission electron micrographs of a semigranulocyte during the early stages of granular activation for hemolymph coagulation in *P. virginalis* showing how granules are transformed. The higher magnification of granule transformation from electron-dense (white arrowheads) into electron-lucent forms resembling fibrous structures (black arrowheads) are shown in Figures B and C. Clotted hemolymph is indicated by asterisks in Fig. 3A. N, nucleus.

as phagocytosis in decapods (Burgos-Aceves et al., 2018; Cerenius et al., 2010; Giulianini et al., 2007; Liu et al., 2020; Smith, 1991). The phagocytic role of hemocytes is crucial for removing pathogenic agents and apoptotic or necrotic cells and for maintaining tissue homeostasis (Liu et al., 2020). Ultrastructural studies of the narrow-clawed crayfish *Pontastacus leptodactylus* have demonstrated that hemocytes are active in the phagocytosis of disintegrating muscle fiber after injury (Branislav Uhrik and Zacharova, 1989).

Several ultrastructural studies have been conducted on the hemocyte-mediated phagocytosis of both biotic and abiotic particles in many crustaceans species including the crayfish *Astacus leptodactylus* (Giulianini et al., 2007), *Procambarus, orconectes* and *P. clarkii* spp. (Lanz et al., 1993; Sternshein and Burton, 1980), *S. ingentis* (Omori et al., 1989a), tiger shrimp *Penaeus monodon* (Sung and Sun, 2002), *C. sapidus* (Bodammer, 1978), American lobster *Homarus americanus,* spiny lobster *Panulirus interruptus*, spider crab *Loxorhynchus grandis* (Hose et al., 1990), Chinese mitten crab *Eriocheir sinensis* (Lv et al., 2014), and common shore crab *Carcinus maenas* (Johnston et al., 1973).

Although the aforementioned studies of decapods focused on the ultrastructural features of the hemocytes, to the best of our knowledge no comprehensive *in situ* ultrastructural study of the coagulation and phagocytosis behavior of hemocytes following injury in decapods exists. Therefore, we performed a transmission electron microscopic study using the marbled crayfish *Procambarus virginalis* as a model organism to illustrate the behavior of hemocytes during the early stages of an injury in decapods, with special emphasis on the morphological features of the development of coagulation and phagocytosis.

2. Materials and methods

Freshwater marbled crayfish were obtained from our own laboratory culture of this species. Only healthy intermolt individuals with fully developed body appendages were used during the experiment. The crayfish were maintained in well-aerated shallow tanks filled with tap water and fed *ad libitum* daily with commercial feed (Granugreen, Sera, Heinsberg, Germany). A total of fifteen animals were used and no animal was killed during the experiment. All animals were returned to the main aquarium following the sampling procedures.

An *in situ* injury was induced by amputating the second pair of walking legs. To do so, the amputation site was first swabbed with 70% ethanol and the legs were then amputated using a sterile surgical blade. Tissue sampling from the injury site was carried out at different time-points (0, 5, 10, 20 min, and 1hr) and samples were processed for transmission electron microscopy (TEM) as described by (Niksirat et al., 2014).

Briefly, the tissue samples were immediately placed in a fixative 2.5% glutaraldehyde in 0.1 M phosphate buffer for 48 h at 4 °C, followed by buffer washing. Next, they were post-fixed for an additional 2 h in 4%

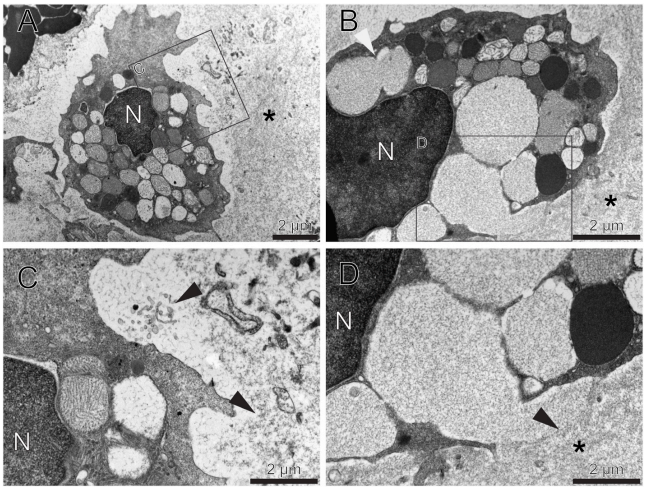

Fig. 4. A–D. Transmission electron micrographs of activated hemocytes showing the degranulation of the cytoplasm via the discharge of electron-lucent materials from the expanded granules into the extracellular environment for clot formation during the later stages of granule activation and hemolymph coagulation in *P. virginalis* (Figs. A, B). Note two expanded merging granules indicated by white arrowhead (Fig B). The higher magnification micrographs show material discharges (black arrowheads) from the expanded granules of the activated hemocytes, which join the hemolymph clot in the extracellular environment (Figs. C, D). Clotted hemolymph is indicated by asterisks in the micrographs. N, nucleus.

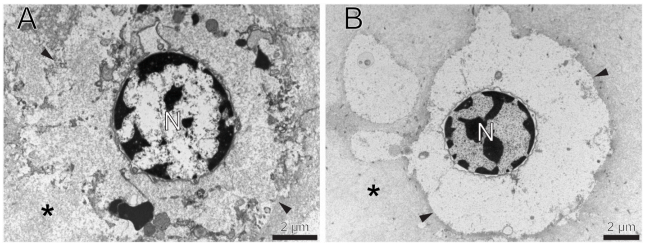

Fig. 5. A–B. Transmission electron micrographs of activated hemocytes after complete degranulation and the release of their cytoplasmic granules into the extracellular environment for clot formation in the hemolymph of *P. virginalis*. Note that the borders of the cytoplasmic zone (black arrowheads) are left after the complete degranulation of the cell. Clotted hemolymph is indicated by asterisks in the micrographs. N, nucleus.

osmium tetroxide at room temperature and then washed again in the buffer, dehydrated through an acetone series (30, 50, 70, 90, 95, and 100% for 15 min each) and finally embedded in resin (EPON). A series of ultra-thin sections obtained using a UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) were mounted on copper grids and double-stained with uranyl acetate and lead citrate. The sections were examined with a 1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at 80 kV in the core facility of the Biology Centre (CAS) laboratory of electron microscopy.

3. Results

From the ultrastructural micrographs we observed that typically hemocytes had two principal roles, coagulation and phagocytosis.

Coagulation: The morphological changes that the hemocytes undergo during coagulation can be described in three stages: i) decondensation of the granule contents in the cytoplasm prior to discharge or release; ii) discharge or release of granular contents into extracellular space; and iii) degeneration and release of the contents of the nucleus.

Phagocytosis: muscle degeneration takes place at the wound site, leading to the subsequent phagocytosis of degenerated muscle tissues by hemocytes.

3.1. Ultrastructural features of hemocytes during coagulation

3.1.1. Structure of intact hemocytes

As previously reported in crayfish, three types of hemocyte identified by electron microscopy were further confirmed by the density gradient centrifugation method (Duan et al., 2014; Soderhall and Soderhall, 2022). We observed three major types of hemocytes, as shown by the control group (Fig. 1A–C). A typical hyaline cell has an irregular profile, characterized by a high nucleus-to-cytoplasm ratio with only a few cytoplasmic granules and poorly developed mitochondria (Fig. 1 A). A semi-granular cell has a round, centrally located nucleus surrounded by cytoplasmic granules of various sizes, and moderately developed mitochondria (Fig. 1 B). Finally, a typical granular cell has a relatively small irregular nucleus packed with large electron-dense cytoplasmic granules (Fig. 1 C).

3.1.2. Activation of the cytoplasmic granules during coagulation

Hemocytes play a key role in coagulation (Fig. 1). They become activated within 5–10 min of an injury occurring. First, the granules of

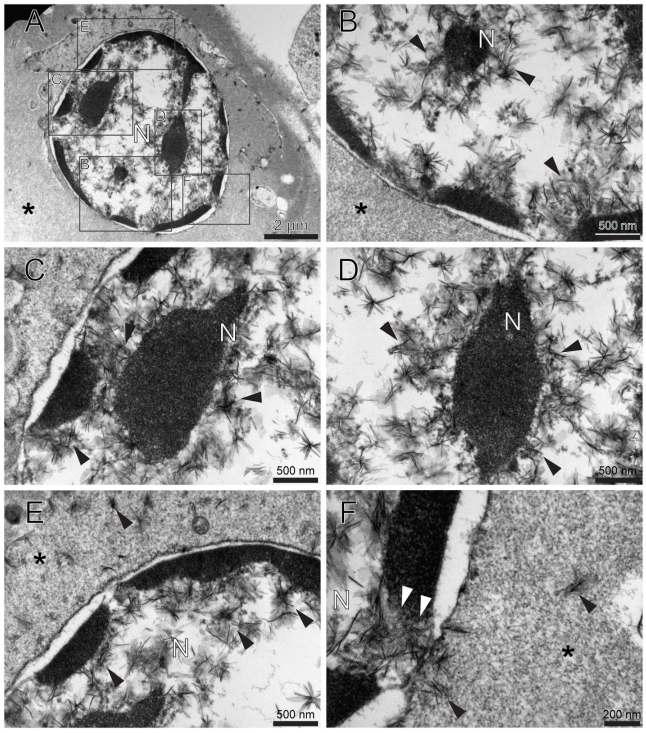

Fig. 6. A–F. Transmission electron micrographs of the nucleus of an activated hemocyte after complete degranulation of the cytoplasm contents showing the degeneration and release of nucleus contents into the extracellular environment during hemolymph coagulation in *P. virginalis*. Black arrowheads show material resulted from the nucleus degeneration that passes through the nuclear pores (white arrowheads) and is released outside. Clotted hemolymph is indicated by asterisks in micrographs. N, nucleus.

the hemocytes decondense and expand, and their contents change from an electron-dense into an electron-lucent state, with some filaments present in the early stages that further degrade into fine particles as the decondensation progresses (Figs. 2 and 3). Then, some of these expanded granules merge and discharge their contents into the extracellular environment to form a clot (Fig. 4). This process continues until all the cytoplasmic granules expand and discharge their contents, at which point the cytoplasm of the cell disappears (Fig. 5). Afterwards, the nucleus of the hemocyte starts to degenerate and release its contents as well into the surrounding clot (Fig. 6). All the major stages of the morphological change that hemocytes undergo during *in situ* coagulation are shown in a schematic illustration (Fig. 7).

3.1.3. Degeneration of muscle tissue and hemocyte phagocytic activity

The ultrastructure of intact muscle tissue from the control group is shown in Fig. 8 A and B. Muscle tissue start to break down and degenerate within 5 min after leg amputation. Muscle fibers lose integrity and become degraded (Fig. 8C–D). The mitochondria swell and the shape of their cristae distorts. Also, electron-dense particles appear inside degenerating organelles such as mitochondria (Fig. 8E–F). Hemocytes arrive at the area of tissue degeneration within 5–10 min and start phagocytizing the degenerating tissue organelles. We observed that hemocytes could absorb small electron-dense particles via their membranes (Fig. 9A–E). In addition, larger organelles were observed to be trapped and digested through the formation of the phagosome in the hemocytes (Fig. 10A–C). Finally, digested particles of degenerating tissues were absorbed and incorporated into the granules and nucleus of phagocytic hemocytes, and were primarily distinguishable as electron-dense particles inside the organelles of the hemocytes (Fig. 11). The process of tissue degeneration and subsequent hemocyte phagocytosis is shown in a schematic drawing (Fig. 12).

4. Discussion

4.1. Hemocyte classification

Two general concepts exist in the literature regarding the classification of hemocytes in decapods (Mengal et al., 2022). The first divides hemocytes into three types, namely, hyaline, semigranular, and granular based on their morphological traits such as degree of granularity (Soderhall and Soderhall, 2022). These can be further divided into subclasses based on their molecular traits determined by transcriptomic studies (Söderhäll et al., 2022). However, these authors also state that at least a fraction of the less granular hemocytes can be differentiated into more granular cells (Chaga et al., 1995).

On the other hand, the second concept states that all morphologically different hemocytes in circulation are actually different developmental stages of a single type of hemocyte. Cells with a lower level of granularity are released by hematopoietic tissue into circulation, in which maturation takes place by increasing the contents of the cytoplasmic granules. The idea of a single cell lineage in crustaceans and arthropods has been proposed in a number of previous studies (Bodammer, 1978;

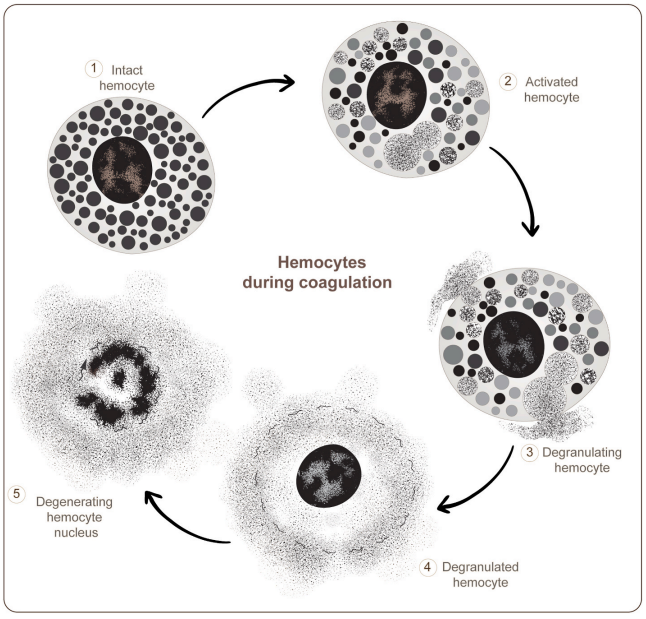

Fig. 7. Schematic illustration depicting different stages of hemocyte change during coagulation. Stage (1) shows an intact hemocyte containing electron-dense cytoplasmic granules. Stage (2) shows an activated hemocyte, in which the cytoplasm contains granules in varying states, undergoing granular transformation from electron-dense to electron-lucent forms in an expanding manner. The expanded electron-lucent granules finally merge and turn into a less electron-dense amorphous material. Stage (3) shows the membrane of the electron-lucent granules as they fuse with the plasma membrane of the hemocyte and discharge their granular components into the extracellular space where they contribute to the formation of the gelatinous clot. Stage (4) shows a hemocyte that has undergone complete degranulation and has an empty cytoplasm devoid of granules surrounded by discharged granular contents. Finally, stage (5) shows a degenerating nucleus, the end stage of a granular hemocyte during coagulation process, in which the contents of the nucleus is disintegrated and released into the clot.

Brehelin et al., 1978; Dumont et al., 1966; Li et al., 2019, 2021; Ravindranath, 1974; Rebelo et al., 2013).

However, both concepts agree that the transformation into more granular cells is possible in decapod hemocytes. Our results suggest that obtaining material via phagocytosis in cells with lower granular contents such as HC and SGC hemocytes and its storage in the cytoplasm can increase the number and size of granules, and can drive the differentiation of cells into more granular cells such as granulocytes. Therefore, phagocytosis is at least partially responsible for this differentiation in decapod hemocytes.

The term hyaline cell is currently used to refer to hemocytes that contain no or only a few cytoplasmic granules. However, in some cases they can be confused with the end-stage of granular hemocytes, which is the left-over nucleus after degranulation (Dolar et al., 2020). Granulocytes are highly unstable cells that degranulate and release their contents during the coagulation process leaving only their nuclei, which have sometimes been mistakenly considered to be hyaline cells by previous studies. Furthermore, given that not all granulocytes react simultaneously, the leftover nuclei of reacted granulocytes and the unreacted granulocytes present in the same sample could be confused and be regarded as two different types of hemocytes (Clare and Lumb, 1994a).

Furthermore, single-cell mRNA sequencing (scRNA-seq) techniques have been used recently in decapods to categorize hemocytes subpopulation and their functions. For example, using scRNA-seq technique in hemocytes of freshwater crayfish, *Pacifastacus leniusculus*, the authors demonstrated that several subtypes of hemocytes exist (Söderhäll et al., 2022). The possibility of several hemocyte subtypes was also proposed in their previous works (Junkunlo et al., 2017, 2020). However, using the same scRNA-seq technique in other decapods such as *Marsupenaeus japonicus* revealed six types of hemocytes (Koiwai et al., 2021), whereas, in white shrimp *Penaeus vannamei,* three major cell-types were identified namely prohemocytes, monocytic hemocytes, and granulocytes (Yang et al., 2022). Although scRNA-seq approaches have some limitations, still use of these techniques can further our understanding of hemocyte subtypes and their functions.

4.2. Morphological changes in hemocytes during in situ coagulation

The results of the present study underscore the crucial contribution made by hemocytes to the coagulation process in a model decapod species. They discharge their cytoplasmic and nucleic contents after decondensation and the expansion of related materials, a process that is accompanied by vast morphological changes to cells.

The activated stage of hemocytes during coagulation is characterized by the presence of variable forms of cytoplasmic granules. Some authors state that cytoplasmic granule transformation is not necessary for hemolymph coagulation and that the granular change from electron lucent to dense forms is the final stage of granule formation (Scharrer, 1972; Seitz, 1972). Conversely, other authors report that low-density granules with microtubular structures containing amorphous flocculent material are transformational phases of the electron-dense granules (Dumont et al., 1966; Hearing and Vernick, 1967; Ratcliffe and Price, 1974; Rowley, 1977; Rowley and Ratcliffe, 1976). Our observations agree with the latter authors that the varying states of granules are in fact transformational phases in which the electron-dense granules change into low-density granules with microtubular structure and, finally, transform into electron-lucent amorphous granules. This process allows highly compacted materials in the granules to be unpacked and released from the cells to participate in the process of clot formation.

Additionally, during the activation of cytoplasmic granules, the clot-promoting enzyme system is activated and released into the extracellular matrix. The contents of the cytoplasmic granules and their *in vitro* release induced by exocytosis (degranulation) have been well studied in

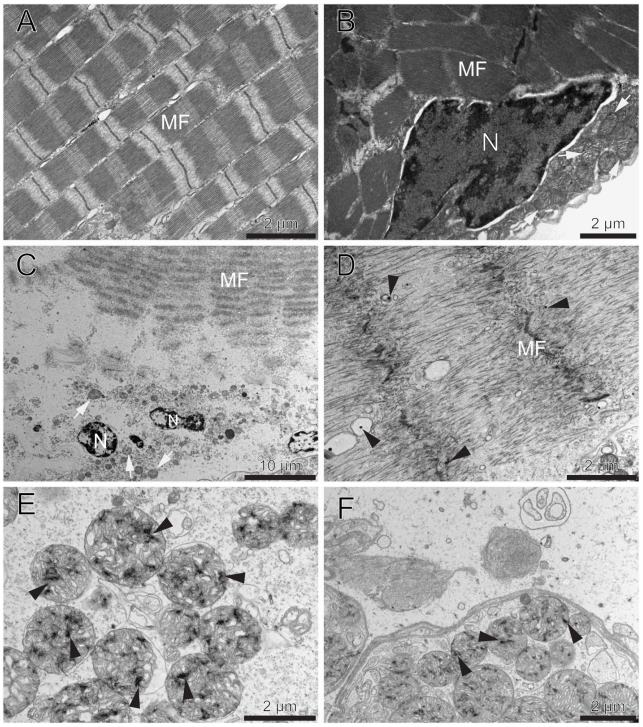

Fig. 8. A–F. Transmission electron micrographs of intact (Figs. A, B) and degenerated (Figs. C, D) muscle fibers and mitochondria that start 5 min after leg amputation (Figs. E, F) in *P. virginalis*. Note how the electron-dense particles can be seen in the degenerating muscle tissue (Fig. D) and in the degenerating mitochondria (black arrowheads) (Figs. E, F). White arrows indicate mitochondria in Figs. B, C. MF, Muscle fibers; N, nucleus.

crayfish and other decapods (Johansson and Söderhäll, 1985, 1989b; Smith and Söderhäll, 1983; Sricharoen et al., 2005). The granules of the hemocytes are packed with clotting factors and several defense molecules such as TGase, various antimicrobial peptides, and components of the pro-phenoloxidase activating system (proPO) (Johansson and Söderhäll, 1996; Junkunlo et al., 2020; Söderhäll and Smith, 1986). The release of these molecules is important in clotting and defense and can be triggered by various degranulation-inducing factors including the β-1, 3-glucan laminarin G and Lipopolysaccharides that induce exocytosis of only SGCs; by contrast, the Ca^{2+} ionophore A23187 and peroxinectin were found to greatly induce exocytosis in both SGCs and GCs from the crayfish (Johansson and Söderhäll, 1985; Sricharoen et al., 2005). Furthermore, components of proPO were observed to induce degranulation in the hemocytes, which as a result may influence the clotting process in *Astacus astacus* (Smith and Söderhäll, 1983). A cell adhesion factor that can mediate adhesion between SGC and GCS hemocytes was found to induce degranulation in the granular hemocytes of crayfish (Johansson and Söderhäll, 1988, 1989a). Therefore, the granule contents can be induced *in vitro* and are rich in various clot-inducing factors and defense molecules.

In the horseshoe crab *Limulus polyphemus* (Arthropoda: Xiphosura), a series of cytological changes were observed by Dumont et al. (1966) during *in vitro* clotting. These changes included hemocyte swelling, the loss of cytoplasmic granule density, the vacuolization of the cytoplasm, and, finally, the release of granular contents into the surrounding plasma (Dumont et al., 1966). These changes in many ways resemble our observations. Solum made similar observations in *L. polyphemus* (Solum, 1970), while Grégoire and Goffinet observed alterations in hemocytes during clotting in a stick insect *Carausius morosus* and reported the discharge of coagulocyte cytoplasmic and nuclear substances into the plasma through micro ruptures of the cytoplasmic membrane (Grégoire and Goffinet, 1975). The involvement of nucleus chromatin contents in the coagulation process has also been observed in the mealworm *Tenebrio molitor* (Stang-Voss, 1970). Similarly, cell lysis in hemocytes has been reported in the hemolymph of certain arthropods during coagulation (Ravindranath, 1980). *In vitro* ultrastructural observations in hemocytes in the crayfish *Procambarus* spp. and *Orconectes* spp., as well as in worms, show that hemocytes undergo cytolysis during hemolymph clotting (Stang-Voss, 1974; Sternshein and Burton, 1980). Similar observations have been reported by Jones, who reported how highly unstable hemocytes rapidly disintegrate and release granular contents into the surrounding plasma (Jones, 1962). Moreover, the controlled release of chromatin from the hemocyte nuclei (known as ETosis) plays an important role in the innate immunity of invertebrates. It has been reported that the chromatin content released from hemocytes in crabs participate in its defense and can effectively protect the host against infection (Robb et al., 2014). Similarly, it has been found that, besides the innate immune response, extracellular nucleic acids can induce

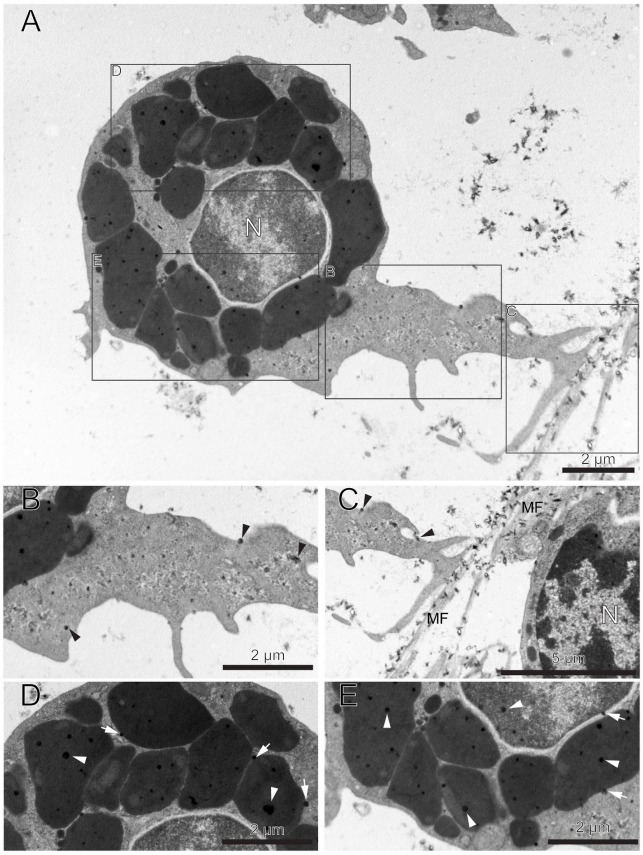

Fig. 9. A–E. Transmission electron micrographs of a large granulocyte phagocytizing small electron-dense particles resulting from the degeneration of muscle fibers 5–10 min after leg amputation in *P. virginalis*. The black arrowheads show where the electron-dense particles are absorbed into the phagocytic hemocyte (Figs. B, C). The white arrows indicate where newly absorbed electron-dense particles enter and are incorporated into the nucleus and granules (Figs. D, E). The white arrowheads indicate electron-dense particles that have already entered the nucleus and granules. MF, degenerating muscle fibers; N, nucleus.

coagulation in the hemolymph of other arthropods such as insects (Altincicek et al., 2008).

In addition, the explosive behavior of these cells can release clotting substances such as TGase into the extracellular environment (Junkunlo et al., 2018, 2020). In the presence of Ca^{2+}, the TGase further crosslinks plasma CP to form the clot (Junkunlo et al., 2018). Besides coagulation, these released materials may also induce lysis in the other explosive cells in the hemolymph and intensify the process of coagulation (Hearing and Vernick, 1967). For instance, components of the proPO system may enhance hemocyte degranulation and lysis, which can result in the release of more TGase and proPO components into nearby hemocytes (Smith and Söderhäll, 1983). Scharrer reported the swelling of cells followed by plasma cytoplasmic membrane rupture and the release of the contents of the granules, which induced hemolymph coagulation in the vicinity of the activated cells (Scharrer, 1972). A similar sequence regarding the release of granular contents into the surrounding extracellular space has also been observed in the granular cells of a crab (Bauchau and De Brouwer, 1974). In the ridgeback prawn *S. ingentis*, hemocyte lysis has been reported which results in the release of granular filamentous material into the extracellular matrix, which further expands to form the clot (Omori et al., 1989b). Earlier *in vitro* ultrastructural observations such as cytoplasmic granular transformations or the explosive or lysis behavior of hemocytes are in many ways equivalent to the decondensation, expansion, and degranulation of hemocytes that we observed *in situ* during coagulation in crayfish. However, due to the *in situ* conditions, the changes we observed in the hemocytes are more natural and detailed and include all stages of the changes. In no past studies were all these stages observed together. It is important to recognize that if hemocytes are under *in vitro* conditions, changes in clot formation do not fully replicate the *in situ* conditions of the coagulation process. Therefore, it is possible that *in vitro* changes in hemocytes during coagulation might not represent the true physiological changes that we observed in our *in situ* study.

Three mechanisms of coagulation in crustaceans were proposed (Perdomo-Morales et al., 2019). Briefly, type A coagulation is achieved by the rapid agglutination of hemocytes with no plasma clotting, where a dense network of hemocytes is enough to form a clot without any need for plasma clotting. Type B coagulation involves hemocyte coagulation followed by plasma coagulation. Type C coagulation is characterized by rapid hemocyte rupture/lysis and immediate clotting of plasma (e.g. in

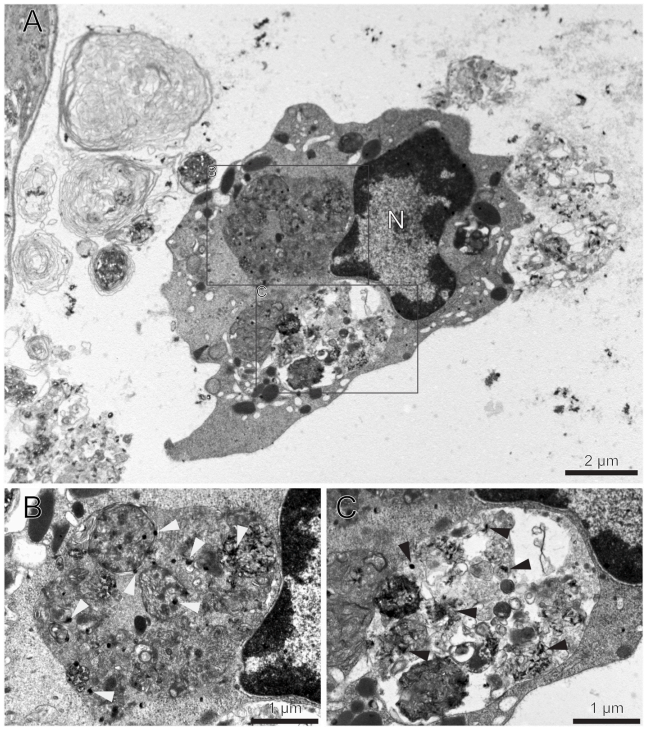

Fig. 10. A–C. Transmission electron micrographs of a phagocytic semigranulocyte on the periphery of degenerating muscle tissue showing various internalized organelles resulting from muscle degeneration in membrane-bound phagosomes (Fig. 10 A). Note the low number of granules in the cytoplasm of the phagocytic hemocyte. Phagosomes and their contents, including degenerated mitochondria, are shown at higher magnifications in Figures B and C. As well, note the electron-dense particles resulting from the degeneration that are trapped inside the phagosomes (white arrowheads in Fig. B and black arrowheads in Fig. C). N, nucleus.

shrimps and spiny lobsters) (Hose et al., 1990). Our observations are consistent with type C coagulation since the hemocytes released their contents through degranulation into the extracellular environment to form the clot.

Our study showed that the process of coagulation in the hemolymph of crayfish can cause rapid morphological changes in the hemocyte cytoplasm and nucleus and, eventually, in the whole cell by discharging its contents into the surrounding environment.

4.3. In situ phagocytic behavior of hemocytes after muscle degeneration

In the present study, massive muscle degeneration was observed after the amputation of the legs, followed by an extensive influx of phagocytic hemocytes that removed a large amount of muscle fibers and other organelles such as mitochondria produced during degeneration in the wound. We observed phagocytic hemocytes with differing numbers of granules in their cytoplasm and noted that phagocytic hemocytes incorporate necrotic bodies resulted from degenerated muscles to their organelles, such as cytoplasmic granules and nucleus.

Phagocytosis is an important immune function used for eliminating pathogens as well as cell debris (Jiravanichpaisal et al., 2006). However, the true phagocytic role of different hemocyte types is unclear, even in very closely related species. For example, in crabs GCs and SGCs the phagocytic activity compared to the HCs was relatively limited (Lv et al., 2014; Matozzo and Marin, 2010; Söderhäll et al., 1986). The SGCs and GCs in penaeid shrimps were reported to be the main types of hemocytes that ingest yeast (Gargioni and Barracco, 1998). Yet, a study of the tiger Shrimp *P. monodon* concluded that HCs were the only type of hemocytes that engulfed latex beads (Sung and Sun, 2002). Furthermore, a more recent study of the crayfish *Cherax quadricarinatus* reported SGCs and GCs as the main phagocytic hemocytes (Li et al., 2018). In some early literature on freshwater crayfish, SGCs were identified as the main phagocytic cells and GCs as non-phagocytic cells, while in crabs HCs were found to be the main phagocytic cells (Smith and Söderhäll, 1983; Söderhäll et al., 1986). A study of the freshwater crayfish *A. leptodactylus* showed that hemocytes of all three types participated in the phagocytosis of foreign particles, with SGCs being the main phagocytic cells. (Giulianini et al., 2007). In addition, a more recent study of crayfish hemocytes has suggested that all three types are functionally active as phagocytic cells at different developmental stages (Li et al., 2021). Our observations showed that granular hemocytes are the main hemocytes that actively ingest necrotic bodies resulting from muscle degeneration. Such disparity in the above-mentioned studies may be due to differences between species and experimental conditions (e.g. *in vitro*), morphological changes in hemocytes after the withdrawal of hemolymph samples (e.g. changes in the pH of the hemolymph), or differing developmental stages in the tested animals.

Muscle degeneration at the site of the injury appears to be an initial stage of wound healing and limb generation. It is known that, like other decapods, crayfish are able to regenerate their limbs after amputation. Therefore, muscle degeneration and the subsequent clearance of the leftover tissue by the phagocytic hemocytes might be an essential

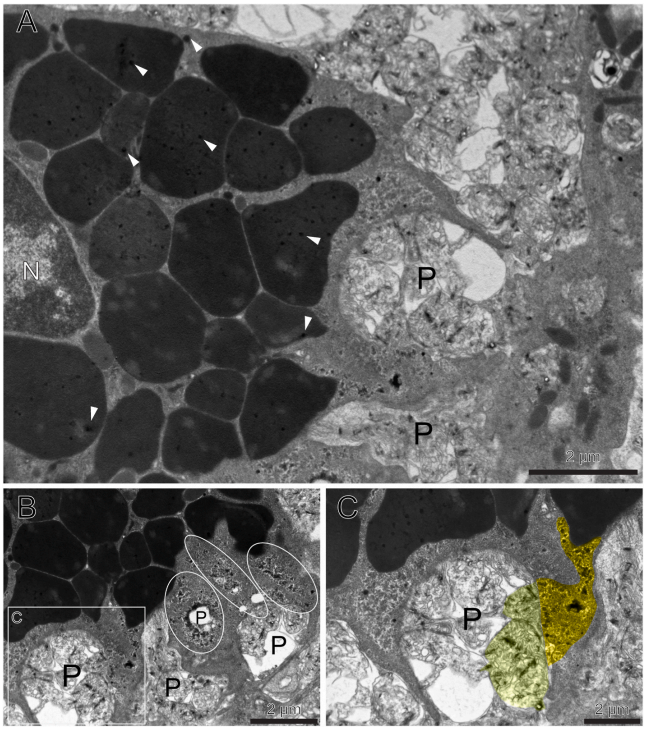

Fig. 11. A–C. Transmission electron micrographs of a phagocytic large granulocyte trapping several mitochondria from degenerated muscle tissue in phagosomes 5–10 min after amputation. Note the numerous newly absorbed electron-dense particles inside the cytoplasmic granules (white arrowheads) in Figure A. The higher magnification micrographs show (Fig. B) the absorption of electron-dense particles (white circles and white square) from phagosomes (P), and their transportation and incorporation into cytoplasmic granules in the phagocytic hemocyte of *P. virginalis* after limb amputation and muscle degeneration. The yellow-highlighted area in Figure C shows one of the paths along which electron-dense particles are transported from the phagosome into the cytoplasmic granules. N, nucleus; P, phagosome.

process for opening space for the arrival, aggregation, and proliferation of the stem cells needed during the process of limb regeneration.

The incorporation of the material from the phagosome into the organelles of the hemocyte can increase the size and number of granules in the cytoplasm and subsequently change the morphology of the whole cell. A study of *P. leptodactylus* demonstrated that the number of cytoplasmic microtubules in the hemocytes transformed after injury were able to engulf material from disintegrating muscle fibers (Branislav Uhrik and Zacharova, 1989). This possibility has also been suggested by previous studies of insects (Brehelin et al., 1978; Ravindranath, 1980). In a similar observation, an *in vitro* culture of crayfish hematopoietic tissue with muscle tissue extract from the same animal can result in differentiation of hemocytes with higher granular contents (Li et al., 2019). This could be the result of the phagocytizing of materials from muscle extract that are incorporated into hemocytes organelles and increased hemocyte granularity. These findings suggest that hemocytes are able to recycle material from degenerating cells and store it in their organelles for future use, possibly for use as part of their other functions such as coagulation, immune reaction, and regeneration.

Therefore, the phagocytosis process can affect cell morphology by incorporating external material into the cytoplasm and nucleus, which may increase the number and size of the granules in the cell.

5. Conclusions

The present *in situ* experiment described in detail all the stages of the morphological changes in hemocytes occurring during coagulation and phagocytosis after injury in a decapod. Our observations showed that cytoplasmic granules expand and release their contents into the extracellular space for the purpose of coagulation. The contents of the nucleus are also discharged and participate in the process of coagulation.

In addition, we observed for the first time that the tiny electron-dense particles resulting from degenerating muscle tissues are ingested and incorporated into the granules and other cellular components of hemocyte via phagocytosis. Therefore, it is possible that after phagocytosis the morphology of hemocytes substantially changes and these cells become more granular.

Therefore, the drastic morphological changes that occur during coagulation and phagocytosis can significantly alter hemocyte morphology such that they can be confused with other types of hemocytes that exist in circulation. For example, degranulated hemocytes in their end stage only have a left-over nuclei, which may have been confused with hyalinocytes in some previous studies.

It is hoped that the results presented here will enhance our understanding of the physiological role of hemocytes in coagulation and phagocytosis during early stages of an injury and help clarify some of the uncertainties that exist regarding the types and development of hemocytes in decapods.

Ethical statement

All experimental procedures involving crayfish were conducted in

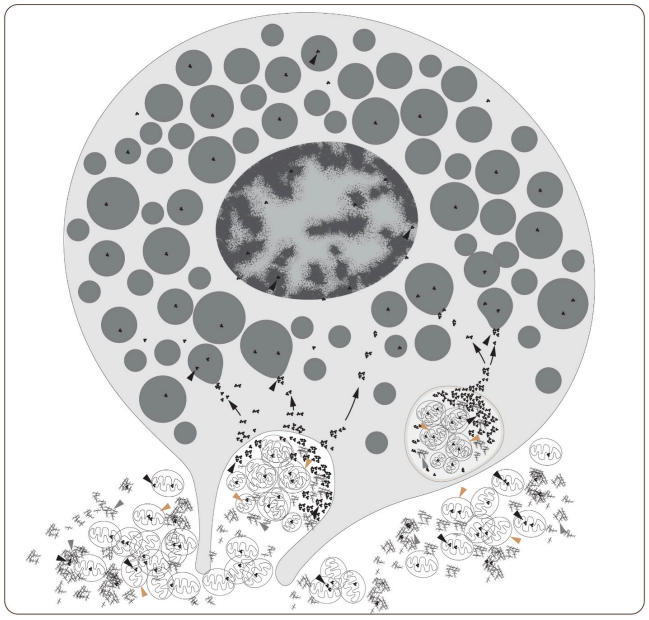

Fig. 12. Schematic illustration depicting a phagocytic granulocyte at the site of degenerating muscle that has trapped several mitochondria from the degenerating muscle in phagosomes. The internalized necrotic bodies in the phagosome, i.e. the mitochondria and muscle tissue fibers, resulted from the degenerated muscle tissue. The internalized bodies are degraded into small electron-dense particles, which are moved (black arrows) and incorporated into other cellular components such as granules and nucleus. brown arrowhead = mitochondria; gray arrowhead = degenerated muscle fibers; black arrowhead = electron-dense particles.

accordance with the principles of laboratory animal care, the national laws and regulations on animal welfare 246/1992, and the institutional animal care guidelines of the Faculty of Fisheries and Protection of Waters of the University of South Bohemia in České Budějovice. All efforts were made to minimize animal suffering.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data availability

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

Acknowledgements

The study was financially supported by the Grant Agency of the University of South Bohemia, (GAJU) projects No. GAJU project (065/2022/Z), 055/2022/Z and 012/2022/Z. We also sincerely thank the assistance of Dr. Michael Lockwood for language correction.

References

Altincicek, B., Stötzel, S., Wygrecka, M., Preissner, K.T., Vilcinskas, A., 2008. Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. J. Immunol. 181, 2705–2712.

Bauchau, A., De Brouwer, M.-B., 1974. Étude ultrastructurale de la coagulation de l'hémolymphe chez les crustacés. Société française de microscopie électronique.

Bodammer, J., 1978. Cytological observations on the blood and hemopoietic tissue in the crab, Callinectes sapidus. Cell Tissue Res. 187.

Branislav Uhrik, K.R., Zacharova, Daria, 1989. The roles of haemocytes during degeneration and regeneration of crayfish muscle fibres. Cell Tissue Res. 255.

Brehelin, M., Zachary, D., Hoffmann, J., 1978. A comparative ultrastructural study of blood cells from nine insect orders. Cell Tissue Res. 195.

Burgos-Aceves, M.A., Abo-Al-Ela, H.G., Faggio, C., 2021a. Impact of phthalates and bisphenols plasticizers on haemocyte immune function of aquatic invertebrates: a review on physiological, biochemical, and genomic aspects. J. Hazard Mater. 419, 126426.

Burgos-Aceves, M.A., Abo-Al-Ela, H.G., Faggio, C., 2021b. Physiological and metabolic approach of plastic additive effects: immune cells responses. J. Hazard Mater. 404, 124114.

Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2018. A potential microRNA regulation of immune-related genes in invertebrate haemocytes. Sci. Total Environ. 621, 302–307.

Cerenius, L., Jiravanichpaisal, P., Liu, H.P., Soderhall, I., 2010. Crustacean immunity. In: Soderhall, K. (Ed.), Invertebrate Immunity, pp. 239–259.

Cerenius, L., Söderhäll, K., 2013. Variable immune molecules in invertebrates. J. Exp. Biol. 216, 4313–4319.

Chaga, O., Lignell, M., Söderhäll, K., 1995. The haemopoietic cells of the freshwater crayfish Pacifastacus leniusculus. Anim. Biol. Leiden 4, 59–70.

Clare, A., Lumb, G., 1994a. Identification of haemocytes and their role in clotting in the blue crab, Callinectes sapidus. Mar. Biol. 118, 601–610.

Clare, A.S., Lumb, G., 1994b. Identification of haemocytes and their role in clotting in the blue crab, Callinectes sapidus. Mar. Biol. 118, 601–610.

Dolar, A., Mayall, C., Drobne, D., Kokalj, A.J., 2020. Modulations of immune parameters caused by bacterial and viral infections in the terrestrial crustacean Porcellio scaber: implications for potential markers in environmental research. Dev. Comp. Immunol. 113, 103789.

Duan, H., Jin, S., Zhang, Y., Li, F., Xiang, J., 2014. Granulocytes of the red claw crayfish Cherax quadricarinatus can endocytose beads, E. coli and WSSV, but in different ways. Dev. Comp. Immunol. 46, 186–193.

Dumont, J.N., Anderson, E., Winner, G., 1966. Some cytologic characteristics of the hemocytes of Limulus during clotting. J. Morphol. 119, 181–207.

Gargioni, R., Barracco, M.A., 1998. Hemocytes of the palaemonids Macrobrachium rosenbergii and M-acanthurus, and of the penaeid Penaeus paulensis. J. Morphol. 236, 209–221.

Giulianini, P.G., Bierti, M., Lorenzon, S., Battistella, S., Ferrero, E.A., 2007. Ultrastructural and functional characterization of circulating hemocytes from the freshwater crayfish Astacus leptodactylus: cell types and their role after in vivo artificial non-self challenge. Micron 38, 49–57.

Grégoire, C., Goffinet, G., 1975. Coagulocyte alterations in clotting hemolymph of Carausius morosus L. Arch. Int. Physiol. Biochim. 83, 707–722.

Hall, M., Wang, R., Van Antwerpen, R., Sottrup-Jensen, L., Söderhäll, K., 1999. The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood. Proc. Natl. Acad. Sci. USA 96, 1965–1970.
Hearing, V., Vernick, S.H., 1967. Fine structure of the blood cells of the lobster, Homarus americanus. Chesap. Sci. 8, 170–186.
Hose, J.E., Martin, G.G., Gerard, A.S., 1990. A decapod hemocyte classification scheme integrating morphology, cytochemistry, and function. Biol. Bull. 178, 33–45.
Jiravanichpaisal, P., Lee, B.L., Söderhäll, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211, 213–236.
Johansson, M.W., Söderhäll, K., 1985. Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. J. Comp. Physiol. B 156, 175–181.
Johansson, M.W., Söderhäll, K., 1988. Isolation and purification of a cell adhesion factor from crayfish blood cells. J. Cell Biol. 106, 1795–1803.
Johansson, M.W., Söderhäll, K., 1989a. A cell adhesion factor from crayfish haemocytes has degranulating activity towards crayfish granular cells. Insect Biochem. Mol. Biol. 19, 183–190.
Johansson, M.W., Söderhäll, K., 1989b. A peptide containing the cell adhesion sequence RGD can mediate degranulation and cell adhesion of crayfish granular haemocytes in vitro. Insect Biochem. Mol. Biol. 19, 573–579.
Johansson, M.W., Söderhäll, K., 1996. The prophenoloxidase activating system and associated proteins in invertebrates. In: Rinkevich, B., Müller, W.E.G. (Eds.), Invertebrate Immunology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 46–66.
Johnston, M.A., Elder, H.Y., Davies, P.S., 1973. Cytology of Carcinus haemocytes and their function in carbohydrate metabolism. Comp. Biochem. Physiol. Physiol. 46, 569–581.
Jones, J.C., 1962. Current Concepts Concerning Insect Hemocytes. American Zoologist, pp. 209–246.
Junkunlo, K., Söderhäll, K., Noonin, C., Söderhäll, I., 2017. PDGF/VEGF-related receptor affects transglutaminase activity to control cell migration during crustacean hematopoiesis. Stem Cell. Dev. 26, 1449–1459.
Junkunlo, K., Söderhäll, K., Söderhäll, I., 2018. Clotting protein – an extracellular matrix (ECM) protein involved in crustacean hematopoiesis. Dev. Comp. Immunol. 78, 132–140.
Junkunlo, K., Söderhäll, K., Söderhäll, I., 2020. Transglutaminase 1 and 2 are localized in different blood cells in the freshwater crayfish Pacifastacus leniusculus. Fish Shellfish Immunol. 104, 83–91.
Junkunlo, K., Söderhäll, K., Söderhäll, I., Noonin, C., 2016. Reactive oxygen species affect transglutaminase activity and regulate hematopoiesis in a Crustacean. J. Biol. Chem. 291, 17593–17601.
Koiwai, K., Koyama, T., Tsuda, S., Toyoda, A., Kikuchi, K., Suzuki, H., Kawano, R., 2021. Single-cell RNA-seq analysis reveals penaeid shrimp hemocyte subpopulations and cell differentiation process. Elife 10, e66954.
Kopacek, P., Hall, M., Soderhall, K., 1993. Characterization of a clotting protein, isolated from plasma of the freshwater crayfish Pacifastacus leniusculus. Eur. J. Biochem. 213, 591–597.
Kumar, B., Deepika, A., Arumugam, M., Mullainadhan, P., Makesh, M., Tripathi, G., C, K., 2013. Microscopic and cytochemical characterisation of haemocytes of the mud crab Scylla serrata (Forskål, 1775) (Decapoda, Portunidae). Crustaceana 86, 1234–1249.
Lanz, H., Tsutsumi, V., Arechiga, H., 1993. Morphological and biochemical characterization of Procambarus clarki blood cells. Dev. Comp. Immunol. 17, 389–397.
Li, F., Chang, X., Xu, L., Yang, F., 2018. Different roles of crayfish hemocytes in the uptake of foreign particles. Fish Shellfish Immunol. 77, 112–119.
Li, F., Xu, L., Hui, X., Huang, W., Yang, F., 2019. Directed differentiation of granular cells from crayfish hematopoietic tissue cells. Fish Shellfish Immunol. 88, 28–35.
Li, F., Zheng, Z., Li, H., Fu, R., Xu, L., Yang, F., 2021. Crayfish hemocytes develop along the granular cell lineage. Sci. Rep. 11.
Lin, X., Söderhäll, K., Söderhäll, I., 2008. Transglutaminase activity in the hematopoietic tissue of a crustacean, Pacifastacus leniusculus, importance in hemocyte homeostasis. BMC Immunol. 9, 58.
Liu, S., Zheng, S.C., Li, Y.L., Li, J., Liu, H.P., 2020. Hemocyte-mediated phagocytosis in Crustaceans. Front. Immunol. 11.
Loof, T.G., Schmidt, O., Herwald, H., Theopold, U., 2011. Coagulation systems of invertebrates and vertebrates and their roles in innate immunity: the same side of two coins? J. Innate Immun. 3, 34–40.
Lv, S.J., Xu, J.H., Zhao, J., Yin, N., Lu, B.J., Li, S., Chen, Y.Y., Xu, H.S., 2014. Classification and phagocytosis of circulating haemocytes in Chinese mitten crab (Eriocheir sinensis) and the effect of extrinsic stimulation on circulating haemocytes in vivo. Fish Shellfish Immunol. 39, 415–422.
Majno, G., Joris, I., 2004. Cells, Tissues, and Disease: Principles of General Pathology. Oxford University Press.
Maningas, M.B.B., Kondo, H., Hirono, I., 2013. Molecular mechanisms of the shrimp clotting system. Fish Shellfish Immunol. 34, 968–972.
Matozzo, V., Marin, M.G., 2010. The role of haemocytes from the crab Carcinus aestuarii (Crustacea, Decapoda) in immune responses: a first survey. Fish Shellfish Immunol. 28, 534–541.
Mengal, K., Kor, G., Kozák, P., Niksirat, H., 2022. Effects of environmental factors on the cellular and molecular parameters of the immune system in decapods. Comp. Biochem. Physiol., A: Mol. Integr. Physiol. 276, 111332.
Niksirat, H., Kouba, A., Kozak, P., 2014. Post-mating morphological changes in the spermatozoon and spermatophore wall of the crayfish Astacus leptodactylus: insight into a non-motile spermatozoon. Anim. Reprod. Sci. 149, 325–334.
Omori, S., Martin, G., Hose, J., 1989a. Morphology of hemocyte lysis and clotting in the ridgeback prawn, Sicyonia ingentis. Cell Tissue Res. 255.
Omori, S.A., Martin, G.G., Hose, J.E., 1989b. Morphology of hemocyte lysis and clotting in the ridgeback prawn, Sicyonia ingentis. Cell Tissue Res. 255, 117–123.
Perdomo-Morales, R., Montero-Alejo, V., Perera, E., 2019. The clotting system in decapod crustaceans: history, current knowledge and what we need to know beyond the models. Fish Shellfish Immunol. 84, 204–212.
Ratcliffe, N., Price, C., 1974. Correlation of light and electron microscopic hemocyte structue in the dictyoptera. J. Morphol. 144, 485–497.
Ravindranath, M., 1974. The hemocytes of an isopod Ligia exotica Roux. J. Morphol. 144, 11–21.
Ravindranath, M., 1980. Haemocytes in haemolymph coagulation of arthropods. Biol. Rev. 55, 139–170.
Rebelo, M.d.F., Figueiredo, E.d.S., Mariante, R.M., Nóbrega, A., de Barros, C.M., Allodi, S., 2013. New insights from the oyster Crassostrea rhizophorae on bivalve circulating hemocytes. PLoS One 8, e57384.
Robb, C.T., Dyrynda, E.A., Gray, R.D., Rossi, A.G., Smith, V.J., 2014. Invertebrate extracellular phagocyte traps show that chromatin is an ancient defence weapon. Nat. Commun. 5, 4627.
Rowley, A.F., 1977. The role of the haemocytes of Clitumnus extradentatus in haemolymph coagulation. Cell Tissue Res. 182, 513–524.
Rowley, A.F., Ratcliffe, N.A., 1976. The granular cells of Galleria mellonella during clotting and phagocytic reactions in vitro. Tissue Cell 8, 437–446.
Scharrer, B., 1972. Cytophysiological features of hemocytes in cockroaches. Z. für Zellforsch. Mikrosk. Anat. 129, 301–319.
Seitz, K., 1972. Zur Histologie und Feinstruktur des Herzens und der Hamocyten von Cupiennius salei Keys.(Aranea, Ctenidae). I. Herzwandung, Bildung und Differenzierung der Hamocyten. Zool Jahrbucher Abt Anat Ontog Tiere.
Sirikharin, R., Junkunlo, K., Söderhäll, K., Söderhäll, I., 2017. Role of astakine1 in regulating transglutaminase activity. Dev. Comp. Immunol. 76, 77–82.
Sirikharin, R., Söderhäll, I., Söderhäll, K., 2018. Characterization of a cold-active transglutaminase from a crayfish, Pacifastacus leniusculus. Fish Shellfish Immunol. 80, 546–549.
Sirikharin, R., Söderhäll, K., Söderhäll, I., 2020. The N-terminal peptide generated after activation of prophenoloxidase affects crayfish hematopoiesis. Dev. Comp. Immunol. 108, 103687.
Smith, V.J., 1991. Invertebrate immunology: phylogenetic, ecotoxicological and biomedical implications. Comp. Haematol. Int. 1, 61–76.
Smith, V.J., Söderhäll, K., 1983. Induction of degranulation and lysis of haemocytes in the freshwater crayfish, Astacus astacus by components of the prophenoloxidase activating system in vitro. Cell Tissue Res. 233, 295–303.
Söderhäll, I., 2013. Recent advances in crayfish hematopoietic stem cell culture: a model for studies of hemocyte differentiation and immunity. Cytotechnology 65, 691–695.
Söderhäll, I., 2016. Crustacean hematopoiesis. Dev. Comp. Immunol. 58, 129–141.
Söderhäll, I., Fasterius, E., Ekblom, C., Söderhäll, K., 2022. Characterization of hemocytes and hematopoietic cells of a freshwater crayfish based on single-cell transcriptome analysis. iScience 25, 104850.
Söderhäll, I., Junkunlo, K., 2019. A comparative global proteomic analysis of the hematopoietic lineages in the crustacean Pacifastacus leniusculus. Dev. Comp. Immunol. 92, 170–178.
Soderhall, I., Soderhall, K., 2022. Blood cell formation in crustaceans. Fish Shellfish Immunol.
Söderhäll, K., Smith, V.J., 1983. Separation of the haemocyte populations of Carcinus Maenas and other marine decapods, and prophenoloxidase distribution. Dev. Comp. Immunol. 7, 229–239.
Söderhäll, K., Smith, V.J., 1986. The prophenoloxidase activating system: the biochemistry of its activation and role in arthropod cellular immunity, with special reference to Crustaceans. In: Brehélin, M. (Ed.), Immunity in Invertebrates. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 208–223.
Söderhäll, K., Smith, V.J., Johansson, M.W., 1986. Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: evidence for cellular co-operation in the defence reactions of arthropods. Cell Tissue Res. 245, 43–49.
Solum, N., 1970. Coagulation in Limulus—Some Properties of the Clottable Protein of Limulus polyphemus Blood Cells. Symposia of the Zoological Society of London, p. 207.
Sricharoen, S., Kim, J.J., Tunkijjanukij, S., Söderhäll, I., 2005. Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish. Dev. Comp. Immunol. 29, 1017–1031.
Sritunyalucksana, K., Söderhäll, K., 2000. The proPO and clotting system in crustaceans. Aquaculture 191, 53–70.
Stang-Voss, C., 1970. Zur ultrastruktur der Blutzellen wirbelloser tiere. Z. für Zellforsch. Mikrosk. Anat. 103, 589–605.
Stang-Voss, C., 1974. On the Ultrastructure of Invertebrate Hemocytes: an Interpretation of Their Role in Comparative Hematology, Contemporary Topics in Immunobiology. Springer, pp. 65–76.
Sternshein, D.J., Burton, P.R., 1980. Light and electron microscopic studies of crayfish hemocytes. J. Morphol. 165, 67–83.
Sung, H.H., Sun, R., 2002. Use of monoclonal antibodies to classify hemocyte subpopulations of tiger shrimp (Penaeus monodon). J. Crustac Biol. 22, 337–344.
Theopold, U., Schmidt, O., Söderhäll, K., Dushay, M.S., 2004. Coagulation in arthropods: defence, wound closure and healing. Trends Immunol. 25, 289–294.
Vafopoulou, X., Laufer, H., Steel, C.G.H., 2007. Spatial and temporal distribution of the ecdysteroid receptor (EcR) in haemocytes and epidermal cells during wound healing in the crayfish, Procambarus clarkii. Gen. Comp. Endocrinol. 152, 359–370.

Vazquez, L., Alpuche, J., Maldonado, G., Agundis, C., Pereyra-Morales, A., Zenteno, E., 2009. Review: immunity mechanisms in crustaceans. Innate Immun. 15, 179–188.

Wang, R., Liang, Z., Hall, M., Söderhäll, K., 2001. A transglutaminase involved in the coagulation system of the freshwater crayfish, Pacifastacus leniusculus. Tissue localisation and cDNA cloning. Fish Shellfish Immunol. 11, 623–637.

Yang, P., Chen, Y., Huang, Z., Xia, H., Cheng, L., Wu, H., Zhang, Y., Wang, F., 2022. Single-cell RNA sequencing analysis of shrimp immune cells identifies macrophage-like phagocytes. Elife 11, e80127.

CHAPTER 4

QUANTIFICATION OF PROTEOMIC PROFILE CHANGES IN THE HEMOLYMPH OF CRAYFISH DURING *IN VITRO* COAGULATION

Mengal, K., Kor, G., Siino, V., Buřič, M., Kozák, P., Levander, F., Niksirat, H., 2023. Quantification of proteomic profile changes in the hemolymph of crayfish during *in vitro* coagulation. Developmental & Comparative Immunology 147, 104760.

My share on this work was 60 %.

Developmental and Comparative Immunology 147 (2023) 104760

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Quantification of proteomic profile changes in the hemolymph of crayfish during *in vitro* coagulation

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ARTICLE INFO

Keywords:
Clot proteomics
Decapods
Innate immunity
Protein

ABSTRACT

Hemolymph is the circulatory fluid that fills the body cavity of crustaceans, analogous to blood in vertebrates. Hemolymph coagulation, similar to blood clotting in vertebrates, plays a crucial role in wound healing and innate immune responses. Despite extensive studies on the clotting process in crustaceans, no comparative quantitative analysis of the protein composition of non-clotted and clotted hemolymph in any decapod has been reported. In this study, we used label-free protein quantification with high-resolution mass spectrometry to identify the proteomic profile of hemolymph in crayfish and quantify significant changes in protein abundances between non-clotted and clotted hemolymph. Our analysis identified a total of two-hundred and nineteen proteins in both hemolymph groups. Furthermore, we discussed the potential functions of the top most high and low-abundant proteins in hemolymph proteomic profile. The quantity of most of the proteins was not significantly changed during coagulation between non-clotted and clotted hemolymph, which may indicate that clotting proteins are likely pre-synthesized, allowing for a swift coagulation response to injury. Four proteins still showed abundance differences ($p < 0.05$, fold change>2), including C-type lectin domain-containing proteins, Laminin A chain, Tropomyosin, and Reverse transcriptase domain-containing proteins. While the first three proteins were down-regulated, the last one was up-regulated. The down-regulation of structural and cytoskeletal proteins may affect the process of hemocyte degranulation needed for coagulation, while the up-regulation of an immune-related protein might be attributed to the phagocytosis ability of viable hemocytes during coagulation.

1. Introduction

Crustaceans possess an open circulatory system in which hemolymph—a circulatory fluid—fills the body cavity and surrounds organs (Grubhoffer et al., 2013). Hemolymph serves as an exchange medium for transporting nutrients, oxygen, and hormones and also for storing amino acids, facilitating development, wound healing, and innate immunity (Vazquez et al., 2009). The hemolymph contains both cellular components—hemocytes—and cell-free plasma, which carry out various defence functions including coagulation, phagocytosis, encapsulation, nodulation, melanization, the activation of antimicrobial peptides (AMPs) and the prophenoloxidase system (proPO) (Jiravanichpaisal et al., 2006; Mengal et al., 2023a, 2023b).

In crustaceans, hemolymph coagulation, analogous to blood clotting in vertebrates, plays an important role in wound healing and immune reactions (Junkunlo et al., 2020; Söderhäll and Smith, 1986; Sricharoen et al., 2005; Theopold et al., 2004). The clot acts as a physical barrier that helps to prevent the spread of pathogens, while the hemocytes release antimicrobial peptides that help to kill or inhibit the growth of invading pathogens and prevent their entry into the hemocoel (Mengal et al., 2023a, 2023b). In crustaceans, hemolymph coagulation is achieved through a combination of cellular (hemocytes) and non-cellular (plasma) components. Clotting factors are stored intracellularly in granules, while clotting protein is present in the extracellular milieu, i.e., plasma (Hall et al., 1999; Junkunlo et al., 2020). Unlike in other non-crustacean invertebrates, the coagulation reaction in crustaceans

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https://doi.org/10.1016/j.dci.2023.104760
Received 5 May 2023; Received in revised form 13 June 2023; Accepted 15 June 2023
Available online 16 June 2023

does not involve a proteolytic cascade. The release of transglutaminase from hemocytes into the hemolymph is sufficient to initiate clotting protein polymerization in the presence of calcium ions, resulting in the formation of the clot (Theopold et al., 2004; Yeh et al., 1999; Hall et al., 1999; KOPÁČEK et al., 1993).

The granules of hemocytes are densely packed with clotting factors, as well as several defense molecules, including transglutaminase (TGase), various antimicrobial peptides, and components of the proPO system (Junkunlo et al., 2020; Söderhäll and Smith, 1986; Sricharoen et al., 2005). The proPO system is an essential component of the immediate immune response in crustaceans, and it is also stored in the hemocyte granules in an inactive state. The proPO system causes the degranulation and lysis of hemocytes, leading to the release of more proPO components and TGase (Smith and Söderhäll, 1983). However, it is worth noting that the proPO system and the clotting reaction do not share a common activation pathway (Hall et al., 1999). The proPO system is activated by a proteolytic cascade triggered by microbial polysaccharides (Söderhäll and Cerenius, 1998; Söderhäll et al., 1996).

Proteomic approaches are powerful tools for identifying and quantifying proteins involved in complex biological processes. Several proteomics studies have been reported to identify differentially abundant proteins in the profiles of the hemocytes of crustaceans against infections (Havanapan et al., 2019; Hou et al., 2016, 2020; Li et al., 2014; Sun et al., 2017). Additionally, proteomics research has been carried out on crayfish hemocytes to detect marker proteins specific to certain hemocyte types. For example, a study employing 2-D electrophoresis on semi-granular and granular hemocytes of the signal crayfish, *Pacifastacus leniusculus*, identified a Kazal-type protease inhibitor (KPI) specific to semi-granular cells and a superoxide dismutase exclusive to granular hemocytes (Wu et al., 2008). Moreover, a comparative proteome analysis was performed between stem cells and mature hemocytes in *P. leniusculus*, revealing several novel putative biomarkers for cell differentiation and cell-specific proteins (Söderhäll and Junkunlo, 2019).

Previous study has also investigated purified components involved in coagulation, such as the plasm clotting protein in *P. leniusculus* (Hall et al., 1999). However, proteomic investigations in decapod crustaceans have been hampered due to insufficient genomic data availability (Gianazza et al., 2021). While proteomic studies on hemocytes and hemolymph under various treatment conditions have enhanced our understanding of the hemocyte and hemolymph proteome, a comprehensive comparative proteomic analysis of non-clotted and clotted hemolymph is required to fully elucidate the proteomic changes that occur during coagulation.

The parthenogenic marbled crayfish *Procambarus virginalis* is considered a model species for studying various biological aspects in decapod crustaceans, such as microscopic anatomy, toxicology, epigenetics, physiology, and development, as well as for human cancer research (Greaves and Maley, 2012; Kor et al., 2023a, 2023b; Scholtz et al., 2003).

In this study, we used a label-free proteomics approach with high-resolution mass spectrometry to identify proteins in the proteomic profile of *P. virginalis*. Our goal was to identify proteins with potential roles in coagulation and immunity in decapod crustacaens. We also quantified the proteomic changes between non-clotted and clotted hemolymph to better understand the molecular functions of crucial proteins contributing to coagulation and immune responses.

2. Materials and methods

2.1. Experimental animals

Intermolt marbled crayfish *P. virginalis* with a carapace length ranging from 3 to 5 cm were selected from the laboratory culture at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice. Six healthy and intact individuals were chosen for the experiment. The animals were kept in aquaria within a recirculation system at a constant temperature of 21–22 °C and were fed *ad libitum* with frozen chironomid larvae and sliced carrots.

2.2. Collection of hemolymph

A sterile 1 mL syringe fitted with a 25G needle was used to collect at least 100 μL of hemolymph from the pericardial sinus of the crayfish. Half of the obtained hemolymph from each animal was immediately inserted into liquid nitrogen as control. The rest of the hemolymph was allowed to coagulate at room temperature for 1 h, and then clot samples were fixed in liquid nitrogen. All samples were then stored at −80 °C until subsequent analysis.

2.3. Protein and peptide extraction for mass spectrometry

The hemolymph samples (n = 12, six non-clotted and six clotted) were dissolved in 90 μl lysis buffer (1% sodium dodecyl sulfate (SDS) and 20 mM tris[hydroxymethyl] aminoethane (Tris), (pH = 7.55) and homogenized on ice by probe sonication with Branson Digital Sonifier® 250-D (Branson Ultrasonics Corporation, Danbury, USA) using 60% amplitude, 5s pulse on x 5 cycles and 5s pulse off x 5 cycles. The samples were then centrifuged (Eppendorf bench top centrifuge) to remove debris and the supernatant, containing proteins, was recovered. The proteins were quantified using Pierce BCA protein assay kit (Thermo Fisher Scientific, Germany).

2.4. HILIC clean-up and on-bead protein digestion

The proteins were first reduced with 10 mM dithiothreitol (DTT) for 45 min at room temperature. This was followed by alkylation with 40 mM iodoacetamide (IAA) for 45 min in the dark. Finally, IAA was quenched with DTT to achieve a final concentration of 20 mM. Fifty μg of proteins were used for hydrophilic interaction liquid chromatography (HILIC) (ReSyn Biosciences, South Africa) clean-up and automated protein on-bead digestion using KingFisher Flex (Thermo Fisher Scientific, Germany) in a 96-well format, as described previously (Siino et al., 2022).

Briefly, the automated procedure involved the following steps: magnetic microspheres (1:10 protein:beads ratio) were incubated and equilibrated in equilibration buffer (15% acetonitrile (ACN), 100 mM ammonium acetate (NH4Ac), pH = 4.5); protein samples were incubated in binding buffer (30% ACN, 200 mM NH4Ac, pH = 4.5) where proteins bind to HILIC beads. To remove unspecific proteins, the beads were then washed twice in 95% ACN. Beads-binding proteins were then incubated with trypsin (Seq grade, Promega AB) at a ratio of 20:1 protein:trypsin dissolved in 50 mM ammonium bicarbonate for 1h at 47 °C. Afterwards peptides were recovered from the plate and dried in a Speedvac (Thermo Fisher Scientific, Germany) prior to C18 desalting procedure.

Peptide desalting procedure was carried out using BioPureSPN™ Mini, PROTO 300 C18 (The Nest Group, Inc., MA, USA). Columns were equilibrated with 100 μl 70% ACN, 5% formic acid (FA) and then conditioning was performed using 100 μl 5% FA. Next, samples were resuspended in 100 μl 5% FA and loaded into the column. Column was washed with 100 μl 5% FA and cleaned peptides were eluted using 100 μl 50% ACN, 5% FA. All centrifugation steps were carried out using an Eppendorf bench-top centrifuge at 50×*g* for 2 min. Cleaned peptides were dried and stored at −20 °C prior to quantification and injection into the mass spectrometer.

2.5. NanoLC mass spectrometry

Cleaned peptide digests were resuspended in 0.1% FA and quantified using the NanoDrop 1000 (Thermo Fisher Scientific, Germany). Four hundred ng peptides were injected through an EvoSep (One LC system EvoSep, Denmark) coupled with a QExactive HF-X mass spectrometer

(Thermo Fisher Scientific, Germany) operating in positive ion mode for data-dependent acquisition (DDA). The analytical column was 15 cm long fused silica capillary (75 μm* 16 cm Pico Tip Emitter, New Objective), packed in-house with C18 material ReproSil-Pur 1.9 μm (Dr. Maisch GmbH, Germany). Peptides were separated using the 58-min whisper method. A top 20 method was used for the MS data acquisition, with an automatic gain control target value of 3×10^6 ions with a maximum fill time of 50 ms and a target resolution of 120000 and a scan range from 375 to 1500 m/z for the MS1 scans. Charge 2–6 ions were selected for MS/MS using higher energy collision-induced dissociation fragmentation (NCE 27), with 15,000 FWHM resolution and a target of 1×10^5 ions with a maximum injection time of 20 ms using an isolation window of 1.2 m/z.

2.6. Mass spectrometry data processing

MaxQuant (www.maxquant.org, version 1.6.17.0) was used to process all MS raw files. Files were searched against the *Procambarus* entries in UniProt as of 20211122 and the *P. pvirginalis*_04 database (Gutekunst et al., 2018) using the following parameters: carbamidomethylation of cysteines was set as fixed modification and oxidation of methionine and protein N-terminal acetylation as variable modifications. Default parameters were used, including precursor mass error tolerance 4.5 ppm and monoisotopic fragments mass error tolerance 0.02 Da and protein filtering at FDR 0.01.

The protein intensity values from the resulting protein groups file were log2-transformed and normalized using NormalyzerDE (Willforss et al., 2019). Cyclic Loess normalization was deemed the most suitable normalization method based on the metrics in the report and because it adjusts for systematic differences in abundance between samples at different abundance levels (Ballman et al., 2004; Smyth, 2005). The transformed data were used for protein differential abundance analysis, and differential abundance was calculated using the empirical Bayes moderated *t*-test (Limma) between the control and clot hemolymph samples in the NormalyzerDE web interface (Willforss et al., 2019). A p-value of less than 0.05 and fold change of more than two were considered significant. Protein sequences with unknown functions were annotated with a BLAST (Basic Local Alignment Search Tool) search in the Universal Protein Resource (UniProt, https://www.uniprot.org/) database to obtain homologous sequences for characterized proteins.

2.7. Gene Ontology (GO) and Protein interaction analysis

The list of identified proteins in the hemolymph samples was subjected to GO functional enrichment analysis using the ShinyGO (v0.77) application tool (bioinformatics.sdstate.edu/go) (Ge et al., 2019). The analysis was performed with the default 'Best matching species' option, and the enriched GO dot plot charts for biological processes and cellular components were selected for the submitted list of genes. The false discovery rate (FDR) cutoff for predictions was set at 0.05. ShinyGO calculates FDR based on the nominal p-value from the hypergeometric test.

The protein-protein interaction (PPI) analysis was performed using the STRING (v11.5) (https://string-db.org/) web server. Nodes in the network are connected with color lines that represent evidence-based interactions for the network edges, including "known interactions" based on experimentally determined curated databases and "predicted interactions" based on gene neighborhood, gene co-occurrence, gene fusion, text mining, protein homology, or co-expression.

The proteome profiling of hemolymph samples in *P. virginalis* was carried out using a mass spectrometry-based workflow, which is depicted in Fig. 1.

3. Results and discussion

Two-hundred and nineteen proteins were identified by MaxQuant search in the *P. virginalis* non-clotted and clotted hemolymph (Supplementary Table 1). Among them, four proteins exhibited significant changes ($p < 0.05$, fold change ≥ 2) during clot formation, including three down-regulated proteins (C-type lectin domain-containing proteins, laminin A chain, and tropomyosin) and one up-regulated protein (reverse transcriptase domain-containing protein) (Fig. 2). To obtain an adequate estimation of protein abundances, the high and low-abundant proteins were sorted based on their protein signal (intensity) values. Furthermore, proteins that play a vital role in coagulation and innate immunity were screened and discussed. The top thirty most high and low-abundant proteins identified in hemolymph are listed in Table 1. The results of the GO biological process and cellular components are shown in Fig. 3, and the results of protein network interactions are shown in Fig. 4.

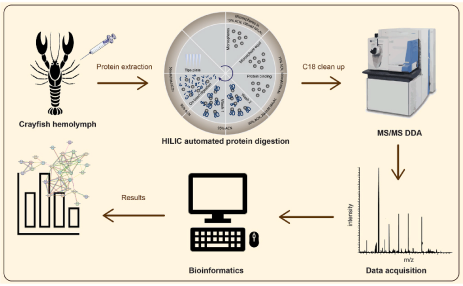

Fig. 1. A schematic overview of the mass spectrometry (MS)-based workflow used for proteome profiling of the non-clotted and clotted hemolymph samples. In this workflow, an automated sample preparation protocol was employed using MagReSyn® HILIC magnetic microspheres for protein clean-up and on-bead digestion.

The proteomic analysis of non-clotted and clotted samples revealed that the abundances of most proteins remained unchanged during the coagulation process. This suggests that most of the proteins required for the coagulation process are pre-synthesized and stored before clot formation. Different mechanisms of coagulation have been proposed for crustaceans, including the Type C mechanism, which involves rapid hemocyte degranulation or lysis and immediate plasma clotting due to the release of clotting factors (Perdomo-Morales et al., 2019), as we observed in our earlier ultrastructural study of coagulation in crayfish (Mengal et al., 2023a). Crustaceans possess robust clotting mechanisms due to their open circulatory system. Our proteomics results suggest that clotting factors are pre-synthesized and stored in hemocyte granules and plasma, facilitating a rapid response to wounds. Upon injury, these clotting factors are immediately released and act on clotting proteins in plasma, resulting in clot formation (Junkunlo et al., 2018). Synthesizing new proteins needed for coagulation after an injury can be time-consuming, particularly in decapods with an open circulatory system where bleeding can be life-threatening. This may explain why only a few proteins significantly changed during clotting, mostly being down-regulated.

Three proteins, namely, C-type lectin domain-containing proteins, Laminin A chain, and Tropomyosin, were down-regulated, while one protein, called Reverse transcriptase domain-containing protein was significantly up-regulated during clot formation. Additionally, we have identified and discussed the key proteins involved in hemolymph clotting and immunity from the top thirty most high and low-abundant proteins in the proteome profile of hemolymph.

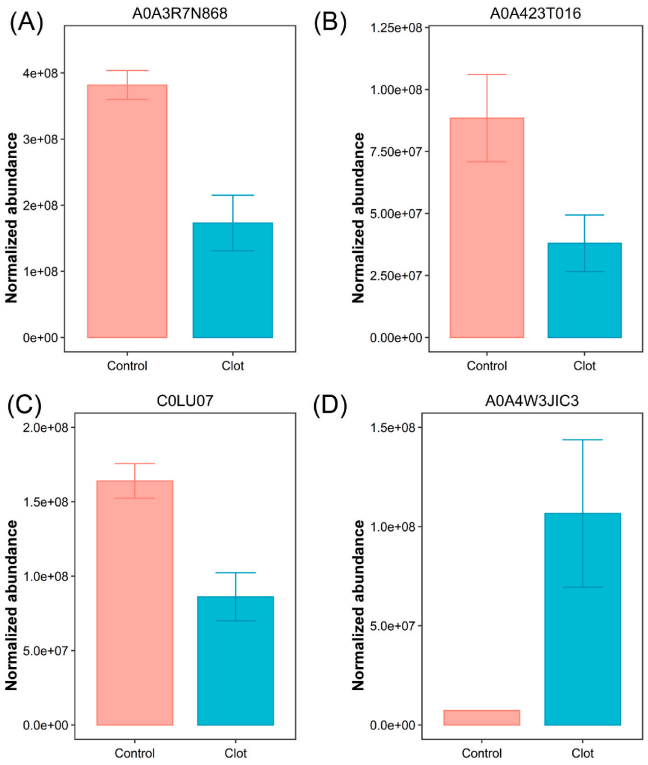

Fig. 2. The bar graphs of the normalized abundances of proteins that were significantly down-regulated (A–C) or up-regulated (D) during clotting. The proteins in each panel are as follows: (A) C-type lectin domain-containing proteins, (B) Laminin A chain, and (C) Tropomyosin. Panel (D) shows Reverse transcriptase domain-containing protein. The normalized abundances are presented as mean ± s. e.m.

3.1. Differentially abundant proteins identified in hemolymph of marbled crayfish

3.1.1. C-type lectin domain-containing proteins

C-type lectin domain-containing proteins (CTLs) are a diverse family of proteins that play important roles in the immune system and coagulation process of crustaceans (Luo et al., 2019; Viana et al., 2022). These proteins are known to have a C-type lectin domain, which is a carbohydrate-binding domain that recognizes specific sugar molecules on the surface of pathogens known as pathogen-associated molecular patterns (PAMPs) (Luo et al., 2019). The existing shrimp transcriptome data have revealed at least seven different types of lectin, comprising C-type, M-type, L-type, P-type, lectins with fibrinogen-like domains, calnexin/calreticulin, and galectins (Wang and Wang, 2013).

In the immune system of crustaceans, CTLs have been shown to play a role in the recognition and clearance of pathogens and hemagglutination. For example, a recombinant lectin LvLec, when used in whiteleg shrimp *Litopenaeus vannamei,* significantly improved the phagocytic abilities of hemocytes, enhanced phenoloxidase, bacteriolytic and hemagglutinating activities (Li et al., 2022). Another study of the mud crab *Scylla paramamosain* showed increased antibacterial and calcium-dependent agglutination activity against both Gram-positive and Gram-negative bacteria in the C-type lectin (designated as Fc-hsL) treated group after a bacterial challenge (Sun et al., 2008). Furthermore, the C-type-lectin (designated as HjCL) in the Japanese bullhead shark *Heterodontus japonicus* caused agglutination of the bacterial pathogen *Edwardsiella tarda* and promoted immediate blood clotting (Tsutsui et al., 2014). This implies that CTLs may serve a role in coagulation and as pattern recognition receptors in antimicrobial defense in crustaceans. Furthermore, as observed in our earlier study of coagulation, hemocytes undergo excessive degranulation, which results in cell lysis similar to apoptosis (Mengal et al., 2023a). It is possible that the activation of apoptotic pathways in hemocytes may lead to the cleavage and degradation of C-type lectins or their associated signaling molecules; this could be one of the reasons that contributed to the down-regulation of C-type lectins during coagulation; further research is needed to confirm this hypothesis.

3.1.2. Laminin a chain

The Laminin A chain is a critical component of laminin, a structural protein abundant in the extracellular matrix of various tissues. Laminin is composed of α, β, and γ chains, and binding of functional sequences like the laminin alpha chain to cellular receptors such as the laminin receptor initiates intracellular signaling that drives cell activities, such

Table 1
Uniprot accession numbers and names of thirty most high and low abundant proteins in the hemolymph of marbled crayfish *P. virginalis*.

High Abundant Proteins		Low Abundant Proteins	
Accession Number	Proteins	Accession Number	Proteins
A0A142BZ28	*Hemocyanin subunit 2*	A0A3R7MK72	*Putative RNA-binding protein 4 isoform X2*
A0A3R7NPL9	*Hemocyanin subunit L2*	A0A5B9GCL9	*Small nuclear ribonucleoprotein E (snRNP-E) (Sm protein E)*
A0A142BZ27	*Hemocyanin subunit 1*	A0A423U6P8	*Alpha2 macroglobulin isoform 3*
A0A5N5TD50	*Hemocyanin A chain*	A0A423TC99	*Vitellogenin domain-containing protein*
A0A5N5TE88	*Hemocyanin A chain*	A0A5B9GAH5	*Ferritin (EC 1.16.3.1)*
Q6RG02	*Vitellogenin [Cleaved into: Vitellin]*	A0A2J7Q0E5	*Beta-glucuronidase (EC 3.2.1.31)*
A0A5B7CMF4	*Beta-1,3-glucan-binding protein*	A0A3R7MWT2	*Putative teneurin-3-like*
A0A386H740	*Clotting protein*	A0A3R7SIW3	*Putative serine proteinase inhibitor*
A0A5B7DUJ6	*Hemocyanin A chain*	A0A423TBN8	*RNA helicase (EC 3.6.4.13)*
P00761	*Trypsin (EC 3.4.21.4)*	A0A5N5TJF1	*Alpha-actinin, sarcomeric*
A0A3R7Q123	*Hemocyanin*	K7QPA1	*Small ubiquitin-related modifier (SUMO)*
A0A3R7MB02	*Hemocyanin*	A0A3R7QZR3	*Alpha-mannosidase (EC 3.2.1.-)*
A0A075BUH1	*Hemocyanin 2*	A0A5N5T2M7	*Cartilage oligomeric matrix protein*
A0A423SGT1	*Hemocyanin subunit L3*	A0A3R7NUJ0	*Putative rho GDP-dissociation inhibitor 2*
A0A386H7H6	*Pacifastin heavy chain*	A0A423TE44	*Fibril-forming collagen alpha chain-like*
A0A1C6ZZL7	*Vitellogenin*	A0A3R7QHC4	*Catalase (EC 1.11.1.6)*
A0A386H7I6	*Alpha-2-macroglobulin-like protein isoform 3*	A0A5B7E1N1	*Eukaryotic translation initiation factor 5A (eIF-5A)*
A0A423TS71	*WD_REPEATS_REGION domain-containing protein*	A0A3R7SZS6	*Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)*
A0A0M4J5L0	*Beta-actin*	A0A5B7FG04	*Secreted protein*
A0A423SB75	*Vitellogenin*	A0A3R7NDA8	*Tail muscle elongation factor 1 gamma*
A0A7T5Y1V8	*Prophenoloxidase*	A0A5B7CPW0	*Laminin subunit beta-1*
A0A318TCI9	*Apolipoprotein A1/A4/E domain-containing protein*	A0A5B7DQN7	*Small nuclear ribonucleoprotein Sm D1 (snRNP core protein D1)*
A0A3R7QXB9	*Laccase 1*	A0A5B7D8D5	*Annexin*
A0A2P1JJ65	*Protein-glutamine gamma-glutamyltransferase (EC 2.3.2.13)*	A0A5N5TMZ3	*60S ribosomal protein L30*
A0A346QR93	*Prophenoloxidase (EC 1.14.18.-)*	A0A1W5LJR1	*Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)*
A0A3R7N8L8	*Pl-crustin 2 (Type Ia crustin crula-6)*	A0A4Y6A8P8	*Heterogeneous nuclear ribonucleoprotein K*
A0A5B7HTA5	*Peroxisomal assembly protein PEX3*	A0A3R7PGP1	*glutamate dehydrogenase*

Table 1 (*continued*)

High Abundant Proteins		Low Abundant Proteins	
Accession Number	Proteins	Accession Number	Proteins
			[NAD(P)(+)] (EC 1.4.1.3)
A0A4S2KIX4	*Histone H2A*	A0A423T909	*Anti-lipopolysaccharide factor isoform 6*
A0A3R7PKU8	*Histone H2B*	F5A6E1	*40S ribosomal protein S18*
A0A3R7MUX2	*Melanization interactin protein*	A0A1D2MJK0	*Putative peptidase C1-like protein F26E4.3*

as adhesion, migration, growth, and differentiation (Castronovo, 1993; Suzuki et al., 2005). Studies of laminin receptor proteins (Lamr) have been conducted in crustaceans. The RNAi-mediated knockdown of PvLamr in *P. vannamei* resulted in 100% mortality within 9 days and a substantial decrease in peripheral hemocyte numbers, though the mechanism behind the hemocyte reduction was unclear (Senapin et al., 2010). Our previous ultrastructural study of coagulation also showed that hemocytes undergo excessive degranulation, leading to high levels of hemocyte lysis and cell death (Mengal et al., 2023a). These observations suggest that the downregulation of the Laminin A chain during coagulation may be linked to the degranulation and cell lysis of hemocytes. Another study of *L. vannamei* revealed that knockdown of Lamr significantly reduced expression of crustacean hematopoietic factor (CHF)-like protein and hemocyte homeostasis-associated protein (HHAP), suggesting Lamr's role in shrimp hemocyte homeostasis through interaction with CHF-like and HHAP proteins (Charoensapsri et al., 2015). Furthermore, the data from human skin cells suggest that laminin LG4–5-derived peptides can improve wound healing and exhibit broad antimicrobial activity (Senyürek et al., 2014). Laminin has also been implicated in regulating core cell behaviors required for wound repair and angiogenesis (Iorio et al., 2015). Although the antimicrobial properties of laminin in crustaceans have been well documented (Busayarat et al., 2011; Liu et al., 2016, 2018), its role in coagulation and wound healing is less explored. It is possible that the structural protein (Laminin) may be responsible for maintaining cell integrity and retaining granules within hemocytes. When it is downregulated during an injury, degranulation is facilitated, resulting in the release of granule content and the formation of clots. Presumably, the non-specific proteases/peptidases may facilitate the selective degradation of laminin proteins, which could result in its observed down-regulation. These proteolytic enzymes may selectively cleave and degrade extracellular matrix proteins like laminin, potentially through an as-yet-unknown mechanism. Further research is needed to understand this mechanism during coagulation.

3.1.3. Tropomyosin

Tropomyosin (Tpm) is a protein that primarily regulates muscle contraction in animals. It acts as a master regulator of cytoskeletal proteins (Gunning et al., 2015), which play a crucial role in cell adhesion, migration, phagocytosis, and wound repair (Cowin, 2006).

In crustaceans, Tpm plays a key role in innate immunity by contributing to the process of phagocytosis. It serves as an important component of the Rab-Complex that regulates phagocytosis in crustaceans (Wu et al., 2008). In kuruma prawn *Penaeus japonicus,* the RNAi and mRNA assays revealed that the Rab-Complex, a protein complex consisting of the PjRab, tropomyosin, and β-actin and a white spot syndrome virus (WSSV) envelop protein VP466, regulates anti-viral phagocytosis (Wu et al., 2008). In this complex, the Rab6 protein can regulate the hemocytic phagocytosis, with tropomyosin serving as a crucial component (Ye et al., 2012). The interaction of Tpm with LcRac1 was also found to be vital to phagocytosis in large yellow croaker

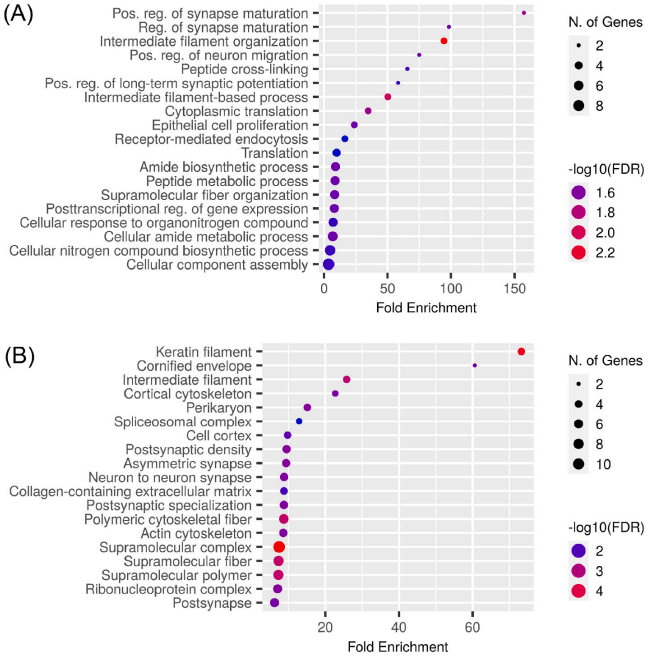

Fig. 3. The dot-plot chart represents the GO enrichment analysis for biological processes (A) and cellular components (B) of the commonly identified proteins in the non-clotted and clotted hemolymph of *P. virginalis*. The chart was created using ShinyGO (v0.77). The biological processes and cellular components in the x-axis are ranked by fold enrichment values. The most significant processes are highlighted with red dots and less significant ones with blue dots based on their log10(FDR) values. Larger dots in the graph correspond to a greater number of proteins involved in the biological process or cellular component. (Detailed information deposited in Supplementary Table 2). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Larimichthys crocea (Han et al., 2019).

Additionally, the role of Tmp in wound healing has been reported in a mouse study. The study found that deletion of the 9D exon from the *TPM3/γTm* tropomyosin gene resulted in improved wound healing and increased cell migration, potentially due to the activation of paxillin/Rac signaling, suggesting Tmp plays an important role in regulating the timing of cell migration during wound healing (Lees et al., 2013). An increase in Tmp levels was also observed in the hemolymph of the Mediterranean mussel *Mytilus galloprovincialis* following an induced injury to the adductor muscle. (Franco-Martínez et al., 2018). Therefore, Tpm can regulate actin filament dynamics and change cell migration rates, thereby affecting wound healing. However, in our current study, Tpm was significantly down-regulated. This may be because the hemocytes involved in coagulation are consumed in the process; as mentioned before, during coagulation, hemocytes undergo rapid degranulation and eventually apoptosis. Thus, the down-regulation of Tpm, being a cytoskeletal protein, could result in the destabilization of hemocyte structure, potentially leading to its lysis during degranulation and coagulation. Further research is needed to determine the specific cause of tropomyosin downregulation during this process.

3.1.4. Reverse transcriptase domain-containing protein

The role of reverse transcriptase domain-containing protein (RTDP) in invertebrates is not well understood and may vary depending on the organism. However, some studies have suggested that cellular reverse transcriptases (RTs) may play a role in the immune response of invertebrates. For example, in insects, cellular RT activity has been linked to the activation of the RNA-mediated interference (RNAi) pathway, which in turn inhibits viral replication, suggesting a role for RT in the defense against viral infections (Goic et al., 2013). It has been proposed that shrimp may also use cellular RT to recognize foreign mRNA of viruses and integrate short cDNA sequences into their genomes through integrases, which could result in the production of immunospecific RNA (imRNA) capable of suppressing viral propagation via RNA interference (RNAi) (Flegel, 2009).

Our study found that RTDP was up-regulated during clot formation in *P. virginalis* hemolymph. During coagulation, most hemocytes undergo rapid degranulation, releasing granular contents for clot formation, and subsequently die through apoptosis, while a distinct group of hemocytes maintain their integrity and remain phagocytically active for an extended period, as noted in our earlier ultrastructural study of coagulation and phagocytosis (Mengal et al., 2023a). Considering the immune-related roles of the reverse transcriptase domain-containing protein, it is likely that the up-regulation of this protein during clotting is from the phagocytic hemocytes. The down-regulated structural proteins may be attributed to the hemocytes that degranulate and eventually undergo apoptosis. These proteins mainly have structural functions related to hemocyte integrity, which may hinder the process of degranulation. Therefore, their down-regulation may be necessary for the release of granular contents to form the clot. Since protein synthesis is not possible inside the cells that have already lysed as a result of degranulation, thus, the up-regulated protein RTDP during clotting may be attributed to the surviving phagocytic hemocytes that synthesize this protein and play immunological roles in phagocytosis.

3.2. High-abundant proteins in the hemolymph proteomic profile

Several high-abundant proteins with critical roles in clotting and the innate immune system of crustaceans were found in both non-clotted and clotted hemolymph samples. In the top 30 most highly abundant

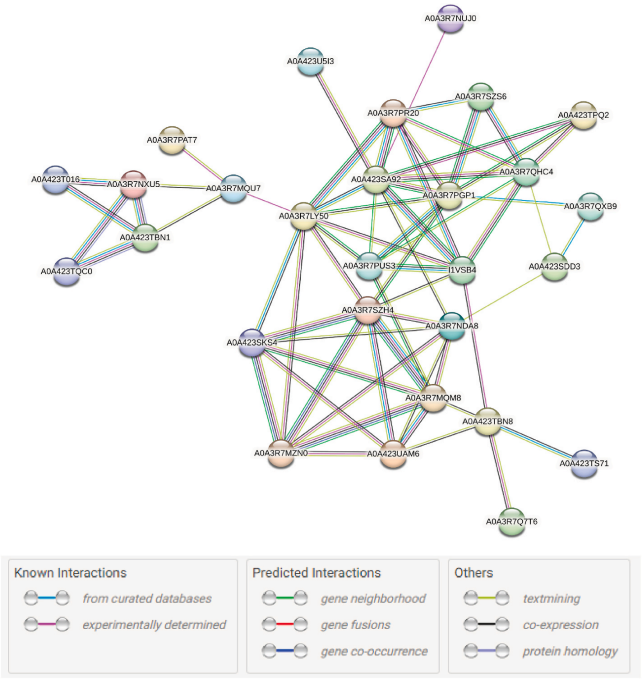

Fig. 4. The protein-protein interaction network displaying the list of identified proteins that were subjected to String (v11.5) analysis to reveal functional interactions for the proteins obtained from hemolymph samples. Each node in the network represents a protein, and each edge represents an interaction that was predicted based on the available evidence. (Detailed information deposited in Supplementary Table 3).

proteins, we discussed the functions of some of the important ones below. Table 1 provides a complete list of the top thirty high-abundance proteins found in the hemolymph.

3.2.1. Clotting factors in the clot and hemolymph

Hemolymph clotting is a crucial immune response involving soluble and cell-derived clotting factors. In our comparative study of hemolymph clotting, we observed that hemocyanin was consistently the most abundant protein in both groups without any significant change in abundance. Additionally, we identified other important clotting factors such as Clotting protein, Vitellogenin [Cleaved into: Vitellin], and Beta-1,3-glucan-binding protein.

In crustaceans, the clotting protein was first cloned and characterized in crayfish (Hall et al., 1999) and later in tiger shrimp *Penaeus monodon* (Yeh et al., 1999). The clotting protein is a homodimeric glycolipoprotein, structurally similar but distinct from vitellogenin and contains a sequence similar to the D-domain of mammalian von Willebrand's factor (Cerenius and Söderhäll, 2004). Interestingly, both vitellogenin and von Willebrand's factor were also found in our results. Additionally, we identified astakine 2, a hematopoietic cytokine that has been shown to inhibit TGase activity and reduce clotting protein crosslinking in crayfish (Sirikharin et al., 2017) that probably regulates hematopoiesis and coagulation in crustaceans. The clotting system in decapods is efficient and rapid due to the pre-existing storage of clotting factors in granulocytes and clotting proteins in the plasma. Our LC/MS results did not reveal a significant difference in clotting factor levels between the non-clotted and clotted hemolymph samples. This suggests that the major clotting factor proteins are not synthesized during the clotting process but are pre-synthesized and stored in the granules of hemocytes and plasma. These clotting factors can be rapidly released from granules when needed to form a clot barrier as quickly as possible. Hemocytes can store different components necessary for coagulation separately from plasma to avoid unnecessary coagulation.

3.2.2. Hemocyanin and prophenoloxidase

Hemocyanin (HC) is a large, copper-containing protein whose primary function is to bind, transport, and store dioxygen molecules in the hemolymph (Coates and Nairn, 2014; Coates and Costa-Paiva, 2020). Apart from its traditional role as a respiratory protein, hemocyanin has been shown to exhibit multiple immunological properties. For example, HC upon activation, displays phenoloxidase activity in chelicerates, including Brazilian whiteknee tarantul *Acanthoscurria geniculata* (Sanggaard et al., 2016), horseshoe crabs *Tachypleus tridentatus* (Nagai and Kawabata, 2000), and in crustaceans, like *P. japonicus* (Adachi et al., 2001), and *P. leniusculus* (Lee et al., 2004). Moreover, it has been reported that HC plays a role in immune modulation by interacting with other signaling pathways or generating antimicrobial immune molecules (Coates and Talbot, 2018). For instance, Havanapan et al., demonstrated that in *Penaeus vannamei* hemocyanin's C-terminal undergoes serine phosphorylation and binds to ERK1/2 during Taura syndrome virus (TSV) infection (Havanapan et al., 2009). In *P. leniusculus*, astacidin 1 - a proteolytic cleavage product of

hemocyanin—has been shown to have antibacterial activity against both Gram-positive and Gram-negative bacteria (Lee et al., 2003). More recently, HC has been reported to act as a pattern recognition receptor (PRR), activating innate immunity in red swamp crayfish *Procambarus clarkii* (Qin et al., 2018). It has been shown that hemocyanin is a component of gametes in crayfish that might be essential for their respiration and immunity (Niksirat et al., 2014, 2015). Additionally, the analysis of the clot proteome revealed that hemocyanin constitutes a significant portion of the hemolymph clot in *A. geniculata*, suggesting its involvement in the coagulation process. However, their study did not compare the clot proteome with fresh hemolymph to detect any differences (Sanggaard et al., 2016).

3.2.3. Histone proteins

In this study, mass spectrometry analysis identified high levels of core histone proteins H2A, H2B, H1, and H3 in the clot and hemolymph. Histones play an essential role as protein components in the architecture of chromatin. The role of histones in the decapod's defense was first identified in *L. vannamei* (Patat et al., 2004). Histones are extracellularly released as part of a defense mechanism known as ETosis, which involves the formation of extracellular traps (ETs) of DNA that entrap and kill microorganisms (Robb et al., 2014). This process has been shown to be triggered by both infection and/or tissue damage (Destoumieux-Garzón et al., 2016). The antimicrobial role of histones and histone-derived proteins has been determined in a number of invertebrates, including *L. vannamei* (Ng et al., 2013, 2015; Patat et al., 2004), shore crab, *Carcinus maenas*, and blue mussel, *Mytilus edulis* (Robb et al., 2014). In addition to activating the innate immune response, extracellular nucleic acids have been found to induce coagulation in the hemolymph of other arthropods, such as insects (Altincicek et al., 2008). Likewise, our earlier ultrastructure study of coagulation showed that granular hemocytes externalized chromatin into the surrounding clot, forming web-like structures that contribute to clot formation and may also have a role in capturing and killing pathogens during coagulation. (Mengal et al., 2023a). It is intriguing to speculate that the high level of histones in the clot and hemolymph indicate that histone proteins are an ancient part of the innate immune system that may enhance innate immune responses and induce coagulation in decapods.

3.2.4. Beta-1,3-glucan-binding protein

Beta-1,3-glucan-binding protein (βGBP) in crustaceans is an important pattern recognition protein (PRP) that plays a key role in the recognition and binding of β-glucan found on the surface of microbes. The binding of βGBP to β-1,3-glucans initiates a cascade of immune responses, including both cell-mediated responses such as phagocytosis, encapsulation, and nodule formation, and humoral responses such as the production of antimicrobial peptides and activation of prophenoloxidase (proPO) system proteins (Jiravanichpaisal et al., 2006). For example, In the field crab *Paratelphusa hydrodromus*, purified Ph-β-GBP enhanced cellular immune responses against pathogens by increasing agglutination, phagocytic activity, and encapsulation in a dose-dependent manner. It also increased prophenoloxidase and serine protease activity, aiding in pathogen clearance (Iswarya et al., 2017). Also, in crayfish and shrimp, it has been reported that βGBP serves a vital role in the activation of proPO activating system proteins which is an important component of the innate immune system involved in melanization, cell adhesion cytotoxic reactions, phagocytosis and encapsulation (Amparyup et al., 2012; Lee et al., 2000). The βGBP is synthesized mainly by the hepatopancreas and secreted to the hemolymph (Yepiz-Plascencia et al., 2000), where it helps to provide continuous surveillance against potential pathogens, allowing for a rapid immune response if necessary.

3.2.5. Vitellogenin

Vitellogenin (VTG) is a protein responsible for carrying lipids such as cholesterol and is primarily associated with major yolk proteins in egg-laying aquatic animals such as crayfish (Niksirat et al., 2014, 2015), sturgeon (Niksirat et al., 2017), rainbow trout (Niksirat et al., 2020) and zebrafish (Niksirat et al., 2021). Recent studies, however, have revealed its non-reproductive functions, such as immunity and antioxidation (Li et al., 2017; Sun et al., 2020). A study conducted in Chinese mitten crab *Eriocheir sinensis*, showed that VTG plays crucial roles in innate immunity, including binding to bacteria, inhibiting bacterial proliferation, and regulating the expression of antimicrobial peptides (Li et al., 2017). Additionally, the survival rate of *E. sinensis* significantly improved after injection with recombinant VTG protein following bacterial infection (Sun et al., 2020).

VTG has also been shown to possess antioxidant abilities. Havukainen et al., reported that it can increase the lifespan of honeybees by enhancing their oxidative stress tolerance and shielding them from oxidative damage (Havukainen et al., 2013). It has been observed that the clotting protein in crayfish is quite similar to vitellogenin. Their sequence similarity suggests that these two proteins may have a common evolutionary origin (Hall et al., 1999). In addition to VTG's involvement in lipid metabolism and reproduction, it may have acquired other functions during metazoan evolution, such as involvement in the clotting cascade and immune response (Hall et al., 1999; Avarre et al., 2007). This further emphasizes the versatility and importance of the VTG protein in decapod crustaceans.

3.3. Low-abundant proteins in the hemolymph proteomic profile

Several low-abundant proteins within the innate immune system of crustaceans were found in both non-clotted and clotted hemolymph samples. In the top 30 most low-abundant proteins, we discussed the functions of some of the important ones below. Table 1 provides a complete list of the top thirty low-abundance proteins found in the hemolymph.

3.3.1. Putative RNA-binding protein

RNA-binding proteins are a broad class of proteins that bind to RNA molecules and play a crucial role in anti-viral immunity in crustaceans. *Trans*-activation response RNA-binding protein (TRBP), a key component of RNA-induced silencing complex, was shown to play a key role in the anti-viral RNA interference pathway in *Marsupenaeus japonicus* (Wang et al., 2012). The use of recombinant TRBP in Chinese white shrimp *Fenneropenaeus chinensis* significantly reduced WSSV proliferation, indicating its importance in the shrimp's antiviral defense (Wang et al., 2009). Another proteomics study reported the significant down-regulation of TRBP under WSSV infection, suggesting that this viral infection might negatively affect the immune defense via the RNAi process in red claw crayfish *Cherax quadricarinatus* (Jeswin et al., 2016).

3.3.2. Alpha2 macroglobulin

Alpha2 macroglobulin (A2M) is a multifunctional, broad-range serine proteinase inhibitor protein that is involved in a variety of immune responses in invertebrates, including the hemolymph clotting system (Hall and Söderhäll, 1994), proPO activating system (Aspán et al., 1990), and phagocytosis (Buresova et al., 2009). A2M serves as an important substrate during the clotting process in crayfish (Hall and Söderhäll, 1994). In *P. leniusculus*, A2M has also been found to have a proteinase inhibitory activity to a limited extent in the proPO system by restricting the activity of the proPO-activating enzyme (ppA) (Aspán et al., 1990). Although A2M has been reported to have a regulatory role to some extent in the proPO system of crayfish and shrimp (Aspán et al., 1990; Ponprateep et al., 2017), it has also been demonstrated that pacifastin, another serine proteinase inhibitor, plays a more significant role in regulating the proPO system in these species (Liang et al., 1997; Sangsuriya et al., 2016). In addition to its role in the proPO system, A2M has also been found to play a role in the cellular immune response. Silencing of the IrAM, an A2M in hard tick *Ixodes Ricinus*, resulted in

reduced phagocytosis of *Chryseobacterium indologenes* pathogen, indicating the role of IrAM in this process (Buresova et al., 2009).

3.3.3. Ferritin

Ferritin is a ubiquitous iron storage protein found in a wide range of organisms and has various functions, such as antioxidant activity, cell activation, regulation of iron metabolism, and immune defense (Niksirat et al., 2020, 2021; Yang et al., 2019). Huang et al. (1996) cloned and characterized ferritin from the hepatopancreas of the freshwater crayfish *Pacifastacus leniusculus*, and found that its primary structure is more similar to vertebrate H-ferritins. In vertebrates, ferritin has been indirectly linked to innate immune response, as its synthesis is regulated by proinflammatory cytokines, suggesting its potential role in innate immunity (Huang et al., 1999; Torti and Torti, 2002).

Studies have explored the function of ferritin in other crustaceans, such as *P. clarkii* and *M. nipponense*. Yang et al. (2019) observed a significant increase in the expression of PcFer mRNA and protein in hemocytes and hepatopancreas of *P. clarkii* exposed to WSSV and *Aeromonas hydrophila*. Similarly, Tang et al. (2019) reported that ferritin expression levels were strongly elevated in river prawn *M. nipponense* after injection of a free radical-generating agent and bacterial infection, suggesting its protective roles in cellular redox homeostasis and antibacterial immunity.

3.3.4. RNA helicase

RNA helicases are involved in various processes that are essential for the innate immune response in crustaceans. They play a vital role in regulating the RNA interference (RNAi) pathway (Phetrungnapha et al., 2015). For instance, knockdown of the RNA helicase gene (Mj-mov-10) led to increased susceptibility to WSSV infection in *M. japonicus*, suggesting that silencing of RNA helicases may disrupt the normal functions of RNAi-related proteins and thus affect the anti-viral defense mechanism (Phetrungnapha et al., 2015). Additionally, RNA helicases are also involved in sensing viral nucleic acids and activating the antiviral immune response in fish. An *in vitro* study on spleen cells (GS) of orange-spotted grouper *Epinephelus coioides,* has shown that infection with red-spotted grouper nervous necrosis virus (RGNNV) induced up-regulation of the expression of RNA helicase ddx3. Moreover, overexpression of ddx3 in GS cells enhanced type I IFN-related anti-viral response and inhibited replication of RGNNV (Liu et al., 2017).

3.3.5. Catalase

Catalase is an enzyme that plays a critical role in the antioxidant defense system of crustaceans. When crustaceans are exposed to stressors, such as pathogens or pollutants, reactive oxygen species (ROS) are generated, leading to oxidative stress. Catalase works to break down hydrogen peroxide, a harmful ROS, into water and oxygen, thereby reducing oxidative stress and preventing cell damage (Zhang et al., 2008; Coates and Söderhäll, 2021). Studies have suggested that catalase may also play a role in the immunity of crustaceans. For example, in the swimming crab *Portunus trituberculatus*, catalase activity was found to increase significantly after the challenge with the pathogenic bacterium *Vibrio alginolyticus* (Chen et al., 2012). Similarly, in the *F. chinensis*, catalase activity was up-regulated in the hemocytes and the hepatopancreas after exposure to the pathogenic virus of WSSV (Zhang et al., 2008). These findings suggest that catalase may be involved in the immune response of crustaceans by protecting cells from oxidative damage caused by pathogens.

3.3.6. Anti-lipopolysaccharide factor

Anti-lipopolysaccharide factors (ALFs) are antimicrobial peptides that play a critical role in antimicrobial defense in crustaceans. ALFs exert their antimicrobial activity by binding to and disrupting the cell membrane structures of the pathogens, leading to their death. A number of ALFs from different crustacean species, including shrimp (de la Vega et al., 2008), crayfish (Liu et al., 2006) and lobster (Beale et al., 2008), have been studied. In *L.vannamei*, LvALF was found to have broad-spectrum antimicrobial activity against bacterial and fungal infections. After the knockdown of LvALF1 in *Vibrio penaeicida* and *Fusarium oxysporum* infected groups of shrimp, the mortality rate significantly increased compared to the control group (de la Vega et al., 2008). ALF has been demonstrated to play a critical role in protecting *P. leniusculus* from WSSV infection both *in vitro* and *in vivo* by interfering with viral replication (Liu et al., 2006). Similarly, after injection of *Vibrio fluvialis* into the American lobster *Homarus americanus* increased ALFHa-1 mRNA abundance in gill, hematopoietic, and hepatopancreas tissues but had no significant effect on the relative abundance of ALFHa-2 mRNA, indicating a specific role of ALFHa-1 in antimicrobial regulation (Beale et al., 2008). In addition to their direct antimicrobial activity, ALFs have also been shown to modulate immune-related genes. For instance, when PcALF was suppressed in crayfish *P. clarkii*, it resulted in the altered expression of several immune-related genes, such as Crustin, Toll, Lectin, and Cactus (Zhu et al., 2019).

4. Conclusion

This is the first study to perform a comparative proteome analysis of non-clot and clot hemolymph in decapods. The abundance of the majority of proteins remained unchanged during coagulation, suggesting that clotting proteins are pre-synthesized and stored in the granules of hemocytes and plasma to enable a rapid response to wounds. Synthesizing new proteins after an injury can be time-consuming, especially in animals with an open circulatory system like decapods, where bleeding can be life-threatening. Therefore, most of the proteins involved in clotting are likely pre-synthesized and stored, enabling a swift response to injury in the form of coagulation. In addition, we found three significantly down-regulated proteins, which two of them are mainly structural proteins related to hemocyte integrity. This indicates that their downregulation could enhance hemocyte lysis and degranulation, which eventually results in clot formation. Lastly, we found an immune related protein that was significantly up-regulated, indicating the synthesis of at least one protein during coagulation, probably by phagocytic hemocytes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The study was financially supported by the Grant Agency of the University of South Bohemia, (GAJU) projects No. GAJU project 065/2022/Z, 055/2022/Z and 012/2022/Z. Frank Lyko and Panagotis Provataris are thanked for providing the *Procambarus virginalis* database. Proteoforms@LU is thanked for providing LC-MS infrastructure.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.104760.

References

Adachi, K., Hirata, T., Nagai, K., Sakaguchi, M., 2001. Hemocyanin a most likely inducer of black spots in kuruma prawn Penaeus japonicus during storage. J. Food Sci. 66, 1130–1136.

Altincicek, B., Stotzel, S., Wygrecka, M., Preissner, K.T., Vilcinskas, A., 2008. Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. J. Immunol. 181, 2705–2712.

Amparyup, P., Sutthangkul, J., Charoensapsri, W., Tassanakajon, A., 2012. Pattern recognition protein binds to lipopolysaccharide and beta-1,3-glucan and activates shrimp prophenoloxidase system. J. Biol. Chem. 287, 10060–10069.

Aspán, A., Hall, M., Söderhäll, K., 1990. The effect of endogeneous proteinase inhibitors on the prophenoloxidase activating enzyme, a serine proteinase from crayfish haemocytes. Insect Biochem. Mol. Biol. 20, 485–492.

Avarre, J.-C., Lubzens, E., Babin, P.J., 2007. Apolipocrustacein, formerly vitellogenin, is the major egg yolk precursor protein in decapod crustaceans and is homologous to insect apolipophorin II/I and vertebrate apolipoprotein B. BMC Evol. Biol. 7, 3.

Ballman, K.V., Grill, D.E., Oberg, A.L., Therneau, T.M., 2004. Faster cyclic loess: normalizing RNA arrays via linear models. Bioinformatics 20, 2778–2786.

Beale, K.M., Towle, D.W., Jayasundara, N., Smith, C.M., Shields, J.D., Small, H.J., Greenwood, S.J., 2008. Anti-lipopolysaccharide factors in the American lobster Homarus americanus: molecular characterization and transcriptional response to Vibrio fluvialis challenge. Comp. Biochem. Physiol. Genom. Proteonomics 3, 263–269.

Buresova, V., Hajdusek, O., Franta, Z., Sojka, D., Kopacek, P., 2009. IrAM—an α2-macroglobulin from the hard tick Ixodes ricinus: characterization and function in phagocytosis of a potential pathogen Chryseobacterium indologenes. Dev. Comp. Immunol. 33, 489–498.

Busayarat, N., Senapin, S., Tonganunt, M., Phiwsaiya, K., Meemetta, W., Unajak, S., Jitrapakdee, S., Lo, C.-F., Phongdara, A., 2011. Shrimp laminin receptor binds with capsid proteins of two additional shrimp RNA viruses YHV and IMNV. Fish Shellfish Immunol. 31, 66–72.

Castronovo, V., 1993. Laminin receptors and laminin-binding proteins during tumor invasion and metastasis. Invasion Metastasis 13, 1–30.

Cerenius, L., Söderhäll, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198, 116–126.

Charoensapsri, W., Sangsuriya, P., Lertwimol, T., Gangnonngiw, W., Phiwsaiya, K., Senapin, S., 2015. Laminin receptor protein is implicated in hemocyte homeostasis for the whiteleg shrimp Penaeus (Litopenaeus) vannamei. Dev. Comp. Immunol. 51, 39–47.

Chen, P., Li, J., Liu, P., Gao, B., Wang, Q., Li, J., 2012. cDNA cloning, characterization and expression analysis of catalase in swimming crab Portunus trituberculatus. Mol. Biol. Rep. 39, 9979–9987.

Coates, C.J., Söderhäll, K., 2021. The stress–immunity axis in shellfish. J. Invertebr. Pathol. 186, 107492.

Coates, C.J., Costa-Paiva, E.M., 2020. Multifunctional roles of hemocyanins. In: Hoeger, U., Harris, J.R. (Eds.), Vertebrate and Invertebrate Respiratory Proteins, Lipoproteins and Other Body Fluid Proteins. Springer International Publishing, Cham, pp. 233–250.

Coates, C.J., Talbot, J., 2018. Hemocyanin-derived phenoloxidase reaction products display anti-infective properties. Dev. Comp. Immunol. 86, 47–51.

Coates, C.J., Nairn, J., 2014. Diverse immune functions of hemocyanins. Dev. Comp. Immunol. 45, 43–55.

Cowin, A.J., 2006. Role of the Actin Cytoskeleton in Wound Healing and Scar Formation. Primary Intention: the Australian Journal of Wound Management vol. 14, 39–42.

de la Vega, E., O'Leary, N.A., Shockey, J.E., Robalino, J., Payne, C., Browdy, C.L., Warr, G.W., Gross, P.S., 2008. Anti-lipopolysaccharide factor in Litopenaeus vannamei (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. Mol. Immunol. 45, 1916–1925.

Destoumieux-Garzón, D., Rosa, R.D., Schmitt, P., Barreto, C., Vidal-Dupiol, J., Mitta, G., Gueguen, Y., Bachère, E., 2016. Antimicrobial peptides in marine invertebrate health and disease. Phil. Trans. Biol. Sci. 371, 20150300.

Flegel, T.W., 2009. Hypothesis for heritable, anti-viral immunity in crustaceans and insects. Biol. Direct 4, 32.

Franco-Martínez, L., Martínez-Subiela, S., Escribano, D., Schlosser, S., Nöbauer, K., Razzazi-Fazeli, E., Romero, D., Cerón, J.J., Tvarijonaviciute, A., 2018. Alterations in haemolymph proteome of Mytilus galloprovincialis mussel after an induced injury. Fish Shellfish Immunol. 75, 41–47.

Ge, S.X., Jung, D., Yao, R., 2019. ShinyGO: a graphical gene-set enrichment tool for animals and plants. Bioinformatics 36, 2628–2629.

Gianazza, E., Eberini, I., Palazzolo, L., Miller, I., 2021. Hemolymph proteins: an overview across marine arthropods and molluscs. J. Proteonomics 245, 104294.

Goic, B., Vodovar, N., Mondotte, J.A., Monot, C., Frangeul, L., Blanc, H., Gausson, V., Vera-Otarola, J., Cristofari, G., Saleh, M.-C., 2013. RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila. Nat. Immunol. 14, 396–403.

Greaves, M., Maley, C.C., 2012. Clonal evolution in cancer. Nature 481, 306–313.

Grubhoffer, L., Rudenko, N., Vancova, M., Golovchenko, M., Sterba, J., 2013. In: Sonenshine, D.E., Roe, R.M. (Eds.), Circulatory System and Hemolymph. Biology of Ticks, second ed., pp. 258–286.

Gunning, P.W., Hardeman, E.C., Lappalainen, P., Mulvihill, D.P., 2015. Tropomyosin – master regulator of actin filament function in the cytoskeleton. J. Cell Sci. 128, 2965–2974.

Gutekunst, J., Andriantsoa, R., Falckenhayn, C., Hanna, K., Stein, W., Rasamy, J., Lyko, F., 2018. Clonal genome evolution and rapid invasive spread of the marbled crayfish. Nature Ecology & Evolution 2, 567–573.

Hall, M., Söderhäll, K., 1994. Crayfish α-macroglobulin as a substrate for transglutaminases. Comp. Biochem. Physiol. Part B: Comparative Biochemistry 108, 65–72.

Hall, M., Wang, R., van Antwerpen, R., Sottrup-Jensen, L., Söderhäll, K., 1999. The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood. Proc. Natl. Acad. Sci. USA 96, 1965–1970.

Han, F., Li, W., Liu, X., Zhang, D., Liu, L., Wang, Z., 2019. Rac1 GTPase is a critical factor in phagocytosis in the large yellow croaker Larimichthys crocea by interacting with tropomyosin. Fish Shellfish Immunol. 91, 148–158.

Havanapan, P.-o., Kanlaya, R., Bourchookarn, A., Krittanai, C., Thongboonkerd, V., 2009. C-terminal hemocyanin from hemocytes of Penaeus vannamei interacts with ERK1/2 and undergoes serine phosphorylation. J. Proteome Res. 8, 2476–2483.

Havanapan, P.-o., Mangkalanan, S., Phungthanom, N., Krittanai, C., 2019. Proteomic analysis and white spot syndrome virus interaction of mud crab (Scylla olivacea) revealed responsive roles of the hemocytes. Fish Shellfish Immunol. 89, 458–467.

Havukainen, H., Münch, D., Baumann, A., Zhong, S., Halskau, Ø., Krogsgaard, M., Amdam, G.V., 2013. Vitellogenin recognizes cell damage through membrane binding and shields living cells from reactive oxygen species. J. Biol. Chem. 288, 28369–28381.

Hou, L., Xiu, Y., Wang, J., Liu, X., Liu, Y., Gu, W., Wang, W., Meng, Q., 2016. iTRAQ-based quantitative proteomic analysis of Macrobrachium rosenbergii hemocytes during Spiroplasma eriocheiris infection. J. Proteonomics 136, 112–122.

Hou, L., Zhou, H., Wan, H., Liu, Z., Wang, L., Cheng, Y., Wu, X., Gu, W., Wang, W., Meng, Q., 2020. TMT-based quantitative proteomic analysis of Eriocheir sinensis hemocytes and thoracic ganglion during Spiroplasma eriocheiris infection. Fish Shellfish Immunol. 96, 126–137.

Huang, T.-s., Law, J.H., Söderhäll, K., 1996. Purification and cDNA cloning of ferritin from the hepatopancreas of the freshwater crayfish Pacifastacus leniusculus. Eur. J. Biochem. 236, 450–456.

Huang, T.-s., Meleförs, Ö., Lind, M.I., Söderhäll, K., 1999. An atypical Iron-Responsive Element (IRE) within crayfish ferritin mRNA and an Iron Regulatory Protein 1 (IRP1)-like protein from crayfish hepatopancreas. Insect Biochem. Mol. Biol. 29, 1–9.

Iorio, V., Troughton, L.D., Hamill, K.J., 2015. Laminins: roles and utility in wound repair. Adv. Wound Care 4, 250–263.

Iswarya, A., Anjugam, M., Vaseeharan, B., 2017. Role of purified β-1, 3 glucan binding protein (β-GBP) from Paratelphusa hydrodromus and their anti-inflammatory, antioxidant and antibiofilm properties. Fish Shellfish Immunol. 68, 54–64.

Jeswin, J., Xie, X.-l., Ji, Q.-l., Wang, K.-j., Liu, H.-p., 2016. Proteomic analysis by iTRAQ in red claw crayfish, Cherax quadricarinatus, hematopoietic tissue cells post white spot syndrome virus infection. Fish Shellfish Immunol. 50, 288–296.

Jiravanichpaisal, P., Lee, B.L., Söderhäll, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211, 213–236.

Junkunlo, K., Söderhäll, K., Söderhäll, I., 2018. Clotting protein – an extracellular matrix (ECM) protein involved in crustacean hematopoiesis. Dev. Comp. Immunol. 78, 132–140.

Junkunlo, K., Söderhäll, K., Söderhäll, I., 2020. Transglutaminase 1 and 2 are localized in different blood cells in the freshwater crayfish Pacifastacus leniusculus. Fish Shellfish Immunol. 104, 83–91.

Kopáček, P., Hall, M., Söderhäll, K., 1993. Characterization of a clotting protein, isolated from plasma of the freshwater crayfish Pacifastacus leniusculus. Eur. J. Biochem. 213, 591–597.

Kor, G., Mengal, K., Buric, M., Kozak, P., Niksirat, H., 2023a. Comparative ultrastructure of the antennae and sensory hairs in six species of crayfish. PeerJ 11, e15006.

Kor, G., Mengal, K., Burić, M., Kozák, P., Niksirat, H., 2023b. Granules of Immune Cells Are the Source of Organelles in the Regenerated Nerves of Crayfish Antennae. Fish Shellfish Immunol., 108787

Lee, S.Y., Lee, B.L., Söderhäll, K., 2003. Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish Pacifastacus leniusculus. J. Biol. Chem. 278, 7927–7933.

Lee, S.Y., Lee, B.L., Söderhäll, K., 2004. Processing of crayfish hemocyanin subunits into phenoloxidase. Biochem. Biophys. Res. Commun. 322, 490–496.

Lee, S.Y., Wang, R., Söderhäll, K., 2000. A lipopolysaccharide- and beta-1,3-glucan-binding protein from hemocytes of the freshwater crayfish Pacifastacus leniusculus. Purification, characterization, and cDNA cloning. J. Biol. Chem. 275, 1337–1343.

Lees, J.G., Ching, Y.W., Adams, D.H., Bach, C.T.T., Samuel, M.S., Kee, A.J., Hardeman, E. C., Gunning, P., Cowin, A.J., O'Neill, G.M., 2013. Tropomyosin regulates cell migration during skin wound healing. J. Invest. Dermatol. 133, 1330–1339.

Li, L., Li, X.J., Wu, Y.M., Yang, L., Li, W., Wang, Q., 2017. Vitellogenin regulates antimicrobial responses in Chinese mitten crab, Eriocheir sinensis. Fish Shellfish Immunol. 69, 6–14.

Li, W., Tang, X., Xing, J., Sheng, X., Zhan, W., 2014. Proteomic analysis of differentially expressed proteins in Fenneropenaeus chinensis hemocytes upon white spot syndrome virus infection. PLoS One 9, e89962.

Li, Y., Pan, L., Yu, J., 2022. The injection of one recombinant C-type lectin (LvLec) induced the immune response of hemocytes in Litopenaeus vannamei. Fish Shellfish Immunol. 124, 324–331.

Liang, Z., Sottrup-Jensen, L., Aspán, A., Hall, M., Söderhäll, K., 1997. Pacifastin, a novel 155-kDa heterodimeric proteinase inhibitor containing a unique transferrin chain. Proc. Natl. Acad. Sci. USA 94, 6682–6687.

Liu, H., Jiravanichpaisal, P., Soderhall, I., Cerenius, L., Soderhall, K., 2006. Antilipopolysaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish Pacifastacus leniusculus. J. Virol. 80, 10365–10371.

Liu, J., Huang, X., Yu, Y., Zhang, J., Ni, S., Hu, Y., Huang, Y., Qin, Q., 2017. Fish DDX3X exerts antiviral function against grouper nervous necrosis virus infection. Fish Shellfish Immunol. 71, 95–104.

Liu, L.-k., Li, W.-d., Gao, Y., Chen, R.-y., Xie, X.-l., Hong, H., Wang, K.-j., Liu, H.-p., 2018. A laminin-receptor-like protein regulates white spot syndrome virus infection by

binding to the viral envelope protein VP28 in red claw crayfish Cherax quadricarinatus. Dev. Comp. Immunol. 79, 186–194.
Liu, W.-J., Li, Y.-C., Kou, G.-H., Lo, C.-F., 2016. Laminin receptor in shrimp is a cellular attachment receptor for white spot syndrome virus. PLoS One 11, e0156375.
Luo, M., Yang, L., Wang, Z.-a., Zuo, H., Weng, S., He, J., Xu, X., 2019. A novel C-type lectin with microbiostatic and immune regulatory functions from Litopenaeus vannamei. Fish Shellfish Immunol. 93, 361–368.
Mengal, K., Kor, G., Kouba, A., Kozák, P., Niksirat, H., 2023a. Hemocyte coagulation and phagocytic behavior in early stages of injury in crayfish (Arthropoda: Decapoda) affect their morphology. Dev. Comp. Immunol. 141, 104618.
Mengal, K., Kor, G., Kozák, P., Niksirat, H., 2023b. Effects of environmental factors on the cellular and molecular parameters of the immune system in decapods. Comp. Biochem. Physiol. Mol. Integr. Physiol. 276, 111332.
Nagai, T., Kawabata, S.-i., 2000. A link between blood coagulation and prophenol oxidase activation in arthropod host defense. J. Biol. Chem. 275, 29264–29267.
Ng, T.H., Chang, S.-H., Wu, M.-H., Wang, H.-C., 2013. Shrimp hemocytes release extracellular traps that kill bacteria. Dev. Comp. Immunol. 41, 644–651.
Ng, T.H., Wu, M.-H., Chang, S.-H., Aoki, T., Wang, H.-C., 2015. The DNA fibers of shrimp hemocyte extracellular traps are essential for the clearance of Escherichia coli. Dev. Comp. Immunol. 48, 229–233.
Niksirat, H., Andersson, L., Golpour, A., Chupani, L., James, P., 2017. Quantification of egg proteome changes during fertilization in sterlet Acipenser ruthenus. Biochem. Biophys. Res. Commun. 490, 189–193.
Niksirat, H., Andersson, L., James, P., Kouba, A., Kozák, P., 2014. Proteomic profiling of the signal crayfish Pacifastacus leniusculus egg and spermatophore. Anim. Reprod. Sci. 149, 335–344.
Niksirat, H., James, P., Andersson, L., Kouba, A., Kozák, P., 2015. Label-free protein quantification in freshly ejaculated versus post-mating spermatophores of the noble crayfish Astacus astacus. J. Proteonomics 123, 70–77.
Niksirat, H., Levander, F., Kouba, A., James, P., 2020. Proteomic changes after fertilization and before first cleavage in rainbow trout Oncorhynchus mykiss. Aquaculture 520, 734951.
Niksirat, H., Siino, V., Steinbach, C., Levander, F., 2021. High-resolution proteomic profiling shows sexual dimorphism in zebrafish heart-associated proteins. J. Proteome Res. 20, 4075–4088.
Patat, S.A., Carnegie, R.B., Kingsbury, C., Gross, P.S., Chapman, R., Schey, K.L., 2004. Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. Eur. J. Biochem. 271, 4825–4833.
Perdomo-Morales, R., Montero-Alejo, V., Perera, E., 2019. The clotting system in decapod crustaceans: history, current knowledge and what we need to know beyond the models. Fish Shellfish Immunol. 84, 204–212.
Phetrungnapha, A., Kondo, H., Hirono, I., Panyim, S., Ongvarrasopone, C., 2015. Molecular cloning and characterization of Mj-mov-10, a putative RNA helicase involved in RNAi of kuruma shrimp. Fish Shellfish Immunol. 44, 241–247.
Ponpateep, S., Vatanavicharn, T., Lo, C.F., Tassanakajon, A., Rimphanitchayakit, V., 2017. Alpha-2-macroglobulin is a modulator of prophenoloxidase system in pacific white shrimp Litopenaeus vannamai. Fish Shellfish Immunol. 62, 68–74.
Qin, Z., Babu, V.S., Wan, Q., Muhammad, A., Li, J., Lan, J., Lin, L., 2018. Antibacterial activity of hemocyanin from red swamp crayfish (Procambarus clarkii). Fish Shellfish Immunol. 75, 391–399.
Robb, C.T., Dyrynda, E.A., Gray, R.D., Rossi, A.G., Smith, V.J., 2014. Invertebrate extracellular phagocyte traps show that chromatin is an ancient defence weapon. Nat. Commun. 5, 4627.
Sanggaard, K.W., Dyrlund, T.F., Bechsgaard, J.S., Scavenius, C., Wang, T., Bilde, T., Enghild, J.J., 2016. The spider hemolymph clot proteome reveals high concentrations of hemocyanin and von Willebrand factor-like proteins. Biochim. Biophys. Acta 1864, 233–241.
Scholtz, G., Braband, A., Tolley, L., Reimann, A., Mittmann, B., Lukhaup, C., Steuerwald, F., Vogt, G., 2003. Parthenogenesis in an outsider crayfish. Nature 421, 806.
Senapin, S., Phiwsaiya, K., Anantasomboon, G., Sriphaijit, T., Browdy, C.L., Flegel, T.W., 2010. Knocking down a Taura syndrome virus (TSV) binding protein Lamr is lethal for the whiteleg shrimp Penaeus vannamei. Fish Shellfish Immunol. 29, 422–429.
Senyürek, I., Kempf, W.E., Klein, G., Maurer, A., Kalbacher, H., Schäfer, I., Wanke, I., Christ, C., Stevanovic, S., Schaller, M., Rousselle, P., Garbe, C., Biedermann, T., Schittek, B., 2014. Processing of laminin α chains generates peptides involved in wound healing and host defense. J. Innate Immun. 6, 467–484.
Sangsuriya, P., Charoensapsri, W., Chomwong, S., Senapin, S., Tassanakajon, A., Amparyup, P., 2016. A shrimp pacifastin light chain-like inhibitor: molecular identification and role in the control of the prophenoloxidase system. Dev. Comp. Immunol. 54, 32–45.
Siino, V., Ali, A., Accardi, G., Aiello, A., Ligotti, M.E., Mosquim Junior, S., Candore, G., Caruso, C., Levander, F., Vasto, S., 2022. Plasma proteome profiling of healthy individuals across the life span in a Sicilian cohort with long-lived individuals. Aging Cell 21, e13684.
Sirikharin, R., Junkunlo, K., Söderhäll, K., Söderhäll, I., 2017. Role of astakine1 in regulating transglutaminase activity. Dev. Comp. Immunol. 76, 77–82.
Smith, V.J., Söderhäll, K., 1983. Induction of degranulation and lysis of haemocytes in the freshwater crayfish, Astacus astacus by components of the prophenoloxidase activating system in vitro. Cell Tissue Res. 233, 295–303.
Smyth, G.K., 2005. Limma: linear models for microarray data. In: Gentleman, R., Carey, V.J., Huber, W., Irizarry, R.A., Dudoit, S. (Eds.), Bioinformatics and Computational Biology Solutions Using R and Bioconductor. Springer New York, New York, NY, pp. 397–420.
Söderhäll, I., Junkunlo, K., 2019. A comparative global proteomic analysis of the hematopoietic lineages in the crustacean Pacifastacus leniusculus. Dev. Comp. Immunol. 92, 170–178.
Söderhäll, K., Smith, V., 1986. The prophenoloxidase activating system: the biochemistry of its activation and role in arthropod cellular immunity, with special reference to crustaceans. In: Immunity in Invertebrates: Cells, Molecules, and Defense Reactions, pp. 208–223.
Söderhäll, K., Cerenius, L., 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. 10, 23–28.
Söderhäll, K., Iwanaga, S., Vasta, G., 1996. New Directions in Invertebrate Immunology. SOS publications, New Haven NJ USA.
Sricharoen, S., Kim, J.J., Tunkijjanukij, S., Söderhäll, I., 2005. Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish. Dev. Comp. Immunol. 29, 1017–1031.
Sun, B., Wang, Z., Wang, Z., Ma, X., Zhu, F., 2017. A proteomic study of hemocyte proteins from mud crab (Scylla paramamosain) infected with white spot syndrome virus or Vibrio alginolyticus. Front. Immunol. 8.
Sun, W., Li, L., Li, H., Zhou, K., Li, W., Wang, Q., 2020. Vitellogenin receptor expression in ovaries controls innate immunity in the Chinese mitten crab (Eriocheir sinensis) by regulating vitellogenin accumulation in the hemolymph. Fish Shellfish Immunol. 107, 480–489.
Sun, Y.-D., Fu, L.-D., Jia, Y.-P., Du, X.-J., Wang, Q., Wang, Y.-H., Zhao, X.-F., Yu, X.-Q., Wang, J.-X., 2008. A hepatopancreas-specific C-type lectin from the Chinese shrimp Fenneropenaeus chinensis exhibits antimicrobial activity. Mol. Immunol. 45, 348–361.
Suzuki, N., Yokoyama, F., Nomizu, M., 2005. Functional sites in the laminin alpha chains. Connect. Tissue Res. 46, 142–152.
Tang, T., Yang, Z., Li, J., Yuan, F., Xie, S., Liu, F., 2019. Identification of multiple ferritin genes in Macrobrachium nipponense and their involvement in redox homeostasis and innate immunity. Fish Shellfish Immunol. 89, 701–709.
Theopold, U., Schmidt, O., Söderhäll, K., Dushay, M.S., 2004. Coagulation in arthropods: defence, wound closure and healing. Trends Immunol. 25, 289–294.
Torti, F.M., Torti, S.V., 2002. Regulation of ferritin genes and protein. Blood 99, 3505–3516.
Tsutsui, S., Dotsuta, Y., Ono, A., Suzuki, M., Tateno, H., Hirabayashi, J., Nakamura, O., 2014. A C-type lectin isolated from the skin of Japanese bullhead shark (Heterodontus japonicus) binds a remarkably broad range of sugars and induces blood coagulation. J. Biochem. 157, 345–356.
Vazquez, L., Alpuche, J., Maldonado, G., Agundis, C., Pereyra-Morales, A., Zenteno, E., 2009. Review: immunity mechanisms in crustaceans. Innate Immun. 15, 179–188.
Viana, J.T., Rocha, R.d.S., Maggioni, R., 2022. Structural and functional diversity of lectins associated with immunity in the marine shrimp Litopenaeus vannamei. Fish Shellfish Immunol. 129, 152–160.
Wang, S., Chen, A.-J., Shi, L.-J., Zhao, X.-F., Wang, J.-X., 2012. TRBP and eIF6 homologue in Marsupenaeus japonicus play crucial roles in antiviral response. PLoS One 7, e30057.
Wang, S., Liu, N., Chen, A.-J., Zhao, X.-F., Wang, J.-X., 2009. TRBP homolog interacts with eukaryotic initiation factor 6 (eIF6) in Fenneropenaeus chinensis1. J. Immunol. 182, 5250–5258.
Wang, X.-W., Wang, J.-X., 2013. Diversity and multiple functions of lectins in shrimp immunity. Dev. Comp. Immunol. 39, 27–38.
Willforss, J., Chawade, A., Levander, F., 2019. NormalyzerDE: online tool for improved normalization of omics expression data and high-sensitivity differential expression analysis. J. Proteome Res. 18, 732–740.
Wu, W., Zong, R., Xu, J., Zhang, X., 2008. Antiviral phagocytosis is regulated by a novel rab-dependent complex in shrimp Penaeus japonicus. J. Proteome Res. 7, 424–431.
Wu, C., Söderhäll, I., Kim, Y.-A., Liu, H., Söderhäll, K., 2008. Hemocyte-lineage marker proteins in a crustacean, the freshwater crayfish. Pacifastacus leniusculus. Proteomics 8, 4226–4235.
Yang, H., Liu, Z., Jiang, Q., Xu, J., An, Z., Zhang, Y., Xiong, D., Wang, L., 2019. A novel ferritin gene from Procambarus clarkii involved in the immune defense against Aeromonas hydrophila infection and inhibits WSSV replication. Fish Shellfish Immunol. 86, 882–891.
Ye, T., Zong, R., Zhang, X., 2012. Involvement of interaction between viral VP466 and host tropomyosin proteins in virus infection in shrimp. Gene 505, 254–258.
Yeh, M.-S., Huang, C.-J., Leu, J.-H., Lee, Y.C., Tsai, I.-H., 1999. Molecular cloning and characterization of a hemolymph clottable protein from tiger shrimp (Penaeus monodon). Eur. J. Biochem. 266, 624–633.
Yepiz-Plascencia, G., Galván, T.G., Vargas-Albores, F., García-Bañuelos, M., 2000. Synthesis of hemolymph high-density lipoprotein β-glucan binding protein by Penaeus vannamei shrimp hepatopancreas. Mar. Biotechnol. 2, 485–492.
Zhang, Q., Li, F., Zhang, X., Dong, B., Zhang, J., Xie, Y., Xiang, J., 2008. cDNA cloning, characterization and expression analysis of the antioxidant enzyme gene, catalase, of Chinese shrimp Fenneropenaeus chinensis. Fish Shellfish Immunol. 24, 584–591.
Zhu, J.-J., Ye, Z.-Z., Li, C.-S., Kausar, S., Abbas, M.N., Xiang, G.-H., Qian, X.-Y., Dai, L.-S., 2019. Identification and molecular characterization of a novel anti-lipopolysaccharide factor (ALF) from red swamp crayfish, Procambarus clarkii. Int. J. Biol. Macromol. 132, 43–50.

CHAPTER 5

General discussion

Marbled crayfish (*Procambarus virginalis*) has recently emerged as a valuable model organism for biological research due to its unique biological features. The hemolymph is an important component of the innate immune system, with hemocytes (the immune cells) present in hemolymph – playing a crucial role in immunity, such as coagulation, phagocytosis, and defense against pathogens and other foreign substances. Therefore, understanding the cellular and molecular characteristics of hemolymph in marbled crayfish is of great importance for elucidating the innate immune system of crustaceans.

Effects of environmental factors on the cellular and molecular parameters of the immune system in decapods

In Chapter 2 of the present study, we reviewed the key components of innate immunity and the effects of various environmental stressors on the immune parameters and the abiotic stress mechanisms of commercial decapod species. The innate immune system of decapod crustaceans relies heavily on hemocytes in the circulating hemolymph. Traditionally, hemocytes in crustaceans have been classified into three morphological types: hyaline cells (HCs), semi-granular cells (SGCs), and granular cells (GCs) (Söderhäll and Smith, 1983; Söderhäll, 2016). However, there is still a lack of consensus among researchers regarding the classification of hemocyte types. As a result, a wide variety of terminologies has been used in the literature to refer to various hemocyte types (Dall, 1964; Mengal et al., 2023b). Additionally, recent studies of bivalves and crustaceans have proposed that hemocyte sub-populations are likely different developmental stages of a single cell type. For instance, a study of the bivalve *Crassostrea rhizophorae* reported that the different subpopulations of hemocytes might, in reality, be different stages of one type of cell, in which the absence of granules (loss of complexity) could be due to degranulation in the event of an immune response (Rebelo et al., 2013). Similarly, a study of hemocytes in the crayfish *Cherax quadricarinatus* suggests that the so-called hemocyte subtypes (i.e., SGC and HC) are probably different developmental stages of a single cell type (GC) lineage (Li et al., 2021).

On the other hand, the involvement of different types of hemocytes in crucial immune processes like phagocytosis and coagulation is still unclear. Studies have shown conflicting results. For instance, in crayfish *Astacus leptodactylus,* it was suggested that all three types of hemocytes are involved in phagocytic activity (Giulianini et al., 2007), while a study of the crayfish *Cherax quadricarinatus* indicated that only SGC and GC perform this phagocytic activity (Li et al., 2018). In the shrimp *Penaeus monodon,* it was found that phagocytic activity was mainly exhibited by HC rather than SGC or GC (Sung and Sun, 2002). While the basic molecular mechanism of clotting in decapods is well understood, the cellular characteristics of the hemocytes that initiate coagulation remain poorly understood. Some studies suggest that hyaline cells are the primary cells that initiate hemolymph coagulation in decapod crustaceans and arthropods, as they have been observed to contain cytoplasmic deposits that are released during lysis (Wood et al., 1971; Ravindranath, 1980; Hose et al., 1990). However, granular hemocytes have also been reported to be involved in hemolymph coagulation (Dumont et al., 1966). Further research is needed to better understand the functional roles of different hemocyte subpopulations in decapod crustaceans.

Moreover, the evidence from the literature review suggested that environmental factors can have a significant impact on the immune parameters and health status of decapod crustaceans (Le Moullac and Haffner, 2000). Various environmental stressors such as temperature, pH, salinity, dissolved oxygen, ammonia concentrations and pathogens can adversely affect the

immune responses of crustaceans and increase their susceptibility to disease (Vargas-Albores et al., 1998; Kathyayani et al., 2019; Jiang et al., 2004; Le Moullac and Haffner, 2000). The immunosuppressive effects of environmental stressors have been well-documented; however, many critical questions regarding the exact mechanisms by which abiotic stressors operate remain unanswered. Understanding how environmental factors, particularly their abiotic components, influence the immune parameters of decapod crustaceans is crucial in mitigating the harmful effects of these stressors and promoting the sustainable production of decapod crustaceans.

In order to gain a clear understanding of hemocyte functions and types, there is an urgent need to explore the hemocyte's behavior during coagulation and phagocytosis using state-of-the-art morphological and molecular techniques, as highlighted in our review. The experimental findings presented in the following studies were based on the gaps identified in the literature review.

Hemocyte coagulation and phagocytic behavior in early stages of injury in crayfish (Arthropoda: Decapoda) affect their morphology

Coagulation plays a vital role in the innate immune response of arthropods and crustaceans, preventing the loss of blood or hemolymph and limiting the spread of pathogens (Jiravanichpaisal et al., 2006; Cerenius and Söderhäll, 2011). It involves the participation of hemocytes, their granular products, and plasma proteins, ultimately leading to the formation of a stable clot.

We conducted an *in situ* study using transmission electron microscopy to investigate the ultrastructural behavior of hemocytes during coagulation and phagocytosis in the early stages of leg amputation injury in marbled crayfish *Procambarus virginalis* (Mengal et al., 2023a). Our results revealed that granular hemocytes were activated first, and the morphology of cytoplasmic granules changed from electron-dense to electron-lucent forms in an expanding manner. The transformed granules containing amorphous electron-lucent material merged and discharged their contents into the extracellular space for coagulation. Additionally, we observed that the contents of the leftover nucleus from degranulated hemocytes participate in the process of coagulation, and this leftover nucleus has been previously confused with hyalinocytes in some studies (Clare and Lumb, 1994; Dolar et al., 2020). This *in situ* experiment results provided a comprehensive description of all the stages of morphological changes in hemocytes during coagulation and phagocytosis after injury in crayfish. Although previous ultrastructural studies on coagulation in arthropods and crustaceans have enhanced our understanding of the process, none of them have described all of these changes altogether *in situ* conditions (Rowley and Ratcliffe, 1976; Rowley, 1977; Ravindranath, 1980; Perdomo-Morales et al., 2019).

The activated stage of hemocytes during coagulation is characterized by variable forms of cytoplasmic granules. Some authors in the past argued that granule transformation is unnecessary for hemolymph coagulation and changes in the granules represent maturation stages (Seitz, 1972; Scharrer, 1972), while others suggested that low-density granules with microtubular structures are transformational phases of electron-dense granules (Ratcliffe and Price, 1974; Rowley and Ratcliffe, 1976; Rowley, 1977). Our observations support the latter view, as the varying states of granules are, in fact, transformational phases where electron-dense granules change into low-density granules with microtubular structures and finally into electron-lucent amorphous particles. This process enables the release of highly compacted materials from the granules to participate in clot formation.

In addition, our findings revealed that leg amputation caused massive muscle degeneration in the place of the wound, followed by a significant influx of phagocytic hemocytes that removed a substantial amount of degenerated muscle fibers and organelles, such as mitochondria, generated from disintegrating muscle. We observed that phagocytic hemocytes contained varying numbers of granules in their cytoplasm and, for the first time, found that these cells incorporate necrotic bodies resulting from degenerated muscles into their organelles, such as cytoplasmic granules and nucleus (Mengal et al., 2023a). The incorporation of material from the phagosome into hemocyte organelles can increase the size and number of granules in the cytoplasm, ultimately changing the morphology of the whole cell. A study on crayfish *P. leptodactylus* demonstrated that the number of hemocyte's cytoplasmic microtubules increase after injury, allowing them to engulf material from disintegrating muscle fibers (Uhrík et al., 1989). Another study observed that *in vitro* culture of crayfish hematopoietic tissue with muscle tissue extract from the same animal resulted in the differentiation of hemocytes with higher granular contents (Li et al., 2019). This may be due to hemocytes phagocytizing materials from muscle extract and incorporating them into their organelles, which increases hemocyte granularity. These findings suggest that hemocytes can recycle material from degenerating cells and store it in their organelles for future use, potentially for other functions such as coagulation and immune reactions.

Therefore, the process of coagulation and phagocytosis can cause drastic morphological changes to hemocytes, altering their appearance and potentially leading to confusion with other types of circulating hemocytes. For instance, degranulated hemocytes in their end stage only have leftover nuclei, which can be mistaken for hyalinocytes in some studies. Additionally, after phagocytosis, the morphology of hemocytes can significantly change, leading to a more granular appearance. Our study provides valuable insights into the physiological role of hemocytes in coagulation and phagocytosis during the early stages of an injury, helping to clarify uncertainties about the types and development of hemocytes in decapods.

Quantification of proteomic profile changes in the hemolymph of crayfish during *in vitro* coagulation

As previously discussed, coagulation plays a vital role in the innate immune response by preventing the loss of blood or hemolymph and limiting the spread of pathogens. The molecular characteristics of coagulation depend on the hemolymph proteins. In our study (chapter 4), we performed the first comparative proteome analysis of non-clotted and clotted hemolymph in the crayfish as a member of decapods.

The proteomic analysis of non-clotted and clotted samples indicated that the abundances of most proteins remained unchanged during the coagulation process, which suggests that the necessary proteins for coagulation are pre-synthesized and stored before clot formation. Crustaceans possess robust clotting mechanisms due to their open circulatory system, and clotting factors are pre-synthesized and stored in hemocyte granules and plasma, allowing for a rapid response to wounds. Clotting factors are released immediately upon injury, acting on clotting proteins in plasma, resulting in clot formation (Junkunlo et al., 2018, 2020). The synthesis of new proteins can be a time-consuming process, particularly in decapods with an open circulatory system where bleeding can be dangerous (Grubhoffer et al., 2013). Therefore, the quantities of only a few proteins, such as C-type lectin domain-containing proteins, Laminin A chain, and Tropomyosin, were down-regulated during clotting, while Reverse transcriptase domain-containing protein was significantly up-regulated.

Studies have shown that C-type lectins (CTLs) play a crucial role in the recognition and clearance of pathogens and hemagglutination in the immune system of crustaceans.

For instance, a recombinant lectin LvLec was found to enhance the phagocytic abilities of hemocytes, as well as phenoloxidase, bacteriolytic, and hemagglutinating activities in shrimp *Litopenaeus vannamei* (Li et al., 2022). Similarly, treatment with a C-type lectin designated as Fc-hsL in mud crab *Scylla paramamosain* led to increased antibacterial and calcium-dependent agglutination activity against both Gram-positive and Gram-negative bacteria (Sun et al., 2008). In addition, our earlier study on coagulation revealed that hemocytes undergo excessive degranulation, resulting in cell lysis similar to apoptosis (Mengal et al., 2023a). It is possible that the activation of apoptotic pathways in hemocytes may lead to the cleavage and degradation of C-type lectins or their associated signaling molecules. This could be one of the contributing factors to the downregulation of C-type lectins during coagulation.

The Laminin A chain is a crucial component of the extracellular matrix in various tissues. Studies have been conducted on laminin receptor proteins (Lamr) in crustaceans. In shrimp *P. vannamei,* RNAi-mediated knockdown of PvLamr resulted in 100% mortality within 9 days and a significant decrease in peripheral hemocyte numbers, although the mechanism behind the hemocyte reduction was unclear (Senapin et al., 2010). Our previous ultrastructural study of coagulation demonstrated that hemocytes undergo excessive degranulation, resulting in high levels of hemocyte lysis and cell death (Mengal et al., 2023a). This suggests that the downregulation of Laminin A chain during coagulation may be linked to the degranulation and cell lysis of hemocytes. Although the antimicrobial properties of laminin in crustaceans have been well documented, its role in coagulation and wound healing is less explored (Busayarat et al., 2011; Liu et al., 2016; Liu et al., 2018). It is plausible that the structural protein, Laminin, maintains cell integrity and retains granules within hemocytes. After an injury and necessity of clot formation, Laminin A chain is downregulated in hemocytes, degranulation is facilitated, and granule content is released from hemocytes, promoting clot formation.

Tropomyosin (Tmp) plays a crucial role in cell adhesion, migration, phagocytosis, and wound repair by regulating cytoskeletal proteins (Gunning et al., 2015). A mouse study found that Tmp can regulate the timing of cell migration and improve wound healing (Lees et al., 2013). Moreover, increased Tmp levels were observed in the hemolymph of *Mytilus galloprovincialis* mussels following injury (Franco-Martínez et al., 2018). However, our study revealed a significant down-regulation of Tmp, which could be due to the consumption and degranulation of hemocytes during the coagulation process.

The role of reverse transcriptase domain-containing protein (RTDP) in invertebrates is not well understood, but some studies suggest a potential role in the immune response (Flegel, 2009; Goic et al., 2013). Cellular reverse transcriptases have been linked to the activation of the RNA-mediated interference (RNAi) pathway in insects, inhibiting viral replication and suggesting a role in defense against viral infections (Goic et al., 2013). Shrimp may also use cellular RT to recognize foreign mRNA and produce immunospecific RNA capable of suppressing viral propagation (Flegel, 2009). Our study found an up-regulation of RTDP during clot formation. During coagulation, some hemocytes undergo rapid degranulation, releasing granular contents for clot formation, and subsequently undergo apoptosis. Meanwhile, another group of hemocytes retain their integrity and remain phagocytically active for an extended period, as previously noted in our ultrastructural study of coagulation and phagocytosis (Mengal et al., 2023a). Therefore, this protein only could be synthesized by phagocytic cells, the cells that retain their integrity and are still alive during *in vitro* coagulation, to enhance the phagocytosis needed as a defense mechanism in the place of wound. The significance of the up-regulation of RTDP during coagulation in crayfish requires further investigation.

Further research is needed to explore the functions of these differentially abundant proteins in the in coagulation and immune response of crustaceans. Overall, this proteomic analysis provides insights into the molecular mechanisms of hemolymph clotting and immunity in

decapod crustaceans and may have implications for the development of disease prevention and control strategies in aquaculture systems.

Proteomics analysis of biofluids, like crustacean hemolymph, often faces challenges due to the masking effect of high-abundant proteins on low-abundant counterparts (Otarigho et al., 2021). This interference can make it difficult to detect low-abundant proteins that may play important roles in biological processes. In crustaceans, hemocyanins, copper-containing proteins, can constitute up to 95% of the total protein content in hemolymph (Horn and Kerr, 1969; Jayasree, 2001). However, identifying low-abundance proteins remains a considerable challenge due to the overwhelming presence of hemocyanin proteoforms (Otarigho et al., 2021). This similar challenge persists in the proteomic analysis of other biological samples, such as serum and plasma, in humans and other mammalians (Paul and Veenstra, 2022).

Moreover, limited genomic resources for Crustacea present an obstacle. The Genome database (https://www.ncbi.nlm.nih.gov/genome/) lists 57 assemblies (410 for organelle DNA) for crustaceans, with no nuclear genome close to completeness for single-copy genes (Gianazza et al., 2021). Large repetitive genomes characterize many decapod crustaceans, complicating genomic analysis (Van Quyen et al., 2020). Consequently, de novo sequence data from MS/MS procedures often lack matching same-species genomic information. Researchers frequently resort to cross-species identification of homologous proteins using similarity searching databases like UniProt.

Additionally, working with crustacean proteomics is challenging due to the limited availability of optimized antibodies for validating proteomics data via western blot or immunohistochemistry. Another limitation of working with hemolymph is the high reactivity and instability of the cells when taken out of the animal or in vitro. To minimize this limitation, we used an *in situ* experimental approach to study cells at their biological spots inside the body. This approach resulted in high-quality samples. However, we faced a major limitation when using transmission electron microscopy (TEM) due to the hard exoskeleton of the crayfish, which made the processes of sample preparation and acquiring micrographs labor-intensive and time-consuming.

In conclusion, the role of hemocytes in the immune response of decapod crustaceans is crucial but still poorly understood. The classification of hemocyte types remains a matter of debate, and further research is needed to explore the process of hemocyte development in decapod crustaceans. Environmental stressors can negatively affect the immune response of decapods, making them more susceptible to diseases. The morphological changes that occur during coagulation and phagocytosis can alter hemocyte morphology. While degranulation during coagulation reduces the granularity of the hemocyte, the ingestion of tiny electron-dense particles from degenerating muscle tissues can make hemocytes more granular. It shows that functions such as phagocytosis can cause development of hemocytes with a higher granularity from a cell with lower granules, such as semi-granulocyte and hyalinocyte and can be considered a proof of the fact that different cells observed in the decapod hemolymph are actually different developmental stages of hemocytes. Also, morphological changes of hemocytes during coagulation and subsequent reduction of their granularity can cause confusion with other types of circulating hemocytes. Additionally, clotting proteins appear to be pre-synthesized and stored, enabling a swift response to injury in the form of coagulation. Although the abundance of most clotting proteins remained unchanged, the downregulation of structural proteins related to hemocyte integrity during coagulation could enhance hemocyte lysis and degranulation, leading to clot formation. The synthesis of a protein during coagulation may be related to the increased level of phagocytosis necessary after injury. Overall, our findings provide a better understanding of the physiological role of hemocytes in coagulation and phagocytosis during the early stages of injury, clarifying

uncertainties about the types and development of hemocytes in decapods. To sum up, this research highlights the following conclusions;

1. The role of hemocytes in the immune response of decapod crustaceans is crucial but still poorly understood. The classification of hemocyte types remains a matter of debate, and further research is needed to explore the process of hemocyte development in decapod crustaceans. Environmental stressors can negatively affect the immune response of decapods, making them more susceptible to diseases.
2. The granularity of immune cells can be affected by hemocytes' phagocytic and coagulation behavior, leading to changes in the number and size of granules in their cytoplasm. This alteration can cause a shift in hemocyte categories, e.g., from semigranulocyte to granulocyte.
3. Most proteins required for coagulation are pre-synthesized, while the downregulated proteins during *in vitro* coagulation primarily serve a structural role in maintaining cellular integrity, and their downregulation can facilitate hemocyte degranulation.

References

Busayarat, N., Senapin, S., Tonganunt, M., Phiwsaiya, K., Meemetta, W., Unajak, S., Jitrapakdee, S., Lo, C.-F., Phongdara, A., 2011. Shrimp laminin receptor binds with capsid proteins of two additional shrimp RNA viruses YHV and IMNV. Fish Shellfish Immunol. 31, 66–72.

Cerenius, L., Söderhäll, K., 2011. Coagulation in Invertebrates. J. Innate Immun. 3, 3–8.

Clare, A.S., Lumb G., 1994. Identification of haemocytes and their role in clotting in the blue crab, *Callinectes sapidus*. Mar. Biol. 118, 601–610.

Dall, W., 1964. Studies on the physiology of a shrimp, *Metapenaeus mastersii* (Haswell) (Crustacea: Decapoda: Peneidae). I. Blood constituents. Mar. Freshw. Res. 15, 145–161.

Dolar, A., Mayall, C., Drobne, D., Kokalj, A.J., 2020. Modulations of immune parameters caused by bacterial and viral infections in the terrestrial crustacean *Porcellio scaber*: Implications for potential markers in environmental research. Dev. Comp. Immunol. 113, 103789.

Dumont, J.N., Anderson, E., Winner, G., 1966. Some cytologic characteristics of the hemocytes of Limulus during clotting. J. Morphol. 119, 181–207.

Flegel, T.W., 2009. Hypothesis for heritable, anti-viral immunity in crustaceans and insects. Biology Direct 4, 32.

Franco-Martínez, L., Martínez-Subiela, S., Escribano, D., Schlosser, S., Nöbauer, K., Razzazi-Fazeli, E., Romero, D., Cerón, J.J., Tvarijonaviciute, A., 2018. Alterations in haemolymph proteome of *Mytilus galloprovincialis* mussel after an induced injury. Fish Shellfish Immunol. 75, 41–47.

Gianazza, E., Eberini, I., Palazzolo, L., Miller, I., 2021. Hemolymph proteins: An overview across marine arthropods and molluscs. J. Proteom. 245, 104294.

Giulianini, P.G., Bierti, M., Lorenzon, S., Battistella, S., Ferrero, E.A., 2007. Ultrastructural and functional characterization of circulating hemocytes from the freshwater crayfish *Astacus leptodactylus*: Cell types and their role after in vivo artificial non-self challenge. Micron. 38, 49–57.

Goic, B., Vodovar, N., Mondotte, J.A., Monot, C., Frangeul, L., Blanc, H., Gausson, V., Vera-Otarola, J., Cristofari, G., Saleh, M.-C., 2013. RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model *Drosophila*. Nat. Immunol. 14, 396–403.

Grubhoffer, L., Rudenko, N., Vancova, M., Golovchenko, M., Sterba, J., 2013. Circulatory system and hemolymph. Biology of Ticks, 2nd ed.; Sonenshine, DE, Roe, RM, Eds, 258–286.

Gunning, P.W., Hardeman, E.C., Lappalainen, P., Mulvihill, D.P., 2015. Tropomyosin – master regulator of actin filament function in the cytoskeleton. J. Cell Sci. 128, 2965–2974.

Horn, E.C., Kerr, M.S., 1969. The hemolymph proteins of the blue crab, *Callinectes sapidus—I. Hemocyanins* and certain other major protein constituents. Comparative Biochemistry and Physiology 29, 493–508.

Hose, J.E., Martin, G.G., Gerard, A.S., 1990. A Decapod Hemocyte Classification Scheme Integrating Morphology, Cytochemistry, and Function. Biological Bulletin 178, 33–45.

Jayasree, S., 2001. Biological properties of a natural agglutinin in the hemolymph of Indian white prawn, Penaeus indicus H. Milne Edwards. Aquaculture. 194, 245–252.

Jiang, L., Pan, L., Xiao, G., 2004. Effects of ammonia-N on immune parameters of white shrimp *Litopenaeus vannamei*. Zhongguo Shui Chan Ke Xue 11, 537–541.

Jiravanichpaisal, P., Lee, B.L., Söderhäll, K., 2006. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. Immunobiology. 211, 213–236.

Junkunlo, K., Söderhäll, K., Söderhäll, I., 2018. Clotting protein – An extracellular matrix (ECM) protein involved in crustacean hematopoiesis. Dev. Comp. Immunol. 78, 132–140.

Junkunlo, K., Söderhäll, K., Söderhäll, I., 2020. Transglutaminase 1 and 2 are localized in different blood cells in the freshwater crayfish *Pacifastacus leniusculus*. Fish Shellfish Immunol. 104, 83–91.

Kathyayani, S.A., Poornima, M., Sukumaran, S., Nagavel, A., Muralidhar, M., 2019. Effect of ammonia stress on immune variables of Pacific white shrimp *Penaeus vannamei* under varying levels of pH and susceptibility to white spot syndrome virus. Ecotoxicology and Environmental Safety 184, 109626.

Le Moullac, G., Haffner, P., 2000. Environmental factors affecting immune responses in Crustacea. Aquaculture. 191, 121–131.

Lees, J.G., Ching, Y.W., Adams, D.H., Bach, C.T.T., Samuel, M.S., Kee, A.J., Hardeman, E.C., Gunning, P., Cowin, A.J., O'Neill, G.M., 2013. Tropomyosin regulates cell migration during skin wound healing. J. Invest. Dermatol. 133, 1330–1339.

Li, F., Chang X., Xu, L., Yang, F., 2018. Different roles of crayfish hemocytes in the uptake of foreign particles. Fish Shellfish Immunol. 77, 112–119.

Li, F., Xu, L., Hui, X., Huang, W., Yang, F., 2019. Directed differentiation of granular cells from crayfish hematopoietic tissue cells. Fish Shellfish Immunol. 88, 28–35.

Li, F., Zheng, Z., Li, H., Fu, R., Xu, L., Yang, F., 2021. Crayfish hemocytes develop along the granular cell lineage. Sci. Rep. 11, 13099.

Li, Y., Pan, L., Yu, J., 2022. The injection of one recombinant C-type lectin (LvLec) induced the immune response of hemocytes in *Litopenaeus vannamei*. Fish Shellfish Immunol. 124, 324–331.

Liu, L.-k., Li, W.-d., Gao, Y., Chen, R.-y., Xie, X.-l., Hong, H., Wang, K.-j., Liu, H.-p., 2018. A laminin-receptor-like protein regulates white spot syndrome virus infection by binding to the viral envelope protein VP28 in red claw crayfish *Cherax quadricarinatus*. Dev. Comp. Immunol. 79, 186–194.

Liu, W.-J., Li, Y.-C., Kou, G.-H., Lo, C.-F., 2016. Laminin Receptor in Shrimp Is a Cellular Attachment Receptor for White Spot Syndrome Virus. PLoS One. 11, e0156375.

Mengal, K., Kor, G., Kouba, A., Kozák, P., Niksirat, H., 2023a. Hemocyte coagulation and phagocytic behavior in early stages of injury in crayfish (Arthropoda: Decapoda) affect their morphology. Dev. Comp. Immunol. 141, 104618.

Mengal, K., Kor, G., Kozák, P., Niksirat, H., 2023b. Effects of environmental factors on the cellular and molecular parameters of the immune system in decapods. Comp. Biochem. Physiol., A: Mol. Integr. Physiol. 276, 111332.

Otarigho, B., Falade, M., William, C.B., 2021. Insights from the shotgun-based proteomics analysis of *Biomphalaria glabrata*. Bioinformation 17, 266–273.

Paul, J., Veenstra, T.D., 2022. Separation of serum and plasma proteins for in-depth proteomic analysis. Separations 9, 89.

Perdomo-Morales, R., Montero-Alejo, V., Perera, E., 2019. The clotting system in decapod crustaceans: History, current knowledge and what we need to know beyond the models. Fish Shellfish Immunol. 84, 204–212.

Ratcliffe, N.A., Price, C.D., 1974. Correlation of light and electron microscopic hemocyte structue in the dictyoptera. J. Morphol. 144, 485–497.

Ravindranath, M.H., 1980. Haemocytes in haemolymph coagulation of arthropods. Biol. Rev. 55, 139–170.

Rebelo, M.d.F., Figueiredo, E.d.S., Mariante, R.M., Nóbrega, A., de Barros, C.M., Allodi, S., 2013. New insights from the oyster *Crassostrea rhizophorae* on bivalve circulating hemocytes. PLoS One. 8, e57384.

Rowley, A.F., 1977. The role of the haemocytes of *Clitumnus extradentatus* in haemolymph coagulation. Cell Tissue Res. 182, 513–524.

Rowley, A.F., Ratcliffe, N.A., 1976. The granular cells of *Galleria mellonella* during clotting and phagocytic reactions in vitro. Tissue and Cell 8, 437–446.

Scharrer, B., 1972. Cytophysiological features of hemocytes in cockroaches. Zeitschrift für Zellforschung und Mikroskopische Anatomie 129, 301–319.

Seitz, K., 1972. Zur Histologie und Feinstruktur des Herzens und der Hamocyten von *Cupiennius salei* Keys.(Aranea, Ctenidae). I. Herzwandung, Bildung und Differenzierung der Hamocyten. Zool Jahrbucher Abt Anat Ontog Tiere.

Senapin, S., Phiwsaiya, K., Anantasomboon, G., Sriphaijit, T., Browdy, C.L., Flegel, T.W., 2010. Knocking down a Taura syndrome virus (TSV) binding protein Lamr is lethal for the whiteleg shrimp *Penaeus vannamei*. Fish Shellfish Immunol. 29, 422–429.

Söderhäll, I., 2016. Crustacean hematopoiesis. Dev. Comp. Immunol. 58, 129–141.

Söderhäll, K., Smith, V.J., 1983. Separation of the haemocyte populations of *Carcinusmaenas* and other marine decapods, and prophenoloxidase distribution. Dev. Comp. Immunol. 7, 229–239.

Sun, Y.-D., Fu, L.-D., Jia, Y.-P., Du, X.-J., Wang, Q., Wang, Y.-H., Zhao, X.-F., Yu, X.-Q., Wang, J.-X., 2008. A hepatopancreas-specific C-type lectin from the Chinese shrimp *Fenneropenaeus chinensis* exhibits antimicrobial activity. Mol. Immunol. 45, 348–361.

Sung, H.H., Sun, R., 2002. Use of monoclonal antibodies to classify hemocyte subpopulations of tiger shrimp (*Penaeus monodon*). J. Crust. Biol. 22, 337–344.

Uhrík, B., Rýdlová, K., Zacharová, D., 1989. The roles of haemocytes during degeneration and regeneration of crayfish muscle fibres. Cell Tissue Res. 255, 443–449.

Vargas-Albores, F., Hinojosa-Baltazar, P., Portillo-Clark, G., Magallon-Barajas, F., 1998. Influence of temperature and salinity on the yellowleg shrimp, *Penaeus californieinsis* Holmes, prophenoloxidase system. Aquacult. Res. 29, 549–553.

Van Quyen, D., Gan, H.M., Lee, Y.P., Nguyen, D.D., Nguyen, T.H., Tran, X.T., Nguyen, V.S., Khang, D.D., Austin, C.M., 2020. Improved genomic resources for the black tiger prawn (*Penaeus monodon*). Marine Genomics 52, 100751.

Wood, P.J., Podlewski, J., Shenk, T.E., 1971. Cytochemical observations of hemolymph cells during coagulation in the crayfish, *Orconectes virilis*. J. Morphol. 134, 479–487.

English summary

The cellular and molecular characteristics of hemolymph in crayfish

The cellular and molecular components of the hemolymph are the major arm of the innate immune system in decapod crustaceans. In-depth knowledge of the hemolymph components, including hemocytes and hemolymph proteins, can enhance our understanding of innate immunity in crustaceans. We utilized transmission electron microscopy and quantitative proteomics to study the cellular and molecular aspects of coagulation and phagocytosis in the hemolymph.

Chapter 2 reviews the cellular and molecular parameters of the innate immune system and the effects of environmental stressors and their abiotic and biotic stress mechanisms in decapod crustaceans. The innate immune system of decapod crustaceans heavily relies on hemocytes in the circulating hemolymph. Generally, three types of hemocytes are accepted based on their morphology; however, there is still a lack of consensus among researchers regarding the classification of hemocyte types. The key innate immune functions such as coagulation and phagocytosis are still poorly understood and require further investigation, especially at a molecular level. Environmental stressors can adversely affect the immune responses of decapod crustaceans, increasing their susceptibility to diseases. However, the abiotic stress mechanism is poorly understood due to the lack of available literature and needs further investigation.

In Chapter 3, transmission electron microscopy was used to investigate the ultrastructural behavior of hemocytes during coagulation and phagocytosis in the early stages of leg amputation injury in marbled crayfish *Procambarus virginalis*. The granular hemocytes were the first to be activated, and the morphology of cytoplasmic granules changed from electron-dense to electron-lucent forms in an expanding manner. The transformed granules containing amorphous electron-lucent materials merged and discharged their contents into the extracellular space for coagulation. We observed that the leftover nucleus from degranulated hemocytes participates in the process of coagulation, which could be confused with hyalinocytes in some previous studies. In addition, leg amputation caused massive muscle degeneration, followed by a significant influx of phagocytic hemocytes that removed a substantial amount of muscle fibers and organelles, such as mitochondria, generated from disintegrating and decaying muscle. Furthermore, we found that phagocytic hemocytes contained varying numbers of granules in their cytoplasm and, for the first time, discovered that these cells incorporate necrotic bodies resulting from degenerated muscles into their organelles, such as cytoplasmic granules and nucleus. The granular hemocytes were found to be the main cells that carry out phagocytic activity in the injury site. This study provides a comprehensive description of all the stages of morphological changes in hemocytes during coagulation and phagocytosis after injury in crayfish for the first time.

In Chapter 4, proteomic analysis of non-clotted and clotted samples indicated that quantities of most proteins remained unchanged during the coagulation process, suggesting that necessary proteins for coagulation are pre-synthesized and stored before clot formation. Due to their open circulatory system, decapod crustaceans possess robust clotting mechanisms. Upon injury, pre-synthesized clotting factors are released, resulting in clot formation. Therefore, only a few proteins, such as C-type lectin domain-containing proteins, Laminin A chain, and Tropomyosin, were down-regulated during clotting, suggesting their possible roles in the structural integrity of cells. Their downregulation could facilitate degranulation, a crucial step for clot formation. Additionally, Reverse transcriptase domain-containing protein was significantly up-regulated during clotting, possibly by the alive phagocytic hemocytes, indicating its role in immunity.

Czech summary

Buněčné a molekulární charakteristiky hemolymfy u raků

Celulární a molekulární složky hemolymfy jsou hlavní součástí vrozeného imunitního systému u desetinožců. Hlubší znalosti složek hemolymfy, včetně hemocytů a proteinů hemolymfy, mohou zlepšit naše chápání vrozené imunity u korýšů. Pro studium celulárních a molekulárních aspektů koagulace a fagocytózy jsme využili transmisní elektronovou mikroskopii a kvantitativní proteomiku.

Kapitola 2 přehledně shrnuje celulární a molekulární parametry vrozeného imunitního systému a účinky environmentálních stresorů a jejich abiotických a biotických stresových mechanismů u desetinožců. Vrozený imunitní systém desetinožců se silně opírá o hemocyty. Obecně jsou přijímány tři typy hemocytů na základě jejich morfologie, avšak stále existuje nedostatek shody ohledně klasifikace typů hemocytů. Klíčové funkce vrozené imunity, jako jsou koagulace a fagocytóza, jsou stále špatně pochopeny a vyžadují další zkoumání zejména na molekulární úrovni. Environmentální stresory mohou negativně ovlivnit imunitní odpovědi desetinožců, čímž zvyšují jejich náchylnost k nemocem. Avšak abiotický stresový mechanismus je špatně pochopen kvůli nedostatku dostupné literatury a vyžaduje další zkoumání.

V kapitole 3 byla transmisní elektronová mikroskopie použita k prozkoumání ultrastrukturního chování hemocytů během koagulace a fagocytózy v raných stadiích zranění amputace nohy u raka mramorovaného *Procambarus virginalis*. Granulární hemocyty byly aktivovány jako první a morfologie cytoplazmatických granulí se změnila z elektronově hustých na elektronově průsvitné formy v expanzivním způsobu. Transformované granule obsahující amorfní elektronově průsvitné materiály se sloučily a vypustily svůj obsah do extracelulárního prostoru pro koagulaci. Pozorovali jsme, že zbývající jádro z degranulovaných hemocytů se podílí na procesu koagulace, což by mohlo být v některých předchozích studiích zaměněno s hyalinocyty. Kromě toho amputace nohy způsobila masivní degeneraci svalů, následovanou významným přílivem fagocytických hemocytů, které odstranily značné množství svalových vláken a organel, jako jsou mitochondrie, generované z rozpadajících se a rozkládajících se svalů. Dále jsme zjistili, že fagocytické hemocyty obsahují různé počty granulí v cytoplazmě a poprvé jsme objevili, že tyto buňky začleňují nekrotická tělíska vzniklá z degenerovaných svalů do svých organel, jako jsou cytoplazmatické granule a jádro. Granulární hemocyty byly nalezeny jako hlavní buňky provádějící fagocytickou aktivitu na místě zranění. Tato studie poskytuje poprvé komplexní popis všech stadií morfologických změn hemocytů během koagulace a fagocytózy po zranění u raků.

V kapitole 4 proteomická analýza sražených a nesražených vzorků ukázala, že množství většiny proteinů zůstalo během koagulačního procesu nezměněno, což naznačuje, že nezbytné proteiny pro koagulaci jsou předem syntetizovány a uloženy před vznikem sraženiny. Díky svému otevřenému oběhovému systému mají desetinožci robustní koagulační mechanismy. Po zranění jsou uvolněny předem syntetizované koagulační faktory, což vede ke vzniku sraženiny. Proto byly pouze některé proteiny, jako jsou proteiny obsahující domény C-typu lektinu, laminin A řetězec a tropomyosin, během srážení sníženy, což naznačuje jejich možné role ve strukturní integritě buněk. Jejich snížení by mohlo usnadnit degranulaci, klíčový krok pro vznik sraženiny. Navíc protein obsahující doménu reverzní transkriptázy byl během srážení významně zvýšen, možná živými fagocytickými hemocyty, což naznačuje jeho roli v imunitě.

Acknowledgments

I would like to express my sincere appreciation to my supervisor, Dr. Hamid Niksirat Hashjin, for his unwavering support, guidance, patience, encouragement, vast knowledge, constructive criticism, insightful advice, and prompt feedback during my research. His visionary guidance in both academic research and personal life has been invaluable to me, and it was an honor to be his student. His youthful spirit, active behavior, and open-mindedness have inspired me in my studies and life. I will always remember and appreciate his tireless efforts to help me fulfill my Ph.D. experience.

I am also grateful to Dr. Fredrik Levander, the head of the Department of Immuntechnology at Lund University, Sweden, and Dr. Valentina Siino for their valuable contributions to my research. I would also like to acknowledge the staff of the Laboratory of Electron Microscopy at the Institute of Parasitology, Biology Center of ASCR, for their valuable contributions. My colleague, Golara Kor, deserves special thanks for her contribution to the experimental activities.

Furthermore, I would like to thank Prof. Pavel Kozák, the Dean of the Faculty of Fisheries and Protection of Waters in Vodňany, for his insightful advice and prompt feedback during my research. The head of the lab, Dr. Miloš Buřič, also deserves recognition for his insightful comments and financial support for the studies conducted. Dr. Antonín Kouba, the Vice Dean for Science and Research, provided excellent support and feedback during my PhD study. I would also like to acknowledge Ms. Lucie Kačerová for her academic support and all my colleagues at the Laboratory of Freshwater Ecosystems and other laboratories of the Faculty of Fisheries and Protection of Waters in Vodňany who assisted me with my experiments and lab work.

Lastly, I want to express my gratitude to my parents and entire family for their love, concern, unconditional spiritual support, and strength throughout my life and studies.

This research would not have been possible without the financial support from the following projects: the Grant Agency of the University of South Bohemia (GAJU) projects No. 065/2022/Z, 055/2022/Z, and 012/2022/Z.

List of publications

Peer-reviewed journals with IF

Kor, G., **Mengal, K.,** Buřič, M., Kozák, P., Niksirat, H., 2023. Comparative ultrastructure of the antennae and sensory hairs in six species of crayfish. PeerJ 11, e15006. (IF 2022 = 2.7, AIS 2022 = 0.709)

Kor, G., **Mengal, K.,** Buřič, M., Kozák, P., Niksirat, H., 2023. Granules of immune cells are the source of organelles in the regenerated nerves of crayfish antennae. Fish & Shellfish Immunology 137, p.108787. (IF 2022 = 4.700, AIS 2022 = 0.605)

Mengal, K., Kor, G., Kouba, A., Kozák, P., Niksirat, H., 2023. Hemocyte coagulation and phagocytic behavior in early stages of injury in crayfish (Arthropoda: Decapoda) affect their morphology. Developmental & Comparative Immunology 141, p.104618. (IF 2022 = 2.9, AIS 2022 = 0.548)

Mengal, K., Kor, G, Kozák, P., Niksirat, H., 2023. Effects of environmental factors on the cellular and molecular parameters of the immune system in decapods. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 276, p.111332. (IF 2022 = 2.3; AIS 2022 = 0.490)

Mengal, K., Kor, G., Kozák, P., Niksirat, H., 2023. Heat shock proteins adaptive responses to environmental stressors and implications in health management of decapods. Aquaculture Reports 30, 101564. (IF 2022 = 3.7, AIS = 0.560)

Mengal, K., Kor, G., Siino, V., Buřič, M., Kozák, P., Levander, F., Niksirat, H., 2023. Quantification of proteomic profile changes in the hemolymph of crayfish during *in vitro* coagulation. Developmental & Comparative Immunology, p.104760. (IF 2022 = 2.9, AIS 2022 = 0.548)

Shabbir, S., Boruah, P., Kulyar, M.F.E.A., Bhutta, Z.A., Nawaz, M., Ashar, A., Mahfooz, A., Khan, M.S., Miao, X., Jabeen, F., **Mengal, K.,** 2023. Nephroprotective effect of *Cinnamon cassia* and *Azadirachta indica* on titanium dioxide nanoparticles. Current Nanoscience 19, 291–303. (IF 2022 = 1.5, AIS 2022 = 0.183)

Babar, A., **Mengal, K.,** Babar, A.H., Wu, S., Shah, M.A., Xu, C., Luo, X., Cai, X., 2021. High altitude hypoxia. Current Proteomics 18, 447–457. (IF 2020 = 0.837; AIS = 0.136)

Tian, H., Liu, J., Chen, X., Li, S., Li, X., **Mengal, K.,** Lu, Y., Wang, D., 2021. Effects of ambient temperature and humidity on body temperature and activity of heifers, and a novel idea of heat stress monitoring. Animal Production Science 61, 1584–1591. (IF 2020 = 1.570, AIS = 0.297)

Babar, A., Mipam, T.D., Wu, S., Xu, C., Shah, M.A., **Mengal, K.,** Yi, C., Luo, H., Zhao, W., Cai, X., Luo, X., 2019. Comparative iTRAQ proteomics identified myocardium proteins associated with hypoxia of Yak. Current Proteomics 16, 314–329. (IF 2018 = 0.768, AIS 2018 = 0.107)

Zhao, W., **Mengal, K.,** Yuan, M., Quansah, E., Li, P., Wu, S., Xu, C., Yi, C., Cai, X., 2019. Comparative RNA-Seq analysis of differentially expressed genes in the epididymides of Yak and cattleyak. Current Genomics 20, 293–305. (IF 2018 = 2.174, AIS 2018 = 0.746)

Zhao, W., Quansah, E., Yuan, M., Gou, Q., **Mengal, K.,** Li, P., Wu, S., Xu, C., Yi, C., Cai, X., 2019. Region-specific gene expression in the epididymis of Yak. Theriogenology 139, 132–146. (IF 2018 = 2.299, AIS 2018 = 0.495)

Abstracts and conference proceedings

Kor, G., **Mengal, K.,** Buřič, M., Niksirat, H., 2022. Ultrastructural and biometrical features of the antenna in six crayfish species. 23rd Symposium of the International Association of Astacology, June 20–25, Hluboká nad Vltavou, Czech Republic.

Kor, G., **Mengal., K.,** Kozák, P., Niksirat, H., 2022. Nerve regeneration in crayfish. NEOBIOTA 2022, 12th International Conference on Biological Invasions. Tartu, Estonia, 12–16 September 2022.

Mengal., K., Kor, G., Kozák, P., Niksirat, H., 2022. Effects of temperature and limb amputation level on limb regeneration parameters in marbled crayfish. 23rd Symposium of the International Association of Astacology, June 20–25, Hluboká nad Vltavou, Czech Republic.

Mengal., K., Kor, G., Kozák, P., Niksirat, H., 2022. Morphological features of hemocytes during early stages of injury in crayfish. NEOBIOTA 2022, 12th International Conference on Biological Invasions. Tartu, Estonia, 12–16 September 2022.

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Seminar days of RIFCH and FFPW	2019
	2020
	2021
	2022

International conferences	**Year**
Mengal, K., Kor, G., Kozák, P., Niksirat, H., 2022. Morphological features of hemocytes during early stages of injury in crayfish. NEOBIOTA 2022, 12th International Conference on Biological Invasions. Tartu, Estonia, 12–16 September 2022. **Mengal, K.,** Kor, G., Kozák, P., Niksirat, H., 2022. Effects of temperature and limb amputation level on limb regeneration parameters in marbled crayfish. 23rd Symposium of the International Association of Astacology, June 20–25, Hluboká nad Vltavou, Czech Republic. Kor, G., **Mengal., K.,** Kozák, P., Niksirat, H., 2022. Nerve regeneration in crayfish. NEOBIOTA 2022, 12th International Conference on Biological Invasions. Tartu, Estonia, 12–16 September 2022. Kor, G., **Mengal, K.,** Buřič, M., Niksirat, H., 2022. Ultrastructural and biometrical features of the antenna in six crayfish species. 23rd Symposium of the International Association of Astacology, June 20–25, Hluboká nad Vltavou, Czech Republic.	2022

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