Crustacean hematopoiesis

Review by

Irene Söderhäll

Department of Comparative Physiology,

Uppsala University, Norbyvägen 18 A, 752 36 Uppsala, Sweden.

Email: Irene.Soderhall@ebc.uu.se

Abstract

Crustacean hemocytes are important mediators of immune reactions, and the regulation of hemocyte homeostasis is of utmost importance for the health of these animals. This review discusses the current knowledge on the lineages, synthesis and differentiation of hemocytes in crustaceans. Hematopoietic tissues, their origins, and the regulation of hematopoiesis during molting, seasonal variation and infection are discussed. Furthermore, studies concerning the molecular regulation of hemocyte formation in crustaceans are also described, and the different lineages and their molecular markers are discussed and compared with several insect species. Signaling pathways and the regulation of hematopoiesis by transcription factors are typically conserved among these arthropods, whereas cytokines and growth factors are more variable and species specific. However, considering the great diversity among the crustaceans, one should be cautious in drawing general conclusions from studies of only a few species.

1. Introduction

Crustaceans form an extremely diverse group, comprising between 40,000 and 60,000 species, and constitute a large proportion of the biomass in the oceans (Giribet and Edgecombe, 2012). The blood cells, or hemocytes, of crustaceans are important players in immune reactions, and these cells contain important immune proteins, such as the components of the prophenoloxidase-activating system (proPO) in the melanization cascade, and they can perform cellular reactions, such as the phagocytosis of small foreign objects or encapsulation of larger intruders. A large number of original studies regarding crustaceans and invertebrate innate immunity in general have been conducted in the freshwater crayfish *Pacifastacus leniusculus*; among their findings, the proteolytic cascade that controls melanization is one of the most important (Cerenius et al., 2010, 2008; Jiravanichpaisal et al., 2006). The proPO system is observed in granular cells (GCs), which function in the circulatory systems of crayfish. GCs and other hemocytes are produced throughout the life of the animal in a specific hemocyte-forming tissue called hematopoietic tissue (HPT) (Lin and Söderhäll, 2011a). When a parasite or foreign object enters the hemocoel (body cavity), certain hemocyte types perform phagocytosis and encapsulate these objects. Because hemocytes are consumed in these processes, there is the need for the rapid synthesis of several new hemocytes during infection. However, the production of hemocytes during normal non-infectious conditions is also needed. Freshwater crayfish belong to the decapods, and among the Crustacea, this order is the most studied with regard to immunology and hematopoiesis. Indeed, only a few species from other groups have been investigated, and none in great detail. Previous studies concerning hematopoiesis in crustaceans other than decapods are summarized in Table 1 (phylogeny simplified from Meland and Willassen, 2007). However, it is noteworthy that the phylogenetic relationships among different crustacean types are controversial and have been revised several times during the past decades; the difficulties in robustly clarifying ancient

relationships within the crustaceans have been discussed in detail (Rota-Stabelli et al., 2013). Thus, the aim of the following review is to provide an overview of the current knowledge about hematopoiesis in crustaceans. Notably, there is significant diversity among the species in the crustacean subphylum, and the limited studies have mainly focused on the economically important decapods. Therefore, general conclusions concerning the entire decapod group should be drawn with great care because studies in a few species may not represent the extensive collection of available crustacean species.

2. Structure of the circulatory system

According to most textbooks, crustaceans have an open circulatory system. However, recent studies have clearly shown that the degree of vascular branching markedly varies among species, and well-developed cardiovascular systems in decapods have occasionally been demonstrated. Thus, the crustacean circulatory system is more accurately described as "incompletely closed" (McGaw and Stillman, 2010; Reiber and McGaw, 2009). The crustacean heart is shaped like a pentagonal chamber, placed dorsally above the gills, and surrounded by a pericardium from which the hemolymph is transported through segmentally paired, laterally arranged ostia with valves into the heart. In less developed crustaceans, such as entomostracans and lower malacostracans, the heart is more primitive and is simply a long, expanded artery with ostia. During embryogenesis, the heart develops from a dorsal vessel. Hemolymph is transported from the heart anterially by non-muscular arteries that penetrate the pericardial membrane and branch into different parts of the head, ultimately delivering the hemolymph to sinuses throughout the entire body. All the sinuses are circumscribed by a membrane and lead the hemolymph to the gills via large ventral sinuses. Efferent arteries then leave the gills and bring oxygenated hemocyanin back to the heart through the ostia (Bauchau, 1981). The decapod crustaceans have a well-developed vascular system with

arterioles penetrating the organs in the body (McGaw and Stillman, 2010). Although the fine arteries are blind ended, the sinuses, at least in some decapods, seem to be defined units formed as discrete but large channels delineated by fibrous connective tissue, making the circulatory system partially closed (McGaw, 2005). However, unlike vertebrates, crustaceans have no separate lymphatic system, and the main transport is provided through a single bodily fluid, the hemolymph. Therefore, the liquid in the crustacean circulatory system is not typically referred to as blood because it is a combination of blood (hemo or haemo [Latin]) and the colorless fluid of the body (lympha – water or clear water [Latin]).

In crustaceans, hemolymph comprises cells, water, and dissolved inorganic salts and proteins, among which the oxygen carrier hemocyanin is the most abundant. The free cells found in hemolymph are accordingly named hemocytes (Johnson, 1980). Thus, hemocytes are the free-moving inhabitants of the circulatory system, which circulate into the smallest arterioles in the brain and penetrate into the tissues surrounded by the sinuses, where they perform their tasks as mediators of immune defense and other functions.

3. Crustacean hemocytes

3.1. Morphology

Carus initially characterized crustacean hemocytes in the early 1800s (*Carus, C. G. Von den aussern Lebensbedingungen der weiss- und kalt-blutigen Thiere. Leipzig, 1824, pp.85-86*), and Bauchau (1981) previously reviewed this and other studies. Hemocytes are divided into different types based primarily on morphological criteria and secondarily on functional properties. Hemocyte types have been described and classified mainly for the decapods. Classifications according to morphology, ultrastructural studies, and staining properties were conducted in several species during the second half of the 1900s (Hose et al., 1990, 1987, 1987; Johansson et al., 2000; Martin et al., 1999; Toney, 1958; Vázquez et al., 1997; Wood

and Visentin, 1967). Bauchau (1981) suggested a classification scheme in decapod crustaceans, dividing the cells into hyaline, semigranular and granular hemocytes, and this classification is used in the following text (Table 2, rewritten with permission from Bauchau, 1981).

Hemocytes have also been described in other crustacean species, although most studies have focused on the decapods. For example, studies in the branchiopod *Artemia* (the brine shrimp) have shown that this species only possesses granular hemocytes (Lochhead and Lochhead, 1941; Martin et al., 1999). Hemocytes in *Artemia* show similarities to amebocytes in primitive chelicerates, such as *Limulus polyphemus* and *Tachypleus* spp., except for the expression of proPO in Artemia hemocytes (Martin et al., 1999). Detailed studies of the cytoskeletons of *Artemia* hemocytes have also been reported, and these hemocytes have been implicated in phagocytosis in these animals (Day et al., 2000).

In decapods, GCs are the largest cell type, usually with a kidney-shaped nucleus and cytoplasm filled with membrane-bound, electron-dense granules. GCs have variable staining properties, and some cells are eosinophilic, whereas others stain less or faintly basophilic (Bauchau, 1981). Whether this differential staining reflects different developmental stages or some functional variability remains unknown, and the proportion varies with different environmental conditions.

Semigranular cells (SGCs) are smaller, typically possessing a larger central nucleus that is surrounded by cytoplasmic granules of different sizes and morphologies. SGCs are occasionally eosinophilic, but typically show faint staining.

Hyaline cells (HCs), are diverse, containing no or few granules, as in *P. interruptus*, to several small granules, as in *Homarus americanus* (Hose et al., 1990). The nature of HCs has been debated; these cells may be immature or prematurely released prohemocytes of the SGC or GC lineage, as suggested (van de Braak et al., 2002) for *P. monodon*. This view is further

substantiated by our studies in *P. leniusculus* (Lin and Söderhäll, 2011b) and might support previous studies in the swimming crab (Hammond and Smith, 2002). In short, crustacean hemocytes are typically classified into three major classes, GCs, SGCs and HCs, based on cytoplasmic granularity, staining properties, density and nuclear size.

3.2. Function

Due to the sensitivity and reactivity of crustacean hemocytes, functional studies have been difficult to perform because coagulation reactions and uncontrolled cell activation regularly occur when mixed hemocyte samples are investigated *in vitro* (Smith and Söderhäll, 1983). Rapid fixation might facilitate morphological studies, but these findings do not provide an understanding of cell function. Thus, conclusions about the functions of different hemocyte types were hampered until a method of cell separation was developed in 1983 (Söderhäll and Smith, 1983). To obtain functional populations of the different cell types, cells were collected in low-pH, anticoagulant-containing citrate EDTA, followed by separation on a Percoll gradient. This methodological breakthrough enabled detailed studies of various hemocyte characteristics, leading to the identification of several proteins that are primarily important for cellular immunological reactions. For example, the proPO-activating system was shown to reside primarily in the granular hemocytes of *Carcinus maenas*, *Cancer pagurus, Macropipus depurator* and *Eupagurus bernhardus* and, to a minor extent, in semigranular hemocytes (Söderhäll and Smith, 1983; for reviews of the ProPO-activating system, see Cerenius et al., 2008; Cerenius and Söderhäll, 2004). Previous findings, such as the identification of hemocyanin immunoreactivity in *C. maenas* hemocytes (Ghiretti-Magaldi et al., 1973), most likely reflected the fact that the purified hemocyanin from the hemolymph of the crab was contaminated with ProPO, which has a similar molecular mass as hemocyanin. Moreover, cell separation techniques facilitated the isolation of a cell adhesion protein, peroxinectin, with

similarities to vertebrate myeloperoxidases (Johansson et al., 1997, 1995; Johansson and Söderhäll, 1988). Peroxinectin contains a cell adhesion integrin-binding motif at the N-terminus and a peroxidase domain at the C-terminus. This protein is synthesized as a propeptide that is cleaved by activation of the proPO-activating enzyme (Lin et al., 2007); upon release, the cleaved form mediates phagocytosis and encapsulation reactions (Johansson et al., 2000; Thörnqvist et al., 1994). During encapsulation, hemocytes adhere to and embed large foreign objects, e.g., fungal hyphae, thereby preventing the spread of infection. In *P. leniusculus*, this reaction is primarily performed by SGCs (Kobayashi et al., 1990).

The roles of the different crustacean hemocyte types in phagocytosis have been debated, and data from different species are different. For example, small and large GCs have been demonstrated to be phagocytotic in *H. americanus, P. interruptus* and *Loxorhynchus grandis* (Hose et al., 1990), whereas HCs in *Carcinus maenas* have been demonstrated to be phagocytotic after coating bacteria with peroxinectin, indicating cell-to-cell cooperation (Smith and Ratcliffe, 1980; Thörnqvist et al., 1994). In, contrast, GCs were found to be phagocytotic in shrimp (Gargioni and Barracco, 1998; Vázquez et al., 1997).

In recent years, RNA interference (RNAi) technology has been developed to help investigate the roles of different proteins in hemocyte immune functions (Maningas et al., 2008; Shockey et al., 2009). Although these experiments are primarily performed *in vivo* and do not provide information about the specific functions of different hemocyte types, these studies have clarified the roles of some proteins in hemocyte functions, such as phagocytosis (Han et al., 2010; Wang et al., 2014), coagulation (Fagutao et al., 2012; Maningas et al., 2008) and viral defense (Li et al., 2015; Visetnan et al., 2015)). These techniques have also been successfully used to confirm the important role of the proPO-activating system as an early defense system (Amparyup et al., 2009; Charoensapsri et al., 2009; Fagutao et al., 2009; Jang et al., 2011). In

summary, the important roles of hemocytes in immune defense have been clarified in the past decades using *in vitro* studies of separated cells and RNAi technology.

3.3. Molecular markers

In humans, the cluster of differentiation (CD) antigens have been successfully used to characterize the functions of different leukocyte types, and monoclonal antibodies against these cell surface proteins and the development of a common nomenclature system have been of vital importance for the development of human/vertebrate immunology research since the 1980s. There were similar attempts in the 1990s to characterize hemocyte types in crustaceans using monoclonal antibodies (Rodriguez et al., 1995). However, no great success in the characterization of cell surface marker proteins for different hemocyte types has been achieved thus far, which likely reflects difficulties in obtaining hemocytes free of contaminating plasma proteins. Crustacean plasma contains an abundance of the oxygen-carrying protein hemocyanin (> 95% of the proteins in plasma), and this protein easily adheres to hemocyte surfaces, thereby facilitating the nonspecific binding of other plasma proteins to cell surfaces. This finding has been clearly illustrated in experiments in *P. japonicus*, in which monoclonal antibodies raised against hemolymph were shown to react with both hemocytes and plasma (Rodriguez et al., 1995). Since then, different approaches have been adopted to identify specific proteins that are exclusively expressed by specific hemocyte types. Techniques for examining mRNA expression in separated cell types, such as in situ hybridization and RT-PCR, have been used to show that prophenoloxidase is primarily expressed in granular hemocytes and, to some extent, in SGCs (Johansson et al., 2000; Söderhäll et al., 2003a). These findings confirmed a 1983 study that produced similar results using enzymatic activity (Söderhäll and Smith, 1983). In situ hybridization was also used to show that the RUNX family transcription factor PlRunt is expressed in mature SGCs and

GCs, whereas less-differentiated HPT cells do not express this transcription factor. However, the expression of PlRunt was induced soon after the injection of laminarin or lipopolysaccharide (LPS) to induce maturation and the release of new hemocytes (Söderhäll et al., 2003a). PlRunt is a member of the RUNX family of transcription factors, which are important for hematopoiesis throughout the animal kingdom, from fruit flies to humans (Wang et al., 2010). Interestingly, PlRunt, referred to as *lozenge* in *Drosophila melanogaster*, has been shown to regulate the expression of prophenoloxidase (Ferjoux et al., 2007; Zou et al., 2008). Similarly, PlRunt is expressed in proPO-expressing hemocytes in crayfish.

To identify additional specific marker proteins for different hemocyte types and develop tools to define the differentiation pathways of these cell types, we performed a proteomics analysis of SGCs and GCs. Using 2D gel electrophoresis, we detected proteins that are specifically expressed in these different hemocyte types (Wu et al., 2008). In granular hemocytes, we detected the specific mRNA expression of superoxide dismutase (SOD) (Wu et al., 2008). Notably, although immunohistochemistry has shown that SOD is present in SGCs and GCs (Johansson et al., 1999), studies of SOD mRNA expression have clearly demonstrated that the transcript is present only in GCs (Wu et al., 2008). SOD is an extracellular enzyme produced by and secreted from GCs as a monomer; however, it subsequently attaches to the outside of SGCs as a dimeric protein (personal observation). These findings clearly showed that immunohistochemistry is not completely reliable for the identification of proteins specific for certain cell types. Subsequently, we identified a mannan-binding lectin specifically expressed in GCs (Wu et al., 2013).

A Kazal-type proteinase inhibitor (KPI) with an unusual structure was found to be specifically expressed in SGCs (Wu et al., 2008). Crustacean hemocytes contain larger numbers of antimicrobial peptides and proteinase inhibitors, particularly of the Kazal type (Rimphanitchayakit and Tassanakajon, 2010). These inhibitors are characterized by one or

several Kazal domains containing six cysteines, forming three intra-domain disulfide bridges (Cerenius et al., 2010). Interestingly, the SGC-specific KPI has an unusual structure with a glycine at the P2 position, and the amino acid at this position has been implicated in interactions between the inhibited proteinase and KPI (Donpudsa et al., 2010). To date, the target proteinase for the SGC-specific KPI is not known. Another protein specific for the SGC lineage is crustacean hematopoietic factor (CHF) (Lin et al., 2011). Only a few proteins specific for different hemocyte types have been identified. However, some of the proteins detected in *P. leniusculus* have been used to identify different differentiation pathways, showing the importance of such "marker" proteins for understanding how hematopoiesis is regulated (Lin et al., 2010; Lin and Söderhäll, 2011b; Wu et al., 2008).

4. Crustacean hematopoietic tissues

4.1. Hematopoietic tissue (HPT)

Hemocyte formation and the tissues in which these cells are formed have been studied since the early 1800s. However, some of these old studies may have been forgotten. The HPT in crustaceans has been assigned several names throughout history, such as lymphogenic, lymphocytogenic, leukopoietic and globulinogenic tissue (Johnson, 1980).

Edgar J. Allen, working at the Plymouth Laboratory of the Marine Biological Association in 1892, made one of the earliest descriptions of this tissue in studies of the brackish water prawn *Palaemonetes varians* (Allen, 1893). This work was followed by Cuenot's studies of *Homarus, Palinurus, Eupagarus, Carcinus,* and *Macropipus* in 1893 (Johnson, 1980). Bruntz examined other crustaceans, identifying hematopoietic organs at various sites in different Eumalacostrata species (Bruntz, 1907). In Isopoda, lobules were described inside the connective tissue in the last 2 thoracic and abdominal segments; in Amphipoda, bilateral lobules were identified in the head; in Stomatopoda, lobules were detected at the ventral

artery surface; and in Cumacea, bilateral lobules were detected at the fifth thoracic segment (Bruntz, 1907). In *Artemia,* HPT was identified bilaterally at the base of each limb (Lochhead and Lochhead, 1941).

The structure of HPT was described in detail in a recent study of the terrestrial isopod *Armadillidium vulgare* (Chevalier et al., 2011). *Armadillidium* HPT was described as small circular organs wrapped in connective tissue, with cells at different maturation stages radially developing from the least-differentiated cell in the center of the circle. Dividing cells were detected in the central area, and more-differentiated cells and cells with granules were more abundant in the external parts of the tissues (Chevalier et al., 2011).

However, most studies have been conducted in decapod crustaceans. Studies of decapod crustaceans have shown that the HPT is located adjacent to or enveloping the ophthalmic artery (or the dorsal median artery) and on the dorsal and/or lateral sides of the cardiac stomach (Charmant, 1973; Ghiretti-Magaldi et al., 1977; Johnson, 1980; Martin et al., 1987). In shrimp, the organization of the HPT may be slightly different. For example, in *Sicyonia ingentis*, the HPT comprises paired epigastric lobules heavily penetrated by branched vessels (Hose et al., 1992). However, in shrimp, there are some reports that HPTs seems to mix with the lymphoid organ (also named as OKA organ), which has been demonstrated to be a filtering and phagocytic organ.

Crustacean HPT generally comprises lobules with hemocyte precursors at different stages of differentiation randomly scattered in layers of connective tissue. These lobules are typically less than 10 cells deep and are covered with fibrous connective tissue at the apical ends, whereas the connective tissue below the lobules is spongy. The lobes are separated from the hemal space by a basal lamina that contains collagen IV (Johnson, 1980). Paired epigastric HPT is present in penaeid shrimps, (Martin et al., 1987), and some species might have

ancillary sites of HPT surrounding the antennal artery and at the base of the maxillipeds (van de Braak et al., 2002). However, there are large variations in lobule morphology between animals and within tissues, making comparisons difficult (van de Braak et al., 2002).

The HPT of *Carcinus* was identified as a cup-shaped, lymphatic gland-like structure (lobule) located at the dorsal side of the stomach. Each lobule is surrounded by a membrane with one open end, and mitosis frequently occurs in the lobules (Ghiretti-Magaldi et al., 1977). The cells in the lobules are small with large nuclei, similar to those described in *P. leniusculus* (Chaga et al., 1995). Ghiretti-Magaldi and coworkers also reported that within lobules, the plasma membranes of adjacent cells often form junctions (Ghiretti-Magaldi et al., 1977). In general, the HPTs in crustacean species studied thus far are similar in having a dorsal location surrounding the ophthalmic artery, with a structure of thin sheets of highly packed cells in lobules surrounded by connective tissue.

4.2. The anterior proliferation center (APC) is an important part of HPT

Allen provided one of the earliest descriptions of a blood-forming organ in *P. varians,* where he described a cephalic aorta surrounded on each side by cells that give rise to blood corpuscles in the area where the aorta bends toward the brain (Figure 1, republished with permission from Allen, 1893).

In 1979, Manfred Gersch reported a new endocrine "cephalic gland" situated between the stomach muscles and the brain in two crayfish species, *Astacus* and *Orconectes limosus* (Gersch, 1979),. The gland was attached to the connective tissue sheets covering the brain (Gersch, 1979). Gersch also described the cells in this gland as having large nuclei surrounded by a thin rim of cytoplasm, and mitosis was frequently observed within the gland. At that time, the cephalic gland was thought to regulate the molting process, based on observations of changes in nuclear size and shape, cytoplasmic staining at different molting stages (Gersch,

1979; Gersch and Birkenbeil, 1979), and the production of ecdysteroids by the gland (Gersch et al., 1979); the latter result was subsequently reinvestigated and modified (Böhm and Gersch, 1983). In this 1983 paper, detailed studies of the previously reported "cephalic gland" were reexamined, and the structure was demonstrated to be a part of the HPT, containing primarily prohemocytes developing into semigranular hemocyte types according to the classification of Bauchau (1981) (Böhm and Gersch, 1983).

The organ described by Allen in 1893 and the former "cephalic gland" were "rediscovered" in 2012 in *P. leniusculus* and named the anterior proliferation center (APC) (Noonin et al., 2012a). This APC was subsequently confirmed in *P. clarkii* (Chaves da Silva et al., 2013). This center of highly proliferating cells is located at the anterior part of the HPT near the brain (Figure 2). The cells in the APC are similarly arranged in lobules, but they constitute a more homogeneous cell population of primarily undifferentiated cells (personal observation). More than twice the number of BrdU-incorporating cells were identified in the APC compared with the posterior parts of the HPT, and this APC area was further shown to have high reactive oxygen species (ROS) production, which is clearly associated with proliferation (Chaves da Silva et al., 2013; Noonin et al., 2012a). The importance of a certain level of ROS production during differentiation has been discussed, and specific roles in regulating stem cell activity in hematopoietic stem cell niches have been shown in mammals (Simon and Keith, 2008). Moreover, detailed studies in *Drosophila* have revealed an important role for ROS production in the induction of lamellocyte differentiation (Owusu-Ansah and Banerjee, 2009; Sinenko et al., 2012). In *P. leniusculus*, ROS production is primarily regulated in the APC, and recent experiments have shown that this ROS production is associated with differentiation, affecting the structure of the extracellular matrix surrounding the hematopoietic precursors (Junkunlo, 2015). A specific, highly proliferating area of the HPT has been described as the APC in *P. leniusculus* and *P. clarkii* (Chaves da Silva et al., 2013;

Noonin et al., 2012b), as the cephalic gland in *Astacus* and *Orconectes limosus* (Böhm and Gersch, 1983), and as the blood-forming organ in *P. varians* (Allen, 1893). However, it is likely that similar structures are also present in other species.

4.3. Cell types in HPT

Decapod crustacean HPTs contain 4-5 different cell types that are distinguished based on morphological criteria obtained through transmission electron microscopy (TEM). Two examples of such studies have been performed in the blue crab, *C. sapidus*, (Johnson, 1980) and the freshwater crayfish, *P. leniusculus* (Chaga et al., 1995). Johnson described four stem-cell types, of which two were considered stem cells: the first type has large and granular nuclei with distinct nucleoli, whereas the second type has large dense nuclei and undergoes little mitosis (Johnson, 1980). These cells might correspond to the type I cells described by Chaga et al. as actual stem cells. These cells might be quiescent and constitute a stem cell reservoir (Chaga et al., 1995; Johnson, 1980). The third cell type described in the blue crab has small nuclei (there are no data concerning mitosis) and is comparable to Chaga's type five; these cells have been classified as semigranular hemocyte precursors (Chaga et al., 1995), but their role remains unknown, and their structure is similar to that of granulocyte stem cells in *S. igentis* (Hose et al., 1992). The last and fourth cell type described by Johnson has large nuclei, is highly mitotic, and is most likely comparable to Chaga's type II cells (Chaga et al., 1995; Johnson, 1980). In *P. leniusculus*, two additional cell types, types III and IV, were further described as potential precursors for granular hemocytes at different stages because these cells contain similar electron-dense granules as mature GCs (Chaga et al., 1995). Similar cell types, such as in *P. leniusculus*, were described in the lobster *H. americanus* (Martin et al., 1993) and the shrimp *S. ingentis* (Hose et al., 1992).

Four cell types with intermediates were characterized in the black tiger shrimp *P. monodon*

using TEM. Inside the lobules, the predominant cell types were types two or three; both cell types reacted with the Mab WSH8 raised against hemocytes and bound to a 35 kDa antigen (van de Braak et al., 2000, 2002), whereas cell types one and four did not react with WSH8 and were only identified in the HPT (van de Braak et al., 2002). *P. monodon* type one cells were considered stem cells that give rise to type two and three cells, whereas type four cells were referred to as interstitial cells. In contrast to other crustaceans, the least-differentiated cells were localized at the exterior of the lobules, whereas more-differentiated cells, types two and three, were localized to the center (van de Braak et al., 2002). Hence, it is clear that additional studies concerning the differentiation process are needed to understand the lineages represented by the different cell types in the HPT and how these cell types develop within this tissue.

4.4. Origin

Allen (1893) described embryonic and larval development in *P. varians*, reporting that the cephalic aorta formed early in the embryo, and before hatching, the cell layers surrounding the aorta were enlarged, giving rise to mesodermal cell masses on either side of the aorta. These cells might be the origin of HPT (Figure 1, reproduced with permission from (Allen, 1893)). In 1908, Kollmann reported a similar origin of HPT cells from mesenchymal cells, some of which become connective tissue cells, whereas others give rise to hemocytes and are detached from the nodules (Kollmann, 1908). However, due to a lack of genetic tools and molecular markers, the specific origins of HPT are not yet clearly understood.

5. Hematopoiesis in crustaceans

5.1. Tissues during molting – seasonal variations

In the beginning of the 1900s, scientists reported large variations in the numbers of circulating hemocytes in crustaceans. Kollmann reported that the number of hemocytes varied markedly depending on the physiological status of the animal, and more granular hemocytes were formed during periods of good nutritional status (Kollmann, 1908). Researchers have detected particularly large variations during the molting cycle (Table 3, where the stages were named according to (Passano, 1960)), and more developing GCs are often observed prior to molting (Smith and Ratcliffe, 1980). Johnson reported a high mitotic index in stages C1-C3, followed by a lower index in C4. Subsequently, in stage D2, the HPT becomes thicker and high mitotic activity is observed, whereas less mitosis is observed in D3-D4, when new hemocytes are released into circulation, resulting in a thin HPT with fewer cells in post-molt stage B (Johnson, 1980). From stage B2 until C, stem cell activity is again initiated.

Hose et al. conducted detailed studies of hemocyte release from the HPT and hemocyte synthesis (mitosis) in the penaeid shrimp *S. ingentis* during the molt cycle (Hose et al., 1992). This work confirmed the earlier studies described above, showing the highest mitotic activity in the D0 stage, with a decline in stages D3-4, reaching the lowest level at stage A, followed by an increase again at stage C (Hose et al., 1992). During stages D to A, the tissue was also identified as rich in cells as described above, likely reflecting the presence of young hemocytes in this tissue, whereas during intermolt (stage C), ongoing mitotic activity and the continuous release of hemocytes makes the tissue slightly thinner (Hose et al., 1992). We made a similar observation in the freshwater crayfish *P. leniusculus* during the molt cycle (personal observation); thus, it is reasonable to conclude that there is an increased need for a large number of circulating hemocytes during this vulnerable stage of the animal's life.

5.2 Reaction to microbial polysaccharides and repeated bleeding

Molting and nutritional status regulate the hematopoietic process. Indeed, hemocytes are primarily involved in immune defense, and the hematopoietic process is essential for survival. Thus, the rates of hematopoiesis and differentiation are highly affected by microbial intruders (Persson et al., 1987). Several studies have shown that the introduction of microbial polysaccharides, such as LPS or β1,3-glucans (pathogen associated molecular patterns, PAMPs), into the hemolymph impact the hemocyte number (Hammond and Smith, 2002; Persson et al., 1987; Söderhäll et al., 2003a). An injection of PAMPs might lead to a rapid decrease in circulating hemocytes and thereby stimulate the synthesis and release of new hemocytes in the HPT (Hammond and Smith, 2002; Söderhäll et al., 2003a). Stimulation through LPS and repeated bleeding or the inhibition of mitosis using vinblastine resulted in increased hemocyte release in the penaeid shrimp *P. monodon* (van de Braak et al., 2002). Injections of PBS or LPS in *P. monodon* stimulated mitosis (van de Braak et al., 2002), and the mitotic index increased 24 hours after LPS compared with PBS, which was measured as mitotic cells identified through H&E staining (van de Braak et al., 2002). Using flow cytometry to measure cells in the S, G2, and M phases of the cell cycle, an early response to laminarin was detected in *P. leniusculus* (Söderhäll et al., 2003a). In the swimming crab (*Liocarcinus depurator*) LPS injection induced the release of cells to circulation in S phase, measured as BrdU incorporation, (Hammond and Smith, 2002); subsequently, this group showed that repeated bleeding could yield a similar result in the spider crab (*H. araneus*). These cells were identified as prohemocytes induced for release in response to the loss of hemolymph (Roulston and Smith, 2011). We observed a similar increase in BrdU-labeled cells in the circulation upon repeated bleeding (personal observation) and further showed that after the injection of laminarin (a β1,3-glucan), proliferating cell nuclear antigen (PCNA) expression was detected in circulating hemocytes and cells with morphologic similarities to immature HPT cells of types II and II according to Chaga (Chaga et al., 1995; Wu et al.,

2008). This result confirms previous studies concerning the rapid release of new hemocytes after the injection of PAMPs (Hammond and Smith, 2002; Söderhäll et al., 2003a).

The sensitivity of the HPT to external stimuli and physiological and nutritional status is extremely important when studying the expression pattern of different genes in hemocytes. The composition of cells in the hemocyte population can change rapidly and largely depends on external factors. Therefore, it is with great caution that conclusions can be drawn about gene expression in mixed hemocytes from various stimuli without considering an analysis of the changes in the cell types in the circulation. This fact was thoroughly discussed and investigated in *Litopeaneus vannamei*, in which the hematopoietic process is classified as an important reaction in the immune response (Bachère et al., 2004). These authors showed the high expression of the antimicrobial peptide Penaeidin 3-1 (Litvan Pen3-1) in a certain hemocyte type, and after microbial challenge the number of this hemocyte type dramatically decreased in circulation and cells penetrating into surrounding tissues. A rapid decrease in Pen3-1-expressing hemocytes was correlated with a similar loss of circulating cells. Approximately 48-72 hours after a challenge, new hemocytes were released from the HPT, restoring the expression of Pen3-1 in hemocytes (Bachère et al., 2004). Similarly, an active hemocyte-producing HPT was detected after the recovery phase in infectious hypodermal and hematopoietic necrosis (IHHN) virus-infected shrimp and after fungal infection (Hose et al., 1984; Lightner et al., 1983). In a subsequent study in *Penaeus japonicas*, a similar rapid increase in newly released cells with ongoing DNA synthesis was detected after LPS injection and infection with *Fusarium* fungi (Sequeira et al., 1996). Not only bacterial or fungal infections affect the HPT; viral infections might also impact hemocyte synthesis. In *P. leniusculus*, the proportion of GCs increased after an infection with white spot syndrome virus (WSSV) (Jiravanichpaisal et al., 2001; Liu et al., 2009). Taken together, several studies have clearly shown the importance of the hematopoietic process, such as induced synthesis,

differentiation and the rapid release of cells during crustacean defense reactions against microbial infections.

5.3. Hemocyte lineages and release

The hematopoietic process can be divided into stem cell division/proliferation, cell determination and differentiation and eventual cell release into the circulatory system. Proliferating cells are typically detected using bromodeoxyuridine (BrdU)- or ^{3}H-thymidine incorporation, which indicates the proportion of S-phase cells, or through mitotic staining with phospho-histone antibodies. Both techniques have been used to describe the HPT in *P. leniusculus*. A high mitotic frequency was identified in the APC and at the lateral edges of the HPT, whereas in the center of the tissue surrounding the ophthalmic artery, low mitotic activity could be detected (Noonin et al., 2012a). This finding is consistent with Ghiretti-Magaldi, who proposed that the walls of the ophthalmic artery do not comprise hematopoietic lobules but reticular connective tissue extending ventrally towards the digestive gland (hepatopancreas) (Ghiretti-Magaldi et al., 1977). Some primitive crustacean species have dividing hemocytes in circulation (*Chirocephalus* and *Argulus*), whereas this phenomenon is exceptional in more advanced species (Bauchau, 1981), and S-phase cells in circulation reflect prematurely released immature hemocytes or prohemocytes (Roulston and Smith, 2011).

As soon as new cells are formed, some signal determines further development into one of two or three different hemocyte types. Several studies have presented different lineage models, and the presence of a specific HC in some crustacean species is debated. A single lineage was proposed for *Pachygraspus marmoratus* (Charmant, 1973) and for other crustaceans (George and Nichols, 1948). Two different hemocyte lineages were suggested for *P. leniusculus,* SGCs and GCs (Chaga et al., 1995), *H. americanus* GCs and HCs (Martin et al., 1993) and *P.*

monodon large and small GCs (van de Braak et al., 2002). HCs have been proposed as pro-stages for the two hemocyte lineages in *P. monodon* (van de Braak et al., 2002), and based on similar observations in *P. leniusculus* (Wu et al., 2008), we agree with this conclusion. Similarly, Ghiretti-Magaldi et al. described the premature release of prohemocytes (hemoblasts) from lobules, giving rise to agranular hemocytes (Ghiretti-Magaldi et al., 1977). In 2002, van der Braak et al. conducted a thorough study that showed evidence consistent with the proposed model suggesting at least two different hemocyte lineages (Lin and Söderhäll, 2011). There is no doubt that there are several developmental stages of cells in the HPT but also that differentiation occasionally continues in circulation (Ghiretti-Magaldi et al., 1977; Roulston and Smith, 2011).

The release of hemocytes from HPT has been studied in *C. maneas* using TEM. Usually the lobules then open at one side where more mature cells are found. The mature hemocytes are detached from the lobules through the open side, as previously described (Chaga et al., 1995; Johnson, 1980). This study showed how young, recently divided hemocytes were released into small hemal lacunae and further transported into the larger hemal space and main hemal sinuses (Johnson, 1980). Release into the hemal spaces in between the lobules was primarily observed in areas where the fibrous connective tissue is thin or missing in the center of the posterior HPT in *P. leniusculus* and *P. monodon* (Noonin et al., 2012a; van de Braak et al., 2002). To determine specific lineages within the HPT, valuable information can be obtained from studies of cell morphology, but these studies are not sufficient. We have previously shown that the cell morphology within the lobules is similar in different areas of the posterior HPT, whereas some areas have more proliferating cells, and other areas have cells which are more mature and primed for subsequent released into the hemal lacunae (Noonin et al., 2012a). Noonin et al. (2012) showed that the expression of an SGC marker was abundant in certain areas where proliferation was lower, and it is clear that cells with morphologies

similar to type III cells, according to Chaga et al. (1995), express this transcript, whereas mature GCs do not (Lin et al., 2011; Noonin et al., 2012a). It is likely that at least two separate lineages within the HPT give rise to SGCs and GCs. Evidence to support this hypothesis is shown in Figure 3, where in situ hybridization for CHF mRNA clearly shows cells with and without CHF expression (a marker for SGCs) (Figure 3). Recent studies have also shown a link between the immune system and adult neurogenesis in crayfish; therefore, cells within the APC and/or HPT likely give rise to neuronal precursors (Beltz et al., 2015, 2011; Benton et al., 2014). However, to obtain a clear picture of how commitment and differentiation are regulated in this tissue, it is absolutely necessary to identify additional markers for the different stages of development into different mature hemocyte types, as illustrated in Figure 3.

5.4 Molecular regulation of hemocyte synthesis, differentiation and release

Compared with *Drosophila* and other model animals, crustacean research is hindered by the absence of genetic tools and molecular markers. Therefore, most of the research performed during the 1900s involved morphological studies. However, during the past decade, RNA interference techniques have made additional molecular studies possible in these animals (Liu and Söderhäll, 2007). Furthermore, we developed a technique to isolate and culture populations of HPT cells *in vitro*, thereby characterizing these cells and performing studies concerning the molecular regulation of hemocyte differentiation (Lin and Söderhäll, 2011b; Söderhäll et al., 2005). Previously, HPT cell cultures have been reported for *Nephrops norvegicus*, but these cultures did not proliferate, and no cell differentiation was observed (Mulford et al., 2000).

Stimulation with microbial polysaccharides or repeated bleeding induces a need for new hemocytes, thereby stimulating cell division in the HPT and the release of immature and

mature hemocytes. One rapid response to such stimulation is the induced expression of certain transcription factors. The transcriptional regulation of hematopoiesis in invertebrates has primarily been studied in *Drosophila melanogaster*, and the RUNX family protein lozenge is a necessary factor for the production of *Drosophila* CCs, a cell type containing the proPO-activating system, similar to the SGCs and GCs in crayfish (Fossett et al., 2003; Minakhina et al., 2011; Muratoglu et al., 2007). The role of RUNX transcription factors in hematopoiesis is highly evolutionarily conserved, and a member of this family (PlRunt) is also the only transcription factor identified thus far, with clear importance in crustacean hematopoiesis (Söderhäll et al., 2005, 2003a). The expression of PlRunt mRNA was also rapidly induced in HPT cells in response to the injection of microbial polysaccharides prior to the release of new hemocytes into circulation, and therefore might be a marker for differentiation within the HPT (Söderhäll et al., 2003a). Similarly, a RUNX factor was demonstrated as important in coelomocyte synthesis in *Asterias rubens* (Oweson et al., 2010).

Using *in vitro* methods to culture cells isolated from the HPT of *P. leniusculus*, we identified and purified a peptide from crayfish plasma with cytokine-like activity that could induce cell division and the partial cell differentiation of a mixed population of primary HPT cells in culture. We named this peptide astakine1 and showed that astakine1 contains a prokineticin (PROK) domain with 10 conserved cysteine residues (Söderhäll et al., 2005). Functional *in vitro* studies were subsequently conducted through the introduction of an easy and reproducible method of RNA interference in cell culture (Liu and Söderhäll, 2007). When LPS or laminarin is injected into a crayfish, there is a rapid increase in the plasma content of astakine1, likely reflecting either regulated release from hemocytes or hemocyte lysis upon injection (Söderhäll et al., 2005). Moreover, 30 minutes after the injection of LPS or laminarin, this “emergency” system induces a burst of ROS production within the APC, eventually leading to the release of hemocytes from HPT. We have shown that this release is

mediated by loosening the extracellular matrix (ECM), enabling cells to enter hemal spaces. The ECM in *P. leniusculus* has not been fully characterized, but has been shown to contain the protein collagen type IV (Junkunlo et, al 2015), a substrate for the crosslinking enzyme transglutaminase (TGase). TGase activity is important for stabilizing the matrix, and ECM loosening is achieved through a decrease in extracellular transglutaminase activity (Lin et al., 2008a). When TGase activity was silenced in HPT tissue culture, cells migrated out of the tissue (Lin et al., 2008a). Furthermore, we showed that astakine1 treatment decreased the extracellular TGase activity. We recently observed that a pulse of high ROS production similarly decreased extracellular TGase activity after 24 hours, thereby stimulating hemocyte release (Junkunlo et.al 2015). This loss of extracellular crosslinking activity further induces hemocyte precursor differentiation, and these cells migrate out of the tissue. Whether astakine1 directly mediates a ROS pulse in the APC is not known, but we are currently investigating the relationship between astakine1 levels and APC or HPT ROS production. The importance of astakine in crustacean hematopoiesis was subsequently confirmed in the tiger shrimp *P. monodon* (Hsiao and Song, 2010). Furthermore, a link between TGase activity and the regulation of astakine translation has been reported in shrimp. A specific part of the 3'UTR of astakine mRNA was shown to interact with the shrimp TGase (STG I) and influence the translation of astakine (Chang et al., 2013).

In a suppression subtraction hybridization (SSH) experiment comparing gene expression in cultured HPT cells with or without the addition of astakine1, a new protein was identified as dependent on the presence of astakine1 in the culture medium. This protein, CHF (crustacean hematopoietic factor), has been characterized, cloned and sequenced (Lin et al., 2011), and shown to be specific for the SGC lineage. When CHF was silenced, an increased number of apoptotic cells was detected, indicating an important role for the survival of the SGC precursors (Lin et al., 2011; Noonin et al., 2012a). However, more functional studies are

needed to fully understand the function of this protein. A cDNA encoding a protein with some similarity to CHF, but also sharing some sequence similarities with neuroparsins, a group of arthropod neuropeptides, was recently cloned in *Litopenaeus vannamei* (Badisco et al., 2007; Charoensapsri et al., 2015). However, some conserved domains in *P. leniusculus* CHF was absent in the *L. vannamei* CHF-like protein (Charoensapsri et al., 2015). Both CHF in *P. leniusculus* and *L. vannamei* contain a single insulin-growth factor-binding protein domain. Interestingly, the *L. vannamei* CHF-like protein was shown to bind to a laminin receptor (Lamr), the silencing of which led to decreases in the expression of *L. vannamei* CHF and the number of circulating HCs (Charoensapsri et al., 2015). These results indicate a role for CHF proteins in hemocyte homeostasis in crayfish and shrimp. Another protein with a putative role in regulating hemocyte number or composition, hemocyte homeostasis-associated protein (HHAP), was initially identified in *P. monodon* and subsequently in *P. leniusculus* and *L. vannamei* (Apitanyasai et al., 2015; Prapavorarat et al., 2010). The sequence of HHAP is not similar to that of CHF-like sequences; instead, these proteins contain a zinc finger CCHC domain. Knockdown experiments in shrimp have shown an increase in apoptosis followed by a decrease in the hyaline hemocyte number. The *P. monodon* HHAP was recently shown to bind to the p20 domain of a shrimp caspase, further confirming a role for HHAP in regulating apoptosis (Apitanyasai et al., 2015). Intriguingly, *L. vannamei HHAP* was shown to bind to Lamr in a manner similar to the CHF-like protein, indicating the involvement of this receptor in hemocyte homeostasis (Charoensapsri et al., 2015). Thus far, the role of HHAP in *P. leniusculus* remains unknown, and its expression level is much lower compared with shrimp, likely reflecting different compositions of hemocyte types in different species and that the shrimp hyaline hemocyte type, regulated by HHAP, is extremely rare or absent in freshwater crayfish. In summary, the current understanding of the regulation of the hematopoietic process in crustaceans still lags behind the knowledge in *Drosophila*, but due to recent

improvements in stem cell culture and RNAi technology there is rapid ongoing progress in this field.

5.5 Proteins in P. leniusculus hematopoiesis – a comparison with insects

Invertebrate hematopoiesis has been studied in detail in the fruit fly and, to some extent, in other insect species. Research concerning the regulation of hemocyte synthesis in crustaceans is hampered by a lack of genomic information. Thus, newly isolated proteins need identification through amino acid sequencing, and then each gene has to be cloned and sequenced, fairly time-consuming work. To obtain a useful library of sequences for further detailed studies of the hematopoiesis in crayfish, we sequenced transcriptomes using Solid- and IonTorrent-based RNA sequencing of samples from hemocytes, HPT, and nervous tissue from *P. leniusculus*. These data were made publically available in the NCBI sequence read archive as BioProject ID: PRJNA259594 at: http://www.ncbi.nlm.nih.gov/bioproject/259594. Because our focus is hematopoiesis in *P. leniusculus*, we performed a manual BLAST search for transcripts of the genes involved in hematopoietic processes in *D. melanogaster* (Gold and Brückner, 2014; Grigorian and Hartenstein, 2013; Lebestky et al., 2000; Morin-Poulard et al., 2013). For an evolutionary perspective, a comparative search for these genes in representatives of other insect phyla was conducted where genomes were available, and the following species were selected: *D. melanogaster* (Diptera), *T. castaneum* (Coleoptera), *A. mellifera* (Hymenoptera), *Bombyx mori* (Lepidoptera), *Pediculus humanus corporis* (Phthiraptera) and *Acyrthosiphon pisum* (Hemiptera). Detailed studies of HPTs from other insects are restricted to Diptera, Lepidoptera and Orthoptera, and among these insects, only *Drosophila* and *Bombyx* have been thoroughly studied (Grigorian and Hartenstein, 2013; Hoffmann et al., 1974; Hoffmann and Porte, 1973; Hoffmann, 1970; Nakahara et al., 2010; Nardi et al., 2003). Furthermore, an old structural descriptive study on the hematopoietic

organ in the coleopteran *Melolontha* is available (Brehélin, 1973), whereas no studies of *Tribolium, Apis, Acyrthosiphon or Pediculus* hematopoiesis have been published. *Drosophila* hematopoiesis has been extensively studied both in terms of morphology and control at the molecular level, and it occurs in two waves: first during embryogenesis in the procephalic mesoderm, and subsequently during the larval stages in the lymph gland (LG), an organ closely associated with the dorsal vessel (Grigorian and Hartenstein, 2013). The different hemocyte types in insects and crustaceans share many properties, but due to a lack of molecular markers for all species, comparisons are primarily performed based on morphological criteria. Three main classes of hemocytes are recognized in *Drosophila*: the plasmatocyte (PC), which is phagocytic; the crystal cell (CC), involved in melanization; and the encapsulating lamellocyte (LC). The GC in crayfish is slightly comparable in function to CCs in Drosophila, whereas SGCs and HCs are partially phagocytic but not strictly comparable to plasmatocytes in flies (Grigorian and Hartenstein, 2013; Lin and Söderhäll, 2011a).

The regulation of hemocyte proliferation and differentiation during the larval stage occurs within the LG. This organ is a bilobed structure with three or more paired lobes in the third instar larvae. The anterior lobe is the largest and can be divided into three distinct areas. The inner region, called the medullary zone (MZ), near the dorsal vessel, comprises tightly packed undifferentiated precursors of hemocytes. Outside the MZ, differentiating cells are located in the cortical zone (CZ), and there is a small area located at the posterior part of the lobes, which functions as a regulatory center, called the posterior signaling center (PSC), for direct hemocyte proliferation and differentiation in MZ and CZ (Krzemień et al., 2007; Mondal et al., 2011; Nakahara et al., 2010). The PSC provides niche signals to prohemocytes, which might stimulate renewal and block differentiation unless these cells are not needed in the circulatory system. PSC cells are determined early in *Drosophila* embryo development and

have been characterized based on the expression of the Notch ligand Serrate (Lebestky et al., 2003), the transcription factors Collier/Knot (Col) and the homeotic Antennapedia gene (Morin-Poulard et al., 2013).

In the *Pacifastacus* transcriptome, we detected Serrate expression, which was also detected in all insects examined. No expression of Antennapedia or any Collier ortholog was detected in the transcriptome; this result further indicates that a *Drosophila*-like PSC might not be present in adult crayfish HPT. However, it seems clear that Antennapedia is present in crustaceans, as this gene was identified in two Branchiopoda (*D. Pulex* and *Artemia franciscana*) and in the amphipod *Parhyale hawaiensis*. In addition, this gene is present in decapoda and was detected in *L. vannamei* as expressed only during the gastrula stage (215 minutes post spawning) in early embryo development (Li et al., 2012).

Hemocyte precursors are present in the MZ of the *Drosophila* LG. Normally, two different hemocyte lineages are detected in the CZ: CCs and plasmatocytes. The GATA transcription factor Serpent (Srp) is crucial for the development of all types of hemocytes and is highly important for niche cell differentiation and control (Lebestky et al., 2000; Tokusumi et al., 2010). Downstream of Srp, the transcription factor Glial cell missing (Gcm) is required for the differentiation of the plasmatocyte lineage, whereas the RUNX-like transcription factor Lozenge (Lz) directs prohemocytes into CCs (Lebestky et al., 2000). Furthermore, the Friend of GATA transcription factor U-Shaped (Ush) acts in combination with a Srp splice variant (SrpNC) to inhibit the development of CCs (Fossett et al., 2003). Recently, Lz expression was shown to necessitate Notch activation of CC development, and this effect partially reflects Notch activation of the transcription factor Klumpfuss (Klu), another zinc-finger protein (Terriente-Felix et al., 2013). In comparison, the *Pacifastacus* transcriptome expressed several GATA factors, one of which was specifically similar to Srp. Previous studies have shown that GATA factors are involved in regulating the expression of the gene encoding the cross-

linking enzyme transglutaminase (TGase) in hemocyte differentiation (Lin et al., 2008b). The main hemocyte lineages in *P. leniusculus,* SGCs and GCs, both express a Lz-like transcript (RUNX-family GenBank: CAD44570.1) required for proPO-expressing cells, such as *Drosophila* CCs and crayfish SGCs and GCs (Lebestky et al., 2000; Söderhäll et al., 2003b). However, an Ush-like sequence was clearly detected, but it remains unknown whether this gene has a similar down-regulatory effect on GCs or proPO-expressing SGCs as in *Drosophila*.

We could not identify any Gcm-like transcripts in *Pacifastacus* in the transcriptome or through the use of several sets of degenerate primers (personal observation), potentially reflecting the fact that no cells comparable to *Drosophila* plasmatocytes are clearly distinguishable in crayfish. In other insects, hemocyte types similar to plasmatocytes and CCs have been identified based on morphology, and in *Bombyx*, two different lineages of HPT were identified based on morphological criteria. However, thus far, no specific molecular markers are designated to the silkworm plasmatocyte or granulocyte lineages (Nakahara et al., 2010).

Interestingly, CHF is not detected in any of the insect genomes, whereas this protein is highly required for the survival of the SGC lineage in *P. leniusculus* and most likely other decapods (Lin et al., 2011). This protein is a small 9-kDa peptide that shares sequence similarity with the N-terminal region of the vertebrate CRIM1 protein.

In *Drosophila,* the JAK/STAT signaling pathway plays a dual role in this regulation, and three cytokines named Unpaired (1-3) act as ligands for Type 1 receptor Dome. Among these, high expression of Upd3 activates JAK/STAT in the MZ, thereby suppressing the differentiation of prohemocytes. However, gain-of-function mutants of Hop show the overproduction of plasmatocytes and the differentiation of lamellocytes (Agaisse et al., 2003; Harrison et al., 1995; Luo et al., 1995). Unpaired cytokines were not detected in the other

investigated insects, *P. leniusculus* or vertebrates, and a BLAST search produced no hits outside Drosophilidae, showing that *Drosophila* uses a unique set of cytokines in the JAK/STAT pathway.

A recent study showed that a fibroblast growth factor receptor (FGFR)-dependent signaling pathway was required and sufficient to induce plasmatocyte differentiation in the CZ (Dragojlovic-Munther and Martinez-Agosto, 2013). This pathway is dependent on the FGFR gene Heartless and the ligands Pyramus and Thisbe in *Drosophila*, neither of which were detected in the *P. leniusculus* transcriptome. Thus, this evidence further indicates that no hemocyte linage comparable to plasmatocytes is present in crayfish. Interestingly, neither Pyramus nor Thisbe was identified in the genomes of *Tribolium*, *Bombyx*, *Apis*, *Pediculus* or *Acyrthosiphon*, whereas Heartless homologs were identified. However, Ush and the ETS protein Pointed, which acts downstream of Heartless-induced differentiation, could be detected in crayfish and all other insects, showing more evolutionary conservation at the level of transcription factors compared with cytokines and receptor ligands.

Directed cell migration is an important process during development and in blood cell recruitment to peripheral areas during an immune response. Mammalian hematopoietic cells and *Drosophila* prohemocytes use PDGF and VEGF pathways to direct the migration of cells during development and the immune response (Duchek et al., 2001). In *Drosophila*, a gene for a PDGF/VEGF receptor homolog (PVR, CG8222) and three ligands (Pvf1-3, CG7103, CG13780 and CG34378) were identified, characterized and implicated in the embryonic migration of hemocytes (Cho et al., 2002). Furthermore, Munier et al. (2002) showed the expression of PVR on the surface of prohemocytes and circulating plasmatocytes. These authors also showed an important role for the Pvf/PVR pathway in hemocyte release through the overexpression of Pvf2, resulting in a massive release of circulating hemocytes. Subsequently, PVR receptor function has been associated with IMD-dependent immune

responses and intestinal immunity and stem cell development (Bond and Foley, 2009; Fukuyama et al., 2013). The functions of the PDGF/VEGF pathways show striking conservation from *Drosophila* to humans, and the receptors have been classified as receptor tyrosine kinase surface receptors. We identified four putative receptors of the PDGFR/VEGFR family in the *P. leniusculus* transcriptome, one of which we named PVR1 due to its similarity with *Drosophila* PVR. In preliminary results from ongoing work, we have shown that PVR1 is highly expressed in hemocytes and to lower extent in the HPT (Junkunlo et.al 2015).

Among the growth factors and cytokines of importance in *Drosophila* hematopoiesis, Upd 1-3, Pvf2, Thisbe and Pyramus were not detected in *P. leniusculus*. However, receptors, signaling pathways and transcription factors are more conserved between flies and crayfish. Astakines have been detected in crustaceans, spiders, scorpions and some insect phyla, but not in Diptera, or Coleoptera. We identified astakine family sequences in *Apis*, *Pediculus* and *Acyrthosiphon* genomes, and after a thorough search, we also identified astakine-like genes in two species of *Bombyx, B. terrestris* (LOC100647082, XM_003401218.1) and *B. impatiens* (LOC100745562), indicating that astakines might also exist in Lepidoptera. However, thus far, the functions of these genes are unknown in insects.

Among insects, apart from *Drosophila*, hematopoiesis has been primarily studied in Orthoptera (Hoffmann et al., 1974; Hoffmann and Porte, 1973; Hoffmann, 1970) and Lepidoptera (Nakahara et al., 2010, 2006, 2003; Nardi et al., 2003).Two extracellular peptides have been identified in *Bombyx mori* that have some impact on hematopoiesis. The paralytic peptide BmPP (NP_001036883.1), showing inhibitory activity, whereas the insulin-like peptide Bombyxin (BAA00246.1) stimulates the proliferation of hematopoietic cells (Nakahara et al., 2006, 2003). None of these peptides were detected in the *P. leniusculus*

transcriptome, again showing differences between species in extracellular hematopoietic growth factors and cytokines.

Clearly, there are similarities and differences between hematopoietic regulation mechanisms in insects and crustaceans. At the level of transcription factors, the roles of GATA and RUNX proteins in hematopoiesis are conserved and are most likely involved in signaling through the JAK/STAT pathway. In addition, proteins in the VEGF/PDGF family of growth factors and receptors have been detected in all studied species. Notably, there are proteins more specific to certain species or group of species, such as the astakines and CHF in crustaceans and Upd in *Drosophila*.

6. Concluding remarks

Crustacean hemocytes are relatively morphologically similar and are typically classified into two or three main groups. The hemocytes circulating in the bloodstream have varying complexity in various crustaceans. Although only a small number of crayfish species have been studied, the site of hemocyte formation, i.e., the HPT, is relatively uniform in structure, as in the localization at the dorsal side of the stomach and enveloping the anterior aorta. In general, an HPT comprising thin sheets of tightly packed precursor cells in lobules, showing high proliferating activity is located in a specific area near the brain and core frontal lobe. Still, there are currently few studies concerning hematopoiesis in crustaceans outside of Decapoda, and importantly, the vast diversity within this phylum and general conclusions should be drawn with care.

The number of circulating hemocytes significantly varies, reflecting external and internal factors, and an increased demand for new hemocytes in cases of infection or during molting when high protection has been documented in several species. Existing studies concerning

how hematopoiesis is regulated at the molecular level are also few. Nevertheless, similarities and large differences in regulation at the molecular level have been observed compared with the well-studied fruit fly. Interestingly, signaling pathways and the regulation of hematopoiesis through transcription factors are highly conserved throughout arthropods (and also among the vertebrates), whereas significant species variety and specificity among proteins acting as growth factors or cytokines have also been identified. In summary, the current understanding of how crustacean hemocytes are synthesized and how the relative number of the different hemocyte types is regulated will hopefully increase in the future with increased access to genomic tools and opportunities to study the effect of modified gene expression. Notably, it is necessary to study older literature to identify previously discovered phenomena that might have been forgotten and could now be explained using modern technology.

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Figure legends

Figure 1. A drawing by Allen (1893) showing a transverse section through the anterior end of the dorsal sac in a young adult *Palaemonetes*, showing the tissue where henocytes are formed (x 240), Ao.c. = cephalic aorta; c= dorsal sac; c.m = cephalic muscle; cor = "blood corpuscle" (hemocyte). Reproduced from Figure 12 in the paper by Edgar J. Allen, 1893, Nephridia and body cavity of some Decapod Crustaceans. The Quarterly Journal of Microscopical Science (or J. Cell Science) s2-34: 403-426, with permission.

Figure 2.

A) Location of hematopoietic tissue (HPT) in P. leniusculus. HPT cells are arranged in lobules surrounded by connective tissue, encased in larger sheets of connective tissue ranging from around the brain and following the ophthalmic artery to the posterior HPT covering the dorsal part of the stomach (photo courtesy of Chadanat Noonin).
B) The entire HPT with surrounding sheets was isolated in one piece. The arrows indicate the APC and the posterior region of the HPT (photo courtesy of Dr. Chadanat Noonin)

Figure 3.

HPT cells isolated from the posterior region of the HPT, where maturation occurs, and these cells were stained to detect CHF protein, a marker for the SGC lineage as previously described (Lin et al., 2011). Note that cells with similar morphology might have different staining, indicating that these cells belong to different lineages. The arrow indicates a SGC precursor, and the arrowhead indicates a GC precursor (photo courtesy of Xionghui Lin).

Table 1. Studies of hematopoietic tissues in crustaceans.

Taxon	Species	HPT location	References
Branchiopoda	*Artemia salina* *Chirocephalus diaphanus* *Phyllopoda*	Bilaterally at the base of each limb No HPT	(Debaisieux, 1952; Lochhead and Lochhead, 1941) (Bruntz, 1907)
Leptostraca	*Nebalia geofroyi*	No HPT	(Bruntz, 1907)
Stomatopoda	*Squilla mantis*	Surface of ventral artery	(Bruntz, 1907)
Euphausiacea		No report	
Mysida	*Mysis charnaelo M. oulgaris*	Surface of cardiac stomach	(Bruntz, 1906)
Syncardia		No report	
Decapoda	*Several species**	APC and HPT surrounding ophthalmic artery	(Allen, 1893)*
Thermosbaenacea		No report	
Spelaeogriphacea		No report	
Amphipoda	*Talitre, Gammarus*	Bilateral lobes in the head	(Bruntz, 1907)
Lophogastrida		No report	
Mictacea		No report	
Stygiomysida		No report	
Tanaidacea		No report	
Cumacea	*Iphinoë*	Bilateral lobules at the fifth thoracic segment	(Bruntz, 1907)
Isopoda	*Oniscus, Asellus, Ligia, Anceus* *Armadillidium*	Lobules in the last 2 thoracic and abdominal segments	(Bruntz, 1907) (Bruntz, 1907) (Chevalier et al., 2011)

* *After the first report by Allen in 1893, numerous studies, as presented in this review, concerning HPT in different decaopds are available.*

Table 2. Hemocyte types, reprinted with permission from Table 1 in Bauchau 1981.

Type	Shape	Nucleus	ER	Free ribosomes	Golgi	Granules	Lysosomes	Mitochondria
Hyaline cell	round to oval	central, round and large	smooth, rough and scarce	present	0 or 1	0 or few		moderate
Semi-granular cell	oval to spindle shaped	central or eccentric, oval and lobed	smooth, rough and abundant	abundant	1 or more	moderate	Present	abundant
Granular cell	oval	eccentric and kidney shaped	smooth, rough and moderate	moderate	0 or 1	abundant	Present	abundant

Table 3. Molting stages of brachyuran crabs (stage named after Passano 1960)

Name	Stage
A1	Newly molt
A2	Soft
B1-B2	Papershell
C1 – C3	Hard
C4	Intermolt
D0 –D1	Proecdysis
D2 – D3	Peeler
D4	About to molt
E	Molt

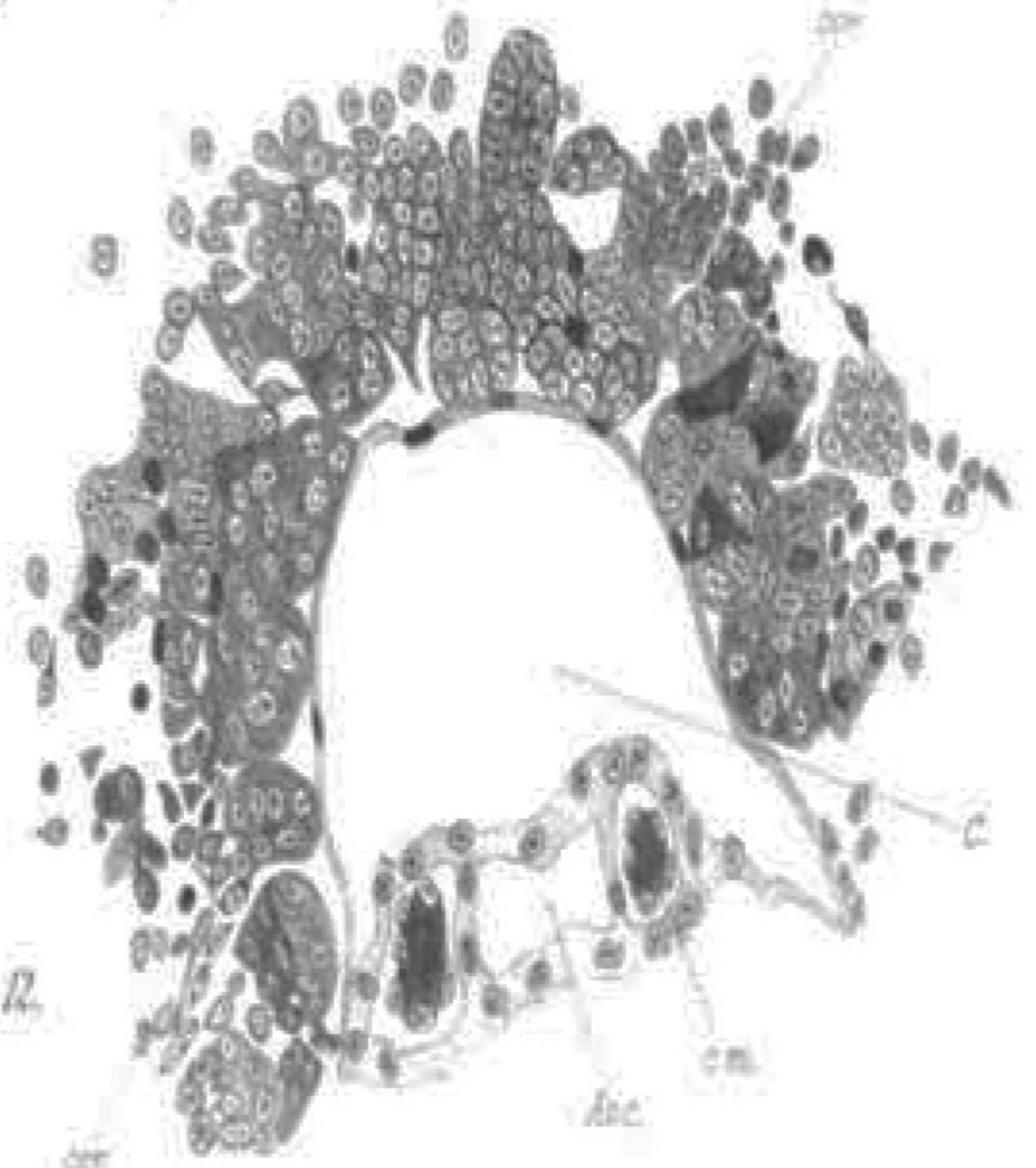
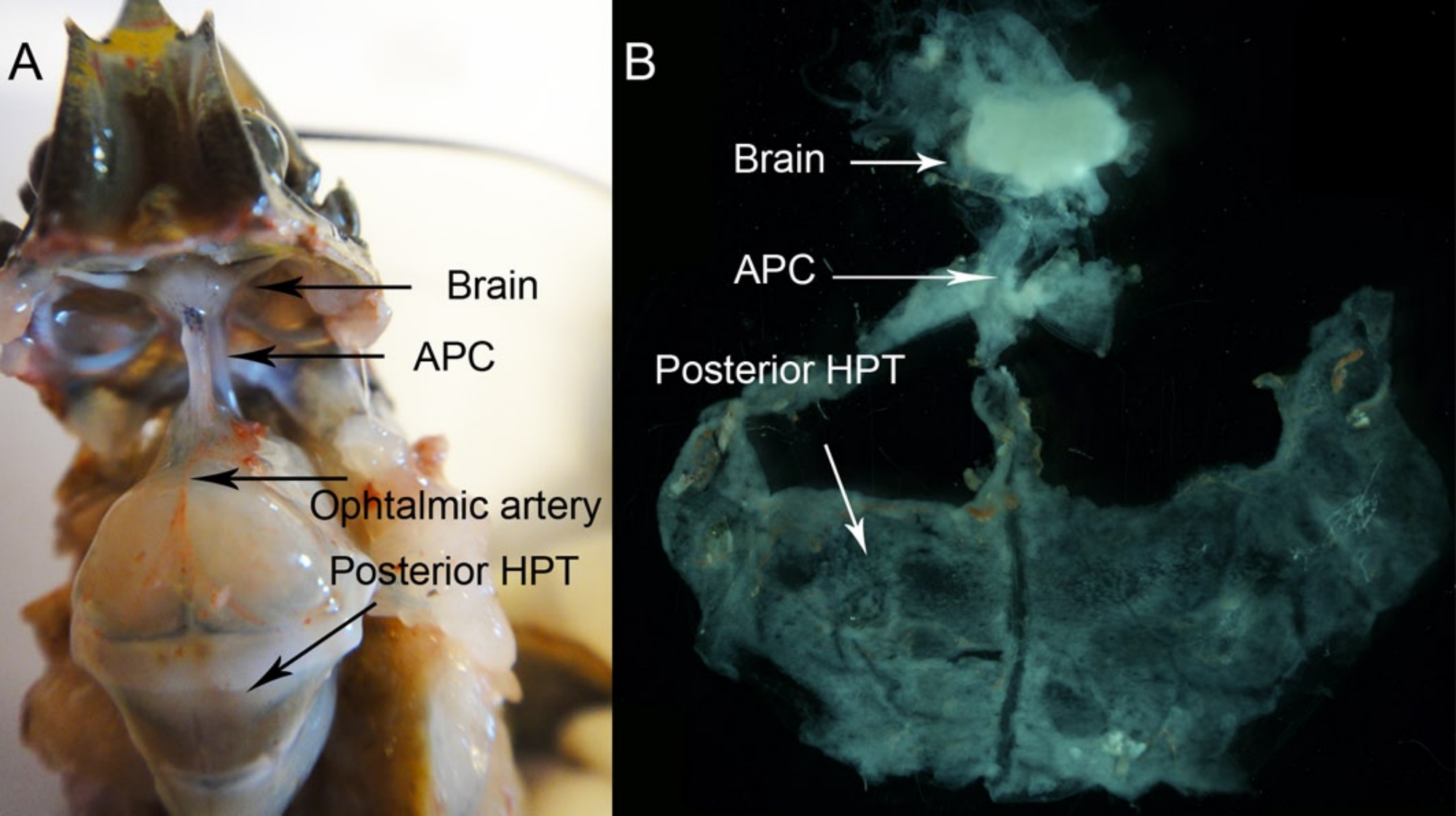


Fig. 12.


A
Brain
APC
Ophtalmic artery
Posterior HPT
B
Brain
APC
Posterior HPT