The humoral immune systems of the American lobster (*Homarus americanus*) and the European lobster (*Homarus gammarus*)

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Abstract

American and European lobsters are both high profile and high value animal species that provide significant economic value in the areas where they are landed. As such, it is important to understand the factors that contribute to the health of the animals and their populations, and how these animals can defend against pathogen impacts, especially the components of their immune systems. It is somewhat surprising to see that there is a considerable amount of published information on the immune systems other commercially important crustacean species such as warm-water marine and freshwater shrimp and crayfish, yet there is relatively little published material on the American and European lobsters. This review highlights the key areas of crustacean immune function and the published evidence as it relates to these important species. This includes; pattern recognition receptors, variable immune molecules, clotting factors, prophenoloxidase, hemocytes and anti-bacterial/anti-microbial components.

1. Introduction

Clawed lobsters are a group of large marine crustaceans of the family Nephropidae. The American lobster *Homarus americanus* and the European lobster *Homarus gammarus* comprise the two living species of the genus *Homarus*. The other eight species contained in this genus are extinct and this is one of three extant genera of clawed lobsters. Both of these species occur in the North Atlantic and both are commercially important. In fact the reliance on the American lobster fishery in some North Eastern states of the US has created what has been defined as a 'gilded trap'.

The apparent 'monoculture' of reliance on lobster landings in Maine is such that more than 80% of the waterfront landings are attributed to lobsters (Steneck, Hughes et al. 2011). The UK landings of European lobster in 2013 were worth £30 million (Seafish 2016). Those for the state of Maine in 2015 for the American lobster were worth \$456 million (DMR 2015).

Immune function is relatively well studied in decapod crustaceans compared to most other invertebrates with the exception of *Drosophila* sp. This is probably associated with their large size and their financial importance through extensive wild fisheries and the significant global aquaculture of shrimps and prawns. Their immune systems contain an array of hemocyte-derived (cellular) and plasma-derived (humoral) immune factors (Cerenius, Jiravanichpaisal et al. 2010, Hauton 2012). Although this distinction can be useful it should be understood that it is an artificial construct and that, in reality, there is considerable integration of these components such that the cellular and humoral components interact to produce the desired immune response.

There are relatively few known infectious diseases of lobsters. A recent review of the diseases of the American lobster highlighted gaffkemia, epizootic shell disease, neoparamoebiasis and bumper car disease as those that may cause notable levels of mortality in the natural environment (Cawthorn 2011). Epizootic shell disease has been a significant issue for the American lobster along the north east coast of the US and Canada (Castro, Cobbs et al. 2012). One of the pathogens associated with shell

disease is *Vibrio fluvialis* reported to induce anti-lipopolysaccharide factor-1 (discussed later) that suggested a molecularly specific response to bacterial pathogens (Beale, Towle et al. 2008). This pathogen has also been associated with limp lobster disease (Tall, Fall et al. 2003). However, little is known about significant disease issues for European lobsters (Behringer, Butler IV et al. 2012, Rowley, Cross et al. 2014, Davies and Rowley 2015). Gaffkemia, a disease associated with the pathogen *Aerococcus viridans* var *homari*, has been identified in European lobsters, especially in pound stocks, although prevalence was low in wild populations (Stebbing, Pond et al. 2012). However, there is considerable concern about the impact of this disease should it become prevalent within European populations (Alderman 1996).

The intention of this review is to compile a current status of our understanding of the immune system of clawed lobsters in broad terms (Table 1.) with occasional reference to the decapod immune system for clarity. While published studies of these animals have been carried out for well over a century, recent genomic and transcriptomic studies, using tools such as microarrays and next generation sequence are rapidly expanding our knowledge of the immune capacity of these commercially important species. Clarification of our current knowledge will assist in identifying possible areas of interest, which may provide clarity for avenues for future research.

2. Pattern Recognition Receptors

Pattern recognition receptors (PRR) are immune factors that assist in identifying microorganisms that are attempting to penetrate, or that have managed to penetrate an organism. They respond to different molecular signatures, or pathogen associated molecular patterns (PAMPs), that are typically present on or released by these microorganisms (Kumagai and Akira 2010). The range of molecular signatures that can be responded to include; lipopolysaccharides, β -1,3-glucans, double-stranded RNA and peptidoglycans. Amongst crustaceans these include β -glucan-binding protein, lipopolysaccharide binding protein, masquerade-like proteins and serine proteinase homologues (Cerenius, Jiravanichpaisal et al. 2010). In clawed lobsters expression of β -glucan binding protein genes has been shown in European lobsters (Hauton, Brockton et al. 2007) and in American lobsters (Hall 2012, Clark 2014). These proteins have been shown to mediate the interaction between glucans and granulocytes in European lobsters (Smith and Söderhäll 1983).

Many invertebrates use lectins to assist in being able to distinguish self from non-self. Lectins are a group of proteins with a high specificity for binding to carbohydrate moieties and have numerous roles in relation to biological recognition at a cellular and molecular level (Vasta, Ahmed et al. 2007). The key difference between the PRR and lectins discussed below are that lectins usually do not possess any catalytic activity of their own. These molecules can facilitate the binding of bacteria and viruses to their target and are able to act as opsonins, tagging particles for subsequent agglutination or phagocytosis and can thereby initiate a range of

diverse cellular events (Vasquez, Alpuche et al. 2009). Lectins can be found in the hemolymph, bound within hemocyte membranes and in cytoplasmic granules (Vásquez, Pérez et al. 1997). It would appear that crustacean lectins have carbohydrate specificity mainly for *N*-acetylated carbohydrates such as *N*-acetylneuraminic acid (sialic acid), *N*-acetyl glucosamine, *N*-acetyl galactosamine and, to a lesser extent, galactose residues (Alpuche, Pereyra et al. 2005). The majority of crustacean lectins are classified as C-type lectins due to their dependence on calcium ions (Vasta, Ahmed et al. 2007). Lectins, or Agglutinins, were first identified in American lobster over a century ago (Noguchi 1903). It has previously been shown that agglutinins of the American lobster have specificity for GalNAc, *N*-acetyl-galactosamine; Neu5Ac, *N*-acetyl-neuraminic acid (Hall and Rowlands 1974). The diversity of the carbohydrate binding domains of C-type lectins gives them ability to orchestrate the agglutination of a wide range of microbes and allows them to function in a similar manner as vertebrate antibodies in providing a certain level of specificity so they can act as nonclonal effector molecules (Cerenius, Jiravanichpaisal et al. 2010). Four plasma lectins were identified in American lobsters with binding specificities for DNA, *N*-acetyl glucosamine and mannan (Battison and Summerfield 2009). These authors considered lectins as potential acute phase proteins, part of the first line of defense in many animal species (for a review of acute phase proteins see (Cray, Zaias et al. 2009)). A number of C-type lectins, galectin, hemolectin, mannose-binding lectin and carcinolectin have also been identified in American lobsters (Clark 2014)

Another group of PRR molecules are the Toll-like receptors. These assist in differentiating self and non-self. A TLR2 has been identified in American lobsters (De Guise, Morsey et al. 2005). More recently another paper indicated expression of an undefined Toll-like receptor following exposure to bumper car parasite *Anophryoides haemophila* (Clark, Acorn et al. 2013). Additional isoforms for Toll, Spatzle (required to activate the Toll pathway in *Drosophila*), Peste, TRAF, Relish and I κ B, all of which are involved in either Toll or immune deficiency pathways have been identified recently in American lobster (Clark 2014).

Masquerade-like proteins are serine proteinases with a range of functions including binding to lipopolysaccharide and glucans and so are able to recognize Gram-negative bacteria and yeast, but not Gram-positive bacteria (Huang, Wang et al. 2000). On binding to these cells the protein is cleaved to generate cell adhesion and opsonic activities, which greatly accelerate phagocytosis and thus clearance. Recently, masquerade-like proteins have been confirmed to be present in American lobster (Clark 2014). There is no published confirmation yet of the presence of any masquerade-like proteins for European lobsters.

3. Variable immune molecules

The dogma within immune research has long held that invertebrates were incapable of mounting an adaptive response to pathogens in the way that vertebrates use antibodies. But slowly it has become apparent that a number of invertebrate taxa possess classes of genes and molecular products that can create the diversity

necessary for a much more specific immune response. One class are Fibrinogen-related proteins (FREPs) which combine fibrinogen and immunoglobulin-like domains in a single protein, at least in mollusks, current known insect FREPs lack the immunoglobulin-like domain (Cerenius and Söderhäll 2013). Another class of variable proteins are the Down syndrome cell adhesion molecules (DSCAMs). These belong to the immunoglobulin superfamily. These molecules appear to bind to bacteria. But the real interest comes from the ability of a limited number of genes to produce thousands of different isoforms through alternative exon splicing events (Watson, Püttmann-Holgado et al. 2005). DSCAMs have been identified in crustaceans where the genes were found to be able to generate 22,000 different unique isoforms (Watthanasurorot, Jiravanichpaisal et al. 2011). More recently, DSCAMs have been noted in American lobster (Clark 2014). Although FREPs have not been indicated in American lobster they have been shown in other crustaceans such as freshwater crayfish (Wu, Soderhall et al. 2011) and freshwater prawn (Zhang, Wang et al. 2014). There is no published evidence for either FREPs or DSCAMs in the European lobster as yet.

4. Clotting factors

It has been shown that there is a transglutaminase-dependent clotting system in crustaceans (Kopáček, Hall et al. 1993, Hall, Wang et al. 1999). In crayfish the clotting protein polymerizes and proceeds in forming the clot in the presence of calcium and transglutaminases that have been released from hemocytes (Maningas, Kondo et al. 2013). In spiny lobster, *Panulirus japonicus*, these transglutaminases

appear to be present in hyaline cells and semigranular cells (Aono and Mori 1996). Little is known about the clotting mechanisms in clawed lobsters. The process has been previously outlined (Martin and Hose 1995). The authors indicate that hyaline cells undergo rapid morphological changes that culminate in cytolysis and release of cytoplasmic factors that trigger coagulation. A recent molecular survey of the American lobster revealed expression of a suite of clotting related factors including; clotting protein precursors, coagulation factors, fibrinogen and fibronectin, and transglutaminases (Clark 2014). There are no published reports for European lobsters. It seems reasonable to expect that the clotting systems in American and European lobsters will be similar to those found in other crustaceans. This molecular insight into potential clotting related molecules of the American lobster provides an interesting starting point.

5. Prophenoloxidase Activating System and Associated Molecules

One of the most important immune mechanisms for removal of pathogens in invertebrates is the melanization cascade. It utilizes circulating hemocytes and the prophenoloxidase (proPO)-activating system to achieve pathogen entrapment and wound healing (Cerenius, Lee et al. 2008). This system plays a central role in the non-self recognition orchestrating humoral components of the immune system and cellular responses by attracting hemocytes, inducing phagocytosis, melanization, cytotoxic reactant production such as toxic quinone substances and reactive intermediates, and particle encapsulation (Cerenius, Jiravanichpaisal et al. 2010). One interesting finding, that doesn't seem common across the arthropods is that

crustacean hemocyanin can, under certain conditions, be cleaved to exhibit phenoloxidase activity (Lee, Lee et al. 2004). It has been reported that several of these activating enzymes were present in a recent study on American lobster (Clark 2014). There are currently no reports in European lobster. Previously, Prophenoloxidase has been reported in American and European lobster (Sugumaran and Nellaiappan 1991). Activation of prophenoloxidase to phenoloxidase requires proteolytic cleavage through the action of a clip-domain serine proteinase often termed the prophenoloxidase-activating enzyme (Amparyup, Charoensapsri et al. 2013). However, these processes need careful control so that they do not become deleterious to the animal (Hauton 2012). And so it is not surprising to find a suite of proteinase inhibitors such as serine proteinase inhibitor and kazal-type proteinase inhibitor present in American lobster (Clark 2014).

6. Hemocytes

Hemocytes play an important role in crustacean immunity mainly through the role of phagocytosis and the efficient uptake of bacteria by circulating hemocytes or static phagocytic cells. Hemolymph is the circulating fluid that carries the various hemocytes and other blood components around the body. It contains a variety of components including; hemocyanin, for oxygen transport, various bacteriocidins, lysins, and agglutinins, prophenoloxidase and alpha 2-macroglobulin (Spycher, Arya et al. 1987). There are usually three morphologically distinct classes of hemocytes observed in the hemolymph in crustaceans, hyalinocytes, semigranular cells and

granular cells, all of which are involved in immobilizing and destroying invasive pathogens (Johansson, Keyser et al. 2000). The granules may also function as a reservoir of immune molecules (Martin and Hose 1995).

One recent reference identified the various cells as follows; hyalinocytes are usually ovoid, 8-16 μm in length with a high nuclear/cytoplasm ratio, relatively few granules (Wahle, Tshudy et al. 2012). They lyse readily in the presence of foreign material releasing clotting factors that immobilize this material (Hose, Martin et al. 1990). Granulocytes are larger, >20 μm long with a low nuclear/cytoplasm ratio and are often separated into small-granule or semigranular hemocytes and large-granule hemocytes. These are responsible for phagocytosis of small particles and encapsulation of larger particles (Paterson, Stewart et al. 1976). Hose et al. suggested that crustacean hemocytes be categorized as hyaline cells, small granule cells and large granule cells and that morphology is a poor indicator of function (Hose, Martin et al. 1990). Specific activities of these cell type notwithstanding, hemocytes have been classified in these general categories for both American and European lobsters (Cuénot 1903, Hearing and Vernick 1967, Cornick and Stewart 1978, Hose, Martin et al. 1990, Battison, Cawthorn et al. 2003). There are variations in these classifications but the basic differentiations remain broadly similar. Battison et al. reported a total of 11 different hemocyte cell types that were classified using a combination of cytoplasmic granule content and staining characteristics, nuclear morphology, and N:C ratio (Battison, Cawthorn et al. 2003). They proposed that the classical breakdown of hemocyte type into the three traditional hemocytes could be further subdivided based on the differential staining

of granules reflecting different functions of the cells and cellular maturity being reflected in the nuclear characteristics (Battison, Cawthorn et al. 2003).

A masquerade-like protein mentioned earlier facilitates the opsonic activity associated with proPO in lobsters promoting phagocytosis and cellular encapsulation of Gram-negative bacteria and yeast through the presence of triggers of the immune system such as lipopolysaccharide and β -1,3-glucans (Cerenius, Jiravanichpaisal et al. 2010).

When the hemocytes of the American lobster respond to a foreign particle they can form tight aggregations or nodules that can enlarge to the point where they become trapped in the vasculature (Martin, Kay et al. 1998).

Interestingly, hemocyanin of crayfish has been indicated to be involved in several functions including immune responses such as an oxygen carrier protein, as a precursor for an antibacterial peptide designated astacidin (Lee, Lee et al. 2003), and a molecule with phenoloxidase function (Lee, Lee et al. 2004). It is unclear if this situation occurs in lobsters.

Finally, one other group of cells that should be mentioned are the fixed phagocytes which have been identified in the hepatopancreas of American lobsters and other decapod crustacean species (Johnson 1987, Factor, Orban et al. 2005). They are shown to remove foreign particles from the blood as it passes through the digestive gland (Factor and Beekman 1990, Factor, Orban et al. 2005).

7. Anti-bacterial/anti-microbial components

Alpha 2-macroglobulins are broad-spectrum anti-proteinases that circulate in the blood or hemolymph. They serve to protect against extraneous proteinases, possibly originating from invading pathogens, as well as endogenous proteinases. They have been identified in American lobsters but have not been confirmed in European lobsters to date (Spycher, Arya et al. 1987). One interesting facet of alpha 2-macroglobulins is that there has been a suggestion that they share a common evolutionary path with complement components C3 and C4, having similar active site structures (Sottrup-Jensen, Stepanik et al. 1985). Complement is a complex network of plasma and membrane-associated serum proteins which can elicit highly efficient and tightly regulated inflammatory and cytolytic immune responses to infectious organisms (bacteria, viruses, parasites), tissue damaged by physical, chemical, or neoplastic insults, and other surfaces identified as 'nonself' (Dunkelberger and Song 2009).

Another group of proteins with anti-bacterial properties are the 'crustins'. Within decapods these proteins have 45-50 amino acid Whey Acidic Protein (WAP) domain at the C-terminus and a 16-24 amino acid signal sequence at the N-terminus. They are 7-14kDa in size that are released by circulating granular hemocytes (Smith, Fernandes et al. 2008). There are several different groups of crustins so far defined in crustaceans (Smith 2011). Both European and American lobsters are reported to have only Type 1 crustins, which have a single WAP-domain, a cysteine-rich and a signal sequence (Christie, Rus et al. 2007, Hauton, Brockton et al. 2007). These proteins appear to have anti-bacterial properties that are predominantly directed at

Gram-positive bacteria (Smith, Fernandes et al. 2008). Additionally, it is supposed that type I crustins also have proteinase inhibitory properties (Smith 2011).

The first reported isolation of antibacterial proteins in lobsters was for the American lobster (Battison, Summerfield et al. 2008). The authors reported two AMPs, CAP-1 (4-6kDa) and CAP-2 (12kDa). CAP-1 displayed bacteriostatic activities towards Gram-negative bacteria as well as protozoacidal activity against two cultured scuticociliate parasites. Cap-2 appeared to have sequence similarities to crustins with the WAP- region being present. A recent report indicated the presence of small (<10kDa) antimicrobial proteins that were present in the carapace of the American lobster (Mars Brisbin, McElroy et al. 2015).

Another group of anti-bacterial factors includes the anti-lipopolysaccharide factors a number of which have been reported in American lobsters (Beale, Towle et al. 2008, Clark 2014). A total of seven isoforms have been identified in this species (Clark, Acorn et al. 2013, Clark, Acorn et al. 2013, Clark, Greenwood et al. 2013). These proteins have wide ranging activities including; antibacterial, antiviral and antifungal actions (Hauton 2012). Four of them have been reported to show increased expression following bacterial or parasite challenge (Beale, Towle et al. 2008, Clark, Acorn et al. 2013, Clark, Acorn et al. 2013, Clark, Greenwood et al. 2013).

8. Conclusion

There is a disparity between the information available on the immune system of the American lobster compared to the information for the European lobster. It is perhaps surprising the gaps that still exist in our understanding of these economically important animals. The advent of advanced analytical techniques such as genomics and transcriptomics are clearly beginning to significantly expand out knowledge of the components of the immune system. One thing that becomes clear in analyzing the immune functions of these animals is the high level of interaction between all these immune components. The various actuators and responders within the immune system orchestrate the timely and efficient removal of foreign particles limiting the impact of pathogenic entities, and the potentially detrimental impacts of these immune factors on the host tissues, before they have an opportunity to spread and debilitate the host animal. The response is finely balanced between sufficient reaction to respond to the invader and limiting the response such that the host can maintain homeostasis in light of the intruder. The continued presence of these animals within modern fauna shows the efficacy of these strategies.

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Table 1. General immune defense mechanisms in clawed lobsters

Mechanism	Downstream action	Targets
Antibacterial/antimicrobial components, including crustins	Pathogen membrane lysis	Bacteria, viruses and fungi
Clotting factors	Clot development and nodule formation	Bacteria and fungi and tissue damage
Hemocytes including: hyalinocytes, granulocytes and semigranulocytes	Bind to bacteria and fungi, can lead to nodule formation, phagocytosis and microbial killing	Bacteria and fungi, tissue damage and wound healing
Lectins/agglutinins	Induce agglutination and phagocytosis via hyalinocytes and semigranulocytes	Non-self particles
Prophenoloxidase activation system (PPAS)	Phagocytosis, encapsulation, cytotoxic reactant production, nodule formation, melanization, wound healing	Bacteria and fungi, tissue damage and wound healing
Pattern recognition receptors (PRRs)	PPAS, and thus phagocytosis, encapsulation, nodule formation	Recognize lipopolysaccharide, peptidoglycan, β -1,3-glucans, double-stranded RNA, mannan
Variable immune molecules	FREPs bind to pathogens, DSCAMs bind to bacteria, both promote phagocytosis	Bacteria, fungi and parasites