School of Pharmacy

Characterisation of the Innate Immune Responses of Marron (*Cherax cainii*)

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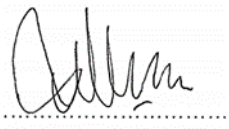
Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: 

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LIST OF ABBREVIATIONS

α_2M	α_2-macroglobulin
AB	Anticoagulant buffer
βGBP	β-1, 3-glucan binding protein
AMPs	Antimicrobial peptides
cAMP	Cyclic antimicrobial peptides
CFS	Crayfish saline
DHC	Differential haemocyte count
EDG	Electron dense granules
FACS	Fluorescence-activated cell sorter
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GCs	Granular cells
HCs	Hyaline cells
HLS	Hemocyte lysate supernatant
Hpt	Hematopoietic tissue
LGBP	Lipopolysaccharide and β-1, 3-glucan binding protein
LGCs	Large granular cells
LM	Light microscopy
LPS	Lipopolysaccharide
NO	Nitric oxide
PAMPs	Pathogen associated molecular pattern
PI	Phagocytic index
PmLec	*Penaeus monodon* lipopolysaccharide-binding lectin
PO	Phenoloxidase
PRPs	Pattern-recognition proteins
PRRs	Pattern-recognition receptors
proPO	Prophenoloxidase
RNAi	RNA interference
RER	Rough endoplasmic reticulum
SGCs	Small granular cells
SRBC	Sheep red blood cells
SSC	Side scatter

TEM	Transmission electron microscopy
TGase	Transglutaminase
THC	Total haemocyte count

ABSTRACT

Cherax cainii (marron) is an indigenous species to Western Australia, and a prominent commercial aquaculture species. The immunological defense of *C. cainii* depends on the innate immune system however, little is known about the specifics of the *C. cainii* defense mechanisms. Therefore, the work herein aims at gaining deeper insights into the *C. cainii* immunological response, with particular emphasis on the cellular component, the haemocyte. Haemocyte responses including morphologic characteristics, nitric oxide (NO) production, population changes and phagocytosis were evaluated in response to inflammatory stimulus from Gram negative bacteria and their endotoxin and the environmental effect of temperature.

Immunological challenge by live and heat-killed *V. mimucus* and lipopolysaccharide (LPS) was conducted on whole *C. cainii* and *in vitro,* through haemocyte culture, at various temperatures. Techniques and technologies to assess responses included light and transmission electron microscopy (TEM), flow cytometry (FCM) and the Griess assay for NO production.

Major findings include the identification of three morphologically distinct haemocyte cell types in keeping with findings in related species: called hyaline cells (HC), small granular cells (SGC) and large granular cells (LGC). Total and differential haemocyte counts changed when marron were cultivated in different water temperatures: total haemocyte counts increased from 1.9 to 4.9 $\times 10^6$ cells ml^{-1} when environment temperature increased from 20 to 30 °C. Hyaline cells represented the most abundant cell type (42.5%), followed by SGCs (35%) and LGCs (22.5%): data from 25 °C. Haemocyte activation in response to live and heat-killed *V. mimucus* and LPS, both *in vivo* and *in vitro*, resulted in a change in ratio of haemocyte type, with HC increasing in number and both SGC and LGC decreasing. HCs in vitro tends to dominate and the SGCs/LGCs fraction is diminished. HCs rise in proportion because granulocytes are extinguished (or convert to HCs) in the course of the immune response. These changes were affected by both incubation time and temperature. However, the *in vivo*'s DHCs, show relatively more granularity than the in *vitro* studies. This is consistent with the idea that the host response is based around the granular fraction. However, phagocytic activity assessed through TEM and FCM revealed that all three haemocyte cell types are involved in phagocytosis of Gram negative bacteria, with HC and SGC demonstrating the

highest activity. Furthermore, haemocyte activation with bacteria resulted in NO production, with highest levels produced by SGCs and LGCs.

These findings are the first in *C. cainii* and demonstrate that circulating haemocyte are a dynamic population of cells involved in the elimination of pathogens. In fact there are heaps of HCs present *in vitro* but these cannot granulate *in vitro* to restore the balance and also the hematopoietic tissue (Hpt) appears to modulate the cell types *in vivo*. The recommended lineages of *C. cainii* haemocytes have been established upon morphological characteristics, since currently only a little information know about the cellular and biochemical signals involved in regulating *C. cainii* haemocyte type *in vivo*. The data is suggestive that there is one cell line forming three distinct haemocyte subtypes of a contiguous nature. The information about the innate immune repertoires of *C. cainii* in response to the invasion of diseases is very important to help further our understanding about the mechanism of freshwater crayfish immunity to disease infection and developing strategies for management of the disease.

CHAPTER 1

LITERATURE REVIEW

1.1. Marron (*Cherax cainii*)

1.1.1. Overview

Freshwater crayfish are truly a wonder of morphological distinction with a marvellously varied group of animals. They have varied ecological habitats, living in four main types: burrow (primarily), fast flowing creeks, ponds and lakes or slow-moving water, and cave (the troglobitic species). Freshwater crayfish have been used as study model organisms in a variety of sciences due to their diversity, coupled with their simplicity of capture and their prominent status in the ecological communities. Several species can grow to relatively large sizes and are plentiful in local areas, attracting the interest of recreational fishers and aquaculturist. The freshwater crayfish family consist of Astacidae, Cambaridae and Parastacidae with each 3, 12, and 14 genera, respectively. Australia has an abundant and wide-ranging freshwater crayfish fauna, all species belonging to the Parastacidae, a southern hemisphere family. Toon et al. (2010) explained that the distribution of family Parastacidae on Australia and New Guinea, South America, Madagascar and New Zealand are not likely to have distributed using oceans, due to harsh limitations of freshwater. Furthermore, Crandall et al. (1999) mentioned that the majority of existed Australian and New Guinean crayfish came from three genera: *Cherax*, *Engaeus* and *Euastacus*. The *Cherax* genus is found in an enormously wide area of Australia landmass, ranging from the farthest south-west, central, eastern and the far north (Austin and Ryan, 2002).

Marron, historically, was assumed as a single species, *Cherax tenuimanus.* This freshwater crayfish was endemic to the southwest of Western Australia (WA) but presently only lives around 60 km (upper reaches) of Margaret River system. Their reduction within this river system and their complete displacement from the lower reaches of Margaret River was due to the introgression of *C. cainii* in 1980s (Austin and Ryan, 2002; de Graaf et al., 2009). Afterwards *C. tenuimanus* has been listed as a critically endangered species under the Western Australian Wildlife Conservation Act 1950 and the IUCN Red List of Threatened Species. *C. cainii* has since occupied Margaret River, and nowadays lives together with the persisting populations of *C. tenuimanus* (de Graaf et al., 2009). Following definite morphological variances and the recognition of substantial genetic differences by Austin and Ryan (2002) and Nguyen et al. (2002), these two species were officially acknowledged as individual taxa. Furthermore, Austin and Ryan (2002) assigned the name *C. tenuimanus* (Smith, 1912) to marron restricted to the Margaret River because the type locality of the original description of marron was the Margaret River and proposed the name *C. cainii*

(Austin, 2002) for the common, widely distributed marron. The amendment of name has been approved by the International Commission on Zoological Nomenclature (Case No. 3267). From here onwards we will refer to the marron restricted to the Margaret River as *C. tenuimanus* and the common, widely distributed marron as *C. cainii*. The systematic classifications of marron then as follows:

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Suborder: Pleocyemata

Infraorder: Astacidea

Family: Parastacidae

Genus: Cherax

Species: *Cherax cainii* (Austin)

1.1.2. Taxonomy

Initially it was thought that the Parastacidae family establish a monophyletic group, On the other hand, the families of Astacidae and Cambaridae seem to be non-monophyletic groupings because of the genus Cambaroides clustering (at present classified as the Cambaridae, from Asia) with the genus Pacifastacus (at present classified as Astacidae, from the north-west United States and south-west Canada (Crandall et al., 2000; Crandall, 2006). Whilst the early studies offer an outstanding framework of phylogenetic data for comparative biology, new research has already established robust phylogenetic relationships both within the families of freshwater crayfish and among the families relative to various potential sister taxa (Figure 1.1). Based on the latest phylogenetic relationships method, crayfish in Australia are actually non-monophyletic, with two genera (Spinastacoides and Ombrastacoides) from Tasmania, establishing a clade with New Zealand and Malagasy (both

monophyletic). Therefore, the main clade of Australia formed of eight genera, which includes three different groups: 1. Euastacus/Astacopsis; 2. Engaewa/Engaeus/Gramastacus/Tenuibranchiurus/ Geocharax and 3. Cherax.

Due to the long terminal branches of Australia compare to the North American fauna, the taxonomy of Australian crayfish as a centre of the southern hemisphere, have some problems (Toon et al., 2010). Crisp and Cook (2007) explained that the long terminal of Southern Hemisphere branches and its increased speciation time can generate monophyletic group because these terminal branches indicate a low rate of diversification in current years, unlike the Northern Hemisphere crayfish. In spite of this, some published phylogeny studies have found paraphyletic genera relationships, for instance Euastacus (Shull et al., 2005) and Engaeus (Schultz et al., 2009). Even though there is some conflict between Southern Hemisphere phylogeny and taxonomy, nearly all differences came from undescribed species instead of true conflicts about taxonomy. Bracken-Grissom et al. (2014) added the information that compared with Northern Hemisphere crayfish, the existing crayfish in the Southern Hemisphere represent much older lineages. These older family may have been assisted by a geographical range activity from the Gondwana break-up in the early Jurassic ~176–201 Megaannum (Ma) that makes a discontinuation of geographical range of individual or whole biota taxon by the physical or biotic barrier to gene flow or dispersal (vicariance) formation. The sequential Gondwana break-up is thought to be an influential factor in the shared biota establishment throughout Southern Hemisphere landmasses (Toon et al., 2010). Recently, this hypothesis has been confused by the discovery of Eocene time's (55 Ma) Parastacid crayfish in British Columbia (Bracken-Grissom et al., 2014). Toon et al. (2010) study also revealed the early-branching of clade from South American crayfish separating from the remaining southern hemisphere crayfish (~215/205 Ma), the existing Madagascar (*Astacoides*) and New Zealand + Australia genera (sister clade to *Astacoides*) come from momentarily afterward (~209/199 Ma).

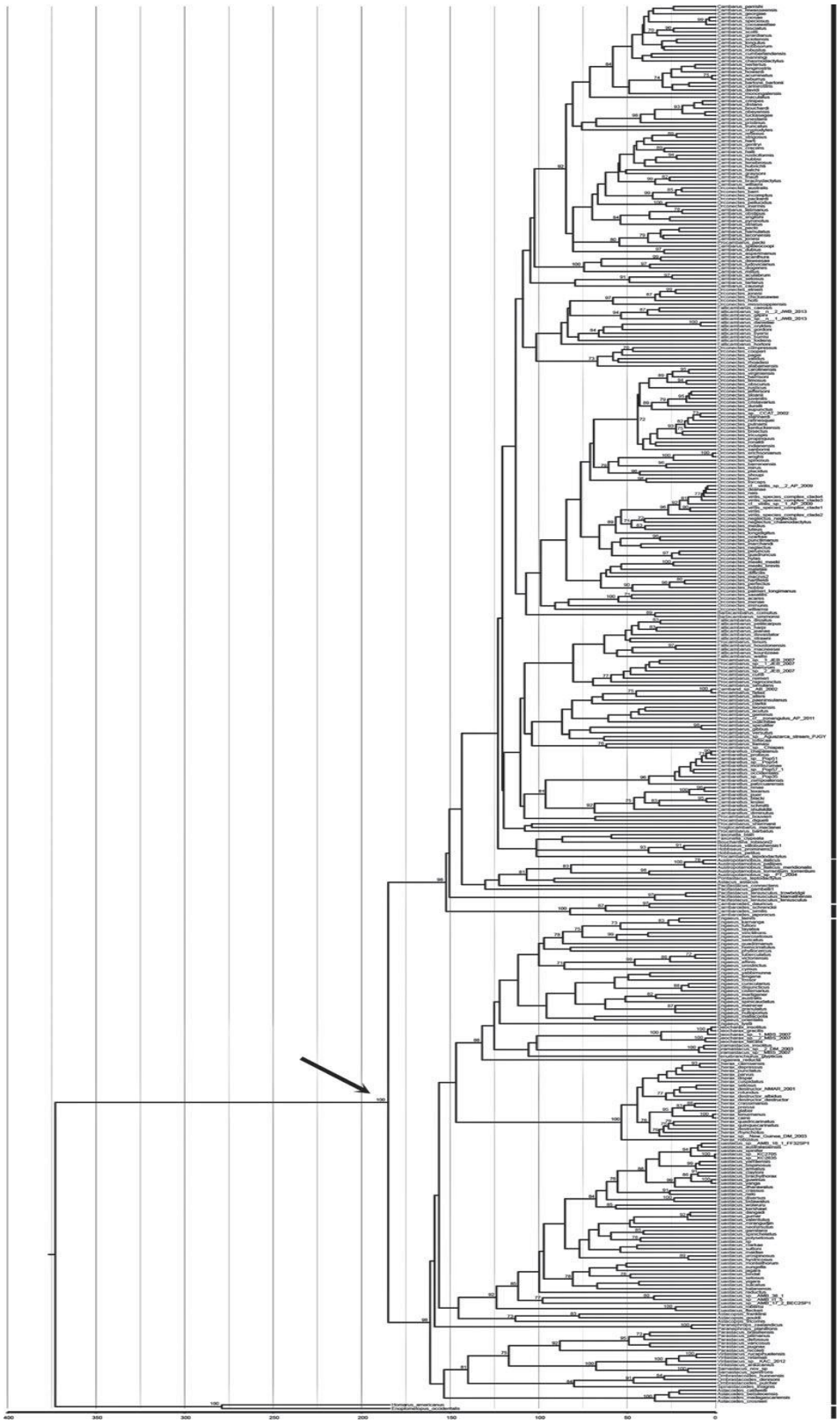


Figure 1.1. Chronogram for the northern and southern hemisphere freshwater crayfish taxa estimated in R8S using the Maximum Likelihood (ML) phylogeny. Node calibrations identical to Toon et al. (2010). Bootstrap support greater than 70% from 100 non-parametric bootstrap replicates shown (Owen et al., 2015).

Marron can reach a 380 mm maximum length and 2.7 kg in weight and are the third largest of freshwater crayfish species in the world (Merrick and Lambert, 1991; Molony et al., 2004). The appearance of five keels on the dorsal surface of the head and two small spines on the telson can immediately differentiate marron from other *Cherax* species. Austin and Ryan (2002) observed that *C. cainii* can be differentiated from *C. tenuimanus* by the well develop but relatively short mid carina, the absence of tufts of long setae on the cephalothorax and abdomen, and in the individuals adult, the absence of large well tuberculation in the areolar region (Figure 1.2).

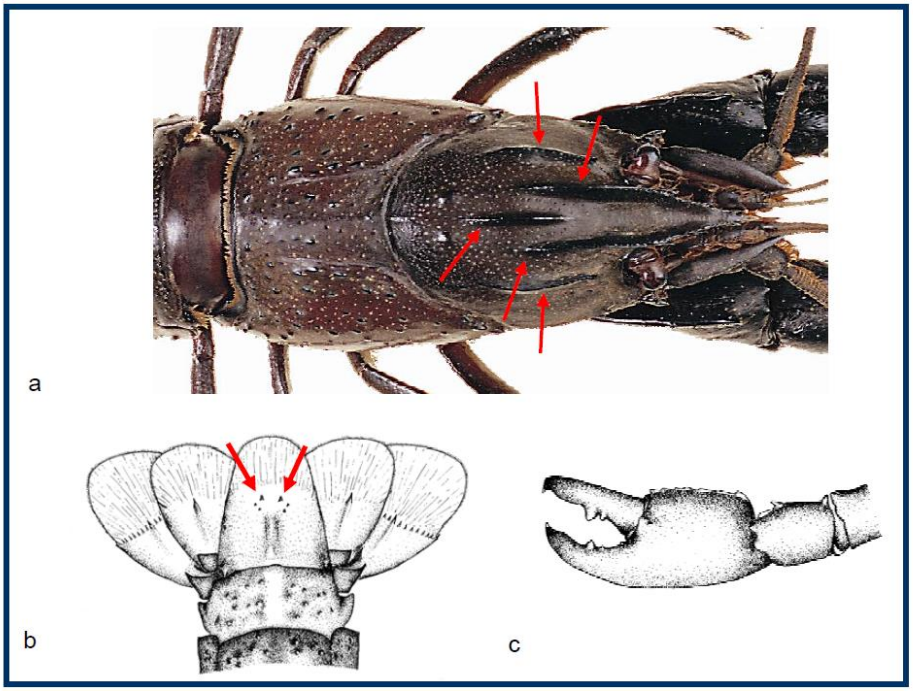


Figure 1.2. Identification of the characteristics of marron (*Cherax cainii*): a) Five keels along their head; b) two small spines on the telson; and c) narrow, pincer-like chelipeds (Fisheries Western Australia, 2004).

1.1.3. Biology

Marron are capable of maturation in two years under favourable conditions, although it normally takes three years (Merrick and Lambert, 1991). Marron can breed in spring every year, primarily due to suitable water temperature and daylight length (Mosig, 1998). Merrick and Lambert (1991) and Mosig (1998) have reported that female marron have between 95 and 900 berries, but average 150 berries. The early development rate of marron is similar to yabbies but keep on growing far beyond yabby size.

Marron are mostly active at night, when they normally feed in shallow water, reducing the risk of predation (Merrick and Lambert, 1991). Marron take cover under their living environment or in deeper water during daylight hours and they are opportunistic feeders; they will eat just about anything put in front of them, eating mostly water vegetation but also meat and small organisms (Merrick and Lambert, 1991; Mosig, 1998). Beatty (2006) demonstrated that marron can live together with the foreign fish, *Gambusia holbrooki*, that live in large numbers in the Hutt River in WA. Lawrence (1998), in reference to aquaculture ponds, reports that in order to prevent marron leaving the ponds and other predators from entering, ponds should have a perimeter fence. This assumes that marron are able to move across land, to some extent, where they can find other water bodies.

1.1.4. Aquaculture

By the early 1990s, new marron farmers in Australia were provided with information to encourage proper site selection, construction and outfitting of ponds (Morrissy, 1992b). Many aquafarmers could not achieve semi-intensive harvests because their ponds were poorly built, un-drainable and without aerators. Basically, pond construction and outfitting characteristics are: reticulated water supply to individual ponds; gravity-draining (bottom) pond outlets with constant water level standpipes; low pond banks (free-boards); consolidated pond floors to allow easy harvest; site and pond outlet drains; overhead predator netting and side fencing; adequate bank access for feeding and harvesting vehicles; time-switched paddle-wheels; and effluent treatment ponds and generally a commercial grow-out pond has 0.1 hectare (Morrissy, 1992a). Management practices that are important for grow-out include: use of single year-class cohorts; annual drain-down harvest followed by restocking; daily demand feeding; daily use of paddle-wheel aerators during the growing season; and personal computer (PC) filing of stocking and harvest data.

Furthermore, Morrissy (2000) added information about annual marron farm schedules, specifically regarding breeding and growth cycles, experiences over-winter harvesting of larger grow-out ponds and restocking with 0+ year old (yo) from smaller spawning-nursery ponds. The smaller spawning-nursery ponds are cleaned and immediately stocked with parent marron for spawning in early springtime. Over the following year, the 0+ yo marron are grown-out to 1+ yo, and other ponds have grown-on of 1+ to 2+ yo and breeding stock. This schedule ensures most efficient practice of all the large ponds in a repeated one-year cycle, including smaller pond use for spawning and nursery stages. That is, grow-out is restricted to a one year cycle, preventing pond duplication for annual harvests, and no pond

is idle for a lengthy period. These practical features became apparent when large commercial farm was practised; other schedules are less efficient or unworkable. This schedule allows quantitative correlations for all the production cycle steps, allowing computerised total farm management of all stocks numbers and pond areas.

Earlier spawning for marron usually was done after late July, young marron are released from parent females at a very small size (~0.06 g) in December-January (early summer) and release by the start of the growing season in September-October (springtime) (Morrissy, 1983). However, marron are an ecotype animal that does not grow rapidly in the first growing season, the most productive phase only starts in the following growing season (Morrissy, 1992b). Then, over the non-growing season of winter (June-August), the large grow-out ponds are gradually harvested of 1+ yo by drain-downs and restocked with 0+ yo from the nursery ponds. With this method, one in ten grow-out ponds can be used profitably, to grow-on small unsaleable 1+ yo marron from the other ponds harvest. Harvest in winter prevents mortalities from daytime heat and soft ecdysial marron handling, in addition the larger 0+ yo marron are more easily counted for stocking. Winter harvesting also circumvents market competition with yabbies, and also marron caught from licensed farm dams that are trapped during the warmer months in WA. However, harvesting of larger marron by labour-intensive trapping during semi-intensive grow-out, still needs to be studied.

1.1.5. Disease

The incidence of diseases due to viruses, bacteria and microsporidia causing pathological conditions in crayfish species have been reported through research in native Australian species (Herbert, 1987; Herbert, 1988; Anderson, 1990; Anderson and Prior, 1992; Ketterer et al., 1992; Owens et al., 1992; Eaves and Ketterer, 1994; Edgerton et al., 1994; Edgerton et al., 1996). The prevalence of potential pathogens has changed in Australia based on the health surveys of red claw crayfish done in 1992 and 1996 (Edgerton and Owens, 1999). Although there have been no significant disease issues in the Australian marron industry recently, irresponsible culture process could provoke virulent diseases, mostly by bacteria and virus, and causing great loss of production.

Pathogenic *Vibrio* members comprising of sucrose negative variants, called *Vibrio mimicus*, has been recognised and to date also considered as aquatic pathogen in crayfish aquaculture (Davis et al., 1981). Two mortality cases in red claw crayfish *C. quadricarinatus* from a commercial crayfish farm in northern New South Wales and southeast Queensland

correlated with systemic *V. mimicus* infections (Eaves and Ketterer, 1994). Several strains of *V. mimicus* have also been isolated from pond waters that produced systemic disease in two freshwater crayfish, the yabby *C. albidus* (from a commercial crayfish farm in WA) and the red claw *C. quadricarinatus* (from experimental culture ponds founded by the University of Queensland (Wong et al., 1995). *V. mimicus* and *V. cholerae* were the main pathogens isolated from all affected red swamp crawfish *Procambarus clarkii*: each case examined was from commercial crawfish operations from Louisiana, United States of America (USA), from 1985 to 1990. (Thune et al., 1991). Association of toxigenic *V. mimicus* with the freshwater prawn *Macrobrachium malcolmsonii* has also been described in Bangladesh (Chowdhury et al., 1986). A novel disease outbreak, identified as *V. mimicus*, distinguished by acute and chronic shaped ulcers in the skin also occurred in the aquatic vertebrate, yellow catfish (*Pelteobagrus fulvidraco*), cultured in Guangdong and Guangxi provinces of South-China in 2011 (Zhang et al., 2014). Raw or improperly cooked *V. mimicus*-infected crayfish when consumed by human can lead to gastrointestinal disease. Outbreaks of seafood-associated gastroenteritis caused by *V. mimicus* have been reported in other parts of the world (Kodama et al., 1984; Shandera et al., 1983). *V. mimicus* isolation from invertebrates and vertebrates, in disease epidemics in several countries, highlights the potential of this pathogen to cause disease, not only for freshwater crayfish but also in those consuming them.

1.2. Ecological innate immune responses in invertebrates

1.2.1. Evolution of innate immune system

During evolution of immune system, two universal immune systems have evolved to protect against pathogen, namely adaptive (acquired) and innate (natural) immunity. The adaptive immune system developed approximately 400 million years ago and found in vertebrates (Thompson, 1995). The mechanisms of vertebrate immune response have progressed sophistically and complicatedly including an immunological memory with production of a large number of antigen-recognition receptors system and innate immune repertoires, for example phagocytosis, natural killer cells and complement system that can recognize and remove pathogen (Hoffmann, 1995; Hoffmann et al., 1996; Carroll, 1998). Despite the fact that the adaptive immune system only developed in vertebrates, invertebrates possess a rapid and effective innate system to recognize and destroy pathogens. The innate immune system is a more ancient defence mechanism phylogenetically and can be found in all multicellular organisms. This immune system is the host first-line defence that helps to reduce early infection and depends on germ line encoded receptors that can identify

conserved microorganisms molecular patterns (Fearon and Locksley, 1996; Fearon, 1997; Medzhitov and Janeway, 1997). The innate immune systems is able to protect against invading pathogens, even though it cannot produce antibodies and has no immune memory. There are a lot of research conducted to study the innate immune function, mechanism and other important roles in invertebrates and all multicellular organisms from humans to plants (Hultmark et al., 1980; Steiner et al., 1981; Medzhitov and Janeway, 1998a; Medzhitov and Janeway, 1998b).

A significant problem in determining an evolutionary association between blood cells of invertebrates and vertebrate is that there are no relic blood cells that have survived. Consequently, researchers must compare among existing species of blood cells. A major difficulty is invertebrate blood cells are very different from their ancestral forms because, as a varied group, they have progressed over millions of years and most likely their cells have also changed by adaptive radiation. The immune cell(s) most likely evolved from a free-living, voyaging and protozoan-like predecessor (Cooper, 1976; Janeway and Medzhitov, 2002; Vinkler and Albrecht, 2011). Millar and Ratcliffe (1989) stated that the wandering phagocytic amoebocytes (archeocytes) in primitive metazoans, which lack a blood system such as sponges, get their diet from the cells that can entrap food (choanocytes) and circulate through the whole body. While in coelenterates and flatworms, relatively simple metazoans deficient in a circulatory system, have a system that can simply carry the products of digestion and respiratory gases throughout the body by diffusion. Those author believed that metazoans evolved body size and complexity, along with coelom development, based on the ability to transport food, gases, and waste materials in their blood vascular system. They also produce haemoglobin as respiratory pigments, either loose in solution or confined in cells (erythrocytes) that can carry and store oxygen. Cooper (1976) has also described that phagocytic cells in metazoans, which have moved around from adjacent connective tissue into the circulatory system, are maybe the only type of blood cell which have been around during the course of evolution, and related to the granulocyte, monocyte, or tissue and organ macrophage of mammals. They eventually differed from their role as food-foraging cells and took a new functional responsibility in immune surveillance as defence cells. Primitive blood cells have further differentiated, because of pressure from the environment and internal systems, eventually generating other cell types with specific functions. Borysenko (1976) explained that it may not be necessary for invertebrates to have a specialized immune system because they have a relatively small size, simple morphology, short life and a lot of progeny which can guarantee the continuation of their species. However, there are adequate

evolutionary pressures to encourage cellular component formation and development with the special function(s) to counteract the threats and elimination by potential pathogens and the somatic mutation effect which can initiate cancer.

1.2.2. Innate immune responses in invertebrates

During evolution, both invertebrates and vertebrates have developed a system, referred to as innate immunity, as their first line of defence against diverse pathogens (Kimbrell and Beutler, 2001; Hoffmann, 2003; Loker et al., 2004). In crustaceans, they are widely depend upon their innate defensive mechanisms, in the absence of an adaptive immune system. Crustaceans possess efficient defence mechanisms in spite of lacking immunological memory and antibodies. Despite the fact that elements signifying memory and specificity are found in invertebrates, no genuine adaptive elements have yet been detected in their innate systems (Wang et al., 2009, Kurtz and Franz, 2003, Schmid-Hempel, 2003; Vazquez et al., 1997; Sierra et al., 2001).

Invertebrates have a very efficient and complex innate immune responses, including cellular and humoral responses. Schmid-Hempel (2003) and Beutler (2004) divided both cellular and humoral components in invertebrate immune responses into two arms: being the afferent (or sensing) and efferent (or effector) arms. Janeway and Medzhitov (2002) stated that the afferent arm utilises receptor-mediated recognition of pathogen-associated molecular patterns (PAMPs), which are very much conserved inside microbial groups and usually not present in the host. Host receptors should become an efferent arm to attach to a pathogen and stimulate a multi-faceted immune response (Beutler, 2004). A wide arrangement of possible innate immune responses are stimulated through a variety of cells that perform responses, such as phagocytosis, cytotoxicity and inflammation (Roch, 1999). Once invaded by pathogens, a series of cellular and humoral immune responses of these defence mechanism are activated.

There are three categories of innate immunity primary components that characterise the immune response effectiveness. Firstly, the organism differentiates between self and non-self; secondly, the organism escalates a protective mechanism that can eliminate pathogens; and thirdly, the organism can identify and destroy its own harmed or unhealthy cells. These conditions direct to the three basic innate immunity elements: phagocytosis (cell-mediated); humoral response activation leading to opsonisation, melanisation and coagulation (cell-free); and humoral antimicrobial compound production (cell-free). The differentiation of

humoral and cellular defences is not distinct as many humoral factors affect haemocyte function.

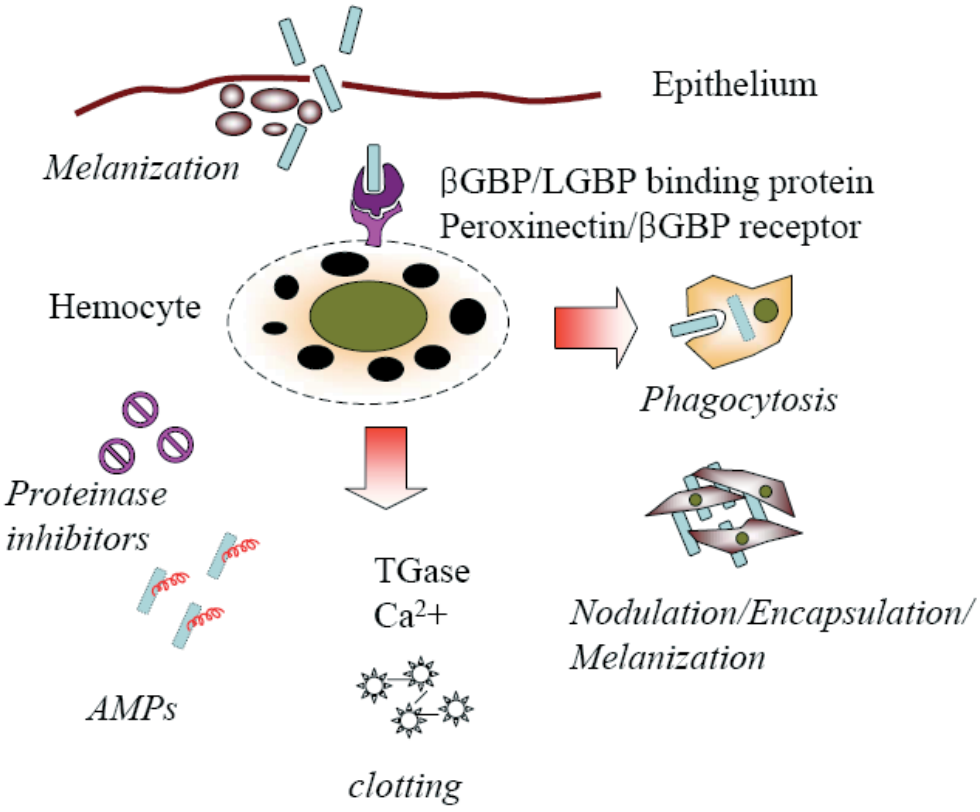


Figure 1.3. Schematic design giving an overview of the defence reactions that occur in crayfish (Kim, 2006).

βGBP: β-1, 3-glucan binding protein; LGBP: Lipopolysaccharide and β-1, 3-glucan binding protein; AMPs: antimicrobial peptides; TGase: Transglutaminase.

Kim (2006) illustrate pathogen entry into crayfish haemolymph which is followed by the reaction of cellular and humoral defence (Figure 1.3). The recognition molecules, such as β-1, 3-glucan binding protein (βGBP) and Lipopolysaccharide β-1, 3-glucan binding protein (LGBP) in haemolymph, participate in binding to microbial cell wall component. These complexes bind to membrane receptors of haemocytes. As a result, the haemocyte degranulates and releases immune molecules which mediate humoral immune responses such as the prophenoloxidase (proPO) system, clotting and antimicrobial activity by antimicrobial peptides (AMPs).

1.2.3. Cellular immune responses

Haemocytes mediate cellular immune responses in the haemolymph, having an important task in crustacean immune defence. Zhang et al. (2011) confirmed that different types of haemocytes participate in cellular immune responses, which are involve in the elimination of pathogens by phagocytosis, confining pathogens in haemocyte aggregates or nodules, and encapsulating the larger pathogens. Haemocytes phagocytose bacteria, yeast and apoptotic cells, while encapsulation or nodulation appears to be the defence mechanism against foreign entities that are too large to be phagocytosed by individual haemocytes. Ratcliffe (1985) and Johansson and Soderhall (1989) detailed that primary cellular immune responses to small microbes is phagocytosis and for larger parasites is encapsulation and nodule formation.

The cellular response mediated by haemocytes is important in early non-self recognition. First, they utilize phagocytosis, encapsulation and nodular aggregation to eliminate foreign entities in the hemocoel (Soderhall and Thornqvist, 1997). Second, haemocytes apply cellular clumping for wound healing and release factors required for plasma gelation to initiate the coagulation processes (Johansson and Soderhall, 1989; Omori et al., 1989; Vargas-Albores et al., 1998), and carry and discharge of the proPO system (Johansson and Soderhall, 1989; HernandezLopez et al., 1996). Moreover, haemocytes are also involved in melanisation, another response that is immediately activated upon injury/infection. This response is normally localized at the injury site or at the invading microorganism's surfaces. Toxic phenol intermediates or melanin, in melanisation, are released which immobilizes and kills the pathogen (Young Lee and Söderhäll, 2002; Soderhall and Cerenius, 1998). The sequence of events of proPO activation is massive aggregation of haemocyte, degranulation and lysis, melanisation and plasma gelation. This activation may become too widespread and be fatal to the host. Therefore, crayfish have protease inhibitors in their haemolymph to inhibit this process: to restrict the action of proPO to the site of infection and inhibit over-activation and unintentional discharge from harmed haemocytes in the absence of pathogens (Hergenhahn et al., 1987; Hergenhahn et al., 1988).

1.2.4. Humoral immune responses

A number of immune-related humoral activities have been reported in numerous invertebrate species. Invertebrate humoral defences are usually known to include a range of factors from serum or plasma which protect against microorganisms, foreign materials or

abiotic particles. These factors encompass natural or inducible bioactive molecules which agglutinate, precipitate or neutralise nonself entities and those that have bactericidal, lytic or bacteriostatic particles. Several fungitoxic, antiviral or cytotoxic agents are regarded as humoral factors (Smith and Chisholm, 1992). Clotting is also part of the humoral factor because crustaceans, like other arthropods, usually use plasma gelation in addition to cell aggregation during haemolymph coagulation (Durliat, 1985). Some reactions can activate the humoral immune system for instance blood clotting by proteolytic cascades, melanin formation, opsonisation and a temporary synthesis of potent antibacterial peptides (Hoffmann et al., 1999). Additionally, any extracellular soluble factors that can enhance the cellular defences (i.e. cytokine-like molecules, opsonins or cell adhesion molecules) integrate into the humoral defence system.

In freshwater crayfish, humoral response involves coagulation and exocytosis of various proteins to the plasma, for example, components of the proPO activating cascade, leading to opsonisation and melanisation (Johansson and Soderhall, 1989; Soderhall and Cerenius, 1998; Cerenius and Soderhall, 2011), and synthesis of antimicrobial peptides (Dimarcq et al., 1997). Smith et al. (1995) explained that bacterial killing by humoral defence molecules can involve opsonisation and/or agglutination which results in neutralisation of the pathogen through a number of mechanisms. These may involve lysis or metabolism disorder through binding cell wall elements or increased permeation of cell membrane, and also inhibition of the pathogen growth. The complement of antimicrobial molecules, including production and storage sites, is species, tissue and even cell specific (Mitta et al., 2000). Jiravanichpaisal et al. (2006a) stated that in order to function, the humoral response utilises pathogen recognition through pattern-recognition receptors (PRRs) that can bind PAMPs or a range of immune effectors capable to neutralize and/or destroy the invading pathogens. Lemaitre and Hoffmann (2007) explained that AMPs assemble in haemolymph to protect against invading pathogens and is an example of humoral defence that synthesize and secrete immune components after pathogen infection.

1.2.5. Immune cell production

Immune cell production (haematopoiesis) is a complex process by which different blood cells are made and circulated from specific tissues. A number of factors are involved in this process, for instance transcription factors and cytokines. Many have been identified throughout the animal kingdom, from invertebrates to vertebrates, and are important in this

dynamic process: to maintain blood cells homeostasis. Invertebrate haematopoiesis offers a simple model system to study blood cells regulation of the innate immune system. Noonin et al. (2012) stated that invertebrate blood cells (haemocytes) are very similar to the myeloid lineage cells in vertebrates, since their main function is to contribute in innate immunity and blood clotting. Haematopoiesis in several other arthropods, for example crayfish, is an ongoing process throughout the whole life of the animals (up to 20 year). According to Soderhall and Thornqvist (1997), circulating haemocytes of most crayfish do not segregate and as a result aged cells must be constantly replaced by new cells into the haemolymph.

Haematopoietic tissues have been found in several crustacean species including the crab *Carcinus maenas*, lobster *Homarus americanus*, crayfish *Pacifastacus leniusculus*, penaeid shrimp *Penaeus stylirostris* and *Penaeus monodon* (Ghirettimagaldi et al., 1977; Martin and Graves, 1985; van de Braak et al., 2002). Martin et al. (1993b) have found haemopoietic nodules in the epigastric region by electron microscopy, and suggested that there are two stem cells types that deliver the different haemocytes in penaeid shrimp, *Sicyonia ingentis*. However, it is considered that crayfish haemocytes originate from a specialised haematopoietic tissue. The crayfish haematopoietic tissue is a separate organ situated in the dorsal part of the stomach and haematopoietic cells are bundled in small lobules found in the interlobular spaces. Five distinct morphological cell types are present in the haematopoietic tissue of crayfish *P. leniusculus*, which give rise to the two primary haemocytes lineages: semi-granular cells (SGCs) and granular cells (GCs) (Soderhall et al., 2005; Wu et al., 2008). A technique for primary culture of crayfish haematopoietic tissue cells was also established, and by using this method, it is now possible to observe the molecular mechanism of crayfish haematopoiesis *in vitro* (Soderhall et al., 2005; Söderhäll et al., 2003). As a result of this technique, astakine 1, a cytokine homolog of the vertebrate prokineticins, was first identified in the freshwater crayfish *P. leniusculus*, and was shown to be critical for new haemocyte formation and release (Soderhall et al., 2005). The total haemocyte number was considerably increased by native or recombinant astakine 1 injections, and silencing of astakine 1 by RNA interference (RNAi) *in vivo*, gave rise to a low haemocyte number following an injection of lipopolysaccharide (LPS) into crayfish.

1.2.6. Effect of environment

Environmental factors affect the health and reproduction of freshwater crayfish, including seasonal variation, temperature, pollutants and diet. However, there are still many gaps in understanding, particularly how these factors impact disease resistance, which influence the

success of the aquaculture industry. Certainly, temperature is a main factor on the metabolic and cellular activities and has a universal effect on the poikilothermic animal biology, but the level to which it affects crustacean immune-competence is largely unknown. In demonstrating how the innate immune system is susceptible to variations in temperature, a number of authors have shown changes in phagocytic activity in some crustaceans species (Parry and Pipe, 2004; Hégaret et al., 2003; Cheng et al., 2004; Chen et al., 2007; Monari et al., 2007). While phagocytic activity is shown to be susceptible to the natural and anthropogenically-induced environmental fluctuation, this immune factor is also subject to seasonal variation activated by adaptations in organism physiology. Some authors have also noticed humoral activity variations in response to temperature or season adaptation. Clotting times, total haemocyte count and plasma protein levels were affected by temperature in the hermit crab, *Uca pugilator* (Dean and Vernberg, 1966). Muramoto et al. (1991) have observed that in summer, agglutinin activity is higher compared to the winter months in the acorn barnacle *Megabalanus rosa*. Although, due to other physiological factors, the Authors could not be sure that this effect was solely due to temperature fluctuations.

Health stressors rarely occur in isolation in nature. Many environmental factors influence the natural environment of organisms, resulting in a complex interrelation of stress factors that can affect an organism's immune system. For freshwater crustaceans, these factors include temperature and water salinity that can fluctuate seasonally. This can affect the disease resistance of organisms and influence population dynamics (Ellis et al., 2011). An important and frequent approach to understand how changing environmental conditions impacted an organism's general immune response is through studying these environmental stressors. Parry and Pipe (2004) revealed the interactive impact of temperature and other stressor in combination on haemocyte phagocytic activity, the total number of circulating haemocytes and differential haemocyte counts of the blue mussel, *M. edulis*. This author also highlighted the necessity to research a greater array of environmental stressors and a wider number of stressors in combination. Duchemin et al. (2007) showed the lowest phagocytic activity occurred during late spring spawning in the Pacific oyster, *Crassostrea gigas*, while highest activity occurred in autumn. Moreover, this study also found that immune parameters changed even between diploid and triploid individual males and females during progression of gametogenesis: the triploids immune response appeared to be less responsive to environmental changes.

1.3. Crayfish haemocytes and their immune functions

1.3.1. Haemolymph, circulating haemocytes and haemocytes differentiation

Crayfish have a primitive open circulatory system (haemocoel) where the blood is described as haemolymph and the blood cells are termed haemocytes, containing haemocyanin-rich plasma (respiratory protein) (Malham et al. 1998). Haemocyte classification, in most of the crustacean species, is generally based on the presence or absence of cytoplasmic granules. According to this criterion, there are three types of circulating haemocytes usually identified in crustacean: hyaline cells (HCs; the smallest cells without apparent granules), SGCs (having small granules) and GCs (abundant cytoplasmic granules) (Bauchau, 1981). Three haemocyte cells have also been recognized from microscopic examinations of haemolymph from tiger shrimp *P. monodon*: (1) granular cells, large number of large granules and low nuclear-to-plasma ratio; (2) SGCs , much smaller quantities, and (3) HCs , without granules but with a high nuclear-to-plasma ratio (Sung et al., 1999; Song et al., 1997). However, there is a deviation from this pattern in the haemolymph of the spider crab, *H. araneus* with a distinct population of small, mostly undifferentiated cells known as prohaemocytes (Roulston and Smith, 2011).

In freshwater crayfish, *Procambarus clarki* (Lanz et al., 1993), HCs with small, spherical cells and none or few granules was reported to be the most abundant type of haemocyte, whereas they are the least abundant in *Astacus astacus* and *P. leniusculus* (Smith and Soderhall, 1983; Soderhall et al., 1986). The SGCs were the first cell to act in response to non-self particles by degranulation *in vivo*, releasing proPO system components into the haemolymph (Johansson and Soderhall, 1985). In the case of fungal or bacterial infections, the discharged proPO system, and a 76 kDa protein, is stimulated by the presence of the components of microbial cell wall (probably through β-l,3-glucan- or LPS-binding proteins) in the haemolymph (Soderhall et al., 1986; Johansson and Soderhall, 1988; Johansson and Soderhall, 1985). The secreted and activated proteins attach to the foreign bodies (Soderhall et al., 1986; Söderháll, 1981). The attachment and release of chemicals by both SGCs and GCs, and possibly the HCs, to large foreign particles is facilitated by the immobilized 76 kDa protein (Johansson and Soderhall, 1988). The formation of a capsule or nodule will thus be controlled by the 76 kDa protein activities. The SGCs, at least *in vitro*, appear to be mainly responsible for encapsulation, removing foreign particles from circulation (Persson et al., 1987). Phagocytosis by HCs (primarily) and SGCs is triggered by the 76 kDa protein combined with the proPO system. Semi-granular cells, when released and activated the 76 kDa protein for

the first time, will also activate the granular cells to degranulate and to discharge greater amounts of the proPO system (Johansson and Soderhall, 1985; Johansson and Soderhall, 1989).

In regards of crustacean haemocytes differentiation, several lineages have been suggested by various researchers. Ghirettimagaldi et al. (1977) suggested only one cell line for haemocytes in the crab *Carcinus maenas*. Three haemocyte types establish a continuous differentiation lineages with granulocytes developing from the hyaline haemocytes (Hose et al., 1990). Two haemocyte lineages classifications were also suggested in some crustacean species, by a combination of morphological, cytochemical and functional features in circulating haemocytes. Two differentiation lineages (a hyaline and a granular) was shown in lobster *Homarus americanus* (Martin et al., 1993). A small and a large granular cell in crayfish *Pacifastacus leniusculus* were shown to come from one precursor cells (Chaga et al., 1995). van de Braak et al. (2002) suggested that small granular and large granular haemocytes were coming from HCs, which were regarded as the young and immature cells in penaeid shrimp *Penaeus monodon*. While Zhang et al. (2006) stated that the HCs were only thought as the SGH precursor cells in Chinese prawn *Fenneropenaeus chinensis*. Hose and Martin (1989) described three distinct haemocyte lines in the decapoda, HCs, small and large granulocytes, and showed that whereas the HCs initiate coagulation, the granulocytes are involved in phagocytosis and encapsulation.

1.3.2. Antimicrobial systems

Production of antimicrobial peptides, the other component of invertebrate innate immunity, is stimulated by signals preserved within broad classes of pathogens (for fungi, Gram-negative and Gram-positive bacteria). Signalling proteins and specific receptors (such as Toll and its associates) are involved but there are no true antibodies (Imler and Hoffmann, 2000). However, the reaction is definite because each of these classes produce a distinct range of peptides. Cociancich et al. (1993) found some potent antibiotics in these peptides that complement to eliminate pathogens, for instance, by altering the penetrability of the pathogen cell membrane.

In crustaceans, two protein types are involved in microbial product recognition, and their cellular functions have been described (Vargas-Albores et al., 2005). The first type is composed of multivalent sugar-binding agglutinins, termed hemagglutinins or lectins. The activities of hemagglutinins have been reported in plasma of *P. monodon* (Ratanapo and

Chulavatnatol, 1990), *P. stylirostris* (Vargasalbores and Ochoa, 1992), *Penaeus japonicus* (Bachere et al., 1995); *Penaeus californiensis* (Vargas-Albores et al., 1996) and *Penaeus indicus* (Maheswari et al., 1997). The second type consists of molecules that are obviously monovalent and do not stimulate agglutination, although capable of binding sugar residues. This second protein discovered in the haemolymph of crustacean has the ability to respond to beta glucan, and consequently named βGBP. Crustacean βGBP was purified and characterized initially in crayfish *P. leniusculus* (Duvic and Soderhall, 1990; Barracco and Amirante, 1992; Duvic and Soderhall, 1992; Thornqvist et al., 1994). Antibodies against crayfish βGBP have been used to discover this protein in other crustaceans (Thornqvist et al., 1994; Vargas-Albores et al., 1996).

1.3.3. Phagocytic system

In all animal phyla, phagocytosis is regarded as a crucial and critical system to eliminate microorganisms or other small particles. In invertebrates, the phagocytic system has been well studied and documented (Bachere et al., 1995). It has been known for a long time that the cells responsible for the removal of foreign material include circulating haemocytes and fixed phagocytes, while larger particles or clumps of bacteria are efficiently encapsulated in crustaceans. Previous studies have observed different functions of each haemocyte type in the invertebrate haemolymph. Therefore, thorough scrutiny is important to determine the role of each haemocyte type in the defence reactions of invertebrates:

- Innate immunity in crayfish *(Parachaeraps bicarinatus)* might be related to an altered activity of the phagocytic cells, since the lack of circulating bactericidins and the failure of serum opsonin to increased clearance rate in their immune system (McKay et al., 1969). In the crayfish haemocytes, *C. maenas* and *P. leniusculus,* the HCs are characterised by the absence of granules, spreading capability and phagocytosis. The SGCs contain small granules and lyses very rapidly when manipulated *in vitro* or reacting with microbial polysaccharides. They are involved in recognition and release of the proPO activating system but also in the encapsulation process (Johansson and Soderhall, 1985). The third cell type is the GCs with large granules and whose main function is the proPO system storage. Soderhall et al. (1994) explained that two proteins, the 76 kDa factor and the βGBP, once it reacts with β-1,3-glucan, can stimulated granular cells to discharge the proPO system. Moreover, in the *C. maenas,* although the contribution of products from any proPO system remains uncertain, the GCs are still involved in antibacterial activity (Chisholm and Smith, 1992). Huang et al. (2000) and Lee and

Soderhall (2001) found a mas-like protein in the haemocyte of crayfish *P. leniusculus* as a mediator for cell adhesion and also acted as an opsonin, which can promote a swift elimination of *Eschericia coli*. On the other hand, although the haemocytes of red claw crayfish *C. quadricarinatus* are also categorized into three forms: HCs, SGCs and GCs only GCs phagocytosed beads and *E. coli* by endocytic pathways (Duan et al., 2014).

- Tiger shrimp (*P. monodon*) in response to the disruption of foreign entities (for example LPS, yeast *β*-glucan and *Vibrio* cells) employ phagocytosis, nodule formation, proPO system activation, and superoxide anion and hydrogen peroxide production (Sung and Song, 1996, 1999; Song and Hsieh, 1994). In another studies, it was shown that SGCs are the cells responsible for foreign particle phagocytosis in shrimp (Bachere, et al., 1995; Soderhall et al., 1994). Granular cells are also capable but with less frequency of phagocytosing foreign material than the SGCs (Hose and Martin, 1989). Granular cells in the shrimp defense system also play a significant role because they have antibacterial activity (Chisholm and Smith, 1995). Hyaline cells were also taken into account as phagocytes (Soderhall et al., 1994). The haemocytes of shrimp *P. monodon* have LPS-binding lectin (PmLec), just like the immulectins in insects, which are able to enhance phagocytosis and encapsulation due to its opsonic activity (Liu, et al., 2007). Nonetheless, the exact role of each haemocyte remains unclear in the shrimp defense system.
- Cornick and Stewart (1973) and Paterson and Keith (1992) have observed a rudimentary antibody or recognition system in haemocytes of the American lobster *H. americanus*.
- The *in vitro* characterization of the different shellfish haemocyte populations was performed after the development of techniques for their isolation by differential centrifugation on Percoll/Ficoll density gradients (Soderhall and Smith, 1983). However, due to the lack of appropriate cell culture methods, most studies dealing with defense cells have used very short-term (hours) cultures. Notwithstanding, (Walton and Smith, 1999) have developed a technique for the culture of hyaline haemocytes from decapods that allow the cells to remain viable and to retain the phagocytic capacity for 14 days. Several *in vitro* assays have been applied on short-term cultures of shellfish haemocytes to assess their antimicrobial abilities, as the degranulation of haemocytes, and activation of the proPO system, phagocytosis, and cytotoxic capacities.
- In the freshwater prawn *Macrobrachium rosenbergii*, the adherent granular haemocyte is the primary cell possessing phagocytic activity which involves a restricted carbohydrate-recognition mechanism as a phagocytosis mediator (Vazquez et al., 1997;

Sierra et al., 2005). Along with other crustacean species, the phagocytosing haemocytes in this organisms also produce lectins, which are kept in vesicles and can be released into the haemolymph or force out into haemocytes membrane (Vasta et al., 1992).

- In *Squilla mantis*, three haemocyte types are known as HC , SGC and LGC (Barracco and Amirante 1992). According to these authors, HC, which can adhere and spread on glass surfaces, are the main cells involved in phagocytic activity. On the other hand, due to their large granules, the SGC and LGC primarily participating in non-self recognition and clearance, coagulation induction and toxic effects. Granular cells, due to their ability to produce lectins, with specificity for b-glycosides, capable of stimulate foreign particles encapsulation, is the first cell type to act as a barrier against microbial entities.

Some researchers, previously, utilized methods such as radio-labelling (Chu, 1988), light microscopy (La Peyre et al., 1995a), and transmission electron microscopy (TEM) (Martin and Graves, 1985; La Peyre et al., 1995b) to conduct research in invertebrate phagocytosis activity. Radio-labelling has the disadvantage of radioactivity and information of phagocytosis activity at the cell population with no distinction between adherence and phagocytosis. Microscopic observation has the inconvenience of time and labour necessities, and limitation to the small numbers of cells examination, but data on the information about individual haemocyte phagocytosis and morphology are detailed and accurate. On the other hand, application of flow cytometry (FCM) has advantages over traditional methods for phagocytosis measurement (Du et al., 2012). Flow cytometry customarily use highly fluorescent beads that make it easy to differentiate between the natural basal fluorescence of non-phagocytic haemocytes and the phagocytic ones which contain fluorescence phagocytosed beads (Sun et al., 2010; Xian et al., 2013).

Interest in innate immunity have increased in recent years. Questions about how these immune system senses infection and thereby initiates a protective immune response is being answered. These basic scientific discoveries are being translated into a more complete understanding of the central role that innate immunity plays in the pathogenesis of many infectious and inflammatory diseases. Although the literature has raised informative findings regarding innate immune characteristics of other crustaceans and crayfish species, the substance of this research has not been investigated within a *C. cainii* immunity. Given that this immune process is commonly served, it is important to critically examine and describe its use within *C. cainii* culture settings in order to determine whether and how it is being used.

Moreover, even though disease is easily diagnosed because of changes in the haemocytes, studies on *C. cainii* haemocytes are still lacking. Recently, most haemocyte investigations in crustacean haemocytes have been done using traditional cytological or biochemical methods, with light microscopy as the most common analytical technique. However, subjectivity is prominent within this technique and can often produce controversies, including on the description of haemocyte morphology, even within the same crustacean species. Therefore, the answer about how the innate immune system senses infection and thereby initiates a protective immune response are still in uncertainty.

This project will therefore gain deeper insights into *C. cainii* haemocyte cell types, their morphological features and functions in immune responses, their phagocytic response to inflammatory stimulus (live and heat-killed *V. mimicus in vitro* and *in vivo* and bacterial lipopolysaccharide *in vitro*) and their capability to produce nitric oxide (after treated with live and heat-killed *V. mimicus in vitro*) and tested over time and at different temperature. This project will using particular methods, such as TEM, FCM and Griess assay over these conventional ones. The basic scientific results will be transformed into a more thorough interpretation of *C. cainii* innate immunity central role in the pathogenesis of infectious and inflammatory diseases.

CHAPTER 2

MATERIALS AND METHODS

2.1. Acclimatization system

The acclimatization system was setup in a purpose-built laboratory designed for aquaculture research in the indoor aquarium facility of the Curtin Aquatic Research Laboratory (CARL), Curtin University Perth Western Australia. The experimental units were cylindrical plastic tanks (80 cm diameter and 50 cm high and 250 L in capacity). The tanks were filled up with freshwater and supplied with constant aeration and a recirculating biological filtration system (Fluval 205, Askoll, Italy). The water in the tank was running continuously, at a rate of approximately 3 L/min. To maintain a constant temperature of 20, 25 and 30±2 °C in the acclimatization tanks, each tank was equipped with a submersible thermostat. The tanks were also provided with sufficient marron shelters in the form PVC pipes of appropriate diameters.

2.2. Animals

The male marron (± 150 gram) were purchased from Aquatic Resource Management Pty Ltd., Manjimup, Western Australia 6258. They were fed with a commercial pelleted diet containing 26% protein, 47-50% carbohydrate, 9% fats and 8.9% ash (Enviroplus Pty Ltd., Perth, Australia) until the experiments commence. Marrons were fed in the late afternoon at 3% of body weight/2 day. Uneaten food was siphoned. Prior to experimental use, animals were acclimated to the laboratory conditions for one week. Only apparently healthy animals were used.

2.3. Preparation of haemocytes

Marron were anesthetized on ice for 15 min, and 2 ml of haemolymph was extracted per animal with a 5-ml syringe and a 25 1/ 2 G needle containing an equal volume of ice-cold citrate/EDTA buffer (0.45 M NaCI; 0.1 M glucose; 30 mM trisodium citrate; 26 mM citric acid and 10 mM EDTA) pH 4.6, as an anticoagulant buffer (AB). Resuspended haemolymph was distributed into separate 10-ml sterile microcentrifuge tubes held on ice and centrifuged at 500xg for 5 min at 4 °C. The supernatant was discarded and each cell pellet was resuspended in 2 ml ice-cold AB. The resuspended samples were transferred into a separate microcentrifuge tube held on ice. A drop of resuspended samples were transferred to a haemocytometer to count the cells with light microscope (Olympus) and trypan blue exclusion. Only cell preparations with viabilities higher than 90% were used. The remaining

resuspended samples were diluted with AB to obtain a final concentration of about 1×10^6 cells mL^{-1}.

2.4. Preparation of the *Vibrio mimicus* stock solution

V. mimicus was a Gram-negative freshwater bacterium isolated from blisters of dead yabbies (*Cherax albidus*). This bacteria was obtained from the Department of Agriculture and Food, Western Australia. The vibrio species was grown at 37 ± 1 °C in Mueller-Hinton agar (MHA, Oxoid Ltd., Basingstoke, Hampshire, England) for 18-24 h to ensure bacteria were in the exponential growth phase, then adjusted to 10^4 CFU/ml with phosphate buffered saline (PBS) according to 1 $OD_{540\ nm}$ = 1.72 x 10^4 CFU/ml. For the phagocytosis assay, one stock solution of the bacteria was harvested by centrifugation and washed three times in PBS before resuspending in PBS and kept in the 4 °C until it was used. Stock solutions were inoculated to fresh agar to ascertain their numbers and their viability before it used. The other stock bacteria solution with the same preparation above then incubated in a water bath at 60°C for 30 minute with occasional shaking before it kept in the 4 °C. Inactivation was confirmed by inoculating the bacterial suspension on MHA for 2 days. If no growth occurred the bacterial suspension was considered heat-killed.

2.5. Transmission electron microscopy

In preparation for transmission electron microscopy (TEM), 1 ml of diluted haemolymph with AB from each 3 marrons was collected. This haemolymph then centrifuged at 500xg for 5 minutes at 4 °C. The haemocytes pellet was first post-fixed in a 5% glutaraldehyde that was prepared in phosphate buffer (pH 7.3) from 2 hours to overnight at 4 °C and then fixed in 1% Dalton's chrome osmic acid in the same buffer at 4 °C for 1 – 1½ h. The fixed cells were dehydrated in a series of ethanol washes followed by propylene oxide to remove residual ethanol previously used for dehydration prior to being processed routinely for conventional embedding in propylene oxide/Epoxy 812 resin (TAAB, UK). Ultrathin sections (approximately 90 nm thick) were cut by using a Reichert Ultracut E (Vienna, Austria) ultramicrotome and a diamond knife were mounted on copper grids. The sections were then double-stained with uranyl acetate and lead citrate. The sections were observed and photographed by a Philips CM100 Biotwin Transmission Electron Microscope (Eindhoven, The Netherlands).

2.6. Flow cytometry

The haemocytes suspensions were analysed by Attune Acoustic Focussing Flow Cytometer (Applied Biosystems) equipped with 2 laser system: Blue and Red Laser with filtered emission at 488 and 638 nm. Photomultiplier bandpass filters for green fluorescence were 488/10 (BL1), 530/30 (BL2), 574/26 (BL3), 690/50 (BL4) and for red fluorescence were 660/20 (RL1) and 780/60 (RL2). Forward scatter (size) data were collected on linear scales and side scatter (granularity) data were collected on logarithmic scales. The voltage settings were FSC 1350, SSC 1450, BL1 1600, BL2 2000, BL3 2000, BL4 2550, RL1 2350 and RL2 1950. Data were FSC/SSC gate, and 10.000 events in total were acquired per sample. Attune Software v1.2 was used to create logical regions for data analyses, and FlowJo software was used to create logical regions to analyse the percentage of cells in each region.

2.7. Griess reaction

Nitric oxide (NO) production by *C. cainii* haemocyte was conducted by the Griess reaction. For NO_2^-/ NO_3^- analysis, experiments were performed using 96-well plates. Each well had around 1 million total haemocytes and 200 µl of final volume. At the end of the LPS incubation period, the samples were immediately frozen and stored at -20 °C. Prior to analysis, the stored samples were thawed at room temperature and were centrifuged 500xg for 5 min at 4 °C. Supernatants were used to measure nitrites as a mean of NO activity *in vitro*. To achieve this objective nitrates were first converted to nitrites using the nitrate reductase enzyme (Boehringer, Spain). After that, the colorimetric Griess reaction for measuring inorganic nitrites was performed. This method was based on the reaction of nitrites (NO_2^-) content of supernatants with 1% (w/v) sulphanilamide in 5% H_3PO_4 and 0.1% (w/v) N-1-(naphtyl) ethylenediamine which was incubated for 10 minute in the dark. It will generate a purple-azo dye product that can be monitored by a spectrophotometer at 540 nm against a suitable reagent blank. The molar concentration of nitrite in the samples was determined from a standard curve generated using known concentrations of sodium nitrite and was represented as μM nitrite.

Chapter 3. Determination of haemocyte cell types and their morphometric characteristics in marron (*Cherax cainii*)

2.8. Two-step density gradient centrifugation using Percoll™

The different types of haemocytes from *C. cainii* were separated by isopycnic centrifugation in discontinuous Percoll™ density gradient. The commercial Percoll™ solution (Pharmacia) was adjusted to 320–360 mOsM kg−1 by addition of 0.5% NaCl. Two-step density gradient centrifugation was attempted to attain enriched populations of the three haemocyte types. Hyaline, small granular and large granular cells could be developed from haemolymph by density gradient centrifugation on 70% (v/v) Percoll™ at 25.000×g for 20 min at 4 °C. At the very top (VT) fraction, laying just under at the haemolymph-gradient interface, contained some flocculated material and high numbers of HCs and SGCs. The proximity of the two bands complicates harvesting the cells without cross contamination. To improve purity, a second step separation on Percoll™ was employed. For this the hyaline cells together with small granular band was aspirated from the 70% Percoll™ and gently layered onto a second gradient of 30, 40, 50 and 60% Percoll™. This second gradient was then spun at 600×g for 15 min at 4 °C. The separated cells were harvested from the gradients with fine bore sterile plastic Pasteur pipettes. Light microscopy was used to identify which cells occupied which fractions and FCM to evaluate the effects of density gradient centrifugation through the two step Percoll™ method by studying the light scattering properties of cells.

Chapter 4. Ultrastructural and functional characterisation of marron (*Cherax cainii*) haemocytes to phagocytic activity at different temperature *in vitro*

2.9. Phagocytosis assay using TEM

Health male marrons (± 150 gr) were anesthetized on ice for 15 min, and 1 mL of haemolymph was extracted each animal with a 5-ml syringe and a 26 1/2 G needle containing 2 mL ice-cold anticoagulant buffer (AB). Haemolymph was immediately mixed with AB gently to inhibit the rapid coagulation. Directly transfer the diluted haemolymph into 5 mL of PBS. Centrifuge at 500xg for 5 minutes at 4 °C. The supernatant was discarded and cell pellet was resuspended in 5 mL of ice-cold PBS. Resuspended haemolymph was distributed into 250 µL sterile teflon tissue culture pot (Savillec). Cell counts and viabilities were determined using a haemocytometer. Approximately 10^6 cells per mL of haemocytes and only cell preparations with viabilities higher than 90% were used. 250 µL of haemocyte samples was incubated with a 100 µL suspension of live and heat-killed (60 ^{o}C, 1 hour) *V.*

mimicus and heat-killed *E. coli*. Samples were incubated for 0, 4 and 8 hours at different temperature (20 and 30 °C) prior to being observed and photographed by TEM. Cells were considered positive when they contained at least one bacterium.

Chapter 5. Phagocytosis by differential involvement of marron *Cherax cainii* haemocytes with live and heat-killed *Vibrio mimicus* as measured by flow cytometry

2.10. Phagocytosis assay using specific fluorophore

Diluted haemolymph sample was mixed with 50 µL 0.5 µM Cell Tracker Deep Red for 45 minutes. Labelled haemocytes are viewed on a fluorescent microscope. Labelled haemocyte samples are distributed into 250 µL sterile teflon tissue culture pot (Savillec). 100 µL of haemocyte samples are incubated with a 100 µL suspension of live or heat-killed *V. mimicus* labelled with FITC. Samples are incubated for 2, 4 and 8 hours at 25 °C. Reaction mixtures washed 3 times with L-15 medium before immediately analysed by flow cytometry.

Chapter 6. Immunological assesment of marron (*Cherax cainii*) haemocytes to bacterial lipopolysaccharide at different temperature *in vitro* using flow cytometric analysis

2.11. Activation of haemocytes

To assess their reactivity, isolated haemocytes in PBS were incubated with Lipopolysaccharide (LPS) from *Escherichia coli* (Sigma). All LPS were prepared as stocks of 10 mg ml^{-1} in saline and final experimental concentrations were 0.25 and 0.5 mg ml^{-1}. Reaction mixtures of 100 µl haemocytes (~10^6 cells ml^{-1} final concentration) and 100 µl LPS or saline controls were incubated at 20, 25 and 30 °C in 1.5 or 2.0 ml teflon tissue culture pot (Savillec). At 0, 2, 4 and 8 hours intervals during the incubation, 200 µl of LPS-treated haemocytes were removed and immediately analysed by flow cytometry.

Chapter 7. Studies on activity of nitric oxide by haemocytes of marron (*Cherax cainii*) challenged with live and heat-killed *Vibrio mimicus* at different temperature

2.12. Haemocytes responses to activators

For this assay, each marron was sampled at the beginning of the experiment (t = 0) and 1 ml of haemolymph per marron was extracted from three different animals. Haemocytes were obtained by puncture through the base of their fifth thoracic leg and mixed with an equal volume of anticoagulant solution. The mixture was then centrifuged 500xg for 5 min at 4 °C.

The resulting pellet containing the haemocytes was re-suspended in PBS, and the number of haemocytes was adjusted to 10^6 cells/ml. Two possible NO activating agents were tested: live *V. mimicus* and heat-killed *V. mimicus*. After haemolymph was extracted, pooled and diluted with AB to obtain a final concentration of about 1 x 10^6 cells ml^{-1}, one hundred micro litres of haemocyte suspension from each marron was incubated with one hundred micro litres of live and heat-killed *V. mimicus* suspension. The haemocyte solutions were incubated for 2, 4 and 8 hours at 20, 25 and 30 ^oC, and then the NO production of *C. cainii* haemocytes was analysed by Griess assay.

2.13. Separation of different haemocytes types using fluorescence-activated cell sorter (FACS)

Haemolymph was extracted and then diluted as described previously (see Section 2.3). All flow cytometric analyses were carried in a Becton-Dickinson FACS Jazz (St. Jose, CA, USA) equipped with both sorting device and cell delivery robotics. Both flow cytometric analysis and cell-sorting were carried out with the 70-mm nozzle. At least 10,000 events were collected. A pre-run on haemocytes was carried out to tune the system into the best scales for separating the various populations on screen. Three separate sorting runs were carried to generate the observed populations. For *C. cainii*, the purity of sorted haemocytes was analysed further microscopically. At least 300 sorted cells were counted for the three cell types and three separate runs were conducted to give the average counts. Three different morphologic haemocyte types (hyaline cells, small granular cells and large granular cells) can be defined base on the relative size (FSC values) and granularity (SSC values) as described previously. After collection, one hundred micro litres of haemocyte were directly incubated with one hundred micro litres of live and heat-killed *V. mimicus* suspension for 2, 4 and 8 hours at 25 ^oC. The NO production of each haemocyte type was analysed.

Chapter 8. The effects of live and heat-killed *Vibrio mimicus* as inflammatory stimulus on marron (*Cherax cainii*) haemocytes at different temperature *in-vivo* using flow cytometric analysis

2.14. Challenge test *in vivo*

The challenge test was conducted two week after acclimation of marron in acclimatization system. A total of 12 marrons, 3 animal from each time points, were injected 50 µl through the base of their fifth thoracic leg with the *V. mimicus* stock solution at a dose of 10^6 CFU/ml per animal using a 25 1/ 2 G needle. Meanwhile, 12 marrons from each time points were

similarly injected with 50 µl saline per animal as the control 'blank' group. After injection, marrons were subdivided randomly into eight tanks of 3 animals each to avoid repeating sampling before being put back into their original tanks. The infected marrons (challenge and control blank groups) were monitored for survival, total haemocyte count and differential haemocyte count at 0, 8, 24 and 48 h post-injection. During time points, haemolymph was withdrawn from each sampled marron and used for the flow cytometry analysis. Marrons were anesthetized on ice for 15 min, and 2 ml of haemolymph was extracted per animal with a 5-ml syringe and a 25 1/ 2 G needle containing an equal volume of ice-cold citrate/EDTA buffer (0.45 M NaCl; 0.1 M glucose; 30 mM trisodium citrate; 26 mM citric acid and 10 mM EDTA) pH 4.6, as an anticoagulant buffer (AB). Diluted haemolymph was distributed into separate 10-ml sterile microcentrifuge tubes held on ice and centrifuged at 500xg for 5 min at 4 °C. The supernatant was discarded and each cell pellet was resuspended in 2 ml ice-cold AB. The diluted haemolymph from each marron was transferred into a separate microcentrifuge tube held on ice. A drop of diluted haemolymph sample was removed to a haemocytometer to count the cells with light microscope (Olympus) and trypan blue exclusion. Only cell preparations with viabilities higher than 90% were used. The remaining diluted haemolymph was diluted with AB to obtain a final concentration of about 1×10^6 cells mL−1, and then used for flow cytometric analysis.

CHAPTER 3

DETERMINATION OF HAEMOCYTE CELL TYPES AND THEIR MORPHOMETRIC CHARACTERISTICS IN MARRON (*Cherax cainii*)

3.1. Introduction

In decapod crustaceans, circulating haemocytes have received considerable attention as the primary mechanism of cell-mediated immunity. Haemocytes play the main role in cellular immune reactions, such as phagocytosis of invading pathogens, immobilization through nodular aggregates, large foreign body encapsulation, and healing processes followed by immediate clotting of the haemolymph (Hose et al., 1990; Soderhall and Thornqvist, 1997). The susceptibility and irregularity of crustacean immune cells, in addition to the limited knowledge of development and differentiation, make it difficult to classify haemocytes in to morphologically well-defined ontogenic classes. Generally they are contrasted to the more morphological and immuno-functional uniform scheme of vertebrate white blood cell classification (Zhang et al., 2006). The ability to separate and isolate haemocyte populations is a major factor not only to characterise haemocytes according to cellular and humoral function, as well as molecular and biochemical studies but also for understanding of cell–cell interactions and developmental studies.

Most haemocyte investigations have been done using traditional cytological or biochemical methods, with light microscopy as the most common analytical technique. However, subjectivity is prominent within this technique and can often produce controversies, including on the description of haemocyte morphology, even within the same crustacean species (Hose et al., 1990) in decapod; (Sawyer et al., 1970; Bodammer, 1978; Mix and Sparks, 1980) in crab; (Bauchau, 1981; Martin and Graves, 1985) in shrimp. Phase contrast or bright field light microscopy (LM) and transmission electron microscopy (TEM) are commonly employed to evaluate granule number and size and also nucleus-to-cytoplasm (N:C) ratio, essential for morphological classification (Ding et al., 2012). Nevertheless, the use of flow cytometer (FCM) over these conventional methods offers several advantages. This method is very sensitive and enables multi-parameter analysis efficiently. Xue et al. (2001) added the information that FCM used auto-fluorescence, fluorescent markers and light scattering parameters to study the size and complexity of the cells, including and some cellular functions.

Crustaceans are increasingly known as potential farming animals in aquaculture industry, thus monitoring their immune function and minimizing stresses that can conceal their immunity is very important. Changes in immune function can be induced by environmental variations. The most important environmental factor is most likely water temperature because it has a direct effect on oxygen intake, metabolism, moulting, growth and survival

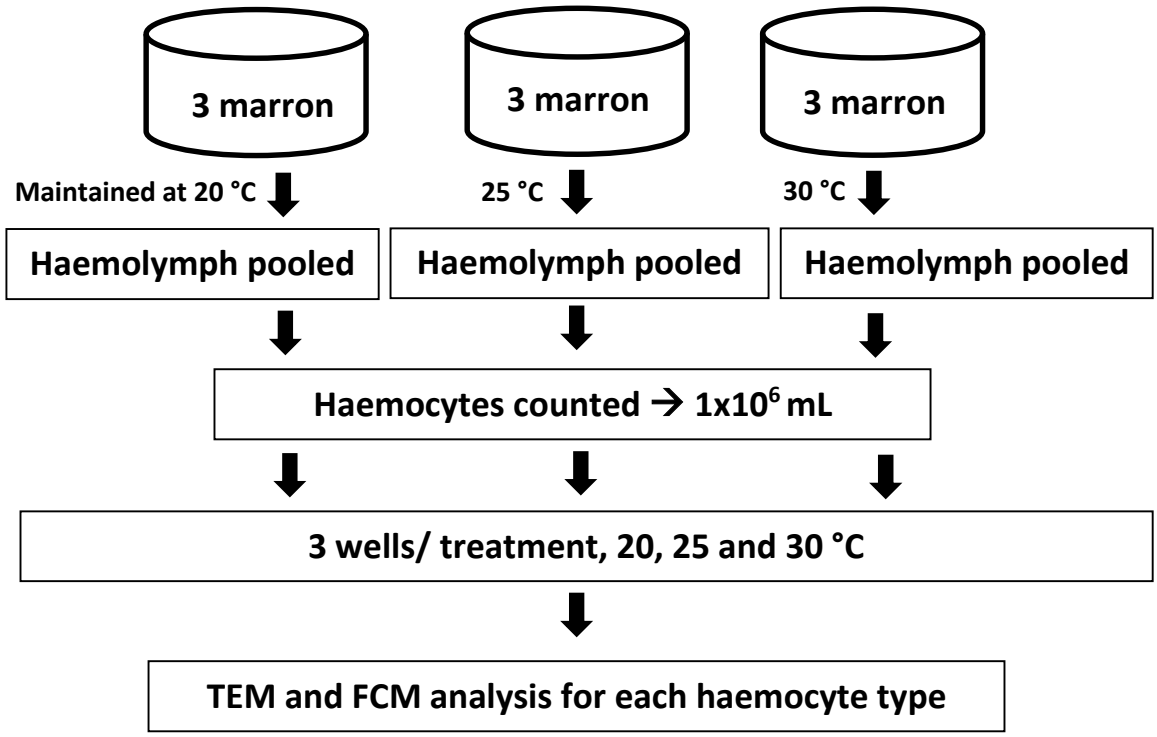
(Chen et al., 1995; Hennig and Andreatta, 1998). Temperature variations are often cause stress for crustaceans, resulting in an immune function reduction as assessed by haemocyte number, proPO activation, phagocytic activities and free oxygen radicals release (Le Moullac et al., 2000). Previously, Vargas-Albores et al. (1998) and Le Moullac and Haffner, (2000) reported that pro-phenoloxidase (pro-PO) cascade to be temperature dependent. Therefore, effects of the temperature variations responses of crustaceans will provide ecologically significant information.

The purpose of this chapter is to identify and morphologically characterise the haemocyte cell types from marron, *Cherax cainii*, and to compare these results to those of the more thoroughly investigated invertebrate species. Similarities and differences among the species were noted and quantified. In addition to morphological observations using a LM and TEM, we also employed the newer technique of FCM.

3.2. Materials and Methods

3.2.1. Experimental outline of different temperature effect at *C. cainii* haemocytes

Experiments assessing the effect of temperature were conducted as outlined below. Three sets of 3 marron in each were maintained at 20, 25 and 30 °C for two weeks prior to being used. Haemolymph from three set was pooled and counted and used for the temperature experiments. Experiments were conducted at 20, 25 and 30 °C and set up in triplicate wells containing one million haemocytes. Transmission electron microscope (TEM) and flow cytometric (FCM) analysis was performed on all three haemocyte cell types (HCs, SGCs and LGCs).



3.2.2. List of Experimental procedures – refer to Chapter 2 for further detail

- 2.1 Acclimatization system
- 2.2 Animals
- 2.3 Preparation of haemocytes
- 2.5 Transmission electron microscopy (TEM)
- 2.6 Flow cytometry
- 2.8 Two-step density gradient centrifugation using Percoll^{tm}

3.3. Results

Classification of the *C. cainii* haemocytes

Three major haemocyte types were identified by LM, TEM and FCM in *C. cainii* based on the number and size of the cytoplasmic granules and N:C ratio. These were hyaline cells (HC), small granular cells (SGC), and large granular cells (LGC).

3.3.1. Determination of *C. cainii* cell types by LM

Three haemocyte types were observed by LM in the haemolymph of *C. cainii* (Figure 3.1). They were identified as HCs, SGCs and LGCs. HCs were the most abundant cells in *C. cainii* followed by SGCs. The least abundant cell type was the LGCs. The relative percentage of circulating haemocyte types from *C. cainii* were HCs (50%), SGCs (29.2%) and LGCs (20.8%)

3.3.2. Determination of *C. cainii* cell types by TEM

The morphological data for this section was derived based on the observation of at least 15 haemocytes of each type (from 3 pooled marron haemolymph).

Hyaline cells (HCs)

Hyaline cells were irregular in shape and had a large nucleus surrounded by a thin cytoplasmic layer, and thus a high N:C ratio (Figure 3.2). These smallest circulating haemocytes have a higher N:C ratio than SGCs and LGCs. The high N:C ratio is useful for their identification. The HCs had relatively few, small rounded granules in their cytoplasm. These granules could be seen with TEM but not under the LM. The nuclei of HCs were variable in shape, being either shape like kidney or ovoid, with or without nucleoli. The cytoplasm possessed free polyribosomes, rough endoplasmic reticulum and few (up to 20 per cell section), small electron dense vesicles about 0.3 μm in diameter.

Small granular cells (SGCs)

Small granular cells were also irregular in shape (Figure 3.3). They characteristically displayed numerous electron dense, small spherical or ovoid granules in a relatively large amount of cytoplasm. The number of electron dense granules were fewer compared to the LGCs but contained more granules than HCs. Granules still only filled less than 30% of the cytoplasmic space. The size of the granules was smaller than the granules of the LGCs (see below). Nuclei were round or kidney shaped, with more euchromatin distribution. There were many large

and small cytoplasmic vacuoles, and pseudopodia were constantly extended peripherally. The large nucleus was located in a central position with an irregular, sometimes lobated and polymorphic profile. The cytoplasm presents a well-developed rough endoplasmic reticulum, Golgi apparatus and small (about 0.5 µm in diameter) round to elongated mitochondria with tabular cristae. The SGCs had a N:C ratio twice than LGCs (see below).

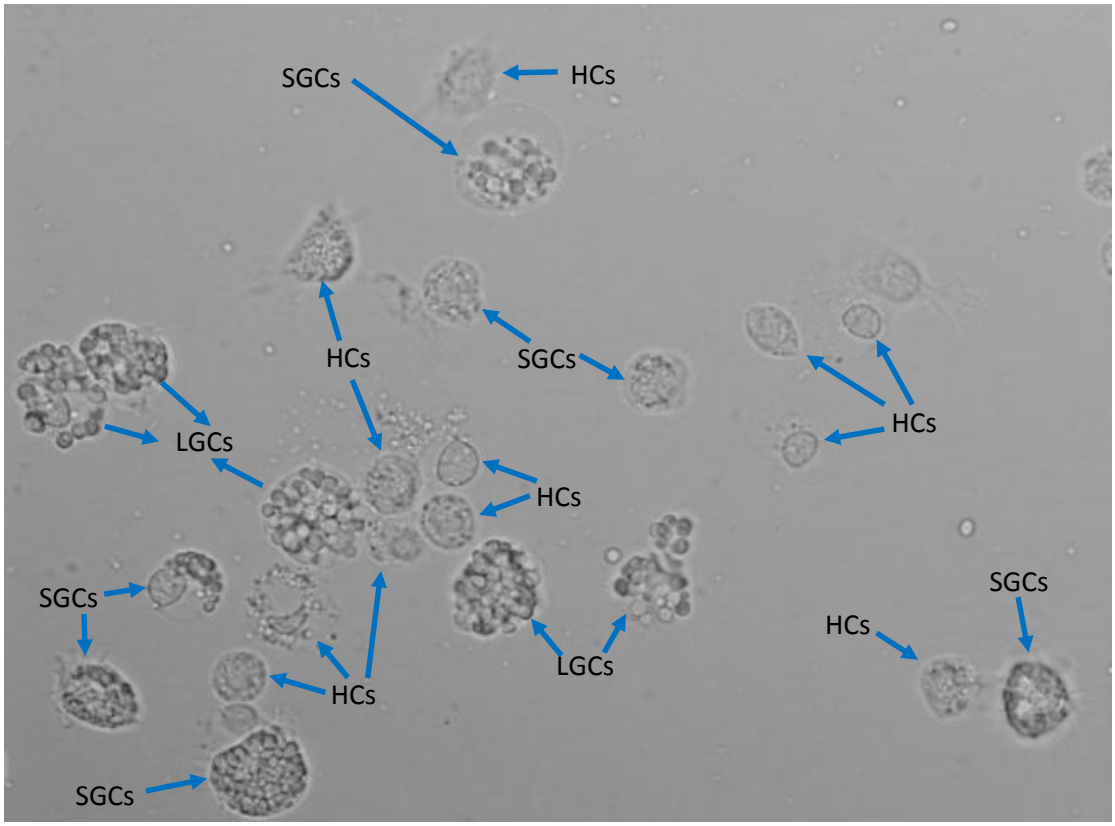


Figure 3.1. Light microscopy of *Cherax cainii* haemocytes: Hyaline cells (HCs), showing a large nucleus and thin cytoplasm, Small granular cells (SGCs) showing different shapes and vacuolated cytoplasm; Large granular cells (LGCs) presenting a number of rounded granules widespread in the cytoplasm.

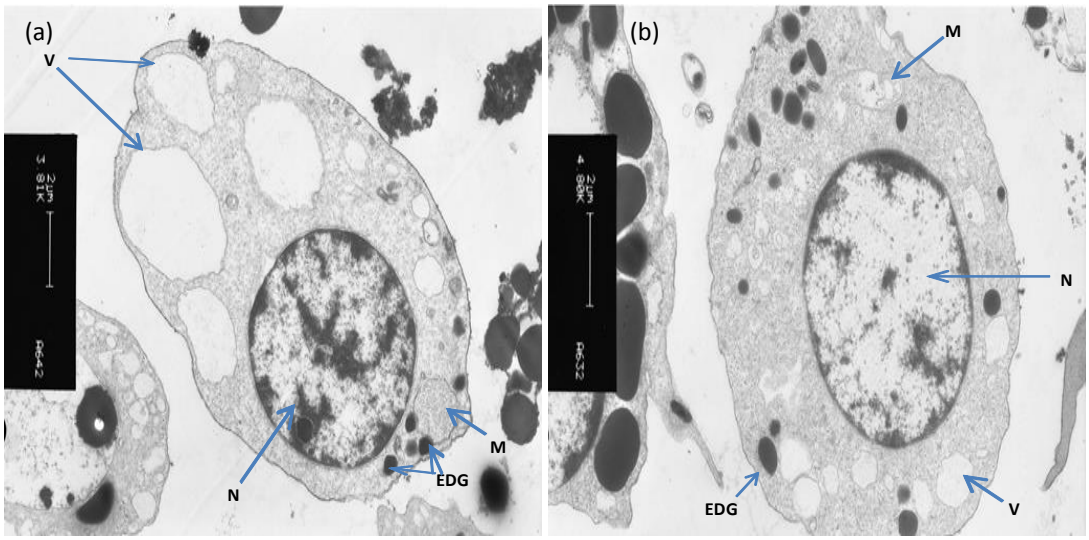


Figure 3.2. Transmission electron micrograph of haemocytes from *Cherax cainii* showing hyaline cells (HCs). EDG, electron dense granules; N, nucleus; V, vacuole and M, mitochondria. Granules are present in hyaline haemocytes although not abundant. Bars, 2 µm (a, b).

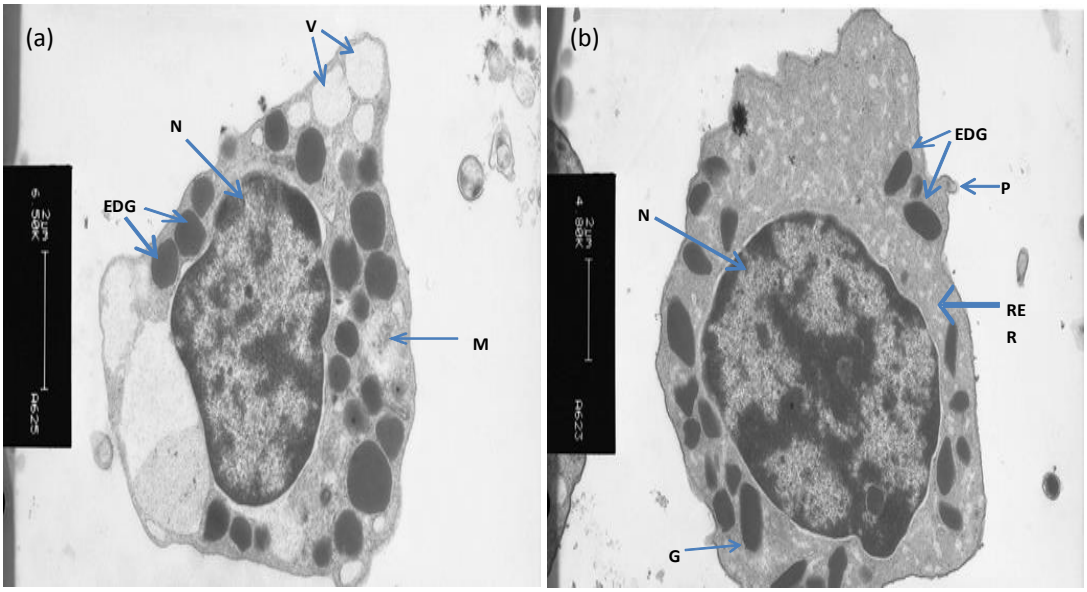


Figure 3.3. Transmission electron micrograph of haemocytes from *Cherax cainii* showing small granular type (SGCs). EDG, electron dense granules; N, nucleus; V, vacuole; RER, rough endoplasmic reticulum; G, golgi and M, mitochondria; P, pseudopodia. Bars, 2 µm (a, b).

Large granular cells (LGCs)

Large granular cells were round or ovoid in shape (figure 3.4). They were characterized by electron dense, large, structure-less, membrane-enclosed granules showing a round to oval-like granule profile with a maximum diameter up to 2.1 µm that fill much of the cytoplasm. The granules were bigger that those in HCs and SGCs, and there were greater in number, occupying more than 50% of the cytoplasmic space. These round or ovoid cells had a relatively small nucleus, with a relatively low N:C ratio. The cytoplasm was filled with numerous large granules that sometimes obscure the nucleus almost entirely. When viewed by TEM, the kidney shaped nucleus of LGCs exhibited condensed chromatin masses and bilobate nucleus. There were fewer organelles, but some large cytoplasmic vacuoles were observed. The pseudopodia also extended peripherally.

3.3.3. Determination of *C. cainii* haemocytes by FCM

Haemocyte populations were successfully resolved into forward scatter (FSC), which measures relative cell size, versus side scatter (SSC), which measures cell granularity with data plots via scatter parameter gating. Three event clusters were observed in the typical hemogram of FSC versus SSC (Figure 3.5a). Gates were drawn around each event cluster (Figure 3.5b), which were provisionally classified as region of SGCs (R1), LGCs (R2) and HC (R3) in one parameter dot-plots in log scale.

3.3.4. Morphometric of the *C. cainii* haemocytes

Based on TEM observations, three haemocyte populations were apparent as for the LM and FCM analyses: HCs, SGCs and LGCs. Morphometric analysis was conducted on haemocytes to quantify various cellular features. These were cell size, nucleus size, granule diameter, number of cytoplasmic granules per cell and N:C ratio (Table 3.1).

Table 3.1. Morphometric analysis of *Cherax cainii* haemocytes features.

Haemocyte type		Hyaline		Small granular		Large granular	
Temperature		20 °C	30 °C	20 °C	30 °C	20 °C	30 °C
Cell size	Length	11.5 (1.0)^{ab}	9.1 (0.2)^{ab}	13.4 (1.5)^{b}	12.1 (0.6)^{b}	13.7 (0.6)^{b}	9.5 (0.3)^{a}
	Width	8.9 (0.3)^{a}	8.2 (0.1)^{a}	8.5 (0.2)^{a}	9.7 (0.8)^{a}	11.1 (0.7)^{ab}	8.1 (0.2)^{a}
Nucleus size	Length	7.7 (0.6)^{ac}	6.5 (0.7) ^{ac}	6.6 (0.4)^{ac}	6.3 (0.5)^{ac}	3.9 (0.6)^{c}	3.8 (0.2)^{c}
	Width	5.5 (0.8)^{c}	5.1 (0.5)^{c}	4.6 (0.4)^{c}	4.6 (0.5)^{c}	3.1 (0.9)^{c}	3.5 (0.04)^{c}
Granule diameter		0.3 (0.1)^{d} - 1.0 (0.3)^{e}	0.5 (0.3)^{d} - 2.1 (0.3)^{f}	0.3 (0.2)^{d} - 1.8 (0.9)^{f}	0.3 (0.1)^{d} - 1.4 (0.4)^{e}	0.3 (0.2)^{d} - 2.0 (0.3)^{f}	0.4 (0.3)^{d} - 2.1 (1.3)^{f}
Number of granules		19 (2.5)^{g}	17 (2.7)^{g}	29 (1.0)^{h}	33 (2.3)^{h}	36 (2.8)^{h}	33 (2.2)^{h}
N:C ratio		45.2 (2.7)^{i}	40.5 (2.4)^{i}	28.3 (0.8)^{j}	25.2 (0.9)^{j}	12.2 (2.6)^{k}	17.3 (1.0)^{k}

Measurements represent the mean (± standard error). Cell and nucleus size are presented in length x width (µm). Granule diameter is in µm. Number of granules is the number per sectioned cell. N:C ratio is in percent. All of the above morphometric data were based on TEM observations from at least 15 haemocyte of each type (from 3 marron, pooled) at different temperature. Data in the same column having the same superscript letter indicate a similar mean which is not significantly different at α level of 0.05.

Some morphological features varied between haemocyte types and were also influenced by temperature. Hyaline cells were the smallest cell compared to SGCs and LGCs. There was no significant difference in cell size between SGCs and LGCs, either at 20 or 30 °C. There was a trend in diminished cell size at the higher temperature but this was only significant for the LGCs population. Hyaline cells had the highest N:C ratios while LGCs had the lowest while also possessing significantly smaller nuclei compared to HCs and SGCs. There was no significant difference in granule size between the three types of haemocytes but LGCs and SGCs possessed abundant, small round granules, significantly greater numbers than HCs.

3.3.5. **Total and differential haemocytes counts**

Three marron were kept for 2 weeks at the different temperatures (20, 25 and 30 °C) and their haemolymph was taken to count their total haemocyte counts (THC) and differential haemocyte count (DHC) using FCM. Total haemocyte count in *C. cainii* increased with higher temperature, going from 1.9 million per ml at 20 °C to 4.9 million per ml at 30 °C (Table 3.2). Large granular cells increases out of proportion as the THC rises with temperature, and both other types decrease in representation. However, the most abundant haemocyte at all 3 temperatures was the HCs, followed by the SGCs, with the LGCs being the least represented in number.

Table 3.2. Total haemocytes count (THC) of *Cherax cainii* at different temperatures.

Temperature (°C)	THC (x 10^6 cells/mL)	HCs (%)	SGCs (%)	LGCs (%)
20	1.978 (0.4)[a]	43.6 (0.6)[a]	37.5 (0.5)[ab]	18.9 (0.9)[c]
25	2.869 (0.5)[ab]	43.2 (1.1)[a]	34.3 (0.4)[bc]	22.5 (1.3)[c]
30	4.924 (0.7)[b]	40.8 (0.9)[a]	33.0 (1.6)[bc]	26.2 (2.7)[bc]

Measurements represent the mean (3 marron, pooled) ± standard error. This data was acquired with FCM. Data in the same column having the same superscript letter indicate a similar mean which is not significantly different at α level of 0.05.

3.3.6. Investigation of a two-step density gradient centrifugation with Percoll™

In this study, we attempted to separate the different types of haemocytes from *C. cainii* by isopycnic centrifugation in discontinuous Percoll™ density gradient. Two-step density gradient centrifugation was attempted to attain enriched populations of the three haemocyte types. Light microscopy was used to identify which cells occupied which fractions. After the first centrifugation in 70% Percoll™, of the whole haemolymph in anticoagulant, the very top (VT) fraction, laying just under at the haemolymph-gradient interface, contained some flocculated material and high numbers of HCs and SGCs (Figure 3.6a). A second fraction, the granulocyte (G) fraction found near the bottom of the centrifuge tube, contained most of the LGCs. In addition, a zone of diffusely dispersed cells (DZ) often extending between the VT and G fractions included variable number of HCs and SGCs. Because the VT fraction contained both HCs and SGCs it was decided to further fractionate this fraction through another Percoll™ gradient. After the 70% Percoll™ run, the VT fraction was aspirated and gently layered onto a second gradient. We investigated a number of Percoll™ gradients for the second separation, which included 30, 40, 50 and 60%. Unfortunately, all of these gradients failed to further adequately separate HCs from SGCs. The 50% Percoll™ gave the best results and we continued analysis with the 70 followed by the 50% Percoll™ gradients. In order to evaluate the effects of density gradient centrifugation through the two step Percoll™ method, the light scattering properties of cells were studied by FCM. After the first Percoll™ gradient of 70%, the VT fraction consisted of 63.1% HCs and 36.8% SGCs. The G fraction was composed of over 56% LGCs. After the second step through 50% Percoll, the VT fraction was comprised of 55.8% of HCs and 41.9% SGCs.

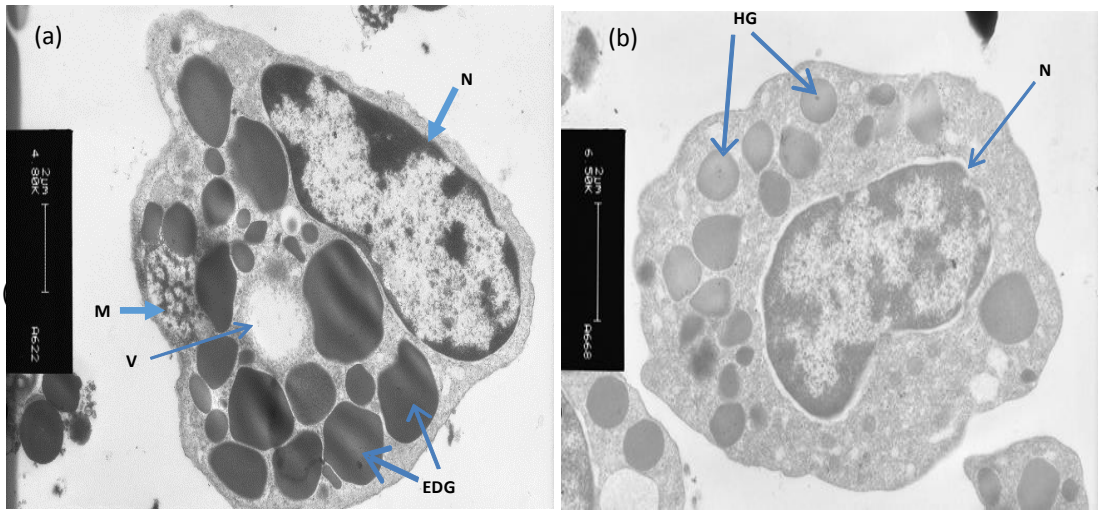


Figure 3.4. Transmission electron micrograph of haemocytes from *Cherax cainii* showing large granular type (LGCs). EDG, electron dense granules; N, nucleus; V, vacuole and M, mitochondria.

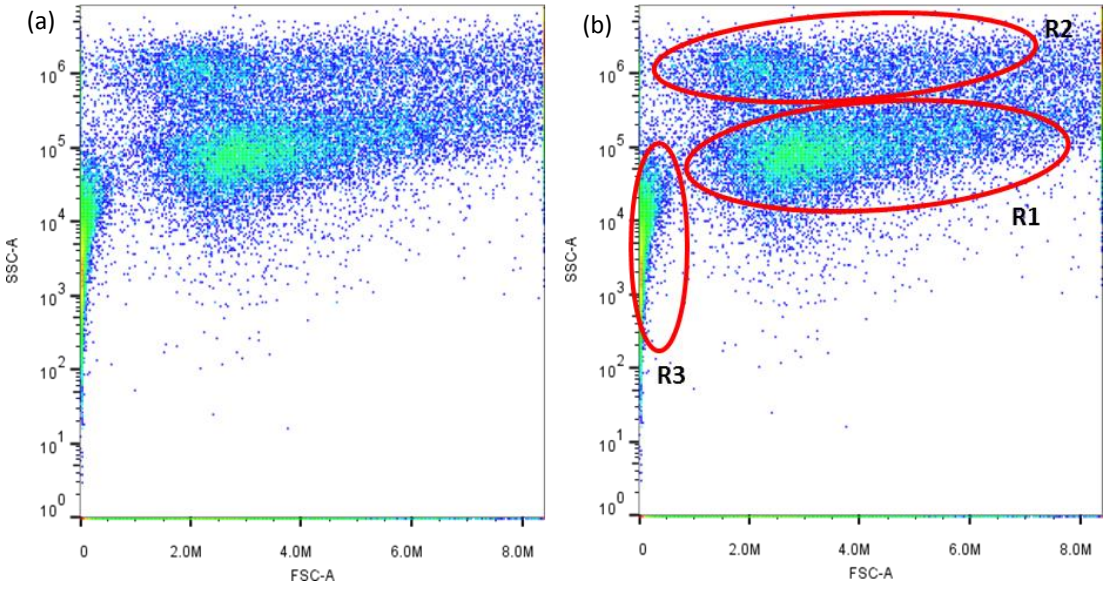


Figure 3.5. Flow cytograms of *Cherax cainii* haemocytes. (a) Dot plot of forward scatter (FSC) in linear scale versus side scatter (SSC) in logarithmic scale. (b) Dot plot of FSC and SSC on haemocytes of *Cherax cainii* showing three putative cell population: R1 (small granular cells), R2 (large granular cells), and R3 (hyaline cells).

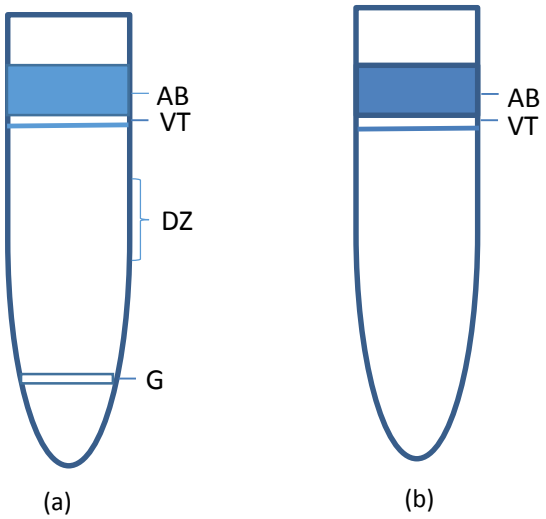


Figure 3.6. Schematic representation showing the sediment profiles of *Cherax cainii* haemocytes recovered by using the two-step density gradient separation method with Percoll™. The positions of the enriched cell bands generated after density gradient centrifugation are indicated for (a) the first sedimentation, 70% Percoll™, and (b) the second sedimentation, 50% Percoll™. Only the VT band was subsequently run through the 50% continuous gradient, containing the hyaline and small granular cell. Abbreviations: VT, very top band of *flocculated* material; G, large granular cells; DZ, diffusely dispersed zone containing some hyaline and small granular cells; AB, anticoagulant buffer.

3.4. Discussion

This chapter investigated the morphology, total and differential cell numbers of haemocytes in marron. Little attention has been given to *C. cainii* haemocytes compared to other crustaceans. After employing the techniques of LM, TEM and FCM to view and analyse the haemocytes of this species, we categorized them into three types of cells. Hyaline cells contain few cytoplasmic granules and SGCs contain a variable number of small granules. The LGCs were filled with large granules. Bauchau (1981) recognized the following type of cells from a variety of crustacean and reviewed it: 1) HCs with none or one Golgi body and none or a few, small granules; 2) SGCs with one or more Golgi bodies and a "moderate" number of small granules; and 3) granular cells with none or one Golgi body and abundant large granules. We have used the same classification, and although is only based on the absence or presence of granule and their relative size, it allowed for consistent haemocytes identification with LM, TEM and FCM (also uses cell size). This system has been used in many crustacean haemocyte studies and is not subject to procedural variations and/or subjective interpretation.

Many classification schemes have assigned equal or greater significance on some characteristics, for instance size, structure, or staining features of the cells. In crayfish, and other crustaceans, the cell size distinctions are insignificant to be of practical use. The structure of the cell is also an insufficient basis for haemocytes classification, since the majority of cells are ovoid *in vivo,* and their structures rapidly change *in vitro* using filipodia and lamellipodia extensions (Johnson 1987). In another study, Dall (1964) used differences in cell shape of the shrimp *(Metapenaeus mastersii)* haemocytes to differentiate the cell types: lymphocytes, thigmocytes, and large granule amoebocytes similar to HCs, SGCs and LGCs, respectively. The variety in haemocyte structures may have been caused by the cells being examined without prior fixation. The pseudopodia occurrence in the last two cell types indicates that they had started extending, since other crustacean haemocytes have been revealed to generate fillipodia and pseudopodia whilst they reside in substrates. Due to the insufficiencies of traditional characteristics (i.e. cell size, shape, and staining characteristics) to classify haemocytes, we assume that haemocytes classification into three types based only on the absence or presence and relative granules size to be the most straightforward and consistent classification presently existing.

C. cainii haemocytes and their distinguishing features can be observed clearly by standard bright field optics. Hyaline cells do not have any apparent granules while SGCs have few small,

non-refractile granules, and LGCs have many large, highly refractile granules that frequently mask the nucleus. However, Ashton-Alcox and Ford (1998) explained that it was quite challenging to quantify the granularity by light microscope. In addition, Martin and Graves (1985) explained that identification of some feature using standard staining methods, such as Wright's stain, haematoxylin and eosin, commonly employed with success vertebrate plasma cells, are not informative because they are often subtle or imprecise, often unreproducible by other researchers and finally, not using properties that allow differentiation of some granules at different maturation phases and different physiological functions. Additionally, when staining is implemented on haemolymph smears, the structure of cells is highly disfigured. In fact, we also attempted to use Wright Giemsa stain with little success (data not shown).

The three haemocytes categories can also be identified using TEM. The accomplishment of this method is dependent on the fixation process of cells examined after removal from the crayfish. This is important because it can make it easier to differentiate circulating cells of the haemolymph from changing morphological cells that are reacting to the clotting initiation or foreign particle presence e.g. syringe or a glass coverslip. The data in this study constitute the first TEM record of *C. cainii* haemocyte populations. Despite its laboriousness, TEM is an important technique for cellular response investigations to environmental parameter and for the monitoring of different features of invertebrate cells. In the study of the correlation of lineages from three haemocyte types in Penaeid and Palaemonid shrimp, TEM data provided a more unitary classification of haemocytes maturation, although the result is still remains an open question (Martin and Graves, 1985; Tsing et al., 1989). Using TEM, agranular haemocytes are rarely observed and have been observed in unusual cases (only spotted in some cross sections of the same cell). Johnson (1987) recognised that the morphology of crustacean haemocytes change rapidly, and make it appear that additional cells categories are present. Three haemocyte types of crab *C. sapidus* have been identified using TEM as HCs, semi granular and granular cells (Bodammer, 1978). The author found that there are some small granules and a well-developed Golgi apparatus in HCs, and suspected that they did not find cells without granules probably due to their relatively low number and their labile nature. Hearing and Vernick (1967) also could not find agranular cells in the lobster haemocyte using TEM, even though they identified three categories of haemocytes with each of them have granules.

Little is known about the use of FCM to analyse the haemocytes of *C. cainii*, even though this technique is faster and provides precise and quantitative data. In many regards, FCM has superseded microscopic evaluation because it offers the ability to rapidly and simultaneously discriminate cellular populations based on a variety of variables, such as relative size, complexity/granularity and fluorescent properties (Shapiro, 2003). However, Ford et al. (1994) explained that the percentage of cells in haemocytes of individual oysters changed two- to fivefold using FCM. Despite the inconsistency in some studies, in this study we verified that FCM with two simple parameters, FSC and SSC, could effectively determine the populations of *C. cainii* haemocytes. Side scatter data collection on a logarithmic scale is an indication that more power is needed to measure a wide granularity range among the haemocytes population. Shapiro (1995) found three factors, other than the cell size, that can influence the measurement of forward scatter data: refractive index differences between cells and suspending medium; the existence of material, inside or upon cells, with strong absorption at the used illumination wavelength; and cells internal structure. Some internal structures, for instance granules or vacuoles, have a tendency to lower forward light scatter; for that reason cells with larger size are placed near to the axis. In the present study, using FCM, we can clearly identified 3 cell populations of *C. caini* haemocytes: LGCs, which had the largest cell size and granule quantities (in R2); SGCs which were intermediate in size and had a smaller granule amount (in R1); and HCs, which had the smallest cell size and the slightest complexity (in R3). In comparison, it is difficult to differentiate between SGCs and LGCs of *C. cainii* based on their size and morphological characteristics by light microscopic investigation, since they are very similar. However these cells can easily be differentiated using FCM. Xian et al. (2009) using FCM on haemocyte of penaeid shrimp, *Litopenaeus vannamei*, and had similar results to ours. So did Comesana et al. (2012), that demonstrated similarity of haemocyte sub-populations for the flat oyster *Ostrea edulis* to that of the haemocytes populations we have demonstrated herein for *C. cainii*. Therefore, FCM offers an informative and efficient method for haemocyte studies.

Additional methods to separate cells such as density-gradient centrifugation and the application of specific fluorescent markers for *C. cainii* haemocytes before FCM may improve the characterization of haemocyte populations. We found that density-gradient centrifugation alone was inadequate to isolate relatively pure populations of haemocytes for further analysis. Bakke (2000) and Sung et al. (1999) have develop antibodies for specific cell surface markers, as for the mammalian systems, for the confirmation of functional haemocyte classifications in crustacean. In our hands, FCM analysis was the preferred

technique to identify *C. cainii* haemocyte populations, and therefore allow the evaluation of changes in the relative proportions of these haemocyte types, certainly superior to LM and TEM.

Total haemocyte count is a practical method to evaluate crustacean physiological status. However, there is a wide range in values published due to different classification schemes and methods used that do not allow an informative comparison between different crustaceans. Environmental factors such as temperature and salinity also affect THC in oyster *Crassostrea virginica in vivo* (Oliver and Fisher, 1995). After cultivating marron at different temperatures for two weeks, we have demonstrated that there was a striking change in THC, with the 30 ^{o}C temperature more than doubling the THC. THC differences in crustaceans are not only due to temperature or salinity differences but can also be caused by other factors such as food availability, sex, diet, captivity length and moult cycle stage (Stewart et al., 1967; Tsing et al., 1989; Baldaia et al., 1984; Cuzin-Roudy et al., 1989; Sequeira et al., 1995).

Through the use of FCM, the differential haemocyte counts (DHC) for *C. cainii* revealed that HCs were the most abundant, followed by SGCs with LGCs being the least in number. An increase in temperature from 20 to 30 ^{o}C resulted in a small but significant, inverse change in SGC and LGC numbers. Variations in DHC have been found among crustacean species influenced by intrinsic (i.e. sex, moult cycle stage) and extrinsic factors (i.e. environment factors, diet, captivity length) (Lorenzon et al., 1999). In earlier microscopic and FCM observations, HCs were the most abundant haemocytes in some Penaeid species (77–89%), (LeMoullac et al., 1997; Owens and O'Neill, 1997) and eastern oyster, *Crassostrea virginica,* haemocytes (Ford et al., 1994; Ashton-Alcox and Ford, 1998). Cardenas et al. (2000) also found that the results from the microscopy and FCM showed similar distribution patterns among the cell types, even though the value of HCs from FCM were about 10% lower. Smith and Soderhall (1983), found from the haemocyte monolayers of freshwater crayfish *Astacus astacus* by microscopy observation consisted of semi-granular ~50% (equivalent to SGCs), granular ~30% (equivalent to LGCs), and HC ~20%. Similarly, haemocyte population types of *L. vannamei* observed using TEM consisted of SGCs as the most abundant type, followed by HCs and LGCs (Heng and Wang, 1998). Sequeira et al. (1995) and Owens and O'Neill (1997), contrary to some reports, found that granulocyte (9–13%) were more abundant than semi-granulocyte (2%), which was similar to other reports (Ford et al., 1994; Ashton-Alcox and Ford, 1998). Amen et al. (1992) found no indication for the existence of different cell type on snail *Lymnaea stagnalis* haemocytes using FCM.

Therefore, herein we have demonstrated, through several techniques, that the haemocytes of *C. cainii* can be classified into 3 population types consistent with other crustaceans. Differential haemocyte count for *C. cainii* revealed that HCs were the most abundant, followed by SGCs with LGCs. Therefore, DHC can be used to evaluate relative changes in haemocyte populations due to environmental factors thus which can affect their immunological system. To be able to compare between different crustacean species, there is a need for standardised techniques. The classification scheme we have represented in this study offers a straightforward and practical method to characterise *C. cainii* haemocytes and also corresponds with current research on haemocytes in other crustacean. The proposed classification would benefit for further development, possibly including other parameters to provide further information on the development and maturation stages of haemocytes and to evaluate their physiological function(s).

CHAPTER 4

ULTRASTRUCTURAL AND FUNCTIONAL CHARACTERISATION OF MARRON (*Cherax cainii*) HAEMOCYTES TO PHAGOCYTIC ACTIVITY AT DIFFERENT TEMPERATURES *IN VITRO*

4.1. Introduction

Phagocytosis is an elemental function for combating pathogens and as an immune fitness indicator in invertebrates (Iwanaga, 2002; Le Moullac and Haffner, 2000). The phagocytic processes (biochemical and molecular) have been well studied and reviewed in the model host systems of mammals and invertebrates: from pathogen recognition *via* receptor–ligand interactions to subsequent digestion and destruction (Garin et al., 2001; Rosales, 2011; Vazquez et al., 2009; Lavine and Strand, 2002; Ratner and Vinson, 1983). Phagocytosis is a non-destructive process for haemocytes in general, unlike melanisation, coagulation, and encapsulation processes which are fatal to the haemocyte. A comprehensive knowledge of the phagocytic process for haemocytes in *Cherax cainii*, which appears to be different to the widely accepted process for mammalian macrophages (Alvarez et al., 1989), will provide further insight in the understanding of the innate immune system.

However, there are contradicting results in the literature relating to the role of crustacean haemocyte types in phagocytosis. For instance, in a study by Johnson (1976) and Johnson (1987) *Paramoeba perniciosa* was phagocytised solely by HCs in the crab *Callinectes sapidus*, whereas both HCs and granular cells phagocytised Gram-negative bacteria. On the contrary, all type of haemocytes in freshwater crayfish *Astacus leptodactylus* appeared to have phagocytic responses, but only SGCs were involved in phagocytosis of all foreign entities: in an *in vivo* assay (Giulianini et al., 2007). Bacterial phagocytosis by *Homarus americanus*, *Panulirus interruptus* and *Loxorhynchus grandis* haemocytes, studied *in vitro*, revealed a participation of both SGCs and LGCs: SGCs demonstrated highest activity (Hose et al., 1990). Gargioni and Barracco (1998) reported that both SGCs and LGCs, but not HCs, in *Macrobrachium rosenbergii*, *Macrobrachium acanthurus* and *Penaeus monodon,* phagocytosed yeast particles *in vitro*. On the other hand, an *in vitro* study by Sung and Sun (2002) demonstrated that the only haemocyte, in *P. monodon*, that phagocytosed latex beads was the HCs.

Various environmental factors have influenced invertebrates, and temperature is always the major physical factor influencing invertebrates in their natural habitat. Variations in temperature are recognised to change host immune functions in invertebrates (Wang et al., 2008; Martin et al., 2010; Seppälä and Jokela, 2011). The Authors have found that acute temperature variations have negative impact on invertebrate phagocytosis. Additionally, the phagocytic capacity of crustacean *Nephrops norvegicus* haemocytes was decreased by approximately 60% in the temperature range of 12-18 ^{o}C (Bodil et al., 2012). Previously Pipe

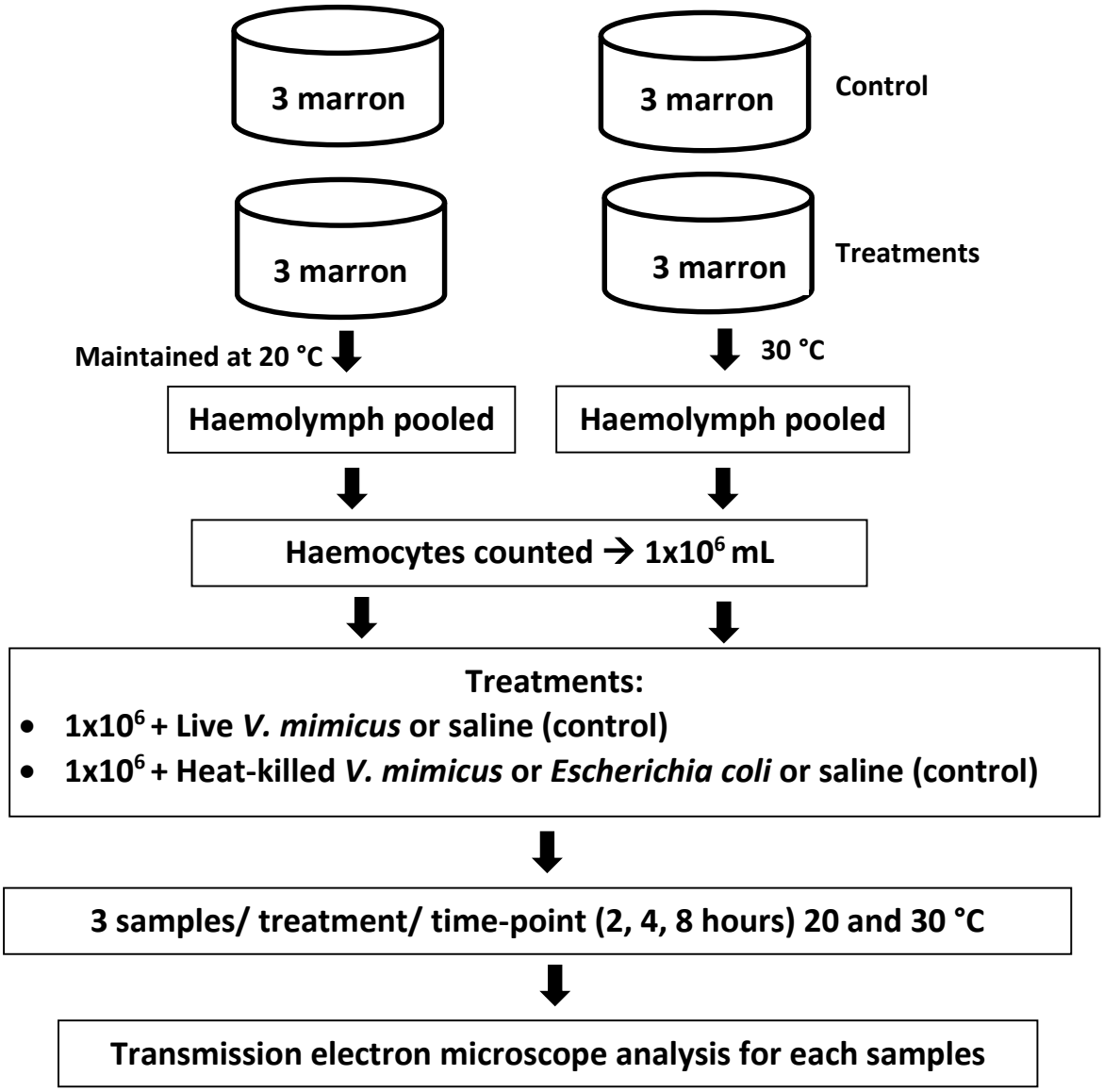
and Coles (1995) found a relationship between temperature variations and diseases incidence in mussels (*Mytilus edulis*). Indeed, the Authors found considerable evidences about temperature variations, diseases and a deterioration of the *M. edulis* immune function. However, there is a little information about temperature variations stimulate an alteration in *C. cainii* immune function.

In Chapter 3, morphological features of *C. cainii* haemocytes were described from a perspective to characterise distinct populations. In this study we further investigate morphological features but with an emphasis on describing the phagocytic process in *C. cainii* haemocytes. The parameters tested were temperature and immunological challenge with live and heat-killed *Vibrio mimicus* and heat-killed *Escherichia coli in vitro*.

4.2. Materials and methods

4.2.1. Experimental outline of phagocytic experiments

The phagocytosis experiment was conducted as outlined below. Four sets of 3 marron were maintained at 20 and 30 °C for two weeks prior to being used. Haemolymph from one set was pooled and counted and used for the control experiments, and the other set of marron was used for the live or heat-killed bacteria experiments. Experiments were conducted at 20 and 30 °C and set up in triplicate samples containing one million haemocytes with live *V. mimicus* or heat-killed *V. mimicus* or *Escherichia coli* or with saline for controls. Separate sets of sample were set up for time-points 2, 4 and 8 hours. Note, for each time-point, a saline control was also performed which enabled baseline corrections. Transmission electron microscope (TEM) analysis was performed on each samples.



4.2.2. List of Experimental procedures – refer to Chapter 2 for further detail

- 2.1 Acclimatization system
- 2.2 Animals
- 2.3 Preparation of haemocytes
- 2.4 Preparation of the *Vibrio mimicus* stock solution
- 2.5 Transmission electron microscopy (TEM)
- 2.9 Phagocytosis assay using TEM

4.3. Results

In order to identify which haemocyte types were involved in the phagocytosis of pathogens, an *in vitro* phagocytosis assay was performed with live and heat-killed *V. mimicus* and heat-killed *E. coli* with *C. cainii* haemocytes. Haemocytes and bacteria, including non-treatment controls, were incubated for 2, 4 and 8 hours at 20 and 30 ^{o}C. The differential haemocyte count (DHC) of *C. cainii* haemocytes significantly changed after these immune challenges, compared to controls. All the haemocyte types underwent drastic morphological changes after the inoculation with bacteria. The alterations were more evident in *C. cainii* haemocytes inoculated with live *V. mimicus*. The major morphological alterations found in all haemocyte types were an increase of multi vesicular structures, an increase in pinocytic activity, and clustering of mitochondria. As shown in the data below, changes to phagocytic activity and haemocyte morphology appeared to, not only, be stimulated by bacteria, but also be affected by temperature.

4.3.1. Non-challenged *C. cainii* haemocytes

In order to make it easier for the reader to compare key features of of treated and untreated haemocytes within this chapter, this section is a summary of haemocyte characteristics form Chapter 3 (please refer to relevant Figures in Chapter 3).

Transmission electron microscopy of non-challenged (treatment control) *C. cainii* haemocytes show HCs with a high N:C ratio and a large nucleus surrounded by a thin cytoplasmic layer. The cytoplasm contains few mitochondria (M) and relatively few small, rounded granules. Small granular cells show irregular membrane surface with filopodic protrusions and a polymorphic nucleus (N). The granular cytoplasm contains few mitochondria (M), few rough endoplasmic reticulum (RER) profiles, and regular sized electron-dense granules (EDG) with different electron-densities close to the plasma membrane. Large granular cells show EDG with round to oval appearance, varying in sizes and electron-density. The cytoplasm has a homogeneous appearance and is poor in organelles.

4.3.2. Phagocytic activity by *C. cainii* haemocytes for live *V. mimicus*

The TEM figures revealed that all three haemocyte types, at both temperatures, contained a number of live *V. mimicus* per cell, mainly in the cytoplasm. Some *V. mimicus* particles were localized in the cytoplasm of HCs within inclusion body, especially at 4 hours after bacteria incubation at 20 ^{o}C (Figure 4.1 a, b). The number of phagocytosed bacteria in the cytoplasm of HCs was higher in the 20 ^{o}C treatment than in the 30^{o}C. However, not all cells were shown to be actively phagocytic at 20 ^{o}C. Bacteria were also found in SGCs in the nucleus (Figure 4.1 c; 4 hours after incubation at 20 ^{o}C) and in cytoplasmic vacuoles (Figure 4.1 d; 4 hours after incubation at 30 ^{o}C). Similar to HCs and SGCs, bacteria were localized and melanised in the vacuoles of the cytoplasm (Figure 4.1 e; 2 hours after incubation at 20 ^{o}C) and were also localized within inclusion bodies in LGCs (Figure 4.1 f; 8 hours after incubation at 30 ^{o}C). All haemocyte types showed some pseudopodic extensions at both temperatures. Intracellular bacteria with variable degrees of disintegration were often seen at 8 hours post-incubation.

Phagocytic activity of *C. cainii* haemocytes showed a significant increase in haemocytes population (from 3 marron) treated with live *V. mimicus* at 30 ^{o}C, compare with 20 ^{o}C, at each time point (Figure 4.2; $p<0.05$). The phagocytic activity amongst LGCs apparently increased in response to higher temperature (30 ^{o}C), with an initial increase at 4 hours after incubated by live *V. mimicus*. The other haemocytes also increased in phagocytic activity with SGCs showing higher percentage than HCs at each time and temperature.

4.3.3. Phagocytic activity by *C. cainii* haemocytes for heat-killed *V. mimicus* and *E. coli*

Transmission electron microscopy revealed that all three types of haemocyte were able to phagocytose heat-killed *V. mimicus* and *E. coli* as early as 4 hours post-incubation at 20^{o}C. As shown in Figure 4.3 a–f, HCs, SGCs and LGCs could phagocyte both types of heat-killed bacteria. Phagocytosed *V. mimicus* in HCs 8 hours post-incubation were localized within inclusion body and melanised in vacuoles in the cytoplasm (Figure 4.3 a). It was noticed that HCs efficiently phagocytosed *V. mimicus* (4 cells), compare to *E. coli* (2 cells) (Figure 4.3 d; 4 hours post-incubation). Similar to HCs, bacterial particles were found in cytoplasmic vacuoles of SGCs: 4 hours post-incubation with *V. mimicus* (Figure 4.3 b) and 8 hours post-incubation with *E. coli* (Figure 4.3 e). In addition, Figure 4.3 e shows melanised *E. coli* in the nucleus of a SGC, at 8 hours incubation. SGCs efficiently phagocytosed *E. coli* and bacteria number varied from 3 to 4 per cells (Figure 4.3 e). Several *V. mimicus* particles were observed in inclusion bodies of LGCs 4 hours post-incubation (Figure 4.3 c) and within the cytoplasm of LGCs after

4 hour incubation with heat-killed *E. coli* (Figure 4.3 f). There was no obvious difference between SGCs and LGCs in their activity to phagocyte heat-killed *V. mimicus* and *E. coli in vitro*. Membrane ruffles resembling macropinocytosis were also observed in the haemocytes containing phagocytosed *V. mimicus* and *E. coli* (Figure 4.3 c, e).

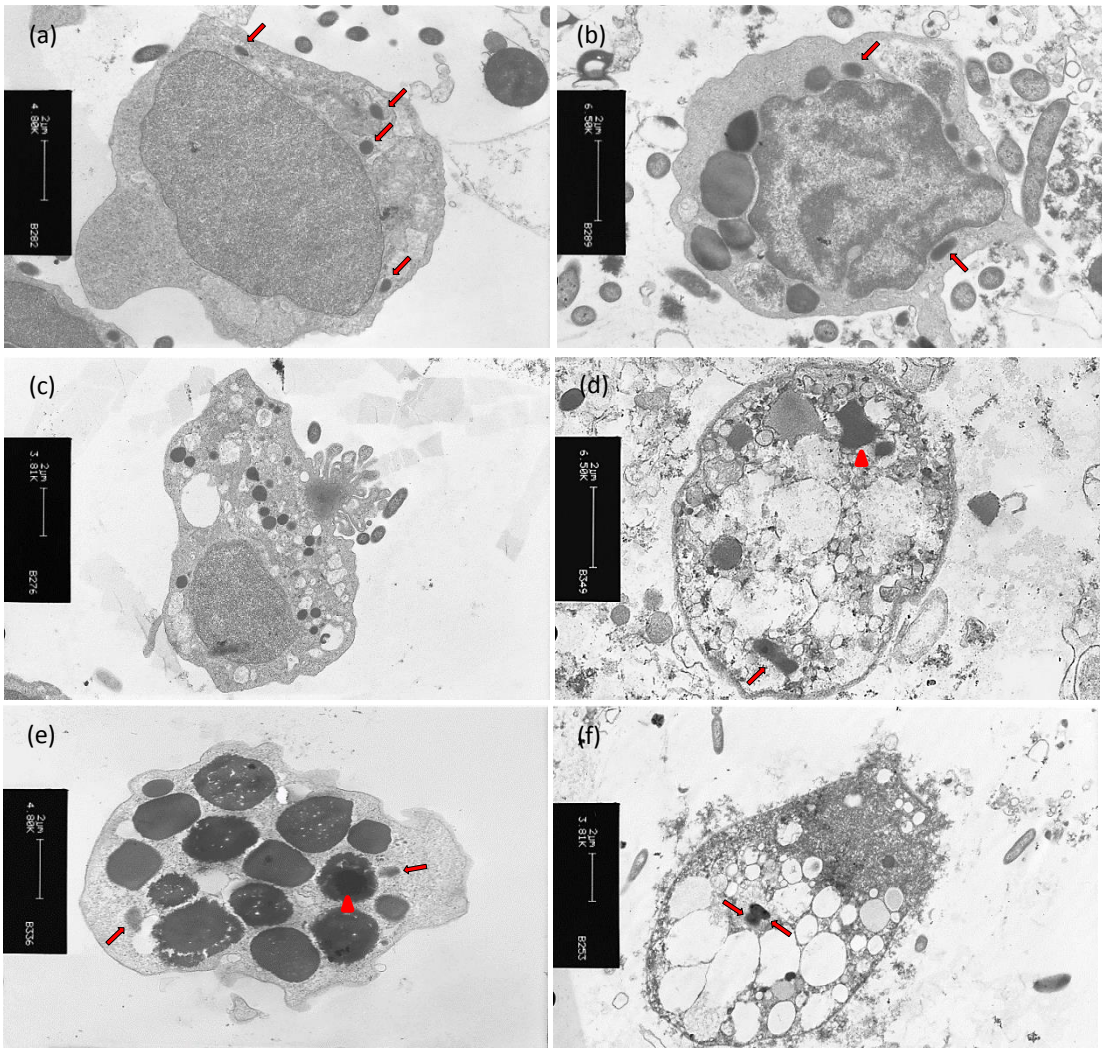


Figure 4.1. Transmission electron micrograph of *Cherax cainii* haemocytes after incubation with live *Vibrio mimicus* cells at 20 °C and 30 °C. Transmission electron micrograph of hyaline (HCs) 4-h (a); small granular (SGCs) 4-h (c); and large granular (LGCs) 4-h (e) at 20 °C and HCs 8-h (b); SGCs 4-h (d); and LGCs 8-hr (f) at 30 °C after incubation *Cherax cainii* haemocytes with live *Vibrio mimicus* cells. Haemocyte cells with phagocytosed (arrow) and melanised (arrowhead) of live *V. mimicus* cells. Bars, 2 µm (a-f).

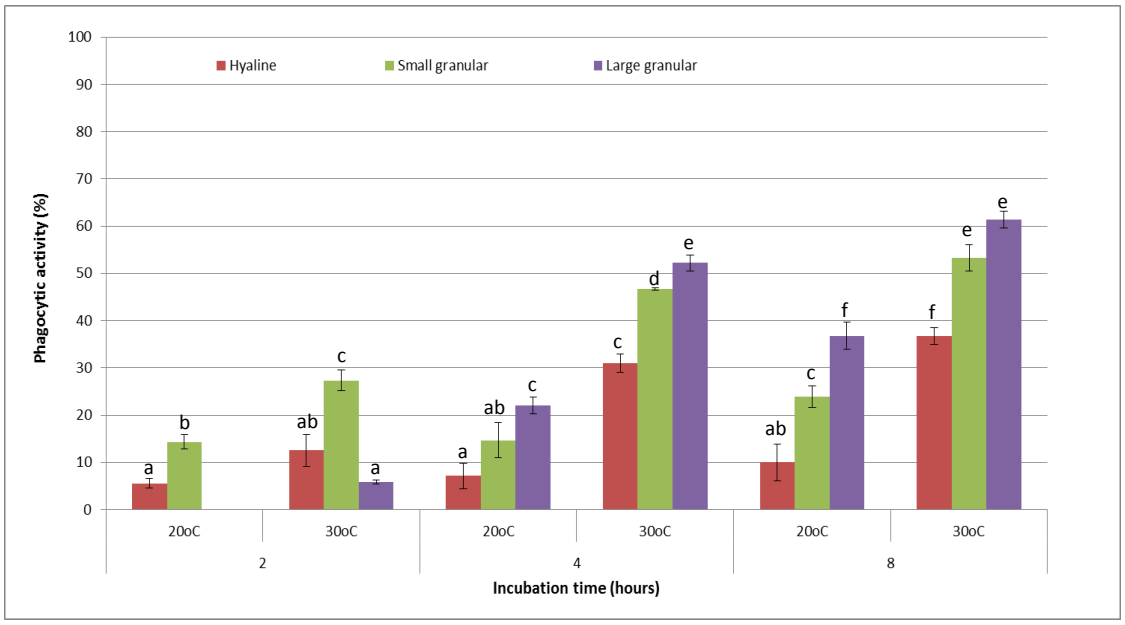


Figure 4.2. Relative percentage of phagocytic activity in *Cherax cainii* haemocytes at increasing times after treated with live *Vibrio mimicus* at different temperature. All of the above data were based on transmission electron microscope (TEM) observations (from 3 marron, pooled) of each haemocyte type at different temperature. Data having the same superscript letter indicate a similar mean which is not significantly different at a level of $p < 0.05$.

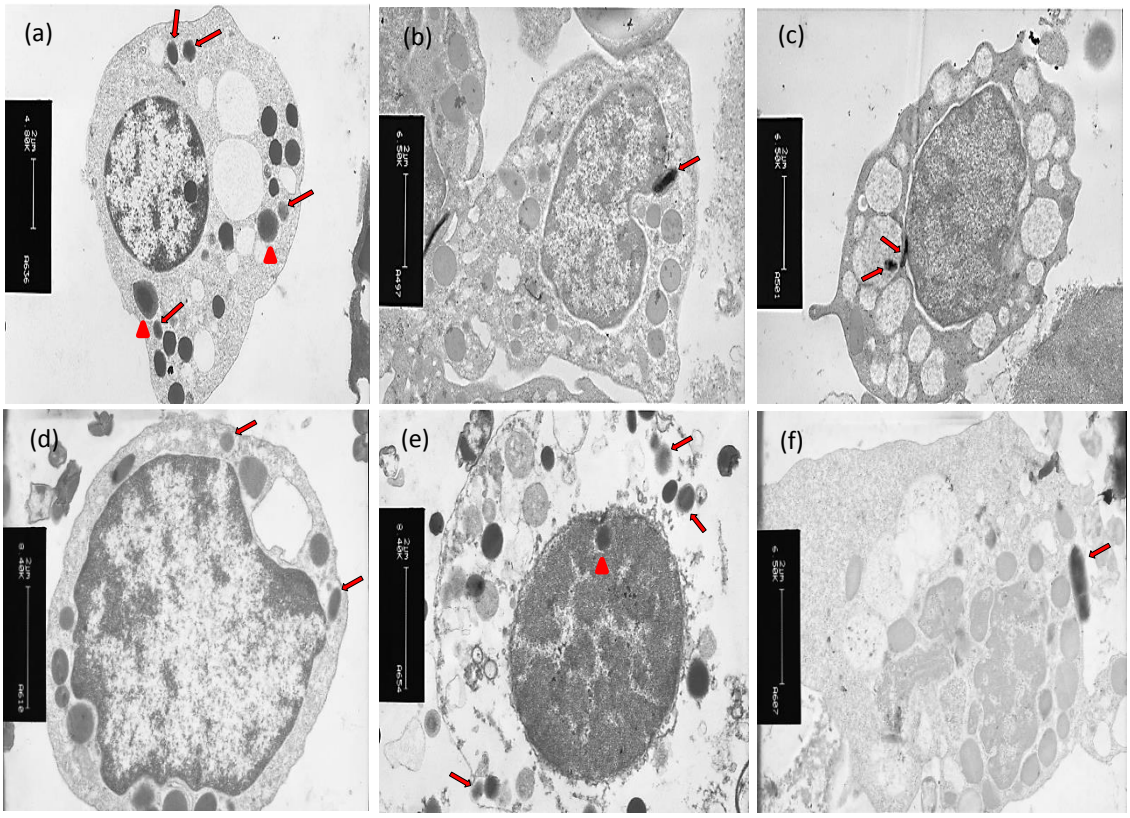


Figure 4.3. Transmission electron micrograph of *Cherax cainii* haemocytes after incubation with heat-killed *Vibrio mimicus* and *Escherichia coli* cells at 20 °C. Transmission electron micrograph of hyaline (HCs) (a,d), small granular (SGCs) (b,e) and large granular (LGCs) (c,f) cells from *Cherax cainii* haemocytes after incubation with heat-killed *Vibrio mimicus* (a,b,c) and *Escherichia coli* (d,e,f) cells at 20 °C. Haemocyte cells with phagocytosed (arrow) and melanised (arrowhead) of heat-killed *E. coli* cells. Bars, 2 µm (a-f).

4.4. Discussion

Phagocytosis is commonly considered one of the most important cell-mediated immune reactions in invertebrates. In this study, based on TEM observations, all three type of *C. cainii* haemocytes were shown to phagocytose *V. mimicus* and *E. coli* cells, with LGCs and SGCs the most active phagocytic cells. There's an abundance of contradictory literature about the role of each crustacean haemocyte type in phagocytosis. For instance, Bauchau (1981) identified HCs and SGCs as the cells that are actively phagocytic of the three recognised haemocyte types in crustacean. Smith and Soderhall (1983); Soderhall and Smith (1983) and Hose et al. (1990) reported on the corresponding function of granular types, with SGCs generally participating in phagocytosis of decapod crustaceans. On the contrary, SGCs and LGCs, but clearly not HCs, were capable of phagocytosing yeast in *M. rosenbergii*, *M. acanthurus* and *P. monodon* (Gargioni and Barracco, 1998). Sung et al. (2000) also observed that HCs were the main phagocytic cells for bacteria elimination from *M. rosenbergii*. Whereas, in the same species of freshwater prawn, Sahoo et al. (2007) found that large ovoid haemocytes were largely phagocytic, while small round cells had a restricted phagocytosis function. Vazquez et al. (1997) also found in the same species that granulocytes were the sole cells that phagocytised chicken red blood cells. The phagocytosis of bacteria by haemocytes of *Homarus americanus*, *Panulirus interruptus* and *Loxorhynchus grandis* revealed that SGCs and LGCs were the most active, resulting in 96% of the activity *in vitro* (Hose et al., 1990). Surprisingly, in another study, it was found that HCs of *Liocarcinus depurator* were the main cell type involved in phagocytosis of the marine bacterium *Psychrobacter immobilis* (Walton and Smith, 1999). Sung and Sun (2002) also found that HCs were the only cell that phagocytosed latex beads *in vitro*. In conclusion, it is clear a comparison of these data, from the above studies, is difficult. The discrepancies may partly arise from the different microorganisms used to induce phagocytosis and the difference methods employed *in vitro*.

The information on phagocytosis and subsequent destruction of microorganisms is limited for *C. cainii* haemocytes, although it is well characterised in mammalian and many other arthropod species. In this study, the phagocytic activity among *C. cainii* haemocytes was relatively high for both bacteria. The semi-quantitative data acquired in this study, is convincing enough to examine phagocytic activity since it is not likely to misinterpret the position of the bacteria in the ultrathin sections of TEM. In a study on haemocyte ultrastructure of *Carcinus maenas*, despite its pathogenic activity, it was shown that only a few milky in appearance disease bacteria were phagocyte by the haemocytes (Eddy et al.,

2007). The morphology of phagocytic haemocytes in *C. cainii* offers interesting insight, suggesting the process is similar to vertebrate and other invertebrate haemocytes (Hirsch, 1965; Foley and Cheng, 1972; Cheng and Foley, 1972). Gupta and Campenot (1996); Gupta (1997) and Conrad et al. (2004) stated that formation of pseudopods, rearrangements of the cytoskeleton, intracellular cAMP levels of amebocytes and exocytosis during phagocytosis in arthropods (*Limulus polyphemus* and *Gromphadorhina portentosa*) are very similar to macrophages and neutrophils of mammals. Our results indicated that long pseudopodia extending from SGCs were found, although we did not find one containing phagocytosed bacteria, it seems indicative of particle engulfment by macropinocitosis. Phagocytosis of both bacteria by *C. cainii* haemocytes may have also occured by a zippering-type phagocytosis, with vacuole-enclosed phagocytosed particles and replenishment of the space made by the discharge granules in phagocytic hemocytes. Swanson and Baer (1995) explained that phagocytic cells of mammals engulfed some particles by three morphological types: zippering phagocytosis (facilitated by Fc receptors), triggering phagocytosis (facilitated by complement receptors) and macropinocytosis (a receptor-independent and actin-dependent process), where the cells phagocytose both particles and a large extra-cellular fluid volume. Phagocytosis of both *V. mimicus* and *E. coli* was not the only cellular response detected. We also observed melanised bacteria, and that process appeared to help destroy the bacteria once internalisation has occurred. Melanisation in invertebrate haemocytes is also an important cellular reaction against pathogens. Borges et al. (2008), found that in the triatomine bug *Rhodnius prolixus,* bacteria melanisation appeared to facilitate bacteria phagocytosis. Soderhall and Thornqvist (1997) also noticed that semi granulocytes and granulocytes are sensitive to foreign particles (particularly glucans, lipopolysaccharides and bacteria), and releasing their granules to discharge prophenoloxidase and other constituent involved in cytotoxicity and melanisation pathways. However, the processes remain unclear in *C. cainii* haemocytes and need further examination.

In this study, we used two Gram-negative bacteria (*V. mimicus* and *E. coli*) as target particles. We found that phagocytic activity of *C. cainii* haemocytes were comparable for both of these two bacterial species. Using different bacteria, Smith and Ratcliffe (1978) noticed a distinct variation in phagocytic index in *C. maenas* after 3 hours incubation with *Moraxella* sp., which was higher than *Bacillus cereus* and *Aerococcus viriden*s. Although, the response mechanisms is still unclear. Armstrong et al. (1990), characterised horseshoe crab amebocytes phagocytosing the particles from carbonyl iron *in vitro*, however, in the presence of bacterial

endotoxin amebocytes failed to show phagocytic behaviour. Smith and Ratcliffe (1978) have also found that Gram-positive bacteria, *Bacillus cereus* and *Gaffkya homari*, were phagocytosed only a small proportion (around 5% and 3%, respectively) for the shore crab *Carcinus maenas* cells *in vitro*. The difference between results may be due to haemocyte physiology and recognition and/or immune effector mechanism differences.

In our study we detected lower phagocytic activity in all types of *C. caini* haemocytes kept at 20 °C. The influence of temperature on the phagocytic process has also been reported in bivalve molluscs and crustaceans. Hégaret et al. (2003) found a major decrease in *Crassostrea virginica* phagocytic activity after an abrupt temperature increase from 20 to 28 °C for 1 week. On the contrary, Carballal et al. (1997) noticed a lower phagocytic haemocytes percentage at 10 °C compared to 20 and 30 °C for *Mytilus galloprovincialis*. Ordas et al. (1999) observed that phagocytosis activity is about the same at 15 and 21 °C in clam *Ruditapes decussatus* and for mussel *Mytilus galloprovincialis*. Alvarez et al. (1989) and Chu and La Peyre (1993) proposed that temperatures above a certain level may result in stress conditions in oyster haemocytes, as a result they are less reactive. The latter group also highlighted the results that temperature could increase the phagocytic activity in eastern oysters (*C. virginica*) haemocyte kept at 20 °C compared to those kept at 10 °C for 68 days, but the activity declined at 25 °C (Chu and Lapeyre, 1993). Furthermore, the filtering capacities and metabolic activities of Mediterranean clam *Chamelea gallina* reduce dramatically at temperatures below 10 °C and over 30 °C (Ramon and Richardson, 1992). Paterson and Stewart (1974) and Uzman et al. (1977) showed that adult American lobsters exhibit a lowered phagocytic ability at 22 and 24 °C compared to 14 °C.

In conclusion, results from this chapter show *C. cainii* haemocytes are active phagocytic cells. Through the *in vitro* phagocytosis assays it was revealed that all the three types of haemocytes are involvement of phagocytic activity, with HCs and SGCs showing the greatest activity at the lower temperature of 20^{o}C. In this study, we have also found that an immunological challenge by *V. mimicus* and *E. coli* stimulated dramatic morphological changes in haemocytes of *C. cainii*. The ultrastructural changes observed in *C. cainii* haemocytes reflect the HCs and SGCs importance in this process, having been shown to be the main phagocytic cells.

CHAPTER 5

PHAGOCYTOSIS BY DIFFERENTIAL INVOLVEMENT OF MARRON (*Cherax cainii*) HAEMOCYTES WITH LIVE OR HEAT-KILLED *Vibrio mimicus* AS MEASURED BY FLOW CYTOMETRY

5.1. Introduction

The innate immune system provides protection from pathogens in invertebrates and in recent years significant progress has been made in the understanding of mechanisms (Huang et al., 1981; Soderhall and Thornqvist, 1997). Like other crustaceans, marron (*C. cainii*) possess an open circulatory system containing haemolymph and haemocytes. The haemocytes have phagocytic activity, which is a most important function, and plays an essential immune role (Yoshino and Granath, 1985). Phagocytosis is described as the ingestion and digestion of large particles (≥0.5-µm) such as cellular debris, intact cells, micro-organisms and macro-molecular aggregates by professional phagocytic cells, including haemocytes. These cells contain receptors on their surface which recognise and bind to target molecules in order to start the phagocytic process. Therefore, phagocytosis is a first line of protection against disease and promotes immune capability in invertebrate (Iwanaga, 2002; Le Moullac and Haffner, 2000).

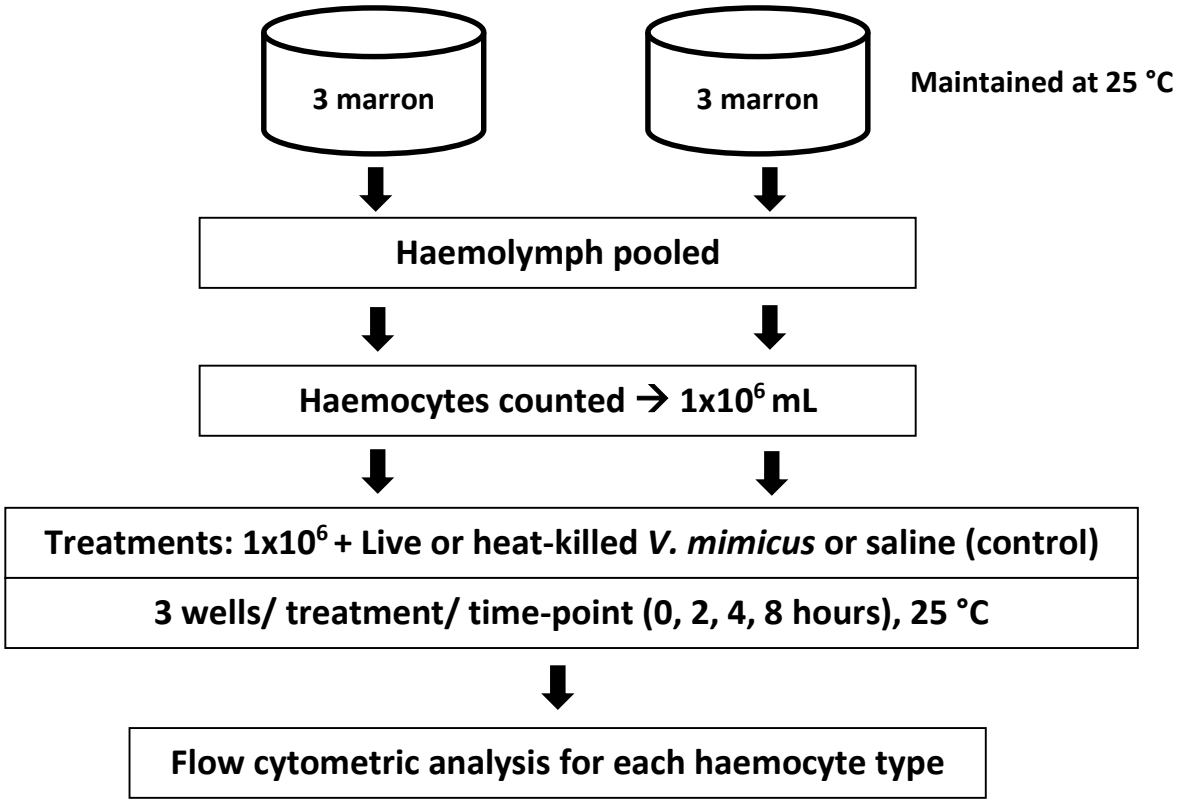
The phagocytic process has been systematically documented in host model systems for mammals and invertebrates: from the molecular identification of the pathogen through receptor–ligand interactions followed by ingestion, digestion and final destruction (Garin et al., 2001; Vazquez et al., 2009; Lavine and Strand, 2002; Ratner and Vinson, 1983). Vazquez et al. (2009), showed that haemocytes are the major phagocytes in freshwater crabs, *Parachaeraps bicarinatus* and *Carcinus maenas*, having the function of disposing of particles in the haemocoel. Phagocytes of *P. bicarinatus* and *C. destructor* specifically identify Gram-negative bacteria, i.e. *Pseudomonas* spp. and *E. coli* (McKay et al., 1969). In the American lobster, *Homarus* americanus, phagocytes particularly eliminate Gram-negative bacteria (Mori and Stewart, 2006). Phagocytes of blue crab, *Callinectes sapidus*, exclude both Gram-negative and Gram-positive bacteria (Cassels et al., 1986). Despite the importance of this process, little is known about phagocytosis of foreign targets by *C. cainii* haemocytes, along with the phagocytic process itself.

The aim of this study was to determine the dynamics of phagocytosis *in vitro* of *C. cainii* haemocytes through a more quantitative technique than employed in Chapter 4. Haemocytes were challenged with live and heat-killed *V. mimicus* and tested over time and at different temperatures.

5.2. Experimental Outline

5.2.1. Experimental outline of phagocytic experiments

The phagocytosis experiment was conducted as outlined below. Two sets of 3 marron were maintained at 25 °C for two weeks prior to being used. Haemolymph from one set was pooled and counted and used for the control experiments, and the other set of marron was used for the live or heat-killed bacteria experiments. Experiments were conducted at 25 °C and set up in triplicate wells containing one million haemocytes with live or heat-killed *V. mimicus* or with saline for controls. Separate sets of wells were set up for time-points 0 (baseline), 2, 4 and 8 hours. Note, for each time-point, a saline control was also performed which enabled baseline corrections. These values were minimal and were subtracted from the treatment values. Flow cytometric analysis was performed on all three haemocyte cell types (HCs, SGCs and LGCs).



5.2.2. Statistical methods for phagocytosis analysis

Standard descriptive statistics (means and standard deviations) were used to summarise the data at each combination of conditions. An analysis of variance (ANOVA) model was used to explore any influence that the treatment, time, and cell type may have exerted on the phagocytosis, for live or heat-killed *V. mimicus* separately. The ANOVA model was applied to the logarithm of the phagocytosis data as the raw measurements appeared to not be normally distributed. The analysis of the log-transformed data was performed using the GLM (general linear model) procedure in the SAS software program (SAS version 9.2, SAS Institute, Cary, NC, USA, 2008). Following the identification of significant main effects, all pairwise interaction terms were included in the model (one at a time), and only included in the final model if they appeared to be significantly associated with the outcome variable. Finally, the differences between the levels of each independent variable (time, treatment, cell type) were examined for statistical significance using post-hoc tests on the adjusted (i.e. logarithm) mean values obtained from the GLM. Following convention, a p-value <0.05 was taken to indicate a statistically significant association in all tests.

5.2.3. List of Experimental procedures – refer to Chapter 2 for further detail

- 2.1 Acclimatization system
- 2.2 Animals
- 2.3 Preparation of haemocytes
- 2.4 Preparation of the *Vibrio mimicus* stock solution
- 2.6 Flow cytometry
- 2.10 Phagocytosis assay using specific fluorophore

5.3. Results

In Chapters 3 and 4 the haemocytes of *C. cainii* were characterised from a morphological aspect. In this chapter we confirm and expand on findings and focus on functional features of *C. cainii* haemocytes in a quantitative manner with respect to their phagocytic activity.

5.3.1. Staining of *C. cainii* haemocytes with specific fluorophore

The investigation of haemocytes with FCM, based solely on size and granularity (i.e. unstained), tended to produce a high background on the flowcytogram that affected the quality of analysis. For that reason we investigated fluorescent cellular stains to improve resolution: resolve dim markers that reposition the outcomes from apparently negative results to explicit results without an increase in background. A potential drawback with the use of fluorescent markers is using them without optimising their concentration. Using concentrations past the point of saturation will give no further positive signal but will increase the background, negating the benefits over analysing unstained cells. In order to find the optimal concentration, serial dilutions of the fluorescent stain were tested. We tested two dyes, CellTracker Red CMPT and Deep Red, to specifically stain *C. cainii* haemocytes. We found best results with CellTracker Deep Red at a concentration of 0.5 µM for 45 minutes (Figures 5.1 and 5.2).

5.3.2. Flow cytometer analysis of haemocytes after exposure to *V. mimicus*

According to the three gates which were set up for FCM, the relative numbers of phagocytic HCs, SGCs and LGCs were determined following incubations with live or heat-killed *V. mimicus*. At this point an explanatory note is necessary regarding the fluorescent signals and determination of phagocytosis. Haemocytes were stained red and the bacteria were stained green. When haemocytes were detected in association with green, this was taken as phagocytic activity. We have no way of determining, with this technology, at what stage the phagocytic process is at e.g. the initial attachment or inside a phagosome. In Figure 5.3 we present data with heat-killed *V. mimicus* for the 2 hours incubation to demonstrate how data was acquired. Figure 5.3a shows HCs for control treatment with the majority of cells in quadrant 1 (Q1, bottom right) with small amount of debris or unstained cells located in Q4 (bottom left). Once HCs were exposed to heat-killed *V. mimicus* (Figure 5.3b), 7.31% of cells were detected in Q2 – these haemocytes, stained red, also contain green signal (from the bacteria) – this is the phagocytic proportion of HCs. The same applies SGCs, showing 23.8%

of phagocytic activity (Figures 5.3c and d) and LGCs showing 35.4% phagocytic activity (Figures 5.3e and f).

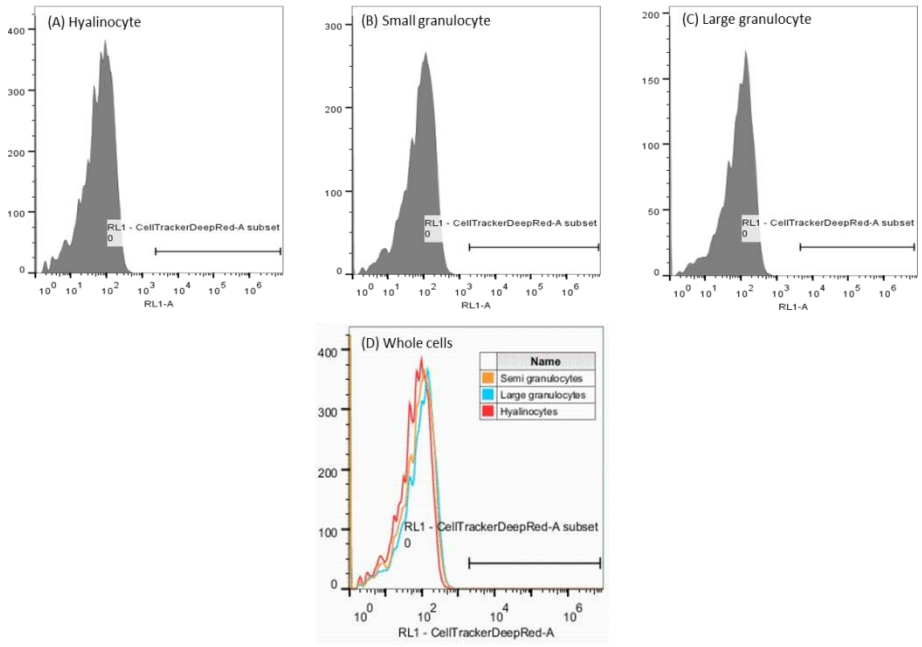


Figure 5.1. The endogenous fluorescence of *Cherax cainii* haemocytes without fluorescent marker. The thick bar represents the position of the marker used to quantify the proportion of cells that had bound with Cell Tracker-Deep Red.

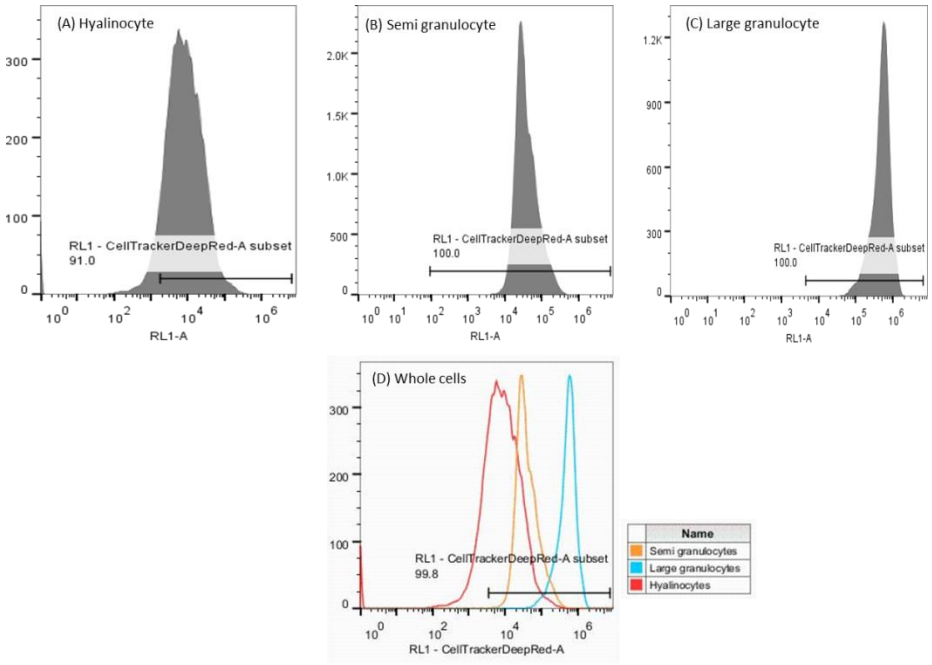


Figure 5.2. *Cherax cainii* haemocytes with fluorescent marker.
The thick bar represents the position of the marker used to quantify the proportion of cells that had bound with Cell Tracker Deep Red.

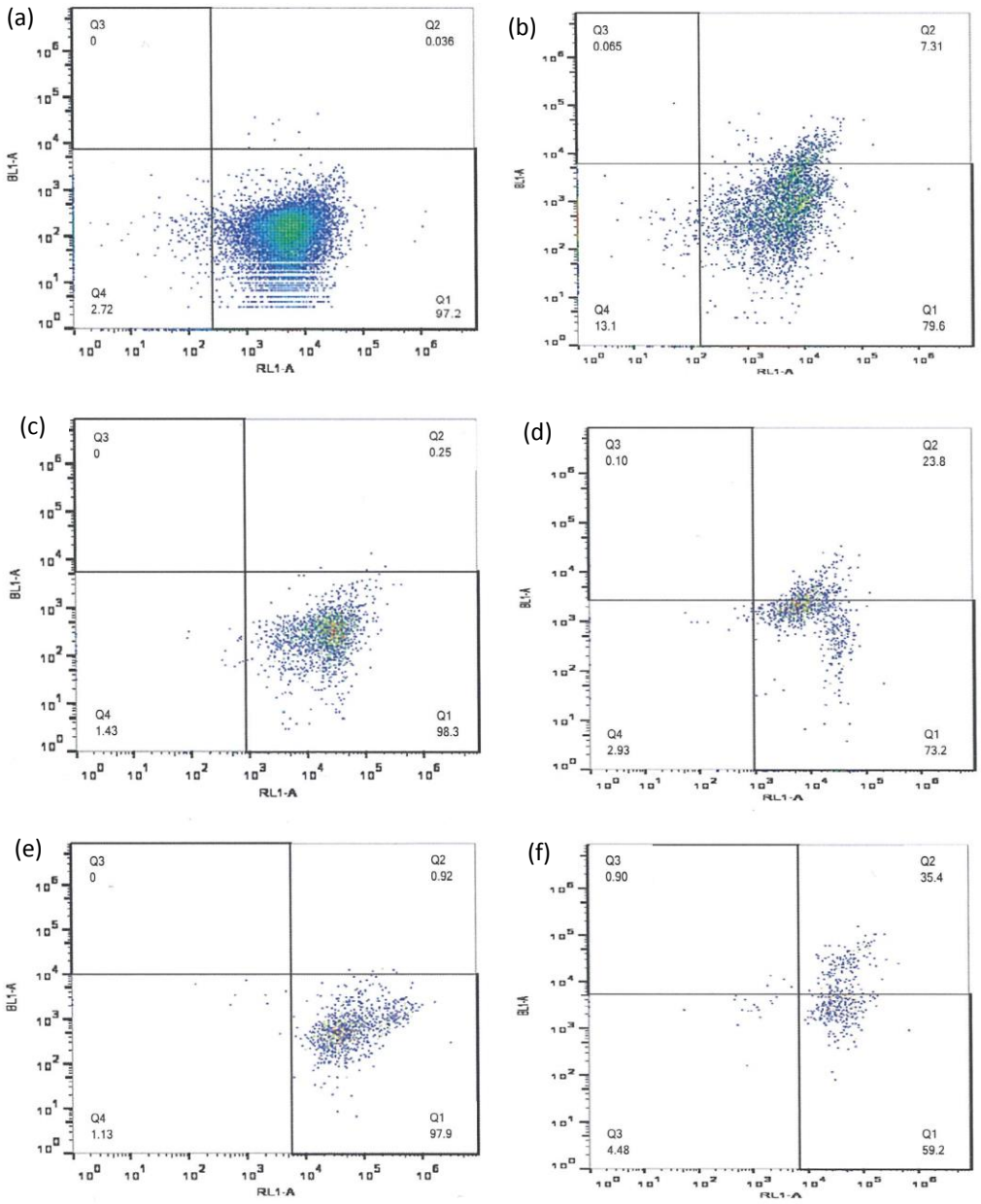


Figure 5.3. Phagocytic activity of *C. cainii* haemocytes analyse by FCM *in vitro*. (a) FCM analysis of *C. cainii* hyaline haemocytes (HCs) stained with Cell Tracker Deep Red (CTDR) exposed with saline (control) in quadrant 1 (Q1). (b) FCM analysis of phagocytic *C. cainii* HCs after 2 hours exposure with heat-killed *V. mimicus* labelled FITC in Q2. (c) FCM analysis of *C. cainii* SGCs stained with CTDR as control in Q1. (d) FCM analysis of phagocytic *C. cainii* SGCs after 2 hours exposure with heat-killed *V. mimicus* labelled FITC in Q2. (e) FCM analysis of *C. cainii* LGCs stained with CTDR as control in Q1. (f) FCM analysis of phagocytic *C. cainii* LGCs after 2 hours exposure with heat-killed *V. mimicus* labelled FITC in Q2. Quadrants 3 (left, up) and quadrants 4 (left, down) mainly consisting of non-cellular populations composed of debris.

5.3.3. Phagocytic activity of haemocytes with live *V. mimicus*

Freshly isolated haemocytes were treated with live or heat-killed *V. mimicus* or without (saline control) for 2, 4 and 8 hours. Baseline readings were carried-out at time-point zero and at each time-point for each cell type (HCs, SGCs and LGCs). Figures 5.4 and 5.5 show the phagocytic mean values with standard deviations for each cell type treated with live or heat-killed *V. mimicus*, respectively. Because these raw data appeared to not be normally distributed, the ANOVA model was applied to the logarithm of the phagocytosis data. A multivariate analysis was performed on differences to identify statistical significance between the levels of each independent variable (time, treatment, cell type). See Appendix 3 for a detailed description of the statistical analysis for section 5.3.3 and 5.3.4.

Treatment with live *V. mimicus* resulted in an increase in phagocytosis for all three cell types compared to no treatment (minimum p value of <0.0009), and a general trend of increased phagocytosis with time (Figure 5.4). There was a statistically significant difference at all time-points between HC and both SGCs and LGCs (minimum p value of <0.0004) and no statistical difference between SGCs and LGCs. The highest levels of phagocytosis were seen at 8 hours but these failed to attain significance from 2 and 4 hours, within each cell type: for LGCs, this was largely due to a high level of sample variation.

5.3.4. Phagocytic activity of haemocytes with heat-killed *V. mimicus*

When treated with heat-killed *V. mimicus*, the pattern of phagocytic activity by the haemocytes was similar to that seen for live *V. mimicus* treatment. Again, there was a statistically significant difference between treatment and no treatment ($p<0.0001$) and between HCs and both SGCs and LGCs at all time-points (minimum p value of <0.05) and only statistical difference between SGCs and LGCs at 8 hours treatment ($p<0.03$) (Figure 5.5).

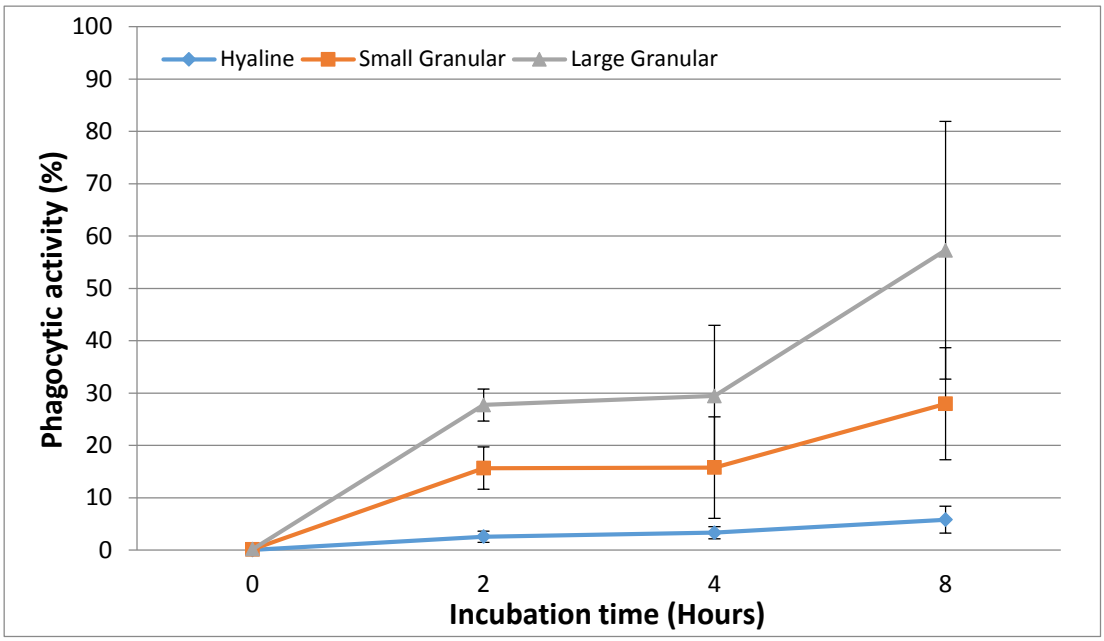


Figure 5.4. Phagocytosis of live *Vibrio mimicus* by differential involvement of *C. cainii* haemocytes at 25 °C *in vitro*. Each bar represents mean value from three replications, including standard deviation.

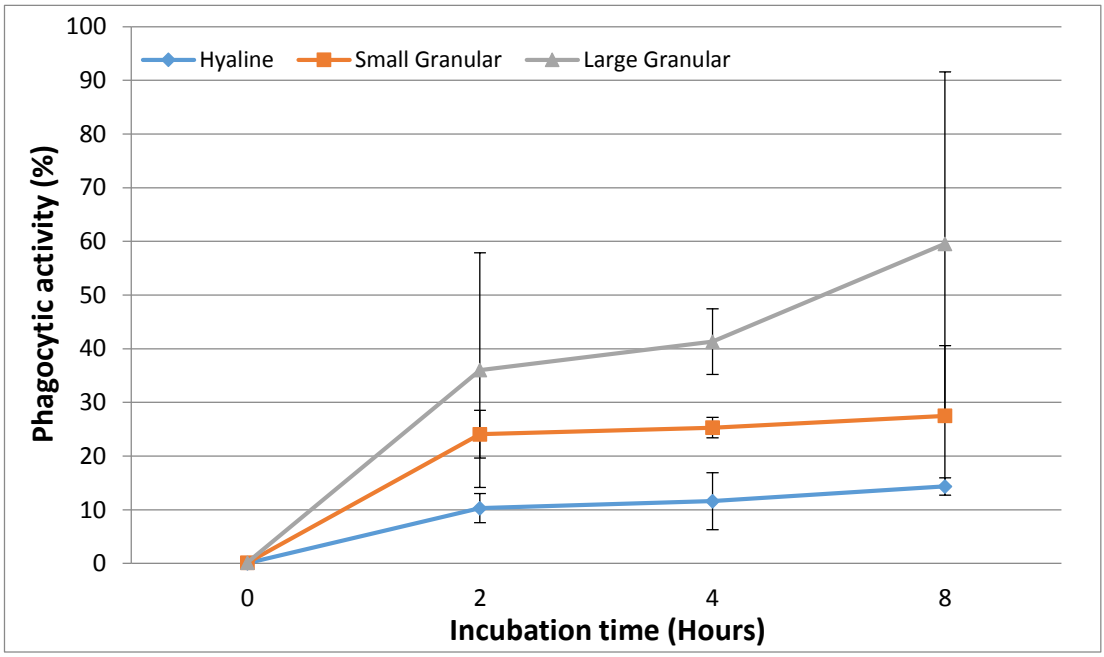


Figure 5.5. Phagocytosis of heat-killed *Vibrio mimicus* by differential involvement of *C. cainii* haemocytes at 25 °C *in vitro*. Each bar represents mean value from three replications, including standard deviation.

5.3.5. Comparisons in phagocytic activity between live and heat-killed *V. mimicus*

The comparison between live and heat-killed *V. mimicus* treatments for each cell type revealed that there was only statistical significance in phagocytic activity between treatment and no treatment and between treatment types (i.e. live vs heat-killed) for HCs alone (minimum p value of <0.004 across the three time-points; Figure 5.6, 5.7 and 5.8). Although there was a trend for higher phagocytic activity due to the heat-killed treatment at 2 and 4 hours for SGCs and LGCs, both treatments resulted in similar phagocytic levels at 8 hours.

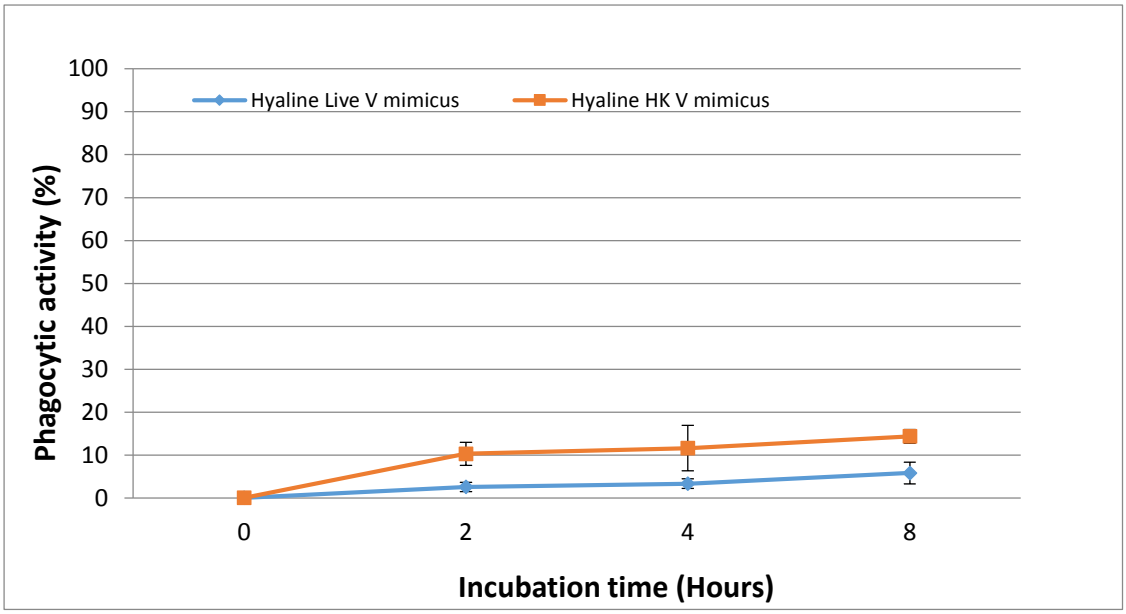


Figure 5.6. Phagocytic activity of live or heat-killed *Vibrio mimicus* by hyaline cells at 25 °C *in vitro.* Phagocytic activities were counted by flow cytometry of each of the 3 replicates per end point. Data are presented as the mean ± SD (bar).

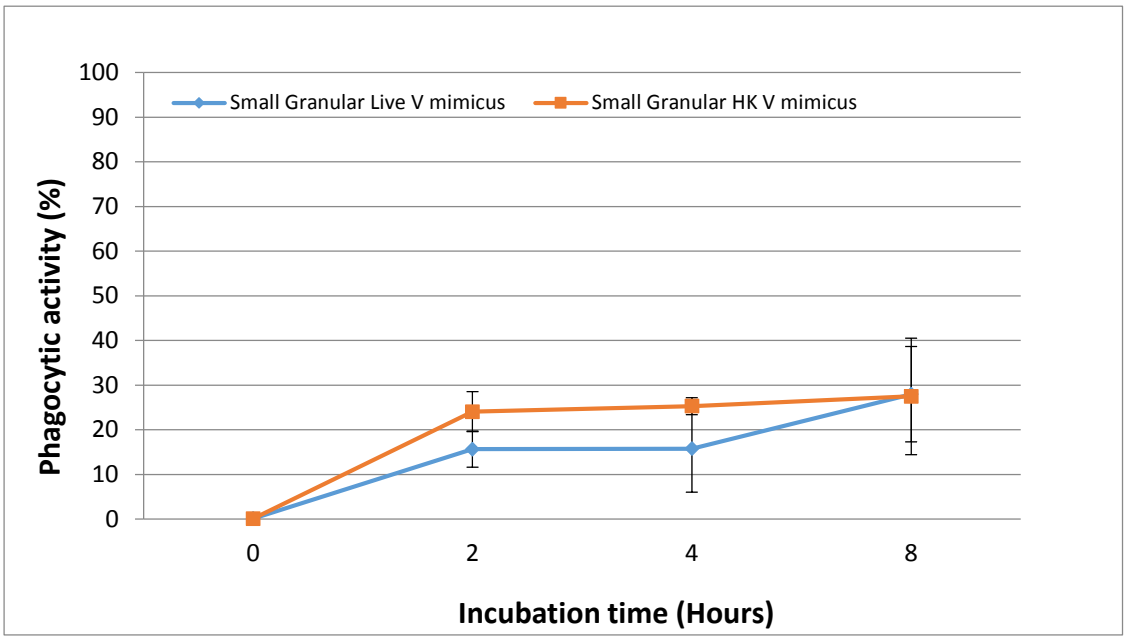


Figure 5.7. Phagocytic activity of live or heat-killed *Vibrio mimicus* into small granular cells at 25 °C *in vitro.* Phagocytic activities were counted by flow cytometry of each of the 3 replicates per end point. Data are presented as the mean ± SD (bar).

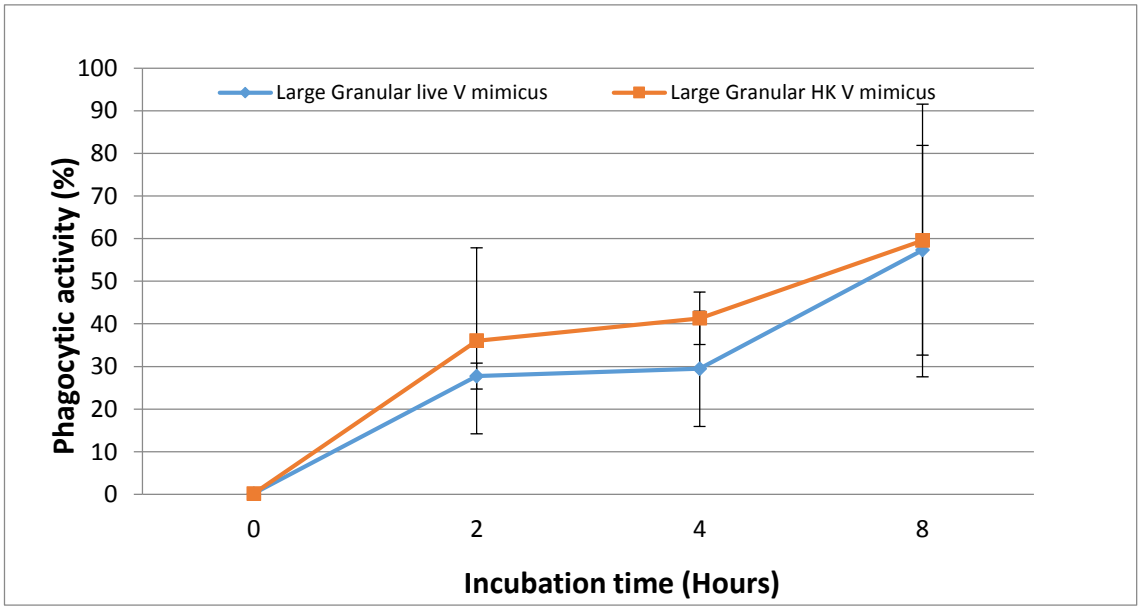


Figure 5.8. Phagocytic activity of live or heat-killed *Vibrio mimicus* into large granular cells at 25 °C *in vitro.* Phagocytic activities were counted by flow cytometry of each of the 3 replicates per end point. Data are presented as the mean ± SD (bar).

5.4. Discussion

The data from the above experiments revealed that the most active phagocytic cells in *C. cainii* were the granular cells (SGCs and LGCs) while HCs were the least active in phagocytosis of live and heat-killed *V. mimicus*, a Gram-negative bacterium. The cell type(s) primarily responsible for phagocytosis in crustaceans is controversial. In the literature, these three haemocyte types are generally recognised in crustacean species and HCs and SGCs are generally regard as the most active phagocytic cell types (Bauchau, 1981). In the crab *Callinectes sapidus*, HCs appear to only phagocytose, while both HCs and GCs engulfed Gram-negative bacteria (Johnson, 1976; Johnson, 1977). Hyaline cells, previously separated by Percoll™ gradient, were regard as the main phagocytic cell type in crab and crayfish (Bell and Smith, 1993; Thornqvist et al., 1994). In a more recent study, HC from *P. monodon* was the haemocyte type that phagocytosed latex beads *in vitro* (Sung and Sun, 2002). Sung et al. (2000), also observed, in *Macrobrachium rosenbergii,* that HCs were the main type of haemocyte for bacteria elimination. Matozzo and Marin (2010), also found yeast cells or zymosan was engulfed only by HCs from crab *Carcinus aestuarii*.

On the other hand, in decapod crustaceans, SGCs were primarily detected having phagocytic activity (Smith and Soderhall, 1983; Soderhall and Smith, 1983; Hose et al., 1990). Both SGCs and LGCs were the haemocytes that phagocytosed bacteria *in vitro* for *Homarus americanus*, *Panulirus interruptus* and *Loxorhynchus grandis*: SGCs were the most active (Hose et al., 1990). Small granular cells and LGCs were also found to be active in phagocytosis of yeast particles *in vitro* and also possessed lysosomal enzymes and prophenoloxidase activity in *M. rosenbergii*, *M. acanthurus* and *P. monodon* (Martin et al., 1993; Gargioni and Barracco, 1998). In an *in vivo* study with the freshwater crayfish *Astacus leptodactylus*, all the haemocyte types were phagocytic but only the SGCs phagocytosed all foreign particles (Giulianini et al., 2007). Sahoo et al. (2007), observed that the most phagocytic cells were large ovoid cells, while the cells with inadequate or restricted capacity for phagocytosis were the small round cells in the *A. leptodactylus*. Again, in the same freshwater prawn species, only granular cells phagocytosed chicken erythrocytes (Vazquez et al., 1997). Therefore, there appears to be discordant results in crustaceans with regards to the role of haemocytes type in phagocytosis. This may be in part(s) due to species variation, the lack of a standardised haemocyte classification system and/or due to the different experimental methods applied, including the target microorganisms.

The phagocytosis levels detected in our study was relatively high compare to some studies on crustaceans. We quantitated phagocytosis rates of 5.9% and 14.5% in HCs cells, 28.3% and 28.9 in SGCs cells and 58% and 60.7% in LGCs cells in *C. cainii* haemocytes after 8 hours exposure with live and heat-killed *V. mimicus*, respectively. Tripp (1966) demonstrated that up to 43.7% cells of cultured oyster were competent of phagocytosis. McKay and Jenkin (1970) observed that about 10% of normal crayfish (*Parachaeraps bicarinatus*) hemocytes demonstrated phagocytosis when exposed to sensitized sheep red blood cells. Pauley et al. (1971) reported that 29% of haemocytes from the sea hare (*Aplysia californica*) showed phagocytosis of chicken erythrocytes. Paterson and Stewart (1974) reported a 2% phagocytosis rate for lobster haemocytes. This may have been accurate but potentially suffered from experimental factors prior or during experimentation. Smith and Ratcliffe (1978), noticed a clear variation in phagocytosis rate after 3 hours in *C. maenas*: 5% for *Bacillus cereus*, 3% for *Aerococcus viridens* and 15% for *Moraxella* sp. Conversely, HCs in *Liocarcinus depurator* displayed a great ability to phagocyte the marine bacterium, *Psychrobacter immobilis*, and the rate of phagocytosis reached 18.8% (Walton and Smith, 1999). A recent study by (Zhu et al., 2005) have documented what looked to be phagocytosis of *Staphylococcus ileaureus* by *Carcinoscorpius rotundicauda* amebocytes, *in vitro* and *in vivo*, nevertheless, the mechanisms related with this reaction remain uncertain. A similar phagocytosis rates of 9% has been recorded for *Acanthoscurria gomesiana* (Fukuzawa et al., 2008) and *Penaeus monodon* (Xian et al., 2010), a phagocytosis rate that is usually quite low among invertebrates. For instance, the rates of phagocytosis of >18% in *Macrobrachium rosenbergii* (Hsu et al., 2005), ~15% in *Polistes dominulus* (Manfredini et al., 2010), >60% in *Rhodnius prolixus* (Borges et al., 2008) and *Galleria mellonella* (Bergin et al., 2005) and between 7.5% and 48% for six arthropod species (Oliver et al., 2011). The difference between these results may be due to differences in properties of haemocyte phagocytosis systems, the technical difficulties related with handling these fragile cells, sized of microba or micro beads and different environmental conditions.

A correlation between target particles and crustacean haemocytes phagocytic activity used in the phagocytic assay has also had a bearing in our study. We found that responses by haemocyte to activation by live and heat-killed *V. mimicus* resulted in different primary immune (phagocytic activity) patterns. The phagocytic activity resulting from heat-killed *V. mimicus* was significantly higher than with live *V. mimicus* only for HCs but failed to reach overall significance for SGCs and LGCs after 8 hours treatment. Phagocytosis of heat-killed *V. mimicus* may probably act as an inducer for early immune reaction. This is despite the fact

that possible disadvantage to using heat-killed bacteria in a phagocytosis assay is that surface proteins in bacteria could be altered. However, heat conditions could be not the same for every bacteria species (Lin et al., 2013). In the case of heat-killed preparation in this study, the *V. mimicus* surface molecules that act as antigens are denatured and more readily released and exposed to identification by pattern recognition receptors (PRRs) leading to enhanced phagocytic activity. Drevets and Campbell (1991), in their experiments to study phagocytosis mediate by macrophage receptors, found that FITC-labeled live and heat-killed *Listeria* resulted in the same percentage of internalized bacteria by macrophage. Therefore, heat-killed *V. mimicus* may preserve less antigenicity but disclosed more LPS, and LPS is a known ‘‘immune potentiator’’ or ‘‘adjuvant’’.

The phagocytic activity of *C. cainii* haemocytes was conducted at 25 ^{o}C, being an average temperate temperature they live in. Temperature is known to have an crucial role in the defence systems of invertebrate, and production of vertebrates antibody (Avtalion, 1969; Tait, 1969). McKay and Jenkin (1970), proposed that the first recognition stages (including foreign material binding to the cell surface) in crayfish phagocytic activity were temperature independent. Bell and Smith (1993) found that acclimation temperature of crayfish *Carcinus maenas* at 13 °C or 19 °C compared to control animals at 6 °C, had a powerful effect on microbial killing of a marine Pseudomonad, *Planococcus citreus* (strain 1-1-1), on *C. maenas in vitro*.

In conclusion, using flow cytometry as a quantitative tool to study *C. cainii* phagocytic activity, the three *C. cainii* haemocyte types were shown to have different capacities to phagocytose bacterial particles. Small granular cells (SGCs) and LGCs were the more phagocytically activity cell type. These assays may provide useful information for the further study of the function of *C. cainii* haemocyte populations and their defence mechanisms.

CHAPTER 6

IMMUNOLOGICAL ASSESMENT OF MARRON (*Cherax cainii*) HAEMOCYTES TO BACTERIAL LIPOPOLYSACCHARIDE AT DIFFERENT TEMPERATURE *IN VITRO* USING FLOW CYTOMETRIC ANALYSIS

6.1. Introduction

Marron (*Cherax cainii*) is an indigenous species to Western Australia, and renowned as a prospective aquaculture species because it is one of the world's largest freshwater crayfish which able to reach 2 kg in weight. Due to the significance of this species in farming, both local and world-wide (South Africa, Zimbabwe, Japan, USA, China and the Caribbean) current research is being conducted in relation to their aquaculture potential (Fotedar, 1998). Marron farming, in order to get higher production, is usually conducted in a semi-intensive freshwater pond where there is need for developed systems to manage high feeding and stocking densities. The industry's sustainability is subject to disease control and the health condition of marron. In order to be effective, methods of disease control have to be part of an integrated health management system. It should be possible to focus in the management system on the most important environmental parameter in light of improved understanding of the relationship between environmental conditions and disease.

Crustaceans have a primitive open circulatory system (haemocoel). The blood is described as haemolymph and the blood cells are known as haemocytes and haemocyanin-rich plasma (respiratory protein) (Malham et al., 1998). In the crustacean immune defence, haemocytes perform an important function. Phagocytosis, encapsulation and nodular aggregation are some of the basic functions of haemocytes to eliminate the foreign substances in the hemocoel (Soderhall, 1992). Furthermore, they can heal the lesion by cellular clumping and release of plasma gelation factors to initiate coagulation (Johansson and Soderhall, 1989; Omori et al., 1989; Vargas-Albores et al., 1998), and for prophenoloxidase (proPO) transporting and circulating system (Johansson and Soderhall, 1989; Hernandez-Lopez et al., 1996). Such essential molecules, like α_2-macroglobulin (α_2M) (Rodriguez et al., 1995; Armstrong et al., 1990), agglutinins (Rodriguez et al., 1995), and antibacterial peptides (Destoumieux et al., 1997; Schnapp et al., 1996; Khoo et al., 1999) can also be produced and released into the haemolymph by haemocytes.

Lipopolysaccharide is an integral element of Gram-negative bacteria outer membrane, which contains highly antigenic and cytotoxic material (Schletter et al., 1995). Lipopolysaccharide causes several effects that potently stimulate the innate immune system (Ulevitch and Tobias, 1995). It triggers haemocytes production in shrimp *Penaeus japonicus* (Sequeira et al., 1996), initiates crayfish proPO release (Soderhall and Hall, 1984), stimulates phenoloxidase (PO) activity of hemocyte lysate supernatant (HLS) from both shrimp *Penaeus monodon* and *Macrobrachium rosenbergii* (Sung et al., 1998), stimulates crayfish haemocytes

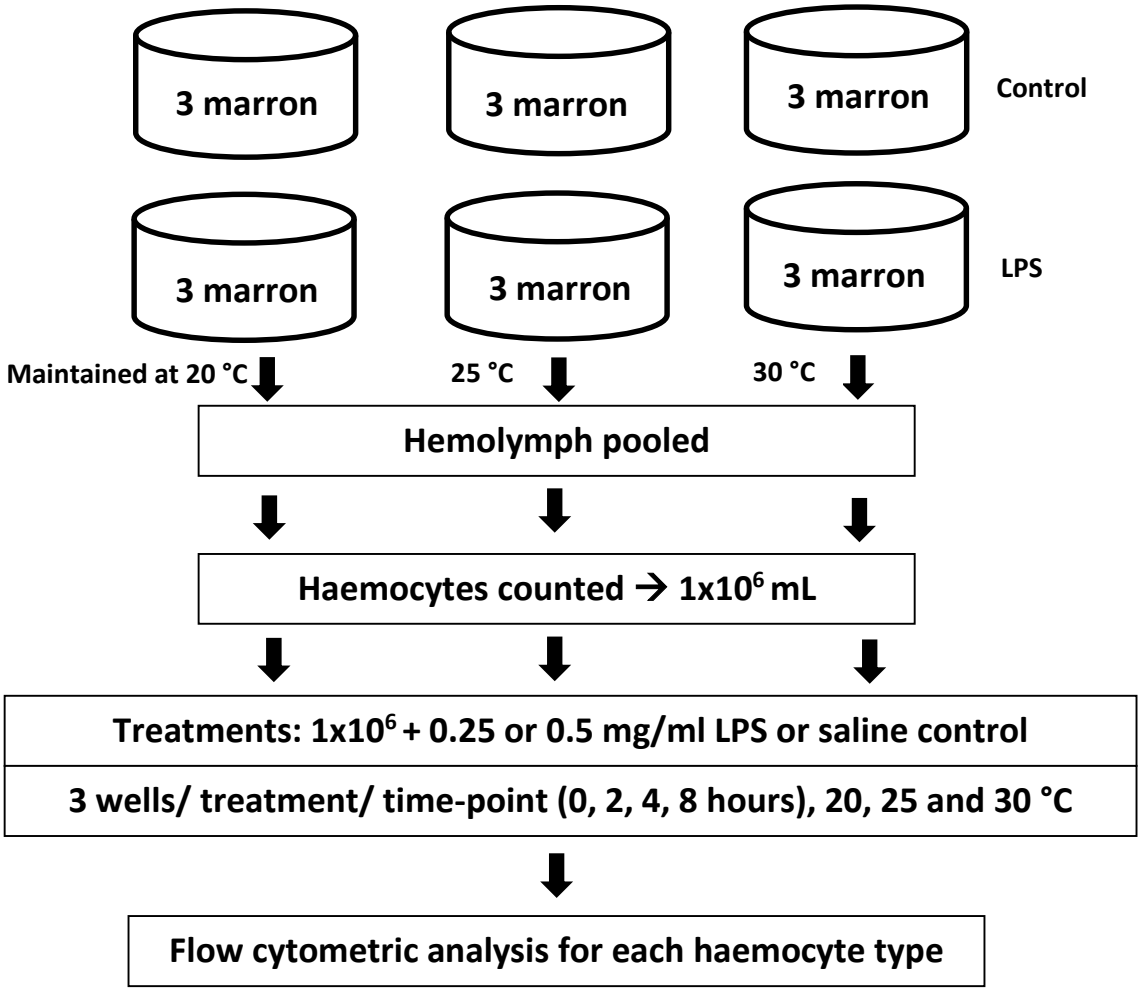
degranulation and release of proPO (Cardenas et al., 2004). Also, it is employed as an immuno-modulator through diet due to its positive impacts on the innate immune system (Takahashi et al., 2000). Conversely, some studies report negative effects of LPS, such as triggering the reduction of circulating haemocytes numbers (Lorenzon et al., 1999; Lorenzon et al., 2002), cause a decrease in the actions of proPO and inhibitory effect on antibacterial activity in *Penaeus monodon* haemolymph *in vitro* (Sritunyalucksana et al., 1999). In fact, the so called 'immunostimulant' effects increased resistance to infection, amelioration of the negative effects of stress and physiological reactions are more difficult to interpret. However, few studies have been performed to elucidate cellular mechanisms (Smith et al., 2003).

Characterization of *C. cainii* haemocytes under inflammatory stimulus (with LPS) will expand our basic knowledge about haemocytes and extend our knowledge about cell-mediated responses of *C. cainii* to stress, set off by inflammation. *C. cainii* haemocytes were also suggested to be responsive to variations of environmental conditions including temperature. Therefore, it is also important to comprehend the response of the immune system in the *C cainii* to different temperature in order to become acquainted with such changes, and be of assistance to comprehend and estimate about how immuno-competence can be changed by environmental variability. Therefore the objective of this study was to investigate the immunological responses of *C. cainii* haemocytes *in vitro* to LPS and to evaluate the impact of temperature variation on haemocytes of *C. cainii* by flow cytometry.

6.2. Experimental Outline

6.2.1. Experimental outline of LPS effect at different temperature experiments

The LPS effect at different temperatures was conducted as outlined below. Six sets of 3 marron were maintained at 20, 25 and 30 °C for two weeks prior to being used. One set from each temperature was used for the control experiments, and the other three set of marron was used for the LPS experiments. Following the 2 week temperature acclimatization, haemolymph from each set were pooled and haemocytes counted. Experiments were conducted at 20, 25 and 30 °C and set up in triplicate wells containing one million haemocytes with 0.25 or 0.5 mg/ml of LPS or with saline for controls. Separate sets of wells were set up for time-points 0 (baseline), 2, 4 and 8 hours. Note, for each time-point, a saline control was also performed which enabled baseline corrections. Flow cytometric analysis was performed on all three haemocyte cell types (HCs, SGCs and LGCs).



6.2.2. List of Experimental procedures – refer to Chapter 2 for further detail

- 2.1 Acclimatization system
- 2.2 Animals
- 2.3 Preparation of haemocytes
- 2.6 Flow cytometry
- 2.11 Activation of haemocytes

6.3. Results

6.3.1. Data analysis

Mixed haemocyte populations were treated *in vitro* with LPS (either 0.25 or 0.5 mg/mL) or without (Control). Treatments were incubated at 20, 25, or 30 ^{o}C for 0 (baseline), 2, 4 or 8 hours. Flow cytometry was employed to quantitate the relative percentages of the three different haemocyte types (HCs, SGCs and LGCs). The data is represented in Figures 6.1, 6.2 and 6.3 as horizontal percentage stack graphs showing the relative proportion of the three haemocyte populations within treatment groups. In order to determine changes in the population profiles, a fixed number of haemocytes were counted, according to their morphology. A change in the relative proportion of one haemocyte type has a concomitant change in one or both of the other haemocyte proportions.

As the primary measure of interest was the profile of the cell types, with each cell type expressed as a percentage of the total composition, one way to assess difference between one particular experimental group and another is to treat the profile as a 3-dimensional vector and calculate the angular separation of results from each experiment from a vector representing the comparison groups. If the percentages of cell types within any test sample are similar to the comparison group, then their angular separation would be near zero. Differences between the profiles, which may be due to a different proportion of any of the cell types, would appear as a difference in their angular separation, the greater the angular separation, the greater the difference between profiles. This enabled us to attain a more objective measure of overall profile changes (all three cell populations) between treatments within the same time point and temperature (Figure 6.4). For p-values of this data see Appendix Figure 1.

6.3.2. Changes in relative haemocyte populations due to treatment with LPS at different temperatures over time

Treatments with LPS at the lowest temperature, 20 ^{o}C (Figure 6.1), resulted in the earliest change in population profile: consisting of a low-moderate change starting from 0 hours (baseline), although the only difference was with the LPS at 0.25 mg/mL. By 4 hours both LPS treatments resulted in overall profile changes compared to the control, to the moderate, progressing to high by 8 hours. There was little difference between both LPS treatments. This overall pattern is quite easy to discern when looking at Figure 6.4. It's important to note

that all changes in haemocyte population profiles resulted in a relative increase in the HCs with a concomitant decrease in SGCs or SGCs and LGCs populations.

There was moderate change in the haemocyte profiles for the treatments incubated at 25 ^{o}C only from 2 hours onwards, and only for the 0.5 mg/mL LPS treatment (Figures 6.2 and 6.4). Interestingly, the profiles were consistent for 2 to 8 hours. The 30 ^{o}C incubations resulted in profile change only starting at 4 hours however, they were moderate and high for 0.25 and 0.5 mg/mL LPS compared to control, with only a low-moderate change between them (Figures 6.3 and 6.4). The highest change was seen for the 30 ^{o}C incubations for 8 hour. From Figure 6.4, the overall patterns of change due to LPS treatments compared to controls and each other can be seen clearly: there was similarity between the 20 and 30 ^{o}C incubations with both LPS treatments being moderate to high compared to control, at 4 and 8 hours, but with little difference between LPS treatments. This contrasted with treatments at 25 ^{o}C, where most of the change, moderate, was only seen for the 0.5 mg/mL LPS treatment.

As stated above, the changes in profile were due to changes in the 3 haemocyte cell types, relative to each other. Whenever there was a change in profile, this corresponded to an increase in HCs with a concomitant decrease in SGCs and LGCs. The exception was the 0.25 mg/mL treatment at 20 ^{o}C, where an increase in HCs resulted in a small increase in LGCs and a decrease only in SGCs, not including the 8 hour incubation (Figure 6.1).

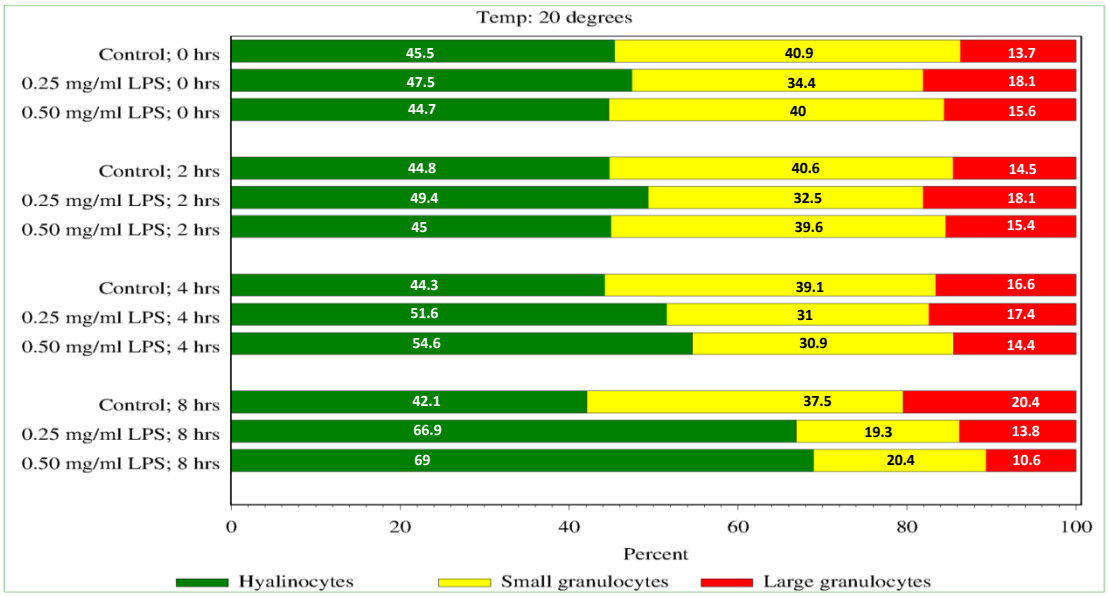


Figure 6.1. The relative cell proportion of *Cherax cainii* haemocytes after incubated with different concentrations of *E. coli* LPS at 20 °C. Relative percentages of each cell type in *C. cainii* at increasing time after incubated with 0.25 or 0.5 mg/ml of *E .coli* LPS or in control haemocytes incubated with saline *in-vitro*. *C. cainii* haemocytes were incubated with treatments and transferred at 0, 2, 4 and 8 hours for analysis by flow cytometry. For each time point 10,000 cells were analysed. For an explanation of data format see Section 6.3.1.

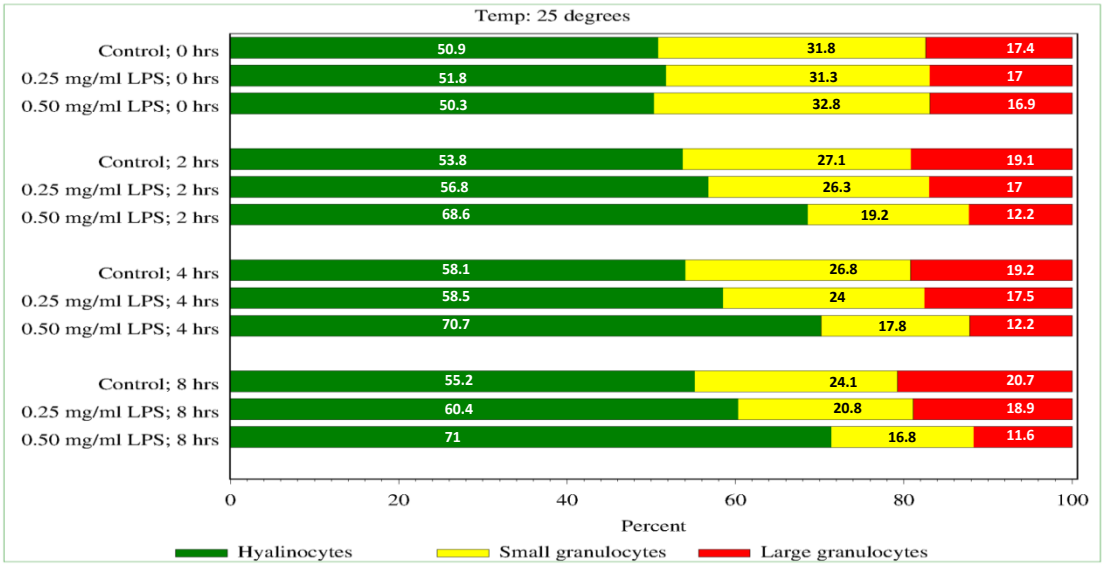


Figure 6.2. The relative cell proportion of *Cherax cainii* haemocytes after incubated with different concentrations of *E. coli* LPS at 25 °C. Relative percentages of each cell type in *C. cainii* at increasing time after incubated with 0.25 or 0.5 mg/ml of *E .coli* LPS or in control haemocytes incubated with saline *in-vitro*. *C. cainii* haemocytes were incubated with treatments and transferred at 0, 2, 4 and 8 hours for analysis by flow cytometry. For each time point 10,000 cells were analysed. For an explanation of data format see Section 6.3.1.

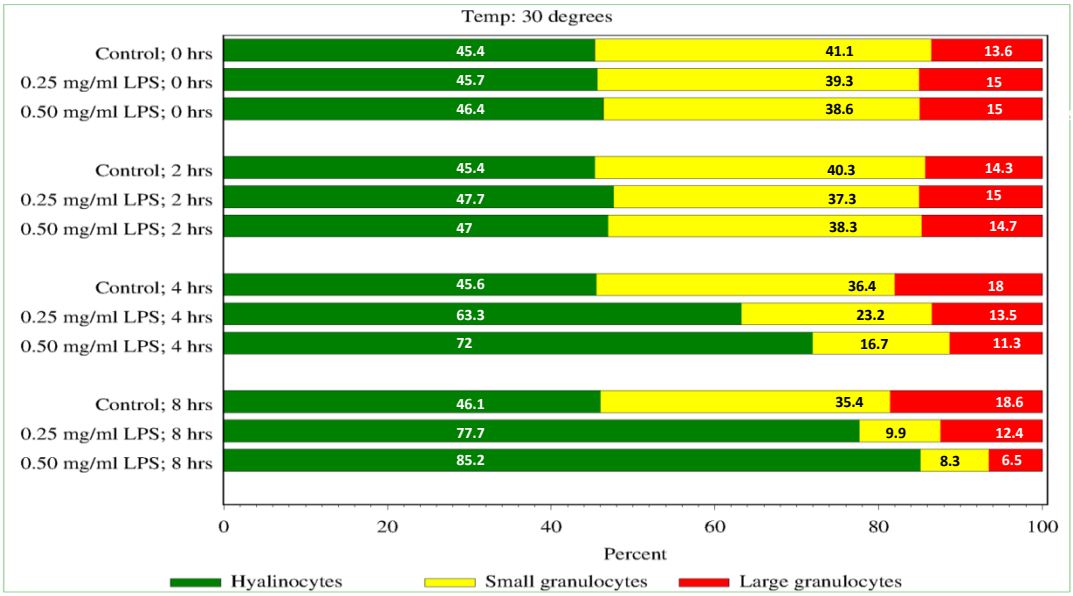


Figure 6.3. The relative cell proportion of *Cherax cainii* haemocytes after incubated with different concentrations of *E. coli* LPS at 30 °C. Relative percentages of each cell type in *C. cainii* at increasing time after incubated with 0.25 or 0.5 mg/ml of *E .coli* LPS or in control haemocytes incubated with saline *in-vitro*. *C. cainii* haemocytes were incubated with treatments and transferred at 0, 2, 4 and 8 hours for analysis by flow cytometry. For each time point 10,000 cells were analysed. For an explanation of data format see Section 6.3.1.

Inc. time (Hours)	Treatment comparisons, Control (C) vs LPS[mg/mL]	Temperature		
		20 °C	25 °C	30 °C
0	C vs 0.25	7.4	1.1	2.2
0	C vs 0.5	2.2	1.2	2.8
0	0.25 vs 0.5	6.0	1.9	1.0
2	C vs 0.25	9.2	3.1	3.6
2	C vs 0.5	1.3	13.4	2.4
2	0.25 vs 0.5	8.1	10.5	1.2
4	C vs 0.25	10.1	4.4	16.3
4	C vs 0.5	11.9	14.4	26.3
4	0.25 vs 0.5	3.5	10.0	7.5
8	C vs 0.25	26.1	5.6	30.6
8	C vs 0.5	27.0	14.1	34.0
8	0.25 vs 0.5	2.9	9.3	5.0

Figure 6.4. Heat map showing change in haemocyte profiles based on mean angular separation (for an explanation of this value see Section 6.3.1). Mixed haemocyte populations were treated *in vitro* with either vehicle (Control), 0.25 or 0.5 mg/mL of LPS for up to 8 hours at either 20, 25 or 30 °C. Values are mean angular separation between treatments within each time point and the colours represent intensity: **Low**, green (0 – 5); **Moderate**, yellow to orange (>5 - < 17); **High**, red (> 17).

6.4. Discussion

Based on our *in vitro* results, the relative proportion of HCs increased with LPS treatment. Because these results were conducted *in vitro*, we cannot expect the production of fresh HCs from precursor cells. Generally, haemocytes of crayfish do not transform in the circulatory system, new haemocytes are produced continuously and proportionally from a distinct organ called hematopoietic tissue (Hpt) (Jiravanichpaisal et al., 2006a; Soderhall and Thornqvist, 1997). In crayfish, haemocytes are released constantly (Chaga et al., 1995; Johansson et al., 2000), but during the course of infection, their number can decrease dramatically (Smith and Soderhall, 1983b; Persson et al., 1987; Johansson et al., 2000; Söderhäll et al., 2003). As a consequence, new haemocytes need to be compensatory and proportionally produced. In the *in vivo* setting, Söderhäll et al. (2003) and Soderhall et al. (2005) found that microbial polysaccharides such as LPS or laminarin, at a high concentration injected into the haemolymph, result in a rapid loss of circulating haemocytes and there is a crucial necessity for new haemocytes. Therefore, Hpt will be stimulated to produce haemocytes and mass released them into the haemolymph.

A more likely reason for the increase in the HCs population may be due to a change in cell size and granularity of SGCs and LGCs. The relative percentage of SGCs and LGCs concomitantly declined with an increase in HCs, after LPS treatment. This result may be linked with the functional immune response of *C. cainii* haemocytes. A similar result in the effect of LPS on haemocytes can also be found in other crustaceans. Cardenas et al. (2004) proposed that degranulation in SGCs and LGCs resulted in a reduction of cell size. This was most likely associated with an active immune reaction by the release of proPO. Lipopolysaccharide can not only trigger cell degranulation but can also result in cell death (Soderhall et al., 1986). As a result in this study, the addition of LPS may not only trigger SGCs or LGCs degranulation, thus making them become a HCs or in the least, appear like one, through flow cytometric analysis, but may also result is a relative reduction in SGCs/LGCs number due to cell death. Johansson and Soderhall (1985) found that LPS activation of these granules, which contain components of the proPO system, results in their release into hemolymph. After initiation by a complex route and foreign molecules proPO turns into its active form, PO, and acts in vital defence activities, such as producing opsonic factors and mediating cell to cell communication in crustaceans. Small and large granular cells have also overlapping functions, both participating in the pro-PO system and both are cytotoxic (Johansson et al.,

2000). Therefore, intact and degranulated granular cells would be identified by flow cytometry as non-granular haemocytes i.e. HCs.

The activation of *C. cainii* haemocytes by LPS *in vitro* displayed a dose-dependent manner. These results have also been found in other crustacean species. Lipopolysaccharide or β-1,3-glucans at higher concentrations (1 μg/mL) caused degranulation of *Limulus* amoebocytes *in vitro*, while individuals have different responses when low dose applied (0.1 μg/mL or less) (Armstrong and Rickles, 1982). Sung et al. (1998) suggested the following dosages for four elicitors in shrimp disease resistance management: 1 mg/ml for LPS and β1,3-1,6-glucan, and 10^7 cells/mL for *Vibrio* cells, disregarding concerns about treatment procedures and concentrations. While LPS is known to have immune-stimulating or potentiating properties on crustaceans (Soderhall and Smith, 1983; Soderhall and Hall, 1984; Smith et al., 1984; Takahashi et al., 2000), it also has negative influences that result in cell death and total haemocytes count (THC) reduction (Lorenzon et al., 1999; Lorenzon et al., 2002; Cardenas et al., 2004). Potent cytotoxic or microbicidal agents (e.g. reactive oxygen, reactive nitrogen and lysins) released by immune-stimulants, may have damaging effects on the host itself (Smith et al., 2003). Therefore, we were cognisant about using suitable LPS doses for potentiating various aspects on *C. cainii* immune system and haemocytes, while maintaining cell death as a minor factor.

The impact of temperature on the immuno-competence of *C. cainii* haemocytes has not previously been reported. Although there was a strong correlation between LPS treatment and time of incubation, we also found that temperature also had an influence on response time and magnitude of response. Results from this study have found that the activity of *C. cainii* haemocytes *in vitro* displayed a temperature-dependant variation which result in a high change in profile values (an increase in HCs and a reduction in SGCs and LGCs) as the temperature increased. However, the contrast between changes in haemocyte activation and population profiles at 25 ^{o}C compared to 20 and 30 ^{o}C is suggestive that *C. cainii* haemocyte activity is temperature-specific. Le Moullac et al. (1998) and Le Moullac and Haffner (2000) found that various environmental factors, such as fluctuations in temperature and dissolved oxygen, can influence the crustacean immune system on a daily basis. Water temperature is probably a major and critical environmental factor because it affects metabolism, oxygen consumption, growth, moulting and survival while it has a direct effect on other environmental parameters such as salinity and dissolved oxygen (Chen et al., 1995; Hennig and Andreatta, 1998). Temperature can also affect clotting times, THC and levels of

plasma protein in the hermit crab, *Uca pugilator* (Dean and Vernberg, 1966). In fact, in Chapter 3 we demonstrate a change in THC in *C. cainii* after only 2 weeks. Changes in temperature at different seasons also affected the antibacterial activity by *C. maenas* haemocytes (Chisholm and Smith, 1994). Owens and O'Neill (1997) found significant changes in the differential haemocyte proportions, although total haemocyte counts were comparable between summer and winter. Winter stocks had increased quantities of non-granular haemocytes accompanied by substantial reductions in granular haemocytes. Similar total haemocyte counts between seasons indicated that there was no haemocytopenia, but there were shifts in the differential counts.

This will be the first FCM study on the defence function of *C. cainii* haemocytes reported. Flow cytometry is a technology that enables the acquisition of quantitatively data in a relatively fast manner, and has proved to be effective for analysis of haemocytes in the *C. cainii*. The FCM approach employed in this study has some advantages compared to other techniques, as it has the ability to evaluate individual cell types, to sort and to harvest a particular kind of cell within a short period of time. The detailed information derived from individual cell analysis allowed the further characterization of the morphology and functions of *C. cainii* haemocytes. This technology could help in presenting *C. cainii* haemocyte characteristics such as size, complexity, viability and phagocytosis, and is used for such a purpose in Chapters 3 and 5.

In conclusion, there was a high correlation between *C. cainii* haemocyte stimulation be LPS and cell morphology, physiology and immunological response. This was despite the fact that it was an *in vitro* study and incubations were for only a short period. Haemocytes were activated, by LPS, in a dose-dependent manner and demonstrated temperature-specific activities, and the major haemocyte profile change was one of increased HCs with a corresponding decrease in SGCs and LGCs. This response was most likely due to due to degranulation of the latter two cell types, 'converting' them into HCs, albeit as identified by FCM based on cell size and granularity.

CHAPTER 7

STUDIES ON ACTIVITY OF NITRIC OXIDE BY HAEMOCYTES OF MARRON (*Cherax cainii*) CHALLENGED WITH LIVE OR HEAT-KILLED *Vibrio mimicus* AT DIFFERENT TEMPERATURE

7.1. Introduction

A large number of infectious diseases affect crustacean aquacultural species impacting on economic sustainability and efforts to characterise and understand these immunological reactions has recently become a priority. Unlike vertebrates, a true system of adaptive immunity is absent in crustacean (Hoffmann et al., 1999). However, Bachere (2000) explain that due to their life in an aquatic environment, which have abundant microorganisms, effective detection and elimination systems for harmful microorganisms have been well established by crustacean using their innate immune responses: haemocyte activity such as release of phenoloxidase, phagocytosis, synthesis of antimicrobial peptides and many others.

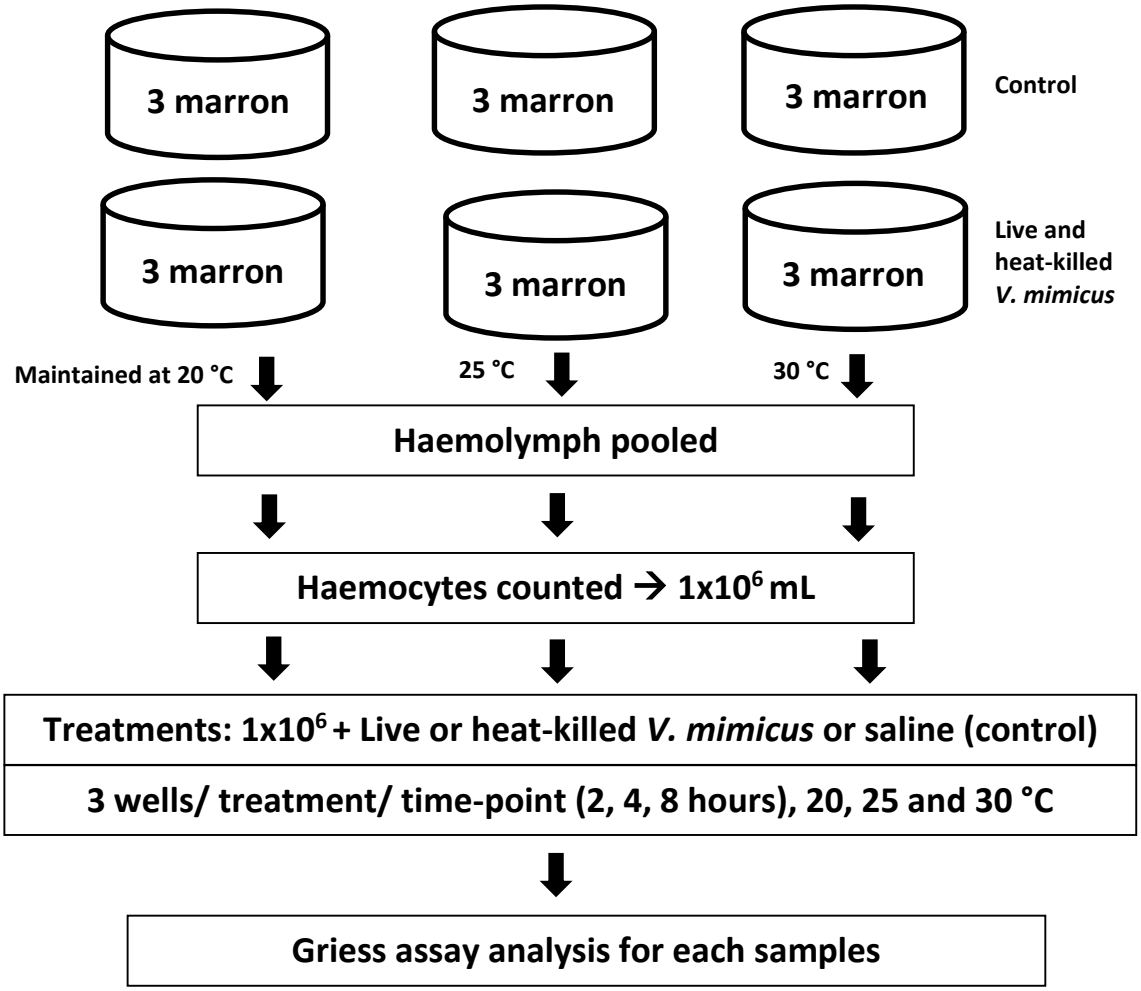
A critical element of the innate immune system is nitric oxide (NO). Nitric oxide is considered an essential signalling molecule involved in various physiological activities, such as signal transmission in the nervous system, regulation of cardiovascular activity, immune defence, and pathogenesis of several diseases (Colasanti et al., 2010). It has been a challenge to detect the content of NO directly in biological samples because it has a short half-life and low concentration. Nitric oxide is one of the few known gaseous signalling molecules. It is highly reactive and the signal lasts only seconds and diffuses across membranes freely (Moncada et al., 1991). Several methods for NO level detection have been applied indirectly. The Griess colorimetric reaction is the most commonly used assay for NO detection, that is quantified through stable reaction products, nitrites (NO_2^-) and nitrates (NO_3^-), from the result of NO degradation (Raman et al., 2008; Rodríguez-Ramos et al., 2008). Recently, the production of NO in several species of invertebrate in response to cytokines, bacterial LPS and parasites has been reported (Wright et al., 2006; Jiang et al., 2006; Yeh et al., 2006; Krishnan et al., 2006).

In this study, we have examined whether Marron *C. cainii* haemocytes have the capability to produce NO after live and heat-killed *Vibrio mimicus* exposure at different temperature over time. We used the Griess assay. For the first time, we have characterised the NO production of individual haemocyte cell types for *C. cainii*, following separation by fluorescence activated cell-sorting (FACS) device.

7.2. Experimental Outline

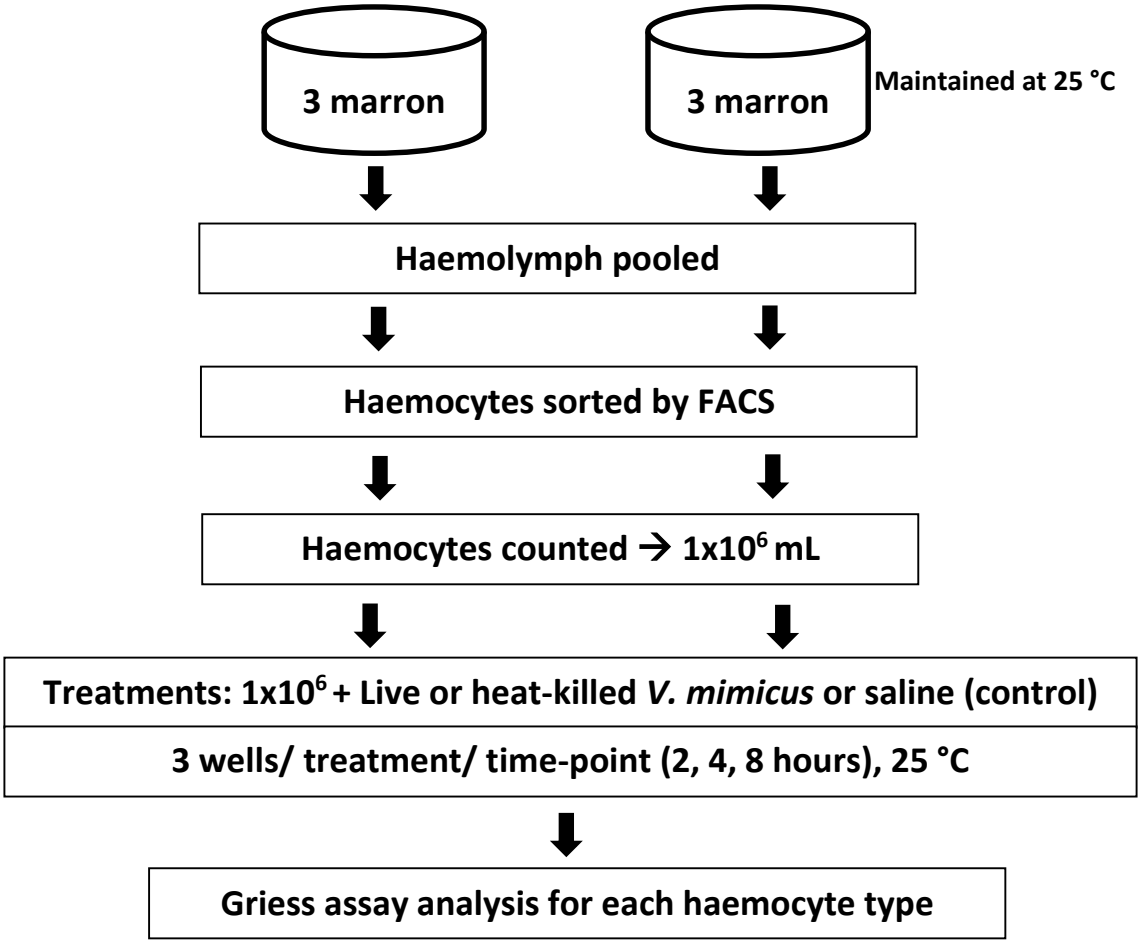
7.2.1. Experimental outline of nitric oxide experiment at different temperatures

The NO experiment at different temperature was conducted as outlined below and pertains to results sections 7.3.1, 7.3.2 and 7.3.3. Six sets of three marron were maintained at 20, 25 and 30 °C for two weeks prior to starting the experiment. One set from each temperature treatment was used for the control experiments, and the other three set of marron was used for the live or heat-killed bacteria experiments. Following the 2 week temperature acclimatization, haemolymph from each set of marron was pooled and haemocytes counted. Experiments were conducted at 20, 25 and 30 °C *in vitro* and set up in triplicate wells containing one million haemocytes with live or heat-killed *V. mimicus* or with saline for controls. Separate sets of wells were set up for time-points 2, 4 and 8 hours. Note, for each time-point, a saline control was also performed which enabled baseline corrections. The Griess assay was performed on each samples.



7.2.2. Experimental outline of nitric oxide with different *C. cainii* haemocytes experiments

The NO experiments on separate *C. cainii* haemocyte types were conducted as outlined below for results section 7.3.4 and 7.3.5. Two sets of three marron were maintained at 25 °C for two weeks prior to being used. Haemolymph from one set of marron was pooled and used for the control experiments, and the other set was used for the live or heat-killed bacteria experiments. Haemocytes were then sorted by FACS. After sorting, each haemocyte type was counted and used in the experiments. Experiments were conducted at 25 °C and set up in triplicate wells containing one million haemocytes with live or heat-killed *V. mimicus* or with saline for controls. Separate sets of well were set up for time-points 2, 4 and 8 hours. Note, for each time-point, a saline control was also performed which enabled baseline corrections. The Griess assay was performed separately on each of the three haemocyte cell types: HCs, SGCs and LGCs.



7.2.3. List of Experimental procedures – refer to Chapter 2 for further detail

- 2.1 Acclimatization system
- 2.2 Animals
- 2.3 Preparation of haemocytes
- 2.4 Preparation of the *Vibrio mimicus* stock solution
- 2.7 Griess reaction
- 2.12 Haemocytes responses to activators
- 2.13 Separation of different haemocytes types using fluorescence-activated cell sorter (FACS)

7.3. Results

In this study live or heat-killed *V. mimicus* was employed to illicit an inflammatory stimulus in *C. cainii* haemocytes to induce NO production *in vitro*.

7.3.1. Nitric oxide production by *C. cainii* haemocytes to live *V. mimicus* at different temperatures

In *C. cainii* haemolymph, live *V. mimicus*-stimulated NO production was significantly higher at 20 and 25 ^{o}C compared with 30 ^{o}C ($p<0.0001$; Figure 7.1; Appendix 5). However, with respect to the timing of treatment, incubation with live *V. mimicus* did not produce any variation in the concentration of NO implying that response is over by 2 hours. In addition, treatment of *C. cainii* haemocytes with live *V. mimicus* caused a significant increase in nitrite production with respect to each control group.

7.3.2. Nitric oxide production by *C. cainii* haemocytes to heat-killed *V. mimicus* at different temperature

The NO levels in *C. cainii* haemocytes after exposure by heat-killed *V. mimicus* at 20 and 25 ^{o}C was significantly higher than at 30 ^{o}C ($p<0.0001$; Figure 7.2; Appendix 5). Incubation with heat-killed *V. mimicus* did not result in any variation in the concentration of NO, with regard to the time points noted except for heat-killed at 30 ^{o}C which showed an increase at 8 hours ($p<0.01$). For the control groups, NO production was relatively stable over the experiments however, their mean values at lower temperature were higher than at 30 ^{o}C ($p<0.0001$). Nitric oxide production in the control group was lower than heat-killed *V. mimicus*-stimulated NO production.

7.3.3. Nitric oxide production by *C. cainii* haemocytes: live and heat-killed *V. mimicus* compared

Both live and heat-killed *V. mimicus* showed similar patterns in NO stimulation, with heat-killed *V. mimicus* showing slightly higher levels than live *V. mimicus* for the 20 ^{o}C and 25 ^{o}C treatments (Figure 7.3). Both 20 and 25 ^{o}C incubation temperatures demonstrated significantly higher NO concentrations compare to 30 ^{o}C ($p<0.0001$). Even though NO concentration was higher for 20 ^{o}C it was not significantly different from 25 ^{o}C. With respect to time-points, there was little difference in NO concentration for live and head-killed *V. mimicus* except for the heat-killed, 30 ^{o}C treatment (see appendix 5 for specifics).

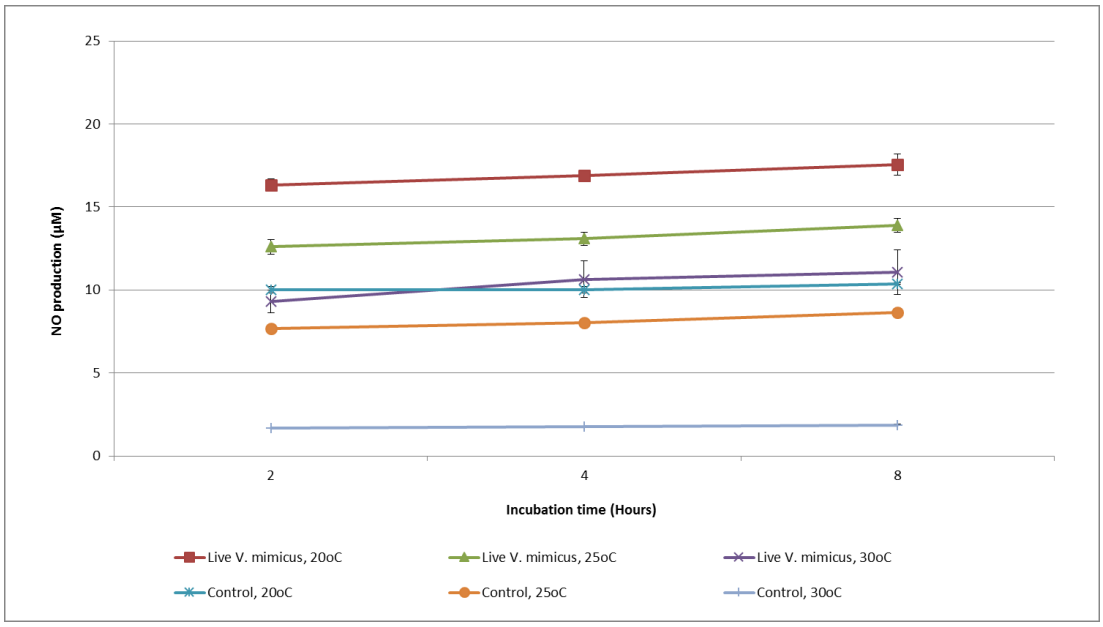


Figure 7.1. Effect of live *Vibrio mimicus* on *Cherax cainii* haemolymph cells nitric oxide production after 2, 4 and 8 hours at different temperature. *C. caini* haemocytes treated with live *V. mimicus* (~10^4 CFU/ml) and in control 75 mM NaCl substituted live *V. mimicus*. Data are shown as mean ± standard deviation.

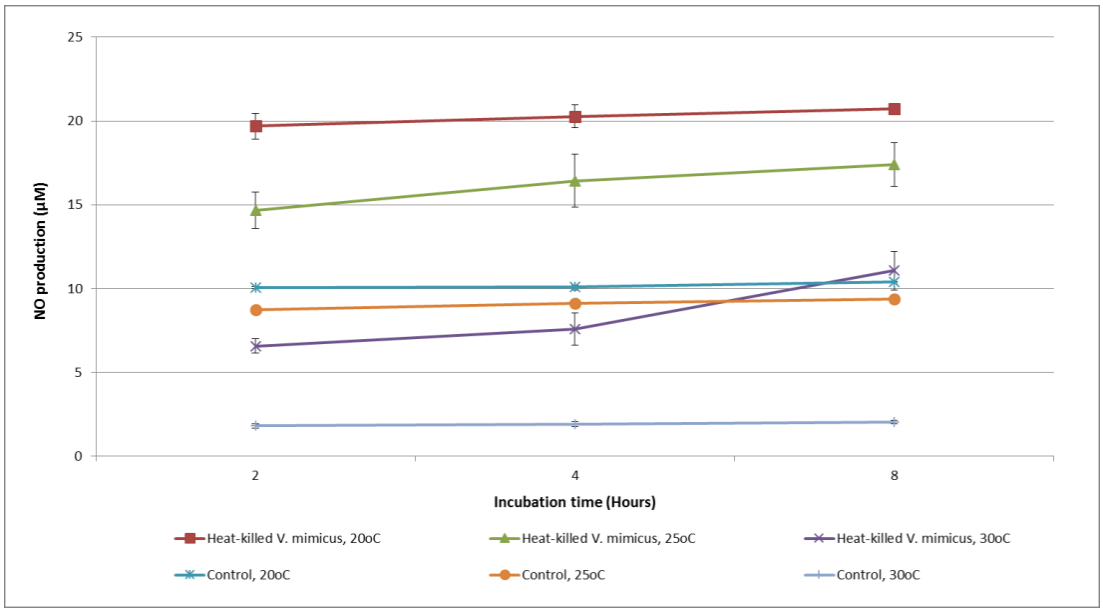


Figure 7.2. Effect of heat-killed *Vibrio mimicus* on *Cherax cainii* haemolymph cells nitric oxide production after 2, 4 and 8 hours at different temperature. *C. caini* haemocytes treated with heat-killed *V. mimicus* (~10^4 CFU/ml) and in control 75 mM NaCl substituted live *V. mimicus*. Data are shown as mean ± standard deviation.

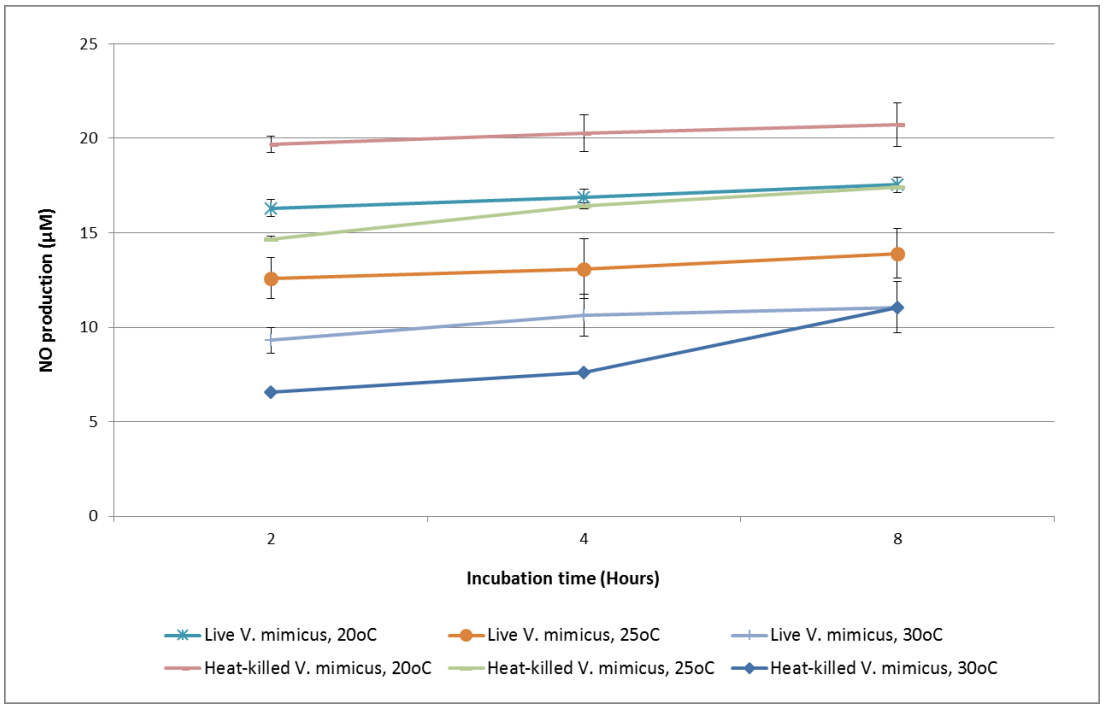


Figure 7.3. Effect of live or heat-killed *Vibrio mimicus* on *Cherax cainii* haemolymph cells nitric oxide production after 2, 4 and 8 hours at different temperature. Data are shown as mean ± standard deviation.

7.3.4. Nitric oxide production by each *C. cainii* haemocytes type after stimulation by *V. mimicus* at 25 °C

Live *V. mimicus* significantly induced the NO production in HCs, SGCs and LGCs after 8 hours at 25 °C ($p<0.0001$; Figure 7.4; see Appendix 6 for details). At this time-point HCs displayed the highest NO value but it was not significantly different from the other haemocyte subpopulations except when compared to the control (an average of all 3 cell type control).

The production of NO by each haemocyte cell type was significantly induced by heat-killed *V. mimicus* compared to the control treatment at all three time-points ($p<0.0001$; Figure 7.5). However, there was no significant difference between each cell type over the three time-points.

7.3.5. Nitric oxide production by each *C. cainii* haemocyte type: live and heat-killed *V. mimicus* compared

Over the first 4 hours of treatment heat-killed *V. mimicus* caused higher levels of NO production by all haemocyte cell types ($p<0.0001$) (Figure 7.6; see Appendix 6 for details). However, live *V. mimicus* treatment at 8 hours resulted in an increase in NO production to levels caused by heat-killed bacteria.

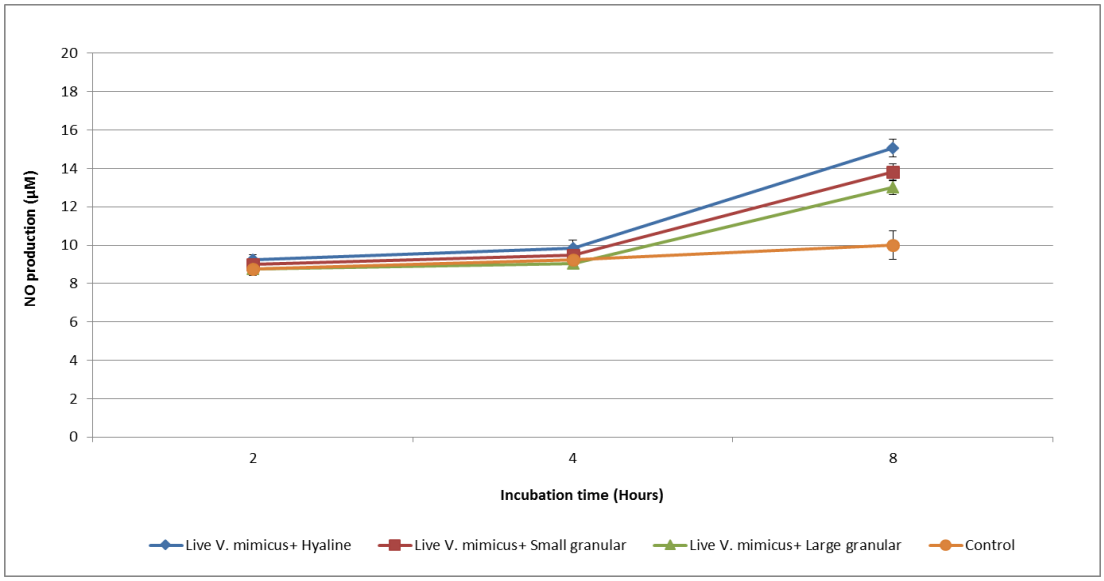


Figure 7.4. NO production in each *Cherax cainii* haemocyte type after 2, 4 and 8 hours *in vitro* exposure to live *Vibrio mimicus* at 25 °C. *C. caini* haemocytes treated with live *V. mimicus* (~10^4 CFU/ml) and in control (an average of three cell types control) 75 mM NaCl substituted live *V. mimicus*. Data are shown as mean ± standard deviation.

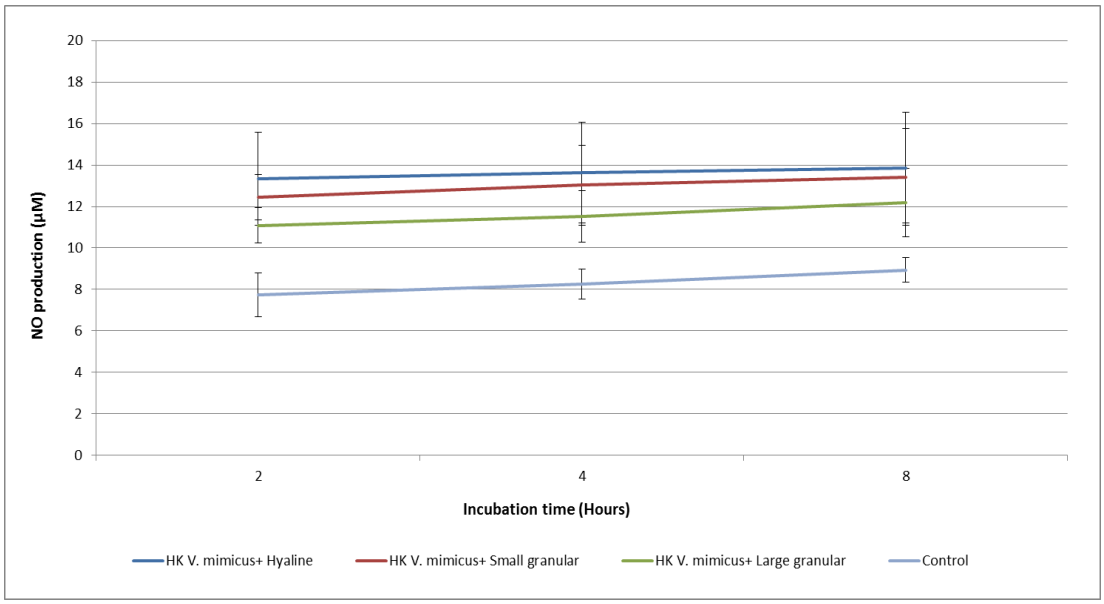


Figure 7.5. NO production in each *Cherax cainii* haemocyte type after 2, 4 and 8 hours *in vitro* exposure to heat-killed *Vibrio mimicus* at 25 °C. *C. caini* haemocytes treated with heat-killed *V. mimicus* (~10^4 CFU/ml) and in control (an average of three cell types control) 75 mM NaCl substituted heat-killed *V. mimicus*. Data are shown as mean ± standard deviation.

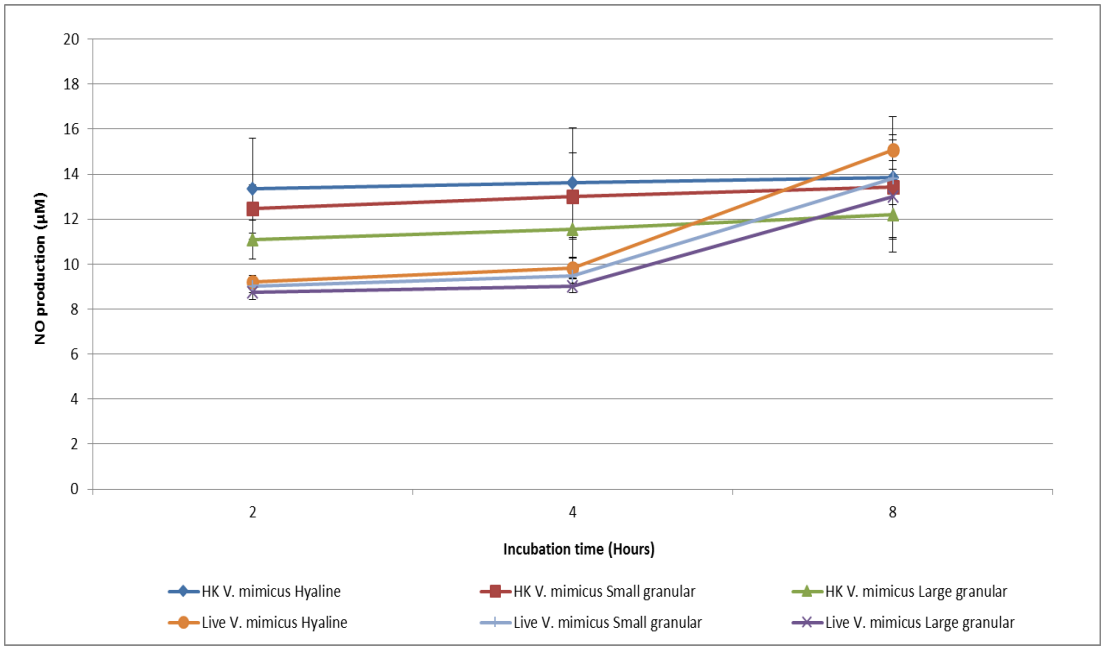


Figure 7.6. NO production in each *Cherax cainii* haemocyte type after 2, 4 and 8 hours *in vitro* exposure to live or heat-killed *Vibrio mimicus* at 25 °C. Data are shown as mean ± standard deviation.

7.4. Discussion

Firstly, we have shown that *C. cainii* haemocytes, as a mixed population of cells produced NO in response to live or heat-killed *V. mimicus,* using a simple colorimetric method commonly used to measure NO production in vertebrate cells, the Griess reaction. Secondly, we separated the haemocytes into the three individual and distinct populations and demonstrated enhanced NO production by *C. cainii* haemocytes compared to control treatment. The concentration of bacteria is critical to illicit a response in haemocytes and the concentration we used, $\sim 10^4$ cfu/ml was adequate to induce NO production. Unexpectedly, heat-killed bacterial resulted in generally higher activation of haemocyte NO production; we had expected a deterioration of NO induction by dead bacterial. Munoz et al. (2000), explained that a loss of in the inductive potential of heat-killed bacteria is most likely caused by heat-induced modifications of membrane components that are recognized by the haemocytes. Even though our results and others, on different invertebrate species, demonstrate the presence NO induction in haemocytes (Conte and Ottaviani, 1995; Franchini et al., 1995; Nakayama and Maruyama, 1998; Arumugam et al., 2000; Gourdon et al., 2001) little is known about the role bacteria play in the induction of this immune system function in invertebrates. Later studies showed that bacterial growth can be inhibited by crustacean haemolymph (Yeh et al., 2006; Destoumieux et al., 2000; Munoz et al., 2000). Yeh et al. (2006) described that bacterial adhesion to haemocytes and bactericidal activity of haemocytes can be promoted and increased by haemocyte-derived NO. Related results were also found in the response of Chinese shrimp haemocytes after injection with *V. anguillarum* (Zhao et al., 2007). The generation of NO in giant freshwater prawn (*Macrobrachium rosenbergii*) haemocytes during phagocytosis has been reported, and there was an enhancement in NO generation (75% increases) during agglutinin-mediated phagocytosis (Raman et al., 2008). The movement of haemocytes toward whole bacteria, bacterial extracts, and bacterial exudates has been demonstrated *in vitro* with haemocytes from *C. virginica*, *V. malleatus*, *M. edulis* and *M. Mercenaria* (Cheng and Rudo, 1976; Cheng and Howland, 1979; Schmid, 1975; Schneeweiss and Renwrantz, 1993; Fawcett and Tripp, 1994). These studies have implicated LPS, low-molecular-weight proteins, and polypeptides as active chemo-attractants from different live bacterial taxa while dead bacterial cells did not. Additionally, when three methods of killing bacteria were used, it was found that killing by heating or by exposure to formalin followed by inoculation of the dead bacteria in the medium on which they had grown, resulted in partial inhibition of their chemo-activating properties, while killing by exposure to ozone failed to inhibit chemo-activation by the dead

bacteria plus growth medium. These observations suggest that heat and formaldehyde partially inhibit haemocyte chemo-activation by denaturation of activation factors in the medium while ozone treatment did not. This is consistent with those obtained *in vitro* for *M. mercenaria* haemocytes (Fawcett and Tripp, 1994). These authors concluded that the chemoattractant secreted by *E. coli* is a dialyzable substance which is inactivated by proteolytic digestion. However they found the chemo-attractants to be heat stabile. Same results have also been reported in the gastropod *V. malleatus*, where heat-killed *Serratia marcescens* induced haemocyte activation *in vitro* (Schmid, 1975). Differing results have been reported by Howland and Cheng (1982) which they isolated two low-molecular-weight proteins from cell envelopes of *E. coli* which they suggested where chemo-attractants for haemocytes of the eastern oyster. They also found that haemocytes were not attracted to dead bacteria and that living *E. coli* were more powerful attractant than either of the isolated cell envelope proteins. This suggests that the enhanced chemo-attraction attributed to live bacteria might be due to soluble secreted materials in the growth medium. Schneeweiss and Renwrantz (1993), isolated an LPS from *E. coli* which induced *in vivo* migration of haemocytes from *M. edulis*. It is doubtful that LPS is responsible for the chemo-activation of oyster haemocytes since the bacteria is immobilised in agar chores. However Jiang et al. (2006) observed that haemocytes of shrimp *Marsupenaeus japonicus* were able to produce NO after stimulation with LPS or with White Spot Syndrome Virus (WSSV). Similar result was also detected in spiny lobster *Panulirus argus* haemocytes which are able to produce NO, after induction by a certain LPS concentration (Rodriguez-Ramos et al., 2010). In addition, Gopalakrishnan et al. (2011) noticed an increase level of NO, up to 3- and 4-fold, in the haemocytes collected from crab (*Scylla paramamosain*) injected with LPS, and this increase was significant during the exposure period. Some recent studies have reported the effect of LPS on NO content and NO synthase when lobster (*P. argus*) and white shrimp (*L. vannamei*) hemocytes were exposed to *E. coli* LPS *in vitro* (Rodriguez-Ramos et al., 2010; Yao et al., 2010). Another *in vivo* study showed that NO concentration in spiny lobster *Panulirus argus* haemolymph increased after 24 hours of the injection with *E. coli* LPS (Rodríguez-Ramos et al., 2008). Similarly, an *in vitro* study on tiger shrimp (*P. monodon*) haemocytes also showed the induction of *E. coli* LPS on NO content in haemocytes (Jian et al., 2013). While the reasons for these differences are not known, they might be attributable to biological differences in divergent taxa.

To extend our understanding about different haemocytes roles in NO production, we analysed NO production in the different *C. cainii* haemocyte types after treatment with live

or heat-killed *V. mimicus*. We found that all the three type of haemocytes (HCs, SGCs and LGCs) produce NO, and to comparable levels. This data provides further evidence that *C. cainii* haemocytes play an important function in killing pathogens. Nitric oxide production during phagocytosis has been shown in haemocyte types (hyaline, semigranular and granular cells) of several crustaceans Soderhall et al. (1985) have found that the NO production capacity in crayfish *Astacus astacus* in granular and semi granular cells may contribute to their cytotoxic function. These two types of *A. astacus* haemocyte have been noted to exhibit cytotoxic activity towards tumour and non-tumour cell line of mammalls. Furthermore, Johansson et al. (2000) demonstrated that each type of freshwater crayfish, *Astacus astacus* haemocytes perform different functions in crustaceans immune defence. Granular and SGCs were recognized as the main phagocytic cell types of this freshwater crayfish. Xian et al. (2013) noticed that without stimulation more NO was produced in granular cells than semigranular and HCs in black tiger shrimp (*P. monodon*) haemocytes. However, zymosan A particles could stimulated the generation of NO in semigranular (equivalent to SGC) and granular cells (equivalent to LGCs), but could not in HCs. These facts indicate that the cells that play a major role in destroying pathogens are both semigranular and granular. A preceding study using white shrimp (*L. vannamei*) haemocytes also demonstrating related findings (Xian et al., 2009). This result implies that the capacity of NO production in semigranular and granular cells in *P. monodon* and *L. vannamei* have relation to their cytotoxic role. However, our results reveal that HCs also produce NO to live and heat-killed *V. mimicus*. Bell and Smith (1993) and Thornqvist et al. (1994) found that the major phagocytic cells were the HCs in crab and crayfish haemocytes, after separation by Percoll™ gradient. However, granular haemocytes, including SGCs and LGCs, in penaeid shrimp were phagocytic and contained lysosome enzymes and possessed pro PO activity (Martin et al., 1993; Gargioni and Barracco, 1998). These findings give new understandings into the crustacean haemocyte type identification and their respective function. Therefore, there is no model or classification scheme that is applicable to all crustacean haemocytes. Furthermore, the different experimental designs can also add to variations in results. Additional studies are needed to further understand the NO activation mechanism and the connection between NO production and phagocytosis in *C. cainii* haemocytes.

Environmental temperature directly impacts aquatic organism's survival, growth, physiology and immunity. These organisms are also indirectly affect by temperature through other abiotic factors (Ponce-Palafox et al., 1997; Wyban et al., 1995). Lightner and Redman (1998), identified three key elements which are pathogens, environmental factors, and organic

immunity, which have bordering associations with emerging diseases in aquaculture systems. Based on the results represented in this study, temperatures seem likely to influence the haemocytes defence capability. Our data revealed a lower level of NO production when exposed to high temperature (30 ^{o}C). These results must be correlated to the cells physiological condition exposed in different temperature. Water temperature changes also result in stress response activation in crustaceans and trigger changes in haemocytes, decreasing total hemocytic proPO (Jia et al., 2014). Ultimately, increased stress due to temperature changes can causes organisms to become more susceptible to pathogens, resulting in disease (Vargas-Albores et al., 1998; Gomez-Jimenez et al., 2000; Cheng and Chen, 2001). Cao et al. (2007) found that haemocytes *of M. galloprovincialis* are half as responsive to LPS antigens in summer compared to winter. This could explain why there is an increased frequency of disease in molluscs during summer (Berthelin et al., 2000). Furthermore, a study by Santarem et al. (1994) reveal that the lowest number of *M. galloprovincialis* circulating haemocytes was in summer, decreasing the immune response already depressed during this season.

In conclusion, the present study shows that live or heat-killed *V. mimicus* can modulate the immune parameters of NO production in *C. cainii* haemocytes and the response was time dependent and temperature dependent. The three types of haemocyte were revealed to possess different competence to NO production. The overall results indicated that bacterial stimulation and their reactions are involved in the *C. cainii* immune system immunomodulation.

CHAPTER 8

THE EFFECTS OF LIVE OR HEAT-KILLED *Vibrio mimicus* AS INFLAMMATORY STIMULUS ON MARRON (*Cherax cainii*) HAEMOCYTES AT DIFFERENT TEMPERATURE *IN-VIVO* USING FLOW CYTOMETRIC ANALYSIS

8.1. Introduction

The immune defence in crustacean is primarily carried out by circulating haemocytes, components of the immune system. Their immune system is also active in regulating other physiological functions including hardening the exoskeleton, repairing wounds, digesting carbohydrates, transporting and storing protein and amino acid, coagulating haemolymph and the confining of offensive organisms by clot formation, phagocytosis and encapsulation (Ratcliffe, 1985; Martin et al., 1993). In decapod crustaceans, species have different total haemocyte count (THC) and differential haemocyte count (DHC) which are responsible for cellular immune defence (Hose et al., 1990). In addition, the values of THC and DHC are highly varied among individual animals for most crustacean species (Johansson et al., 2000; Sung and Sun, 2002).

Total cell counts and DHC vary among crustacean species and are known to be affected by a variety of stressors, including bacterial infections which can result in a dramatic decline in circulating haemocytes (Lorenzon et al., 2001; Lorenzon et al., 2002). Smith and Ratcliffe (1980), observed that when the shore crab, *Carcinus maenas* (L.), was injected with bacteria *in vivo* haemocytopenia ensued, with bacteria being rapidly removed from the circulation. However, THC returned to normal after 24 hours due to haemopoiesis. Similar outcomes have been reported after injection of a variety of substances in lobster *Homarus americanus* and in the crayfish *Parachaeraps bicarinatus* (Stewart and Cornick, 1972; McKay et al., 1969). Persson et al. (1987) also reported similar findings in crayfish *Pacifastacus leniusculus* (Dana) infected with the fungus *Aphanomyces astaci*.

The Gram-negative bacterium, *V. mimicus*, was use as a model pathogen for the present study. *Vibrio mimicus* was identified as the causative agent in rapid and fatal systemic infections of the cultured red swamp crawfish *Procambarus clarkii* in The United States (Thune et al., 1991). Mass deaths in yabby *Cherax albidus*, during postharvest, was attributed to *V. mimicus* epidemics at a commercial Australian farm (Morrissy and Fellows, 1993). Eaves and Ketterer (1994), have isolated this bacteria from systemic infections of cultured red claw crayfish *Cherax quadricarinatus* that caused stock mortalities. Various reports have shown a variety of measurable changes to the immune system of *C. cainii* after inoculation with live *V. mimicus* (Sang et al., 2009; Sang and Fotedar, 2010; Nugroho and Fotedar, 2013; Ambas et al., 2013). Conversely, *V. mimicus* has also been isolated from fresh and brackish water prawns that were evidently healthy in Bangladesh and India (Chowdhury et al., 1986; Nair et al., 1991).

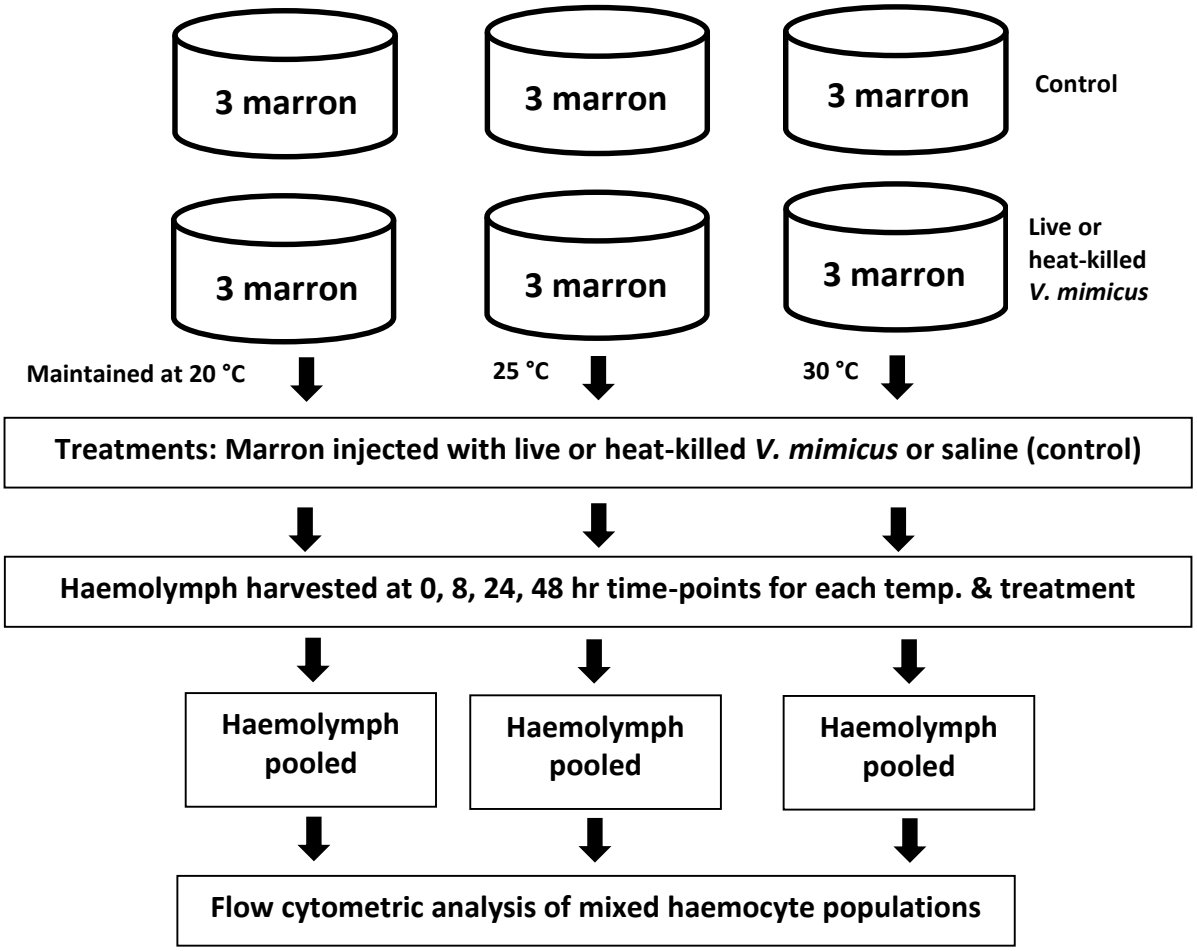
Reports have been published on studies of crustacean haemocytes using FCM. These include cell classification and proliferation in *Penaeus japonicas* and other shrimp species (Sequeira et al., 1995; Sequeira et al., 1996; Lee et al., 2001; Yip and Wong, 2002), cell sorting in *P. penicillatus*, *P. monodon*, and *P. japonicus* (Yip and Wong, 2002), and changes to cell size and granularity after stimulation with zymosan or LPS in *Litopenaeus vannamei*, shrimp species and *Palaemon elegans* (Xian et al., 2009; Lorenzon et al., 1999; Lorenzon et al., 2002). Flow cytometry was also employed to detect changes in cell proportions of the three commonly identified haemocyte cell types in response to disease agents. These include the behaviour of haemocyte sub-populations in the Caribbean spiny lobster *Panulirus argus* in response to *P. argus* Virus 1 (Li and Shields, 2007), and classification and detection of haemocyte changes induced by *Spiroplasma eriocheiris* on *Procambarus clarkii* haemocyte (Ding et al., 2012).

Animals are stressed when a factor, called a stressor, causes their internal physiology to deviate from homeostasis. An increased understanding of the mechanism of stress and physiological responses in marron, as a result of bacterial infection, may lead to enhanced farming practices. The well-being and survival of animals should result in higher yields and quality of product. The first objective of this study was to investigate the cellular responses of *C. cainii* haemocytes following *in vivo* injection of live and heat-killed *V. mimicus*. Metabolic and cellular processes are also affected by temperature which is an important factor in crayfish. Therefore, the second objective was to investigate the effects of bacterial infection with respect to different temperatures. Flow cytometry was used to evaluate changes.

8.2. Experimental Outline

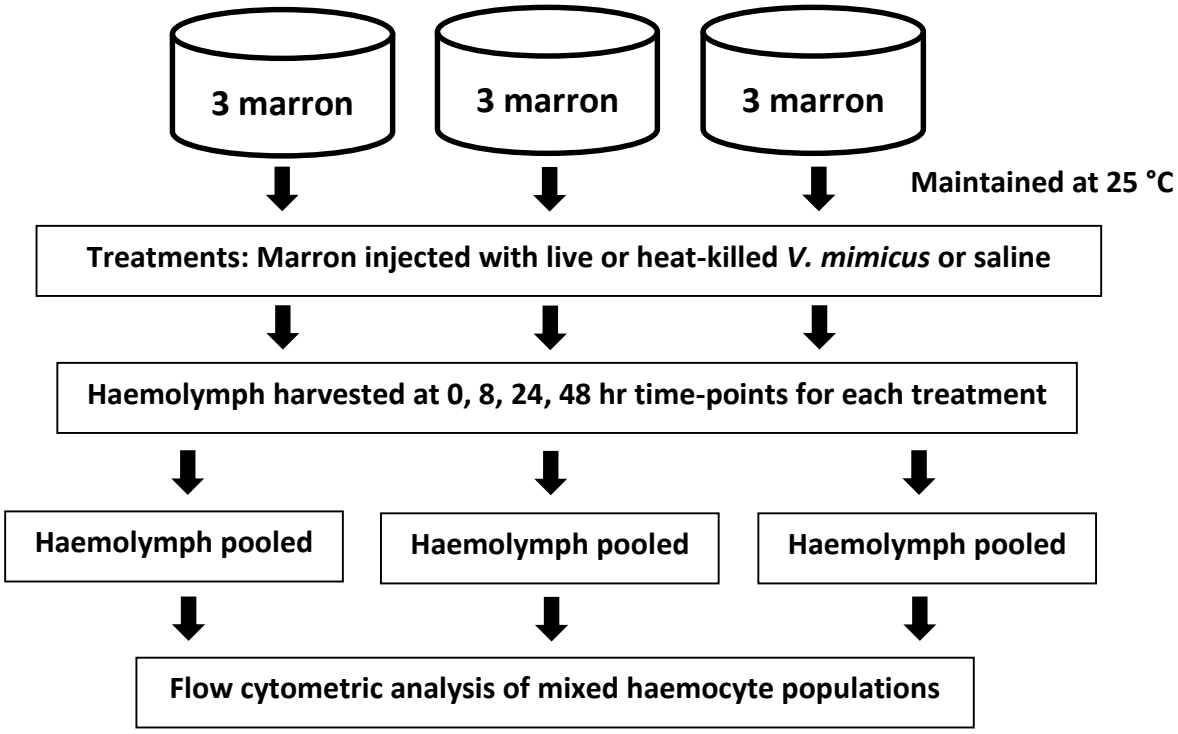
8.2.1. Experimental outline of *in vivo* effects of live and heat-killed *V. mimicus* at different temperatures

The live and heat-killed *V. mimicus* effect on haemocytes *in vivo* experiments were conducted as outlined below. Six sets of three marron were maintained at 20, 25 and 30 °C for two weeks prior to being used. Marron were injected with live or heat-killed bacteria or saline. Following in vivo incubations at the respective temperatures, haemolymph was harvested at 0 (base-line), 8, 24 and 48 hours. Haemolymph from each set of 3 marron was pooled. Three sets were used for the control experiments, and the other three sets were used for the live and heat-killed bacteria experiments. Following washes, the mixed haemocyte populations were analysed with FCM to identify and quantitate all three haemocyte cell types (HCs, SGCs and LGCs).



8.2.2. Experimental outline of *in vivo* phagocytosis experiments

The phagocytosis experiment was conducted as outlined below. Two sets of three marron were maintained at 25 °C for two weeks prior to being used. Marron were injected with live or heat-killed *V. mimicus* (that had been fluorescently labelled with FITC) or saline. Following *in vivo* incubations haemolymph was harvested at 0 (base-line), 8, 24 and 48 hours. Haemolymph from each set of 3 marron was pooled. One set of marron was used for the control experiment, and the other sets were used for the live and heat-killed bacteria experiments. Following washes, the mixed haemocyte populations were analysed with FCM to identify and quantitate phagocytic activity of all three haemocyte cell types (HCs, SGCs and LGCs).



8.2.3. List of Experimental procedures – refer to Chapter 2 for further detail

- 2.1 Acclimatization system
- 2.2 Animals
- 2.3 Preparation of haemocytes
- 2.4 Preparation of the *Vibrio mimicus* stock solution
- 2.6 Flow cytometry
- 2.14 Challenge test *in vivo*

8.3. Results

8.3.1. Data analysis

Mixed haemocyte populations were treated *in vivo* with live or heat-killed *V. mimicus* or without (saline, Controls). Marron were kept at 20, 25, or 30 ^{o}C for 2 weeks. Following injection of either live, heat-killed or saline, haemolymph was collected at 0, 8, 24 and 48 hours following treatment. Flow cytometry was employed to quantitate the relative percentages of the three different haemocyte types from a mixed population (HCs, SGCs and LGCs). The data is represented in Figures 8.1, 8.2 and 8.3 as horizontal percentage stack graphs showing the relative proportion of the three haemocyte populations within treatment groups. In order to determine changes in the population profiles, a fixed number of haemocytes was counted, according to their morphology. A change in the relative proportion of one haemocyte type has a concomitant change in one or both of the other haemocyte proportions.

Note - The same analysis was applied to data in this chapter as in Chapter 6. Figure 8.4 is a heat map showing the overall change in haemocyte profiles based on mean angular separation. For p-values of this data see Appendix Figure 2.

8.3.2. Changes in relative haemocyte populations due to treatment with live and heat-killed *V. mimicus* at different temperatures over time

Treatments with live *V. mimicus,* at the lowest temperature 20 ^{o}C (Figure 8.1), resulted the greatest change, although only at low to moderate levels for overall haemocyte profiles (see Figure 8.4, heat map). There were only minor changes between heat-killed treatments and controls. Most of the change for the live bacteria treatment was seen in the HCs (decrease) and LGCs (increase) early on, with the opposite response for HC at 24 and 48 hours and primarily a reduction in SGCs.

The greatest change in haemocyte profiles were seen at 25 ^{o}C. This change was mostly seen for the live *V. mimicus* treatments, resulting in moderate high levels of changes (Figures 8.2 and 8.4). In this treatment, HCs increased in proportion while both SGCs and LGCs decreased and this was consistent for 8 to 48 hours. Again, the heat-killed *V. mimicus* resulted in a change compared to control.

The treatments at 30 °C incubations was the least changed, with only a low to moderate change in profile at 8 hours for the heat-killed treatment. However, they were low for both *V. mimicus* treatment compared to control, with only a low to moderate change between them (Figures 8.3 and 8.4). From Figure 8.4, the overall patterns of change due to *V. mimicus* treatments compared to controls and each other can be seen clearly: there was similarity between the 20 and 30 °C incubations with both *V. mimicus* treatments being low to moderate compared to control, at 8 and 24 hours, but with little difference between *V. mimicus* treatments. This contrasted with treatments at 25 °C, where most of the change was only seen, but only for the live *V. mimicus* treatment.

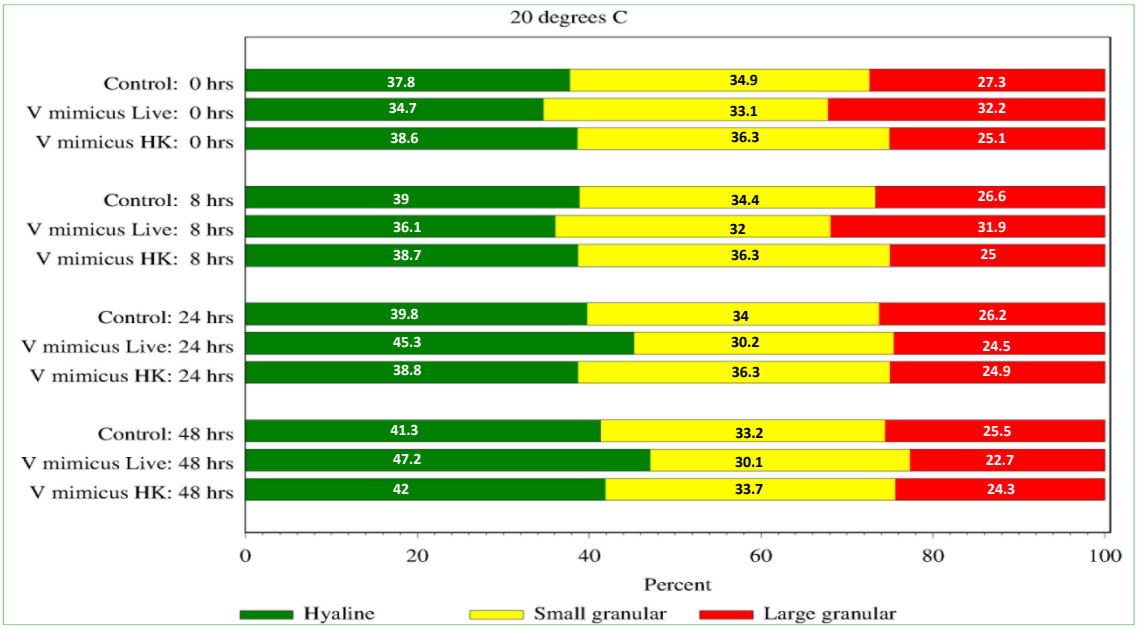


Figure 8.1. The relative cell proportion of *Cherax cainii* haemocytes after injected with live or heat-killed *Vibrio mimicus* at 20 °C. Relative percentages of each cell type in *C. cainii* at increasing time after injected with live or heat-killed *V. mimicus* or in control haemocytes injected with saline *in vivo*. For each time point 10,000 cells were analysed. For an explanation of data format see Section 8.3.1.

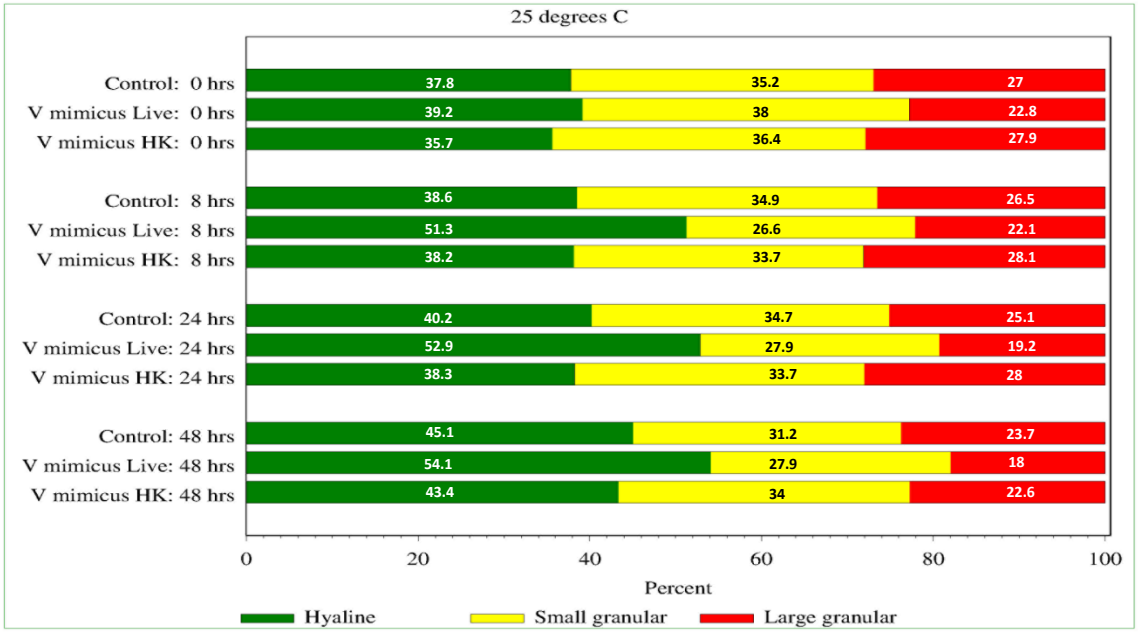


Figure 8.2. The relative cell proportion of *Cherax cainii* haemocytes after injected with live or heat-killed *Vibrio mimicus* at 25 °C. Relative percentages of each cell type in *C. cainii* at increasing time after injected with live or heat-killed *Vibrio mimicus* or in control haemocytes injected with saline *in vivo*. For each time point 10,000 cells were analysed. For an explanation of data format see Section 8.3.1.

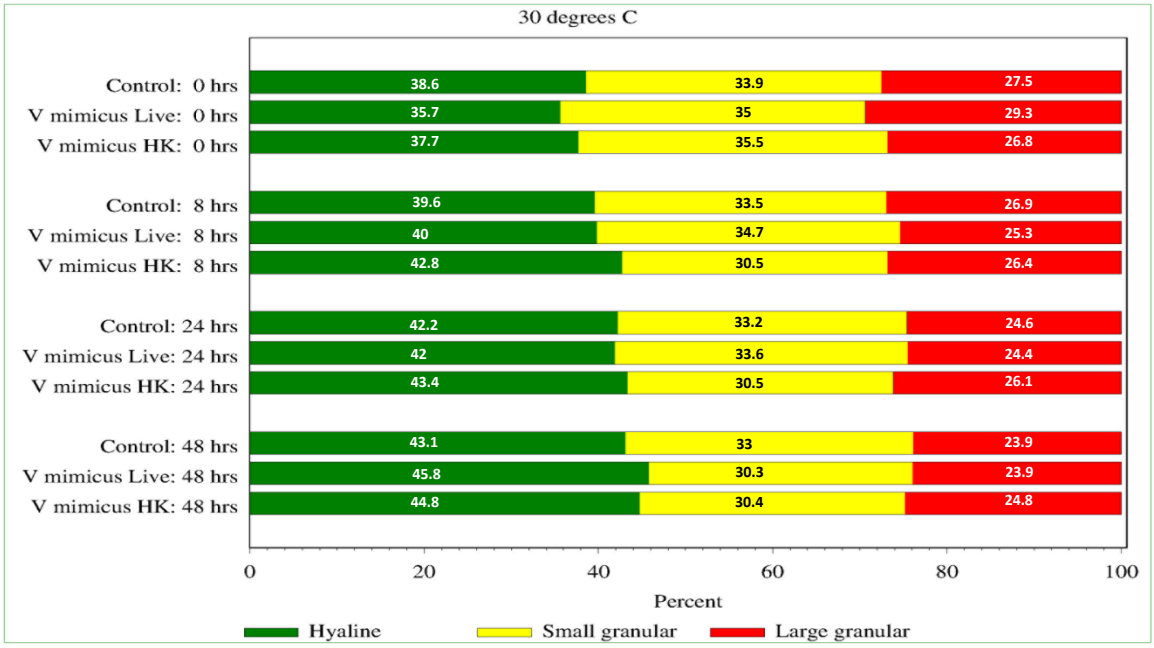


Figure 8.3. The relative cell proportion of *Cherax cainii* haemocytes after injected with live or heat-killed *Vibrio mimicus* at 30 °C. Relative percentages of each cell type in *C. cainii* at increasing time after injected with live or heat-killed *V. mimicus* or in control haemocytes injected with saline *in vivo*. For each time point 10,000 cells were analysed. For an explanation of data format see Section 8.3.1.

Time post-injection	Treatment comparisons	Temperature °C		
		20	25	30
0	Live vs Control	5.92	5.03	3.58
0	HK vs Control	2.8	2.59	1.84
0	Live vs HK	8.63	6.2	3.27
8	Live vs Control	6.35	14.74	2.01
8	HK vs Control	2.48	1.99	4.2
8	Live vs HK	8.43	14.96	5.21
24	Live vs Control	6.58	14.17	0.46
24	HK vs Control	2.79	3.51	3.25
24	Live vs HK	8.64	16.59	3.68
48	Live vs Control	6.7	9.88	3.61
48	HK vs Control	1.38	3.2	2.99
48	Live vs HK	6.12	11.78	1.29

Figure 8.4. Heat map showing change in haemocyte profiles based on mean angular separation (for an explanation of this value see Section 8.3.1). The relative cell proportions of *C. cainii* after injection with live, heat-killed or saline control at 0, 8, 24 and 48 hours, and at 20, 25 and 30 °C. Values are mean angular separation between treatments within each time-point and the colours represent intensity: **Low**, green-yellow (0 – 5); **Moderate**, orange (>5 - < 10); **High**, red (> 10).

8.3.3. Phagocytic activity of *C. cainii* haemocytes with live *Vibrio mimicus* at 25 °C

In the *in vivo* phagocytosis assays of live *V. mimicus,* all the haemocytes types appeared to be involved in phagocytosis. Large granular cells mounted the strongest phagocytic response at 48 hour post-injection, while the other haemocyte types (HCs and SGCs) did not show significant phagocytic activity compare to controls (Table 8.1). However, phagocytic activity was low overall, with a maximum of 0.317% after 48 hours post-injection with live *V. mimicus*.

Table 8.1. Phagocytic activity (% of counted cell) of *C. cainii* haemocytes with live *Vibrio mimicus* at 25 °C *in vivo*.

Hours	HCs		SGCs		LGCs	
	Live *V. mimicus*	Control	Live *V. mimicus*	Control	Live *V. mimicus*	Control
0	0	0	0.001	0.001	0.002	0.001
8	0.003	0.002	0.008	0.006	0.032	0.025
24	0.003	0.003	0.013	0.008	0.041	0.032
48	0.007	0.003	0.018	0.016	0.317	0.045

This data was acquired with FCM and 10,000 events in total were acquired per sample.

8.3.4. Phagocytic activity of *C. cainii* haemocytes with heat-killed *Vibrio mimicus* at 25 °C

The same pattern was also seen for the *in vivo* phagocytosis after heat-killed *V. mimicus* treatments as for the live treatments: LGCs showed the highest phagocytosis activity with HCs and SGCs showing negligible levels (Table 8.2). Furthermore, all levels of phagocytosis were low. In addition to shown some measurable phagocytic activity at 48 hours, LGCs also showed activity as early as 24 hours.

Table 8.2. Phagocytic activity (% of counted cell) of *C. cainii* haemocytes with heat-killed *Vibrio mimicus* at 25 °C *in vivo*.

Hours	HCs		SGCs		LGCs	
	Heat-killed *V. mimicus*	Control	Heat-killed *V. mimicus*	Control	Heat-killed *V. mimicus*	Control
0	0.001	0	0.001	0.001	0.001	0.001
8	0.003	0.001	0.004	0.002	0.032	0.006
24	0.008	0.003	0.032	0.008	0.317	0.008
48	0.01	0.004	0.045	0.011	0.463	0.018

This data was acquired with FCM and 10,000 events in total were acquired per sample.

8.4. Discussions

In the present study, *C. cainii* haemocytes showed different variations for the three cell categories after they were challenged with bacteria *in vivo*. The general response of was an increase of HCs paralleled by a decline in the number of granular cells after treatment with live *V. mimicus* injection. In our experimental design, a relative reduction in circulation of one haemocyte type will result in a relative increase in one or both of the other cell types and *vice versa*. The three cell type classifications varied considerably, possibly due to differential defence involvement corresponding to the pathogen. Our data show a relative decrease in SGCs and LGCs following bacteria injection, with a concomitant increase in HCs, possibly correlated to *C. cainii* defence mechanisms against *V. mimicus* infection. Bayne (1990) and Soderhall et al. (1994) stated that different crustacean haemocyte types have been associated with defence against invading diseases. Soderhall et al. (1986) suggested that SGCs are the first type of haemocyte to counter foreign particles *in vivo* by degranulation and discharge of the proPO system into the plasma in crayfish. Once the system is activated, it will trigger further degranulation by peroxinectin, which then amplified the proPO system secretion for both SGCs and LGCs. However, Cajaraville and Pal (1995) mentioned that HCs of bivalve mollusc *Mytilus galloprovincialis*, although showing characteristics of undifferentiated cells and less active than granular cells, were capable of phagocytosis. The HCs involvement in phagocytosis was not through antimicrobial peptides as such immune effectors are stored into the granules of granulocytes in mussel (Mitta et al., 1999). This phenomenon can be compared to findings in the crab, where the granules in highly activated phagocytes became sparse and finally disappeared. It was suggested that these granules were responsible for the production of a fibrous layer, surrounding the rosette of phagocytes in the hepatic haemolymph vessel (Johnson, 1976). Similar reactions, but to a much higher intensity, have also been observed in the lymphoid organ in shrimp *P. monodon* experimentally infected with white spot syndrome virus (van de Braak et al., 2002). The DHC of freshwater prawn *M. rosenbergii* failed to change after injection with *Aeromonas hydrophila*, except a small variation in HCs after 7 days and an increase in small, round granular cells at 24 h with increase in bacteria dose as compared to control *in vivo* (Sahoo et al., 2007). Mercier et al. (2009) emphasized that a change in the different haemocyte proportions of Pacific whiteleg shrimp *L. vannamei* could have significant impact on differential immune functions and on the whole immunocompetence of this animal. The Authors found a change in the HCs proportion to be a likely process for the increased clotting time showed 1 h after a handling stress exposure. A report from Comesana et al. (2012) on

DHC in flat oyster *Ostrea edulis* infected with *Bonamia ostreae* in Cork Bay (South Ireland, June 2005) showed similar results to Mercier et al. (2009): a significant increase in HCs and a decrease in granular cells proportions in *O. edulis* infected by *B. ostreae* (da Silva et al., 2008). Previously, Cochennec-Laureau et al. (2003) presumed that *O. edulis*, possessing higher granular cells proportions, can counteract *B. ostreae* more effectively. However Chang et al. (2005) and Aladaileh et al. (2007) considered that an increase in HCs proportion, after infection by *B. ostreae*, could enhance *O. edulis* haemopoietic activity to destroy the pathogen. These results suggest that either DHC affects the susceptibility to *B. ostreae* or disease causes variations in *O. edulis* DHC (or both).

Based on this *in vivo* study, we found that *C. cainii* haemocytes present weak phagocytic activities (0.3-0.5%) after 48 hours treated with live or heat-killed *V. mimicus*. This results is quite different to our *in vitro* results (See Chapter 5), in which we observed phagocytosis rates of 5.9% and 14.5% in HCs, 28.3% and 28.9% in SGCs and 58% and 60.7% in LGCs after 8 hours exposure with live or heat-killed *V. mimicus*, respectively. Even though the phagocytic value is different, there is agreement that LGCs were the most active phagocytically, albeit at a lower level *in vivo*. Martin et al. (1993b) found that haemocytes from penaeid shrimp, *Sicyonia ingentis*, 48 hours after injection with *Aerococcus viridans*, only showed 1.4% phagocytosis rate *in vivo*. Giulianini et al. (2007) in order to overcome some drawbacks in phagocytosis *in vivo*, used only artificial beads with different diameters injected into freshwater crayfish, *Astacus leptodactylus*. The Authors found that after treatment, HCs, SGCs, medium granule haemocytes and LGCs presented only low phagocytic activity, with maximums of 0.1-1.4%; 0.2-3.6%; 0.4% and 0.1-0.4% (respectively). These numbers were from total circulating haemocytes 2 hours post-injection of 1 and 3 µm diameters latex beads. The leukocytes phagocytic activity of pufferfish, *Takifugu rubripes,* treated with *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22) (Lpp) showed a significant difference compared with that of the control ($P < 0.01$; 0.05) at 4, 8, 24 and 72 hours post administration (Biswas et al., 2013). Similar to Biswas result, administration of lactic acid bacterium (LAB) probiotics to kelp grouper, *Epinephelus bruneus* and freshwater fish, *Labeo rohita*, lowered the loads of bacterial pathogen (Harikrishnan et al., 2010; Giri et al., 2013). A reason why the phagocytic activity is low in the *in vivo* state, as Martin et al. (1993) points out, is that haemocytes in penaeid shrimp are only capable of phagocytosis after adherence or attachment to the foreign entity, but this fails if the animal is constantly under stress. This is similar to the required activation of macrophages in vertebrates (Goldenberg et al., 1984). This may have

been a contributing factor for the low phagocytic activity detected in our *in vivo* experiments within this chapter.

Reports about the impact of temperature on the immune capability of poikilotherm such as *C. cainii* are rare. But typically, temperature is a major factor in metabolic and cellular activities and changes in temperature are recognised to change host immune functions in poikilotherms (Wang et al., 2008; Martin et al., 2010). In this study, we found that temperature had a significant influence on response time and magnitude of response on *C. cainii* haemocytes *in vivo*. Similarly, temperature can affects and modify the cellular and biochemical processes, including defence-related activities of oysters (Oliver and Fisher, 1995; Feng and Canzonier, 1970; Pipe and Coles, 1995; Chu and Lapeyre, 1993). Phagocytosis inhibition was observed in oysters *Crassostrea virginica* and *Mercenaria mercenaria* haemocytes when they were held at low temperatures (Foley and Cheng, 1975; Alvarez et al., 1989; Tripp, 1992; Pipe and Coles, 1995; Chu and Lapeyre, 1993; Carballalet al., 1997). There is a positive correlation between temperature and locomotion rate of *C. virginica* haemocytes. The increasing of phagocytosis activity at high temperatures is believed to be due to the haemocyte locomotion increment which in turn increased the haemocyte number attacking foreign entities (Fisher and Tamplin, 1988). Temperatures above a specific level can lead to haemocyte stress in marine bivalve mollusks that make them less responsive (Pipe and Coles, 1995; Alvarez et al., 1989; Chu and Lapeyre, 1993). Pauley et al. (1971), found that the sea hares *Aplysia californica* cleared bacteria more rapidly when they were reared at higher temperatures rather than at lower temperatures. Our results corroborate those results, where the environmental temperature influenced the phagocytic activity of *C. cainii* haemocytes. However, Seppälä et al. (2011) found that the great pond snail (*Lymnaea stagnalis*) subjected to acute thermal changes did not impact on their phagocyte haemocytes. The similar result found by Bodil et al. (2012) indicates that the Norway lobster, *Nephrops norvegicus* haemocytes is well-adapted to function under various temperatures (5, 10, 12, 14, 16, and 18 ^{o}C). The Authors found that these lobsters exhibit exceptional acclimation ability, even to temperatures higher than their normal habitat.

The *C. cainii* haemocyte populations delineated by light-scatter flow cytometry underscore persistent questions about cell lineages, but FCM also offers a powerful technique for answering them. Our results correspond to the view that HCs, SGCs and LGCs cells characterise three different phases in the single haemocyte type differentiation, HCs signifying the youngest cells that just released into circulation, and SGCs cells representing a

more advanced phase just before full differentiation to LGCs cells. To support this theory, it was also found that there is a strong relationship between HCs and SGCs proportions. This relationship could be expected, since most of the haemocytes developed from this cell types and thus their proportions changes should be related. Furthermore, the lack of relationship between HCs and LGCs cells could signify that LGCs cells are aged cells, and because these cells are disappearing from the haemolymph ultimately, there is only limited relationship with their ancestors. The relationships between the types of *C. cainii* haemocyte still need to be studied in detail.

In summary, the results herein provide further understanding of the functional integration of the pathogen recognition and subsequent activation of defence mechanisms, namely phagocytosis activity. *C. cainii* haemocytes are a dynamic population of cell, influenced by different environmental condition, affecting the elimination of pathogens. The data is suggestive the haemocytes in *C. cainii* are essentially one cell line which differentiates into three distinct cell types, based on morphological and functional analysis.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSION

9. Discussion and conclusion

Australia has an abundant and wide-ranging freshwater crayfish fauna. Due to the commercial value and recreational importance, investigations into the genus *Cherax* has generally been conducted on three species: red claw, *C. quadicarinatus* (von Martens), yabby *C. destructor* (Clark) and marron *C. cainii* (Austin) (Huner, 1994). Marron grown in semi-intensive freshwater ponds are at risk from various microorganisms including protozoan, parasites and bacteria and including other contamination that can damaging their market value. The industries sustainability is subject to disease handling and the health condition of marron. In order to be effective, disease control methods have to be part of an integrated health management system. Some systems are available but they require further development if they are to be truly ecologically and financially sustainable. It should be possible to focus management systems on the most important environmental parameter, in light of improved understanding of the relationship between environmental conditions and disease. However, even with the most efficient management systems there will always be some occasions when the marron are subject to stress, for example during transfer between hatchery and the pond. Marron may also be stressed as a result of climatic or unforeseen problems with the water source. At present, if marron are stressed, farmers will reduce feeding and may even administer drugs to reduce opportunist bacterial infections. It would be preferable to bolster the marron's own defence mechanisms thereby giving the animals the best chance of resisting opportunist infections. Therefore, the main goal of this body of work presented herein is to shed further understand of *C. cainii* immunological response to key stressors.

The following describes aspects of the *C. cainii* innate immunity involving haemocytes mechanisms from *in vitro* and *in vivo* experiments, based on the results described in Chapters 3, 4 and 5. There is some difference found in the phagocytic responses in *C. cainii* haemocytes using bright-field LM and TEM versus FCM. We found that HCs and SGCs where the most phagocytic cells with TEM (Chapter 3 and 4). Clearly these cell types were involved in immune action and/or activation, possibly in phagocytosis. However, it may not be easy to assign one function to a specific cell type, particularly in mixed haemocyte experiments. Cajaraville and Pal (1995) support the result that HCs of bivalve mollusc *Mytilus galloprovincialis* have phagocytic ability, even though they were not as active as granular cells. However, phagocytosis was not found in HCs of giant freshwater prawn *Macrobrachium rosenbergii* (Liang et al., 2011). Interestingly in Chapter 5, using FCM, we

found SGCs and LGCs were more phagocytic *in vitro*. This may be in part(s) due to the different experimental methods applied, including the target microorganisms. This discrepancy might result from analysing fresh cell suspensions in FCM instead of the fixed haemocyte smears used in TEM. Other Authors have also found a similar discrepancy with haemocytes of mussel *Mytilus galloprovincialis*. Carballal et al., (1997) examined these mussel's haemocytes using TEM and reported that HCs showed characteristics of undifferentiated cells and not phagocytosis. However, other Authors have reported that HCs of the same mussel observed using FCM also participated in the phagocytic response (Parisi et al., 2008).

Phagocytosis assays in crustaceans are usually performed *in vitro* with consequent cell adhesion and altered morphology (Hose et al., 1990; Gargioni and Barracco, 1998; Sung and Ru Sun, 2002). In this study (Chapter 8), even though the phagocytic value is different, there is agreement that LGCs were the most active phagocytically, albeit at a lower level *in vivo*. In an *in vivo* study with the freshwater crayfish *Astacus leptodactylus*, all the haemocyte types examined with TEM were phagocytic but only the SGCs is identified as the primary line of defence against foreign particles (Giulianini et al., 2007). In Chapter 8, most of the change was due to live bacteria, whereas in the *in vitro* setting there was a bigger response to heat-killed bacteria. These observations suggest that the enhanced chemo-attraction attributed to live bacteria might be due to soluble secreted materials in the growth medium. Schneeweiss and Renwrantz (1993), isolated an LPS from *E. coli* which induced *in vivo* migration of haemocytes from oyster *Mytilus edulis*. It is doubtful that LPS is responsible for the chemo-activation of oyster haemocytes since the bacteria is immobilised in agar chores. On the other hand, heat-killed bacteria, to some extent should inhibit haemocyte chemo-activation due to denaturation of soluble activation factors in the medium. However, there is still induction of haemocyte in the short term *in vitro*, possibly by other, less natural factors released into solution due to the damage caused by heating. Similar results have also been reported in the snail *Viviparus malleatus*, where heat-killed *Staphylococcus aureus* induced haemocyte activation *in vitro* (Schmid, 1975). However, Cheng and Rudo (1976), Cheng and Howland (1979) and Howland and Cheng (1982) found that haemocytes of the eastern oyster (*Crassostrea virginica*) were not attracted to dead bacteria and that living *Escherichia coli, Bacillus megatherium,* and *Micrococcus varians* were more powerful attractant *in vitro*. While the reasons for these differences are still not known, it is believed that *in vivo* data is the more accurate data.

The increased percentage of HCs was potentially due to changes in cell size of the other haemoctyes, namely SGCs and LGCs (Chapter 6 and 8). This suggests that changes in cell size are correlated to an active immune reaction. Reaction to the non-self particles such as bacteria and LPS also can make granular cell releasing their granules or undergo lysis. This can happen because SGCs and LGCs contain highly refractive granules as their main morphologic characteristic. These granules, which may contain the proPO system, are ready to be released after the cells are stimulated by pathogens. Soderhall and Smith (1983) and Johansson and Soderhall (1989) found that degranulation of intracytoplasmic granules in crustacean decapods is actually highly significant, indicating release of the proPO system resulting from the recognition of foreign material. Cardenas et al. (2004) also suggested that the cell size reduction due to LPS effect on white river crayfish *Procambarus zonangulus* was due to degranulation of granular cells, which coincided with an increase in non-granular haemocytes. As a result, proPO is released into the haemolymph and converted to its active form which not only results in melainzation of foreign invaders but also activities other immune functions like opsonisation and as mediator for cell to cell communication. However, LPS and bacterial treatments at a high concentration, can result in a rapid circulating haemocytes loss and therefore new haemocytes are produced. Consequently, new cells are produce in greater numbers in haematopoietic tissue (Hpt) and released into haemolymph. It is known from Söderhäll et al. (2003) that bacterial polysaccharides injection into the haemolymph will stimulate higher proliferation in the Hpt and new haemocytes released into circulation. We hypothesise that, when foreign particles are encountered, *C. cainii* haemocytes seem to settle first, primarily in the Hpt, in order to phagocytose. If haemocytes settle out of circulation onto the sites of bacterial attachment on the haemocoel, they would continue to be available for elimination of the secondary bacterial challenge. After phagocytosis, the haemocytes might leave their content with the degrading foreign material in the extracellular compartments.

Nitric oxide is an important and ubiquitous signalling molecule in animal kingdom (Colasanti et al., 2010). We revealed that NO production in HCs, SGCs and LGCs of *C. cainii* haemocytes is promoted by live and heat-killed *V. mimicus*. *C. cainii* haemocytes possess different NO response to live and heat-killed *V. mimicus*, nevertheless SGCs and LGCs were the prime cells for the production of NO. These facts indicate that *C. cainii* haemocyte cells may have an important function in their innate immune responses (Chapter 7). Mar Costa et al. (2008) mentioned that an exposure of haemocytes, from the Mediterranean mussel *M. galloprovincialis* and carpet shell clam *Ruditapes decussates,* to β-glucan has resulted in the

upregulation in NO production. Additionally, Costa et al. (2009), demonstrated a dramatic increase in NO production from haemocytes of Mediterranean mussel, *Mytilus galloprovincialis*, incubated with *Micrococcus lysodeikticus* and *Vibrio anguillarum*; showing that NO is an essential and inducible factor in the invertebrate immune system.

Previously, the impact of temperature on the immuno-competence on *C. cainii* haemocytes was not known. Based on the results from our studies (Chapter 6 and 8), it was found that *C. cainii* haemocyte activity was temperature-specific, since the treated haemocytes incubated at 25 ^{o}C showed the earliest change in profiles. It was also noticed that the activity of *C. cainii* haemocytes *in vitro* displayed a temperature-dependant variation which result in a large change in haemocyte profile values (Chapter 6), as the temperature increase. An inhibition of phagocytic activity was also noticed in the *C. caini* haemocytes kept at the lower temperature (Chapter 4). Carballal et al. (1997) noticed a lower phagocytic haemocytes percentage at 10 °C than at 20 °C and 30 °C for mussel *Mytilus galloprovincialis*. However, Alvarez et al. (1989) and Chu and La Peyre (1993), proposed that temperatures above a certain level may result in stress conditions in oyster haemocytes, as a result they are less reactive. In our point of view, these results must be correlated to the cells physiological condition exposed to different temperatures. Results in this study about *C. cainii* innate immune responses to environmental changes, along with other renewed studies about ecology, evolutionary biology and population biology, it is now known as ecological immunology (Sheldon and Verhulst, 1996; Schmid-Hempel, 2003; Rolff and Siva-Jothy, 2003). This area of immunology is rapidly increasing in popularity, compared to traditional immunological study, that has mainly concentrated on the physiological and molecular systems of host-pathogen relations in optimum situations (Hoffmann, 2003; Tirape et al., 2007; Rolff and Siva-Jothy, 2003). There has been a lot of literature produced since the beginning of this study area. Even though most of the studies have been done with vertebrates, they are particularly focused on the effects of stress on the immune response (Zuk et al., 2004; Rolff and Siva-Jothy, 2003). However, because there are a lot of invertebrate species (five to ten million) compare to around 45,000 existent vertebrates, the number of study on invertebrate immunology is growing rapidly, especially on the study of immune responses to environmental factors (Hoffmann, 2003).

One of the important features of crayfish immunity that needs further study is the location where haemocytes are produced and which are the stem cells they are generate from. Even though a number of studies have been published (Ghirettimagaldi et al., 1977; Hose and

Martin, 1989; Hose et al., 1990; Martin et al., 1993; van de Braak et al., 2002; Zhang et al., 2006). However, studies using isolated Hpt and/or cell lines are required to confirm the production and maturation system of crayfish haemocytes. Furthermore, the connection between the described haemocytes and the haemocyte precursor cells in *C. cainii* is yet uncertain, and while there are no molecular markers of the different haemocyte precursors at presented, the recommended lineages of *C. cainii* haemocytes have been established upon morphological characteristics. The ultrastructural figures indicate that HCs are a fairly undifferentiated type of haemocyte (Chapter 3 and 4). On the contrary, SGCs and LGCs show features of a well-differentiated haemocyte type. Our understanding of function certainly reflects what we see, i.e. granularity, especially without a clear special role for the hyalinocytes in response to immunological challenges. Observed rises in HCs proportions are as likely then to result from responses of granulocytes: the degranulation or death of SGCs and LGCs in the course of an infection. It is not actually the aim of this thesis to explain the differentiation of *C. cainii* haemocytes but the contiguous scheme or one lineage is the simplest explanation using the information here. Therefore, it was suggestive that there was one haemocyte cell lines which has three distinct cell types, with granular cells developing from HCs in *C. cainii*. This proposition is important since we only know currently a little information about the cellular and biochemical signals involved in regulating different *C. cainii* haemocyte type in vivo. In spite of this, some genetic study about haemocytes of fruit fly (*Drosophila melanogaster*), which have two different classes of haemocytes (plasmatocytes and crystal cells), have found that actually both types originate from one type of haemocyte precursor expressing the GATA protein Serpent (Srp) (Lebestky et al., 2000). The cells that expressing Srp pool bring about plasmatocytes along with crystal cells, which are able to be recognized by their early expression of distinctive transcription factors. Plasmatocytes particularly express the glial cell missing (Gcm) transcription factor, while crystal cells are differentiated by their Runt family protein, Drosophila lozenge (Lz) expression (Lebestky et al., 2000; Rizki and Rizki, 1959). Drosophila Lz is necessary for crystal cell determination, since Drosophila Lz functional loss also cause crystal cells loss during embryo and larval stages (Canon and Banerjee, 2000). An understanding of this activity, through knowledge of haemocyte production processes is important if we want to stimulate innate immunity in this significant invertebrates group. It would still need to be studied that the "lineage" shows a coherent evolution in gene expression and molecular organisation in crustacean.

In conclusion, the information about the innate immune repertoires of marron in response to the invasion of diseases is very important to help further our understanding about the mechanism of marron immunity to disease infection and developing strategies for management of the disease. Thorough comprehension about the innate immune responses of this freshwater crayfish to environmental variations is also crucial in order to inform our understanding about the interactions between host and pathogen, and how they are influenced by such variations. This will assist us, in turn, to comprehend and predict how environmental variability may affect immuno-competence in *C. cainii* that could impact at the population level. In this context, greater awareness and understanding of host-pathogen-environment interactions will be very important to ensure the long-term survival of marron aquaculture.

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APPENDIX

Time (Hours)	Temperature (°C)	Treatment comparison Control (C), LPS (0.25 & 0.5 mg/mL)	Mean angular separation	p-value	R squared value
0, baseline	20	C vs 0.25	7.4	<0.001	75.30%
		C vs 0.5	2.2	0.0212	
		0.25 vs 0.5	6.0	<0.001	
	25	C vs 0.25	1.1	0.236	
		C vs 0.5	1.2	0.914	
		0.25 vs 0.5	1.9	0.076	
	30	C vs 0.25	2.2	0.026	
		C vs 0.5	2.8	0.005	
		0.25 vs 0.5	1.0	0.682	
2	20	C vs 0.25	9.2	<0.001	97.90%
		C vs 0.5	1.3	0.015	
		0.25 vs 0.5	8.1	<0.001	
	25	C vs 0.25	13.4	<0.001	
		C vs 0.5	3.1	<0.001	
		0.25 vs 0.5	10.5	<0.001	
	30	C vs 0.25	3.6	<0.001	
		C vs 0.5	2.4	<0.001	
		0.25 vs 0.5	1.2	0.11	
4	20	C vs 0.25	11.9	<0.001	75.30%
		C vs 0.5	10.1	<0.001	
		0.25 vs 0.5	3.5	<0.001	
	25	C vs 0.25	4.4	<0.001	
		C vs 0.5	14.4	<0.001	
		0.25 vs 0.5	10.0	<0.001	
	30	C vs 0.25	16.3	<0.001	
		C vs 0.5	26.3	<0.001	
		0.25 vs 0.5	7.5	<0.001	
8	20	C vs 0.25	26.1	<0.001	97.00%
		C vs 0.5	27.0	<0.001	
		0.25 vs 0.5	2.9	<0.001	
	25	C vs 0.25	5.6	<0.001	
		C vs 0.5	14.1	<0.001	
		0.25 vs 0.5	9.3	<0.001	
	30	C vs 0.25	30.6	<0.001	
		C vs 0.5	34.0	<0.001	
		0.25 vs 0.5	5.0	<0.001	

Appendix Figure 1. Heat map showing change in haemocyte profiles based on mean angular separation. Mixed haemocyte populations were treated *in vitro* with either vehicle (Control), 0.25 or 0.5 mg/mL of LPS for up to 8 hours at either 20, 25 or 30 °C. Values are mean angular separation between treatments within each time point and the colours represent intensity: **Low**, green (0 – 5); **Moderate**, yellow to orange (>5 - < 17); **High**, red (> 17). The p-values were calculated from the analysis of the Log (angle), but the means shown in the table are the raw angles between the groups (in degrees). Because the replicates were generally very similar, many of the differences between groups are statistically significant, except at baseline, there were some similarities between groups. Most other groups have profiles of cell types which are quite different from each other.

Time (Hours)	Temperature (°C)	Treatment comparisons	Mean angular separation	p-value	R squared value
0, baseline	20	Live vs Control	5.92	0.0001	60.37%
		HK vs Control	2.8	0.0001	
		Live vs HK	8.63	0.0001	
	25	Live vs Control	5.03	<0.0001	
		HK vs Control	2.59	<0.0001	
		Live vs HK	6.2	<0.0001	
	30	Live vs Control	3.58	0.0065	
		HK vs Control	1.84	0.0065	
		Live vs HK	3.27	0.0065	
8	20	Live vs Control	6.35	0.3233	74.55%
		HK vs Control	2.79	0.3233	
		Live vs HK	8.43	0.3233	
	25	Live vs Control	14.74	<0.0001	
		HK vs Control	1.99	<0.0001	
		Live vs HK	14.96	<0.0001	
	30	Live vs Control	2.01	0.005	
		HK vs Control	4.2	0.005	
		Live vs HK	5.21	0.005	
24	20	Live vs Control	6.58	<0.0001	77.17%
		HK vs Control	2.79	<0.0001	
		Live vs HK	8.64	<0.0001	
	25	Live vs Control	14.17	<0.0001	
		HK vs Control	3.51	<0.0001	
		Live vs HK	16.59	<0.0001	
	30	Live vs Control	0.46	<0.0001	
		HK vs Control	3.25	<0.0001	
		Live vs HK	3.68	<0.0001	
48	20	Live vs Control	6.7	<0.0001	75.74%
		HK vs Control	1.38	<0.0001	
		Live vs HK	6.12	<0.0001	
	25	Live vs Control	9.88	0.0324	
		HK vs Control	3.2	0.0324	
		Live vs HK	11.78	0.0324	
	30	Live vs Control	3.61	<0.0001	
		HK vs Control	2.99	<0.0001	
		Live vs HK	1.29	<0.0001	

Appendix Figure 2. Heat map showing change in haemocyte profiles based on mean angular separation. Mixed haemocyte populations were treated *in vivo* with either vehicle (Control), live or heat-killed *Vibrio mimicus* for up to 48 hours at either 20, 25 or 30 °C. Values are mean angular separation between treatments within each time point and the colours represent intensity: **Low**, green-yellow (0 – 5); **Moderate**, orange (>5 - < 10); **High**, red (> 10). The p-values were calculated from the analysis of the Log (angle), but the means shown in the table are the raw angles between the groups (in degrees). Because the replicates were generally very similar, many of the differences between groups are statistically significant, except at baseline and 8 hours, were there were some similarities between groups. Most other groups have profiles of cell types which are quite different from each other.

Appendix 3 (Chapter 5; section 5.3.3. and 5.3.4.)

STATUS=Heat-killed V mimicus

The MEANS Procedure; Analysis Variable: Phagocytosis

TREAT	HOURS	CELL	N Obs	N	Minimum	Median	Mean	Std Dev
Control	0	Hyaline	6	6	0.03	0.03	0.04	0.01
		Large	6	6	0.11	0.13	0.16	0.07
		Small	6	6	0.00	0.14	0.15	0.11
	2	Hyaline	3	3	0.03	0.03	0.04	0.02
		Large	3	3	0.15	0.40	0.55	0.49
		Small	3	3	0.11	0.16	0.20	0.11
	4	Hyaline	3	3	0.03	0.05	0.06	0.03
		Large	3	3	0.41	0.84	0.79	0.35
		Small	3	3	0.22	0.29	0.47	0.37
	8	Hyaline	3	3	0.00	0.08	0.11	0.12
		Large	3	3	0.77	0.85	1.44	1.09
		Small	3	3	0.12	0.46	1.17	1.54
V mimicus + FITC	2	Hyaline	3	3	7.41	10.90	10.34	2.69
		Large	3	3	20.40	27.30	36.57	22.29
		Small	3	3	19.60	25.00	24.27	4.35
	4	Hyaline	3	3	7.18	10.30	11.66	5.29
		Large	3	3	35.10	44.70	42.10	6.13
		Small	3	3	23.80	26.00	25.77	1.86
	8	Hyaline	3	3	12.60	15.30	14.47	1.62
		Large	3	3	16.40	29.90	60.73	12.08
		Small	3	3	30.20	60.30	28.93	30.75

STATUS=Live V mimicus

Analysis Variable: Phagocytosis

TREAT	HOURS	CELL	N Obs	N	Minimum	Median	Mean	Std Dev
Control	0	Hyaline	3	3	0.03	0.03	0.04	0.02
		Large	3	3	0.11	0.16	0.20	0.11
		Small	3	3	0.11	0.13	0.16	0.08
	2	Hyaline	3	3	0.03	0.03	0.04	0.02
		Large	3	3	0.24	0.56	0.46	0.19
		Small	3	3	0.15	0.17	0.22	0.11
	4	Hyaline	3	3	0.04	0.07	0.07	0.02
		Large	3	3	0.45	0.67	0.70	0.26
		Small	3	3	0.07	0.13	0.32	0.38
	8	Hyaline	3	3	0.07	0.09	0.10	0.02
		Large	3	3	0.38	0.77	0.74	0.35
		Small	3	3	0.22	0.24	0.33	0.17
V mimicus + FITC	2	Hyaline	3	3	1.98	2.00	2.61	1.07
		Large	3	3	26.30	26.50	28.20	3.12
		Small	3	3	11.40	17.00	15.90	4.06
	4	Hyaline	3	3	2.06	3.99	3.39	1.15
		Large	3	3	15.00	35.70	30.17	13.29
		Small	3	3	8.70	12.90	16.10	9.42
	8	Hyaline	3	3	2.97	7.25	5.93	2.57
		Large	3	3	30.80	65.50	58.03	24.37
		Small	3	3	18.30	27.30	28.30	10.54

Analysis by status (live/heat killed)

---------- STATUS=Heat Killed V mimicus ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
TREAT	2	Control VM + FITC
HOURS	4	0 2 4 8
CELL	3	Hyaline Large granular Small granular

Number of Observations Read	45
Number of Observations Used	44

Analysis by status (live/heat killed)

---------- STATUS=Heat Killed ----------

The GLM Procedure

Dependent Variable: logphag

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	332.5369363	30.2306306	200.32	<.0001
Error	32	4.8292460	0.1509139		
Corrected Total	43	337.3661823			

R-Square	Coeff Var	Root MSE	logphag Mean
0.985685	38.12270	0.388476	1.019016

Source	DF	Type I SS	Mean Square	F Value	Pr > F
HOURS	3	316.7088313	105.5696104	699.54	<.0001
CELL	2	14.7794173	7.3897087	48.97	<.0001
HOURS*CELL	6	1.0486877	0.1747813	1.16	0.3527

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HOURS	3	311.8249262	103.9416421	688.75	<.0001
CELL	2	12.7306162	6.3653081	42.18	<.0001
HOURS*CELL	6	1.0486877	0.1747813	1.16	0.3527

Analysis by status (live/heat killed)

---------- STATUS=Heat Killed V mimicus ----------

The GLM Procedure
Least Squares Means

HOURS	CELL	logphag LSMEAN	LSMEAN Number
0	Hyaline	-3.29507536	1
0	Large	-1.87793003	2
0	Small	-1.81430221	3
2	Hyaline	2.31106507	4
2	Large	3.48318533	5
2	Small	3.17790912	6
4	Hyaline	2.38854805	7
4	Large	3.73254231	8
4	Small	3.24732271	9
8	Hyaline	2.66746322	10
8	Large	4.00856547	11
8	Small	3.29881393	12

Least Squares Means for effect HOURS*CELL
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logphag

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		0.7885	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	0.7885		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001		0.0008	0.0101	0.8086	<.0001	0.0059	0.2695	<.0001	0.0039
5	<.0001	<.0001	<.0001	0.0008		0.3430	0.0016	0.4376	0.4625	0.0150	0.1074	0.5651
6	<.0001	<.0001	<.0001	0.0101	0.3430		0.0182	0.0900	0.8282	0.1174	0.0134	0.7056
7	<.0001	<.0001	<.0001	0.8086	0.0016	0.0182		0.0002	0.0108	0.3858	<.0001	0.0072
8	<.0001	<.0001	<.0001	<.0001	0.4376	0.0900	0.0002		0.1359	0.0020	0.3907	0.1810
9	<.0001	<.0001	<.0001	0.0059	0.4625	0.8282	0.0108	0.1359		0.0769	0.0224	0.8721
10	<.0001	<.0001	<.0001	0.2695	0.0150	0.1174	0.3858	0.0020	0.0769		0.0002	0.0551
11	<.0001	<.0001	<.0001	<.0001	0.1074	0.0134	<.0001	0.3907	0.0224	0.0002		0.0323
12	<.0001	<.0001	<.0001	0.0039	0.5651	0.7056	0.0072	0.1810	0.8721	0.0551	0.0323	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Analysis by status (live/heat killed)

---------- STATUS=Live V mimicus ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
TREAT	2	Control VM + FITC
HOURS	4	0 2 4 8
CELL	3	Hyaline Large granular Small granular

Number of Observations Read	36
Number of Observations Used	36

Analysis by status (live/heat killed)

---------- STATUS=Live V mimicus ----------

The GLM Procedure

Dependent Variable: logphag

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	190.4698072	17.3154370	89.43	<.0001
Error	24	4.6470656	0.1936277		
Corrected Total	35	195.1168728			

R-Square	Coeff Var	Root MSE	logphag Mean
0.976183	32.41023	0.440032	1.357693

Source	DF	Type I SS	Mean Square	F Value	Pr > F
HOURS	3	161.1689862	53.7229954	277.46	<.0001
CELL	2	28.4915247	14.2457623	73.57	<.0001
HOURS*CELL	6	0.8092963	0.1348827	0.70	0.6548

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HOURS	3	161.1689862	53.7229954	277.46	<.0001
CELL	2	28.4915247	14.2457623	73.57	<.0001
HOURS*CELL	6	0.8092963	0.1348827	0.70	0.6548

Analysis by status (live/heat killed)

---------- STATUS=Live V mimicus ----------

The GLM Procedure
Least Squares Means

HOURS	CELL	logphag LSMEAN	LSMEAN Number
0	Hyaline	-3.23352645	1
0	Large	-1.72643022	2
0	Small	-1.87793003	3
2	Hyaline	0.90723880	4
2	Large	3.33539332	5
2	Small	2.74231060	6
4	Hyaline	1.17411679	7
4	Large	3.32235593	8
4	Small	2.66840463	9
8	Hyaline	1.69747820	10
8	Large	3.98790209	11
8	Small	3.29500409	12

Least Squares Means for effect HOURS*CELL
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logphag

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.0003	0.0009	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	0.0003		0.6770	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	0.0009	0.6770		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001		<.0001	<.0001	0.4648	<.0001	<.0001	0.0377	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001		0.1118	<.0001	0.9714	0.0757	0.0001	0.0819	0.9114
6	<.0001	<.0001	<.0001	<.0001	0.1118		0.0002	0.1195	0.8388	0.0077	0.0020	0.1371
7	<.0001	<.0001	<.0001	0.4648	<.0001	0.0002		<.0001	0.0004	0.1582	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	0.9714	0.1195	<.0001		0.0812	0.0001	0.0763	0.9399
9	<.0001	<.0001	<.0001	<.0001	0.0757	0.8388	0.0004	0.0812		0.0124	0.0012	0.0940
10	<.0001	<.0001	<.0001	0.0377	0.0001	0.0077	0.1582	0.0001	0.0124		<.0001	0.0002
11	<.0001	<.0001	<.0001	<.0001	0.0819	0.0020	<.0001	0.0763	0.0012	<.0001		0.0657
12	<.0001	<.0001	<.0001	<.0001	0.9114	0.1371	<.0001	0.9399	0.0940	0.0002	0.0657	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Analysis by cell type

---------- CELL=Hyaline ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
STATUS	2	Heat Killed Live
HOURS	4	0 2 4 8

Number of Observations Read	27
Number of Observations Used	27

Analysis by cell type

---------- CELL=Hyaline ----------

The GLM Procedure

Dependent Variable: logphag

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	165.6632679	23.6661811	175.51	<.0001
Error	19	2.5619513	0.1348395		
Corrected Total	26	168.2252192			

R-Square	Coeff Var	Root MSE	logphag Mean
0.984771	249.9443	0.367205	0.146915

Source	DF	Type I SS	Mean Square	F Value	Pr > F
STATUS	1	0.0024215	0.0024215	0.02	0.8948
HOURS	3	163.3289723	54.4429908	403.76	<.0001
STATUS*HOURS	3	2.3318742	0.7772914	5.76	0.0056

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STATUS	1	4.9750273	4.9750273	36.90	<.0001
HOURS	3	146.3367114	48.7789038	361.76	<.0001
STATUS*HOURS	3	2.3318742	0.7772914	5.76	0.0056

Analysis by cell type

----------CELL=Hyaline ----------

The GLM Procedure
Least Squares Means

STATUS	HOURS	logphag LSMEAN	LSMEAN Number
Heat Killed	0	-3.29507536	1
Heat Killed	2	2.31106507	2
Heat Killed	4	2.38854805	3
Heat Killed	8	2.66746322	4
Live	0	-3.23352645	5
Live	2	0.90723880	6
Live	4	1.17411679	7
Live	8	1.69747820	8

Least Squares Means for effect STATUS*HOURS
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logphag

i/j	1	2	3	4	5	6	7	8
1		<.0001	<.0001	<.0001	0.8152	<.0001	<.0001	<.0001
2	<.0001		0.7989	0.2492	<.0001	0.0002	0.0012	0.0548
3	<.0001	0.7989		0.3639	<.0001	<.0001	0.0007	0.0326
4	<.0001	0.2492	0.3639		<.0001	<.0001	<.0001	0.0044
5	0.8152	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
6	<.0001	0.0002	<.0001	<.0001	<.0001		0.3845	0.0163
7	<.0001	0.0012	0.0007	<.0001	<.0001	0.3845		0.0970
8	<.0001	0.0548	0.0326	0.0044	<.0001	0.0163	0.0970	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Analysis by cell type

---------- CELL=Large granular ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
STATUS	2	Heat Killed Live
HOURS	4	0 2 4 8

Number of Observations Read 27
Number of Observations Used 27

Analysis by cell type

---------- CELL=Large granular ----------

The GLM Procedure

Dependent Variable: logphag

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	181.1809379	25.8829911	130.76	<.0001
Error	19	3.7609969	0.1979472		
Corrected Total	26	184.9419348			

R-Square	Coeff Var	Root MSE	logphag Mean
0.979664	24.43433	0.444913	1.820850

Source	DF	Type I SS	Mean Square	F Value	Pr > F
STATUS	1	3.6124713	3.6124713	18.25	0.0004
HOURS	3	177.2858838	59.0952946	298.54	<.0001
STATUS*HOURS	3	0.2825828	0.0941943	0.48	0.7028

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STATUS	1	0.0729801	0.0729801	0.37	0.5509
HOURS	3	165.5003168	55.1667723	278.69	<.0001
STATUS*HOURS	3	0.2825828	0.0941943	0.48	0.7028

Analysis by cell type

---------- CELL=Large granular ----------

The GLM Procedure
Least Squares Means

STATUS	HOURS	logphag LSMEAN	LSMEAN Number
Heat Killed	0	-1.87793003	1
Heat Killed	2	3.48318533	2
Heat Killed	4	3.73254231	3
Heat Killed	8	4.00856547	4
Live	0	-1.72643022	5
Live	2	3.33539332	6
Live	4	3.32235593	7
Live	8	3.98790209	8

Least Squares Means for effect STATUS*HOURS
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logphag

i/j	1	2	3	4	5	6	7	8
1		<.0001	<.0001	<.0001	0.6356	<.0001	<.0001	<.0001
2	<.0001		0.5007	0.1644	<.0001	0.6887	0.6630	0.1808
3	<.0001	0.5007		0.4567	<.0001	0.2879	0.2729	0.4906
4	<.0001	0.1644	0.4567		<.0001	0.0795	0.0743	0.9552
5	0.6356	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
6	<.0001	0.6887	0.2879	0.0795	<.0001		0.9717	0.0884
7	<.0001	0.6630	0.2729	0.0743	<.0001	0.9717		0.0827
8	<.0001	0.1808	0.4906	0.9552	<.0001	0.0884	0.0827	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Analysis by cell type

---------- CELL=Small granular ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
STATUS	2	Heat Killed Live
HOURS	4	0 2 4 8

Number of Observations Read 27
Number of Observations Used 26

Analysis by cell type

---------- CELL=Small granular ----------

The GLM Procedure

Dependent Variable: logphag

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	134.7621039	19.2517291	109.89	<.0001
Error	18	3.1533634	0.1751869		
Corrected Total	25	137.9154674			

R-Square	Coeff Var	Root MSE	logphag Mean
0.977136	26.81448	0.418553	1.560923

Source	DF	Type I SS	Mean Square	F Value	Pr > F
STATUS	1	0.4752016	0.4752016	2.71	0.1169
HOURS	3	133.9173511	44.6391170	254.81	<.0001
STATUS*HOURS	3	0.3695513	0.1231838	0.70	0.5624

Source	DF	Type III SS	Mean Square	F Value	Pr > F
STATUS	1	0.4620888	0.4620888	2.64	0.1217
HOURS	3	128.4907940	42.8302647	244.48	<.0001
STATUS*HOURS	3	0.3695513	0.1231838	0.70	0.5624

Analysis by cell type

---------- CELL=Small granular ----------

The GLM Procedure
Least Squares Means

STATUS	HOURS	logphag LSMEAN	LSMEAN Number
Heat Killed	0	-1.81430221	1
Heat Killed	2	3.17790912	2
Heat Killed	4	3.24732271	3
Heat Killed	8	3.29881393	4
Live	0	-1.87793003	5
Live	2	2.74231060	6
Live	4	2.66840463	7
Live	8	3.29500409	8

Least Squares Means for effect STATUS*HOURS
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logphag

i/j	1	2	3	4	5	6	7	8
1		<.0001	<.0001	<.0001	0.8374	<.0001	<.0001	<.0001
2	<.0001		0.8413	0.7276	<.0001	0.2187	0.1533	0.7358
3	<.0001	0.8413		0.8819	<.0001	0.1568	0.1075	0.8906
4	<.0001	0.7276	0.8819		<.0001	0.1208	0.0816	0.9912
5	0.8374	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
6	<.0001	0.2187	0.1568	0.1208	<.0001		0.8312	0.1232
7	<.0001	0.1533	0.1075	0.0816	<.0001	0.8312		0.0833
8	<.0001	0.7358	0.8906	0.9912	<.0001	0.1232	0.0833	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix 4 (Chapter 5; section 5.3.5.)

STATUS=Heat-killed V mimicus

The MEANS Procedure; Analysis Variable: Phagocytosis

TREAT	HOURS	CELL	N Obs	N	Minimum	Median	Mean	Std Dev
Control	0	Hyaline	6	6	0.03	0.03	0.04	0.01
		Large	6	6	0.11	0.13	0.16	0.07
		Small	6	6	0.00	0.14	0.15	0.11
	2	Hyaline	3	3	0.03	0.03	0.04	0.02
		Large	3	3	0.15	0.40	0.55	0.49
		Small	3	3	0.11	0.16	0.20	0.11
	4	Hyaline	3	3	0.03	0.05	0.06	0.03
		Large	3	3	0.41	0.84	0.79	0.35
		Small	3	3	0.22	0.29	0.47	0.37
	8	Hyaline	3	3	0.00	0.08	0.11	0.12
		Large	3	3	0.77	0.85	1.44	1.09
		Small	3	3	0.12	0.46	1.17	1.54
V mimicus	2	Hyaline	3	3	7.41	10.90	10.34	2.69
		Large	3	3	20.40	27.30	36.57	22.29
		Small	3	3	19.60	25.00	24.27	4.35
	4	Hyaline	3	3	7.18	10.30	11.66	5.29
		Large	3	3	35.10	44.70	42.10	6.13
		Small	3	3	23.80	26.00	25.77	1.86
	8	Hyaline	3	3	12.60	15.30	14.47	1.62
		Large	3	3	16.40	29.90	28.93	12.08
		Small	3	3	30.20	60.30	60.73	30.75

STATUS=Live V mimicus

Analysis Variable: Phagocytosis

TREAT	HOURS	CELL	N Obs	N	Minimum	Median	Mean	Std Dev
Control	0	Hyaline	3	3	0.03	0.03	0.04	0.02
		Large	3	3	0.11	0.16	0.20	0.11
		Small	3	3	0.11	0.13	0.16	0.08
	2	Hyaline	3	3	0.03	0.03	0.04	0.02
		Large	3	3	0.24	0.56	0.46	0.19
		Small	3	3	0.15	0.17	0.22	0.11
	4	Hyaline	3	3	0.04	0.07	0.07	0.02
		Large	3	3	0.45	0.67	0.70	0.26
		Small	3	3	0.07	0.13	0.32	0.38
	8	Hyaline	3	3	0.07	0.09	0.10	0.02
		Large	3	3	0.38	0.77	0.74	0.35
		Small	3	3	0.22	0.24	0.33	0.17
V mimicus	2	Hyaline	3	3	1.98	2.00	2.61	1.07
		Large	3	3	26.30	26.50	28.20	3.12
		Small	3	3	11.40	17.00	15.90	4.06
	4	Hyaline	3	3	2.06	3.99	3.39	1.15
		Large	3	3	15.00	35.70	30.17	13.29
		Small	3	3	8.70	12.90	16.10	9.42
	8	Hyaline	3	3	2.97	7.25	5.93	2.57
		Large	3	3	30.80	65.50	58.03	24.37
		Small	3	3	18.30	27.30	28.30	10.54

Analysis by cell type

---------- CELL=Hyalinocytes ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
STATUS	2	Heat-killed Live
TREAT	2	Control V mimicus
HOURS	4	0 2 4 8

Number of Observations Read	45
Number of Observations Used	44

Analysis by cell type

---------- CELL=Hyalinocytes ----------

The GLM Procedure

Dependent Variable: logphag

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	255.3573674	51.0714735	206.99	<.0001
Error	38	9.3759272	0.2467349		
Corrected Total	43	264.7332946			

R-Square	Coeff Var	Root MSE	logphag Mean
0.964583	-49.96363	0.496724	-0.994172

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TREAT	1	247.7405307	247.7405307	1004.08	<.0001
STATUS	1	1.9230691	1.9230691	7.79	0.0082
HOURS	3	5.6937675	1.8979225	7.69	0.0004

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TREAT	1	186.3596072	186.3596072	755.30	<.0001
STATUS	1	2.6826104	2.6826104	10.87	0.0021
HOURS	3	5.6937675	1.8979225	7.69	0.0004

Analysis by cell type

---------- CELL=Hyalinocytes ----------

The GLM Procedure
Least Squares Means

TREAT	logphag LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Control	-2.91337846	<.0001
V mimicus	1.70946524	

STATUS	logphag LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Heat-killed	-0.35182030	0.0021
Live	-0.85209292	

HOURS	logphag LSMEAN	LSMEAN Number
0	-1.04651598	1
2	-0.81218726	2
4	-0.54720302	3
8	-0.00192019	4

Least Squares Means for effect HOURS
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logphag

i/j	1	2	3	4
1		0.3275	0.0412	0.0001
2	0.3275		0.1992	0.0004
3	0.0412	0.1992		0.0124
4	0.0001	0.0004	0.0124	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Analysis by cell type

---------- CELL=Large granulocytes ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
STATUS	2	Heat-killed Live
TREAT	2	Control V mimicus
HOURS	4	0 2 4 8

Number of Observations Read	45
Number of Observations Used	45

Analysis by cell type

---------- CELL=Large granulocytes ----------

The GLM Procedure

Dependent Variable: logphag

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	225.3847060	45.0769412	169.03	<.0001
Error	39	10.4005067	0.2666797		
Corrected Total	44	235.7852127			

R-Square	Coeff Var	Root MSE	logphag Mean
0.955890	59.62884	0.516410	0.866041

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TREAT	1	212.3729662	212.3729662	796.36	<.0001
STATUS	1	0.0838681	0.0838681	0.31	0.5781
HOURS	3	12.9278717	4.3092906	16.16	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TREAT	1	142.1755431	142.1755431	533.13	<.0001
STATUS	1	0.0154167	0.0154167	0.06	0.8113
HOURS	3	12.9278717	4.3092906	16.16	<.0001

Analysis by cell type

---------- CELL=Large granulocytes ----------

The GLM Procedure
Least Squares Means

TREAT	logphag LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Control	-0.79432794	<.0001
V mimicus	3.18025161	

STATUS	logphag LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Heat-killed	1.21168028	0.8113
Live	1.17424339	

HOURS	logphag LSMEAN	LSMEAN Number
0	0.15362019	1
2	1.26473674	2
4	1.58113823	3
8	1.77235217	4

Least Squares Means for effect HOURS
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logphag

i/j	1	2	3	4
1		<.0001	<.0001	<.0001
2	<.0001		0.1415	0.0209
3	<.0001	0.1415		0.3700
4	<.0001	0.0209	0.3700	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Analysis by cell type

---------- CELL=Small granulocytes ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
STATUS	2	Heat-killed Live
TREAT	2	Control V mimicus
HOURS	4	0 2 4 8

Number of Observations Read	45
Number of Observations Used	44

Analysis by cell type

---------- CELL=Small granulocytes ----------

The GLM Procedure

Dependent Variable: logphag

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	236.7219556	47.3443911	115.53	<.0001
Error	38	15.5726630	0.4098069		
Corrected Total	43	252.2946186			

R-Square	Coeff Var	Root MSE	logphag Mean
0.938276	144.4172	0.640162	0.443273

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TREAT	1	229.8039509	229.8039509	560.76	<.0001
STATUS	1	1.5111301	1.5111301	3.69	0.0624
HOURS	3	5.4068746	1.8022915	4.40	0.0094

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TREAT	1	180.5789758	180.5789758	440.64	<.0001
STATUS	1	1.8207869	1.8207869	4.44	0.0417
HOURS	3	5.4068746	1.8022915	4.40	0.0094

Analysis by cell type

---------- CELL=Small granulocytes ----------

The GLM Procedure
Least Squares Means

TREAT	logphag LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Control	-1.43937990	<.0001
V mimicus	3.03994267	

STATUS	logphag LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Heat-killed	1.00487164	0.0417
Live	0.59569113	

HOURS	logphag LSMEAN	LSMEAN Number
0	0.35035108	1
2	0.65520580	2
4	0.81921837	3
8	1.37635030	4

Least Squares Means for effect HOURS
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: logphag

i/j	1	2	3	4
1		0.3347	0.1412	0.0022
2	0.3347		0.5340	0.0089
3	0.1412	0.5340		0.0395
4	0.0022	0.0089	0.0395	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix 5 (Chapter 7; section 7.3.1-3.)

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Treat	2	Control V mimicus
Time	3	2 4 8
Condition	2	Heat-killed Live
Temp	3	20 25 30

Number of Observations Read	108
Number of Observations Used	108

The SAS System
The GLM Procedure

Dependent Variable: NO Conc NO Conc

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	2936.257380	489.376230	324.69	<.0001
Error	101	152.226461	1.507193		
Corrected Total	107	3088.483841			

R-Square	Coeff Var	Root MSE	NO Conc Mean
0.950712	11.64870	1.227678	10.53918

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Condition	1	22.753943	22.753943	15.10	0.0002
Temp	2	1445.207315	722.603657	479.44	<.0001
Treat	1	1453.913847	1453.913847	964.65	<.0001
Time	2	14.382276	7.191138	4.77	0.0105

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	22.753943	22.753943	15.10	0.0002
Temp	2	1445.207315	722.603657	479.44	<.0001
Treat	1	1453.913847	1453.913847	964.65	<.0001
Time	2	14.382276	7.191138	4.77	0.0105

The SAS System
The GLM Procedure
Least Squares Means

Condition	NO Conc LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Heat-killed	10.9981823	0.0002
Live	10.0801743	

Temp	NO Conc LSMEAN	LSMEAN Number
20	14.3655914	1
25	11.6411411	2
30	5.6108025	3

Least Squares Means for effect Temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: NO Conc

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		<.0001
3	<.0001	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Treat	NO Conc LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Control	6.8700960	<.0001
V mimicus	14.2082607	

Time	NO Conc LSMEAN	LSMEAN Number
2	10.0985862	1
4	10.5267454	2
8	10.9922034	3

Least Squares Means for effect Time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: NO Conc

i/j	1	2	3
1		0.1421	0.0026
2	0.1421		0.1108
3	0.0026	0.1108	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix 6 (Chapter 7; section 7.3.4 and 7.3.5.)

The SAS System
The GLM Procedure

Class Level Information

Class	Levels	Values
Condition	2	Heat-killed Live
Treat	2	Control V mimicus
Cell	3	Hyalinocytes Large granulocytes Small granulocytes
Time	3	2 4 8

Number of Observations Read	108
Number of Observations Used	108

The SAS System

The GLM Procedure

Dependent Variable: NO Conc

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	1279.721490	213.286915	188.66	<.0001
Error	101	114.184531	1.130540		
Corrected Total	107	1393.906021			

R-Square	Coeff Var	Root MSE	NO Conc Mean
0.918083	15.67015	1.063269	6.785310

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Condition	1	1161.865460	1161.865460	1027.71	<.0001
Cell	2	8.724425	4.362213	3.86	0.0243
Treat	1	47.854131	47.854131	42.33	<.0001
Time	2	61.277473	30.638736	27.10	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	1161.865460	1161.865460	1027.71	<.0001
Cell	2	8.724425	4.362213	3.86	0.0243
Treat	1	47.854131	47.854131	42.33	<.0001
Time	2	61.277473	30.638736	27.10	<.0001

The SAS System
The GLM Procedure
Least Squares Means

Condition	NO Conc LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Killed	3.5053688	<.0001
Live	10.0652510	

Cell	NO Conc LSMEAN	LSMEAN Number
Hyalinocytes	7.14025973	1
Large granulocytes	6.44449320	2
Small granulocytes	6.77117676	3

Least Squares Means for effect Cell
r > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: NOConc

i/j	1	2	3
1		0.0066	0.1439
2	0.0066		0.1954
3	0.1439	0.1954	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Treat	NOConc LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
Control	6.11965698	<.0001
V. mimicus	7.45096282	

Time	NOConc LSMEAN	LSMEAN Number
2	6.09838852	1
4	6.42366047	2
8	7.83388071	3

Least Squares Means for effect Time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: NOConc

i/j	1	2	3
1		0.1973	<.0001
2	0.1973		<.0001
3	<.0001	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.