ABSTRACT

Title of Document:	UNDERSTANDING MOLECULAR MECHANISMS REGULATING THE INITIAL SHELL-HARDENING PROCESS OF THE BLUE CRAB *CALLINECTES SAPIDUS*: INVOLVEMENT OF PROPHENOLOXIDASE AND THE TANNING HORMONE BURSICON.
	Javier Vicente Alvarez Zepeda, Doctor of Philosophy 2015
Directed By:	Associate Professor, J. Sook Chung, University of Maryland Center for Environmental Science. Institute of Marine and Environmental Technology

Phenoloxidase, the integral enzyme for the prophenoloxidase cascade system in insects and crustaceans is essential in the melanization of pathogens and wound healing. Upon proteolytic activation of the zymogen prophenoloxidase, the active enzyme phenoloxidase catalyzes phenol substrates to sclerotize the protein matrix of arthropod cuticles. The hard exoskeleton protects and supports the body of crustaceans; however, it presents several challenges to continuous growth. To grow,

crustaceans must periodically molt by shedding their exoskeletons (ecdysis). Ecdysis is triggered by the action of a set of hormones, which also initiate the previous deposition of a new soft cuticle beneath the hard exoskeleton. The flexible and soft new cuticle is expanded by the isosmotic intake of water during and immediately after ecdysis. Animals can then display their molt-related somatic growth. At the same time the unarmored crustaceans undergo the concurrent shell-hardening process to build a new exoskeleton.

In crustaceans, the molecular and physiological mechanisms involved in the initial shell hardening process have been poorly understood compared to the corresponding process in insects. This dissertation addresses the molecular mechanisms which regulate the initial shell-hardening process of crustaceans, using the blue crab *Callinectes sapidus* as research model. To this end, the research focuses on the hemocyte prophenoloxidase and the neurohormone bursicon to define their roles and interactions in the regulation of the initial shell-hardening. A differential expression of prophenoloxidase in hemocytes is found and the role of prophenoloxidase in the shell-hardening process of crustacean is established. Unraveling the shell-hardening process of *C. sapidus* will add our understanding of the molt-related somatic growth of this and other economically valuable decapod crustacean species. This knowledge may contribute to increases in productivity of crustacean fisheries and aquaculture by the development of biotechnology to extend or reduce the soft-shell stage of crustaceans.

UNDERSTANDING MOLECULAR MECHANISMS REGULATING THE INITIAL SHELL-HARDENING PROCESS OF THE BLUE CRAB *CALLINECTES SAPIDUS*: INVOLVEMENT OF PROPHENOLOXIDASE AND THE TANNING HORMONE BURSICON

By

Javier Vicente Alvarez Zepeda.

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2015

Advisory Committee:
Associate Professor J. Sook Chung, Chair
Professor Allen R Place
Professor Gerardo Vasta
Professor Bradley Stevens
Dr. Gary H Wikfors

Dedication

To my family ……

My wife, who gives unconditional love to my life

My daughter, who is soulful and adds emotions to my world

My son, who has become the bravest fighter of life, just in front of my eyes

….. They do yours all my quests

Acknowledgements

This dissertation research was carried out at the Institute of Marine and Environmental Technology (Baltimore, Maryland), University of Maryland Center for Environmental Science.

I am especially grateful to my adviser Dr. J. Sook Chung, who opened the door of her lab and allowed me to step into the fascinating field of crustacean molecular biology and endocrinology. She made this study possible by her invaluable advice and stimulating ideas and technical help.

I am also thankful to my committee, Drs. Place, Wikfors, Stevens, and Vasta for their invaluable discussions and guidance.

Nothing is possible without friendship and partnership. Thanks to my lab coworkers (Gib and In Sook), who provided me with the best friendly environments and science discussions.

Thanks to the Fulbright program and Chilean Government for funding support.

Finally, the greatest thanks go to my wife, daughter and son. Together we made this journey possible.

Table of Contents

List of Tables

Tables

Supplemental Tables

List of Figures

Figures

Supplemental Figures

List of Abbreviations

µg	micro grams
µl	micro liters
AE	After ecdysis
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Burs	Bursicon (the *Italized* form refers to transcripts)
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA, deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
Ddc	DOPA decarboxylase
DHC	Differential hemocyte counting
DOPA	L-3,4-dihydroxyphenylalanine
dsRNA	Double strand RNA, ribonucleic acid
DU	Durometer units
EDTA	Ethylenediaminetetraacetic acid
FSC	Forward scattered light
Gs	Granulocytes
HLS	Hemocyte lysate supernatant
HPO	Hydrogen peroxidase or peroxidase
hrs	Hours
Hs	Hyaline cells or hyaline hemocytes
ICC	Immunocytochemistry
NADA	N-acetyldopamine
NBAD	N-beta-alanyldopamine
ng	nano grams
nt	Nucleotides
ORF	Open reading frame
PBS	Phosphate buffered saline
PBSG	DPBS or Dulbecco's Phosphate Buffered Saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PPAE	Prophenoloxidase-activating enzyme
PPO	Prophenoloxidase
PTX	Phosphate buffer with Triton X-100
qPCR	Quantitative polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNAi	RNA interference
s	seconds
SE	Standard error

SGs	Semigranulocytes
SSC	Side scattered light
TGC	Thoracic ganglion complex
TH	Tyrosine hydroxylase
THC	Total hemocyte counting
UTR	Untranslated region

General introduction

Crustaceans, as do all arthropods, undergo the molting process repeatedly during their lifespan to complete their growth, sexual maturation and reproduction [1,2]. Molting is a cycle that involves the synthesis of a new soft cuticle beneath the hard exoskeleton. Next, the exoskeleton needs to be shed during a step called ecdysis to expand the new pliable cuticle and reach the somatic growth [1,2]. Several physiological pathways, such as cuticle deposition, calcium and protein reabsorption, and osmotic intake of water, must work in coordination to have successful ecdysis [3,4]. This physiological coordination is hormonally controlled through interactions among hormones and neurohormones and determine different molt cycle stages [3–10]. After ecdysis, the new soft cuticle needs to be transformed quickly into a hard exoskeleton to recover mobility, body support, defense and feeding capabilities that the hard external armor confers to arthropods. The physiological pathways that activate this transformation are called the shell-hardening process [11–17].

The shell-hardening process in crustaceans involves two sequential processes, beginning with the sclerotization of the new cuticles and ending with mineralization, the incorporation of calcium carbonate into the cuticle [11,12,14,15,17,18]. The sclerotization occurring in the two first layers of the cuticle is mediated by the enzymatic reaction of phenoloxidases (POs) that catalyze phenols as a substrate to cross-link chitin and protein fibers [2,11,18]. In crustaceans, unlike insects, this process is not well understood.

POs are copper binding enzymes that catalyze the hydroxylation of monophenols or the oxidation of diphenols to the correspond quinones, or both [19–21]. Multiple forms of POs are found in several arthropod species [17,21–32]. In insects such as *Nephotettix cincticeps*, *Tribolium castaneum*, *Calliphora vicina*, and *Bombyx mori* the sclerotization process is complex and POs and other enzymes act in concert to stiffen the new cuticle: tyrosinases, laccases, tyrosine hydroxylases, and peroxidases seem to be part of the whole insect sclerotization system [12,17,21,25,31,33–35]. Tyrosinases are known with the general name of prophenoloxidase (PPO), the zymogen form that is activated into the functional PO by the action of serine proteases (prophenoloxidase activating enzymes) [26,36]. In crustaceans, it is believed that the PPOs expressed in hemocytes initiate the shell-hardening process [37,38]. This PPO is an essential component of the innate immunity system of crustaceans involved in melanization, blood coagulation, encapsulation of pathogens, and wound healing [39–43].

Only PPOs have been characterized in crustaceans [44–47] and the presence of laccases remains to be confirmed. Tyrosine hydroxylases have been reported in crustaceans, but unlike insects, they have not been directly associated with the shell-hardening process [48–53].

Hemocyte aggregations are found in the connective tissues just below cuticle and underneath the crustacean hypodermis specifically during premolt and ecdysis [37,38]. Hence it has been suggested that the hemocyte PPO may be transported into the hypodermis during early premolt and ecdysis, to initiate the shell-hardening of the new soft cuticle during postmolt [37,38]. Evidence that PPO is transported exist in

insects [25,30]. A trans-epithelial transporter system mobilizes the insect PPO from the hemolymph into the hypodermis to be localized later into the cuticle [25]. In crustaceans, however, the transport of hemocyte PPO into the new cuticle has not yet been examined.

Based on the information presented, it is possible to consider that a coordinated series of events needs to occur to reach the shell-hardening process. The precise timing of each event is essential and should be addressed below a major control. In insects, the cuticle hardening involves sclerotization and melanization, a process called tanning [11]. Bursicon is a heterodimer neurohormone (α and β at a 1:1 ratio) that regulates the sclerotization and melanization of the insect cuticle after ecdysis [54–59]. Bursicon is released during ecdysis and mediates the activity of insect tyrosine hydroxylase [60,61]. In crustaceans, little is known about the control of postmolt events, but the recent findings of a crustacean bursicon suggest that this hormone may have roles similar to insect bursicons [62–65]. Indeed, the release of bursicon is found in the hemolymph of *Callinectes sapidus*, the concentration of which peaks at the completion of ecdysis [62].

Chung *et al.* [62] suggested that crustacean bursicon might participate in the shell-hardening process of crustaceans. The hormone could play a role in hemocyte granulation underneath the hypodermis during ecdysis. This information suggests that crustacean bursicon may regulate the sclerotization process. More specifically, it may regulate the activity of hemocyte PPO and PPO cascade system, if the hemocytes are involved in the shell-hardening process.

Defining the role of crustacean PPO and bursicon in the cuticle hardening process, are essential for understanding the initial shell-hardening process in crustaceans. This study tested the hypothesis that hemocyte PPO could be a component of the shell-hardening process of crustaceans, using the blue crab *C. sapidus* as a model. It also investigated if crustacean bursicon is involved in the activity of hemocyte PPO during postmolt. The results provide evidence that hemocyte PPO is involved in the shell-hardening process of crustaceans. It was also found there are three main types of hemocytes that distribute in the hemolymph and contain PPO in a molt stage-dependent manner, suggesting particular roles for each hemocyte type during the molting cycle. Bursicon may not directly mediate the activity of PPO in hemocytes, but the homodimer forms of this protein may be related to the shell-hardening process.

.

Chapter 1: Background and significance of the research

1.1. Molting and crustacean cuticle

Crustacean somatic growth requires the shedding of the exoskeleton through a periodic molt process. The molting cycle and molt are orchestrated by various hormones and neurohormones (ecdysone, molt-inhibiting hormone, crustacean hyperglycemic hormone, and crustacean cardioactive peptide hormone, etc.) which act in a concerted manner [4–7,9,62,66]. The molt cycle involves the synthesis and deposition of a new soft cuticle during the premolt, the shedding of the old exoskeleton (ecdysis), and hardening of the new cuticle at postmolt [1,2,18].

Four layers form the crustacean external cuticle (exoskeleton): the epicuticle, exocuticle, endocuticle, and membranous layer (Figure 1A). Tanned lipoproteins and calcium carbonate constitute the epicuticle, the outer layer. The exocuticle and endocuticle contain chitin-protein fibers and calcium carbonate, but unlike the exocuticle, the chitin-protein fibers are not tanned in the endocuticle. Endocuticle is the thickest layer and is followed by the membranous layer. The membranous layer is composed only of chitin (> 70%) and protein fibers without any mineral material. As the membranous layer is the last to be laid, completion of its secretion marks the end of postmolt and the beginning of intermolt [2,18,67].

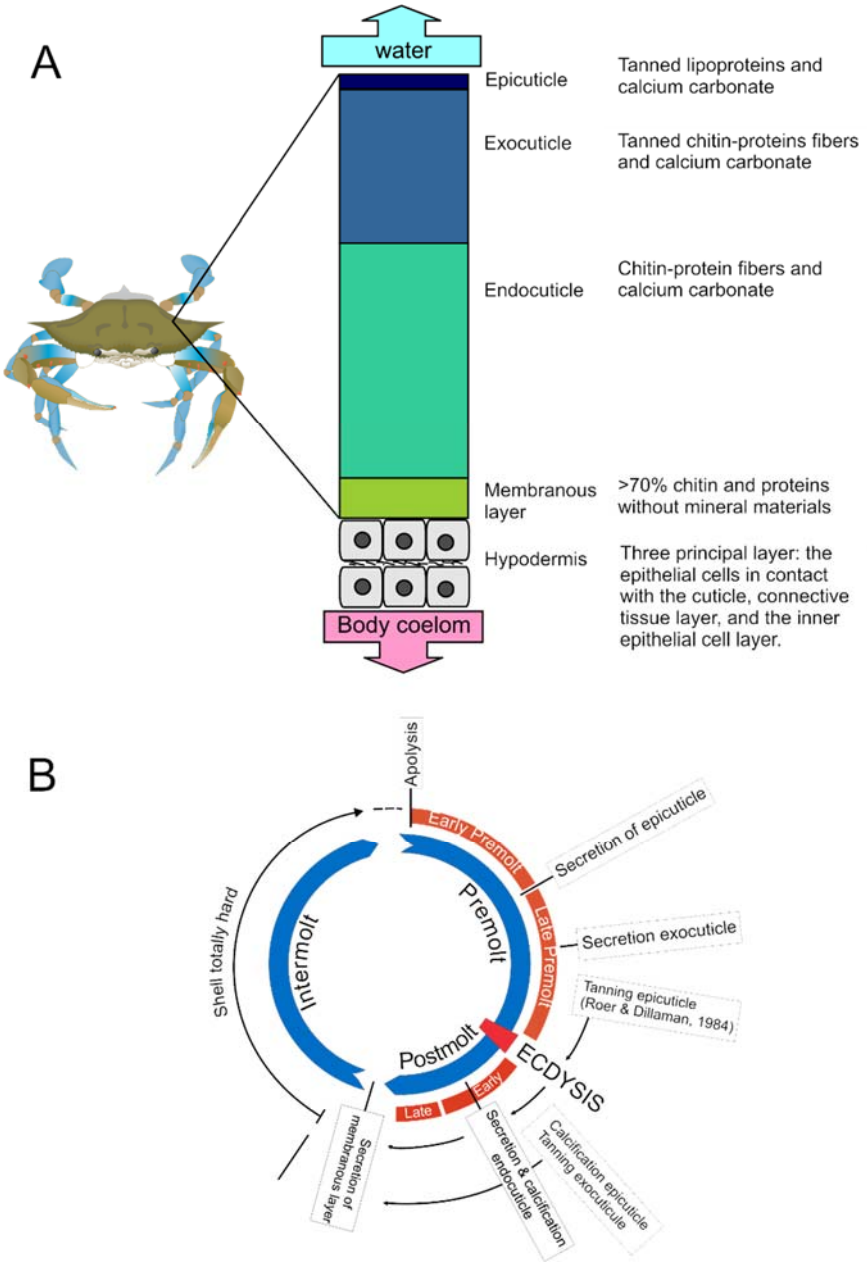


Figure 1. At the intermolt stage the exoskeleton of crustacean is hard and formed by (A) a set of 4 different layers. B) The layers are secreted by hypodermal cells at specific time through premolt and postmolt. Epicuticle and exocuticle are secreted before ecdysis (pre-ecdysial layers). Endocuticle and membranous layer are secreted during postmolt (post-ecdysial layers). During postmolt the shell-hardening process transforms the three outer layers in the hard exoskeleton. The diagrams are based on Smith and Chang (2007).

[The blue crab illustration is used with permit: Chip Cherry, IAN Image Library http:ian.umces.edu/imagelibrary].

The molt cycle is largely divided into four stages: premolt, ecdysis, postmolt, and intermolt [1,2,68]. The hypodermis cells secrete the new cuticle (Fig. 1B) before and after ecdysis (premolt: D_1 and D_2 stages; postmolt: A and B stages). At the beginning of early premolt, marked as "apolysis," the hypodermis synthesizes proteolytic enzymes to re-absorb the component materials of the membranous layer, thus separating the hypodermis from the rest of the old hard cuticle. Then, the outer cells of the hypodermis begin to secrete the new epicuticle. Upon the completion of laying the new epicuticle, the exocuticle is then secreted [2,18]. In contrast to the epi- and exocuticle, the endocuticle and membranous layer are deposited by hypodermal cells after the ecdysis (Fig. 1B) [2,15,67].

The hardening process of the new cuticle is gradual and takes place over different time scales: the sclerotization of epicuticle and exocuticle occurs before and after ecdysis, respectively [2,18]. The calcification occurs in the top three outer layers, starting about two hours after ecdysis when the body expansion is completed (timing may change among the different species and size of animals) [2,15,18,69–72]. The body expansion is the distension of the crustacean soft body into the maximal size by the isosmotic intake of water [2,15,67] using an active influx of ions into hemolymph via gills and gut [73]. Then the animals display the molt-related somatic growth.

1.2. Shell-hardening process

In crustaceans, the shell-hardening process involves both the initial sclerotization and subsequent calcification [2,13,15,69–72]. Sclerotization involves enzymatic catalysis in which phenolic precursors are oxidized and incorporated into

the organic matrix of cuticles. These catalytic processes result in the cross-linking between proteins and chitin fibers [17,21], forming a stiff organic network. The calcification process involves the deposition of calcium carbonate ($CaCO_3$) and calcium phosphate [$Ca_3(PO_4)_2$] within the organic network. This mineralization process is dependent on the type of proteins integrated in the cuticle, the previous sclerotization, and chitin concentration [15,69,70,74–79].

Cuticle sclerotization has well-been studied in insects [11,12,17,21,27,35], and it is recognized as the last hormonally regulated step of ecdysis [60,61]. In addition to the hormonal regulation of the cuticular tanning, laccase and tyrosinase enzymes, and dopa-decarboxylase (Ddc) play crucial roles in the "cross-linking" of proteins and chitin (Figure 2) [11,12,14,17,21]. In the first part of the phenol cascade, tyrosinase and/or tyrosine hydroxylase converts tyrosine into 3,4-dihydroxyphenylalanine (DOPA). Then, the enzyme Ddc decarboxylates DOPA into dopamine, which is the substrate for the synthesis of melanin, N-acetyldopamine (NADA) and N-β-alanyldopamine (NBAD). NADA and NBAD can be enzymatically oxidized by tyrosinase and laccase [21] and becoming the precursors of *ortho*- and *para*-quinones. Quinones initiate the last part of the cuticle sclerotization [17,21].

PO converted from the inactive zymogen (PPO) by prophenoloxidase-activating enzyme (PPAE), seems to be the major component of the phenol cascade in the sclerotization of arthropods' shells [17,44,80]. PPO also plays an important role in the innate immunity of insects and crustaceans [43,80,81]. It catalyzes the melanin synthesis, participates in blood coagulation, and encapsulates parasites and

pathogenic microorganisms thru the PPO cascade system [23,43,80–83]. In the innate immunity of crustaceans, the phenol cascade occurs as in insects [43], but the involvement of this pathway in the shell-hardening process has not yet been well-defined.

1.3. The phenoloxidase enzymes

Phenoloxidase is a common name for closely related copper containing oxidases. The two main POs are tyrosinases and laccases. However, hydrogen peroxidase (HPO) and tyrosine hydroxylase (TH) may also be included in the same group of POs due to its phenol activity. It is likely that HPOs are marginal implicated in the cuticle sclerotization process, but THs appear to participate at the first step (Fig. 2) of insect cuticle tanning [12,14,17,20,21,48,50,51,84].

Tyrosinase enzymes are type III copper proteins in which the dioxygen-copper binding site belongs to the hemocyanin family [20,85,86]. Tyrosinases can both hydroxylate monophenols to *ortho*-diphenols (i.e. L-tyrosine to L-DOPA) and oxidize the *ortho*-diphenols to *ortho*-quinones (i.e..L-DOPA to dopamine) [21,87,88]. However, it is reported that their hydroxylation capacity to monophenols is somewhat limited [89]. Tyrosinases act through a pathway that does not generate free radicals and cannot oxidize *para*-diphenols. Their activity is inhibited by phenylthiourea and thiourea [21]. Moreover, the tyrosinases from hemocytes lack a signal peptide, indicating they are not secreted proteins [47,89]. These hemocyte tyrosinases are usually synthesized as zymogens (proenzymes) and known as PPOs, which need to be converted to the active PO form before they exert their enzymatic activity. To

activate PPOs, the cleavage of its N-terminus takes place by the action of serine-like proteases, often called, in crustaceans and insects, PPAE [26,36].

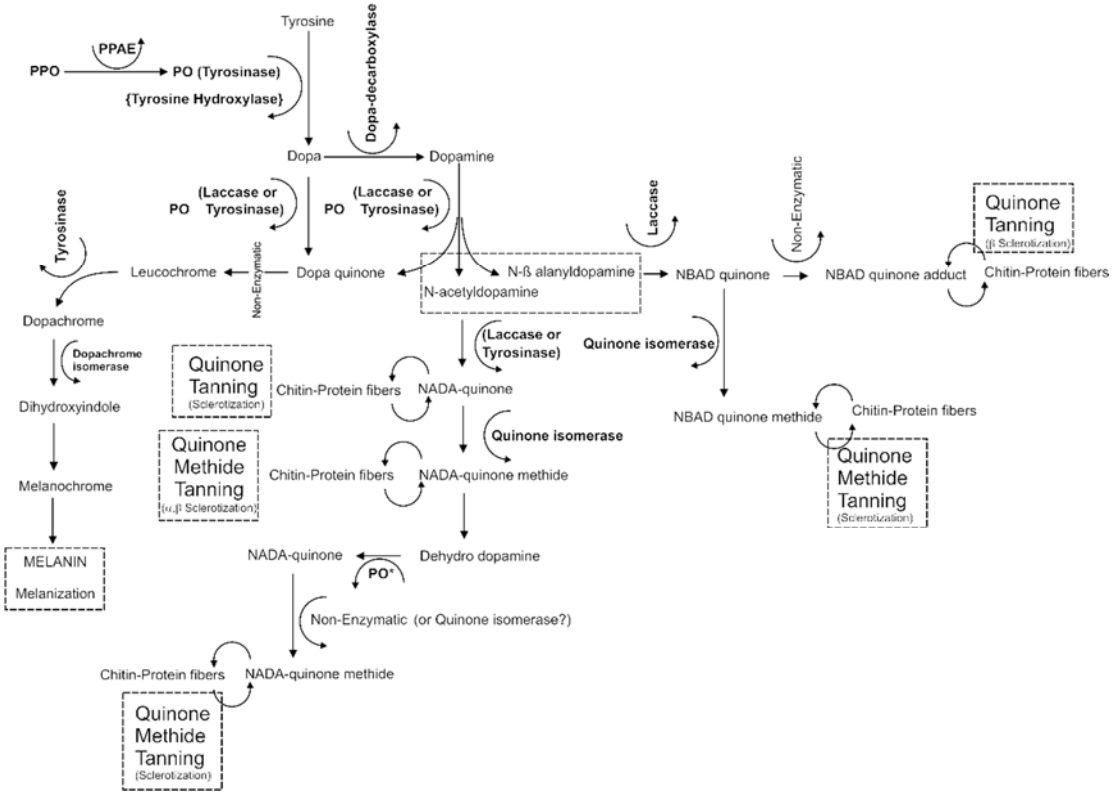


Figure 2. Proposed diagram that illustrates a putative sclerotization process in arthropods (modified and based on insects; Andersen and Peter, 1996; Andersen, 2012, 2010; Sugumaran, 1998). POs convert the phenol precursors in the corresponding quinones, which spontaneously react with the proteins and chitin fibers. This reaction produces as a result the cross-linking of the organic component of the cuticle, the sclerotization. Tyrosinase (PPO) can hydroxylate tyrosine to DOPA and oxidize dopamine to NADA. Independently, laccase can oxidize dopamine to NADA or NBAD. In each step of the pathway where the catalyst is shown as PO*, the precise nature of the enzyme is undefined. PPAE = PPO-activating enzyme.

Laccases, also called multi-copper oxidases (MCOs), are multi-copper containing enzymes. Most of these enzymes contain a copper ion in a type I site and three copper ions in type II/type III [87]. Laccases can oxidize *ortho*- and *para*-

diphenols to the corresponding quinones using a free radical pathway, but they are unable to hydroxylate monophenols substrates [12,17,21,87]. They are usually associated with cuticles and protein matrixes, and are more thermostable than cuticle tyrosinases. Their activities can be inhibited by azides or fluorides, but not by phenylthiourea [17,21,27]. The majority of laccases do not need proteolytic activation to perform their enzymatic activity. Hence, they are not produced as zymogen [21].

THs are well-known enzymes which catalyze the hydroxylation of tyrosine into DOPA as precursor of dopamine. Unlike tyrosinases and laccases, THs are iron-containing mixed-function oxidases, and therefore require oxygen and the cofactor tetrahydrobiopterin to catalyze the substrate [90]. Although it usually shows a low enzyme activity [91], the phosphorylation at Ser40 of THs increases its activity [91]. This post-translational modification mediates the TH enzyme activity [91]. Furthermore, their enzyme activity is inhibited by iron-chelators and tyrosine analogues [52]. The arthropod forms of TH differ from the counter parts of vertebrates by requiring a more alkaline pH and higher affinity to the pterine cofactor [52]. All THs are neuro-expressed enzymes [52]. TH are clearly identified in insects and related to cuticle sclerotization [48,50,51]. However, in crustaceans, the studies of THs have been focused on their enzymatic synthesis of dopamine as a neurotransmitter and not their role as component of the cuticle shell-hardening process [53].

The functional phenol activity of HPOs suggests that they may involve in the sclerotization of certain insect cuticles. Its importance in the cuticle tanning process

seems to be so far speculative [17,21,92]. HPOs have a PO activity that depends on hydrogen peroxide and that it could be acting on *ortho*-diphenols and *ortho*-quinones [14,21,92].

1.4. Sources of PPO and PO activity

In most arthropods, hemocytes are the main known source of PPO [38,93–95]. There are three main types of hemocytes widely accepted in crustaceans, based on morphology and chemo-analyses: granulocytes, semigranulocytes, and hyaline cells [37,82,96–98]. The composition of both the hemocyte population in the hemolymph and its PO activity vary during the molt cycle [95]. The hemocyte PPOs perform a fundamental role in the innate immunity as the final target enzyme in the PPO cascade system [80,99].

Nevertheless, it appears that the cellular source for PPO is controversial. In a study on the shell-hardening process of *Uca pugilator*, the fiddler crab, Vacca and Fingerman (1983) stated that the hyaline hemocytes were the source of PPO during ecdysis. Later, other authors described granulocytes (semi and granular) as the exclusive PPO hemocyte source. Most of these studies, however, focus on the immune role of hemocytes at the intermolt stage rather than throughout molting cycle [82,97,98,100–105]. Hose *et al.* (1990) reported that the granulocytes contain PO activity whereas a small group of hyaline cells found in *Panulirus interruptus* and *Loxorhynchus grandis* also showed PO activity. Therefore, it remains to be determined whether a particular type of hemocyte exhibits a molt stage-dependent PO activity.

On the other hand, arthropod hemocyanin exhibits PO activity like tyrosinases, because they contain a hemocyanin domain carrying the dioxygen-copper binding site [19,20]. The enzymatic properties and immune role of hemocyanin have been observed by *in vitro* proteolytic activation [85,106,107]. In the Chelicerata group of arthropods (the horseshoe crabs) does not carry PPOs or POs [108]. Their hemocyanin is the protein with PO activity in their blood. Thus, it has been suggested that hemocyanin may be involved in the cuticle sclerotization, possibly in a limited capacity [38,86].

Cryptocyanin is similar to hemocyanin in terms of protein sequence, size, and structure, but without binding oxygen or PO activities. Cryptocyanin increases its concentration in the hemolymph during premolt to be the primary protein [84]. Its concentration drastically declines during ecdysis, while the localization of this protein is found beneath the new cuticle [109]. Therefore, the role of cryptocyanin has been suggested as a transporter of hormones, ions, catecholamines or PPO [84,109].

Recently, a novel crustacean PPO has been reported in kuruma prawn, *Marsupenaeus japonicus* [47]. This PPO, associated with melanization during immune response, is expressed in the hepatopancreas and not hemocytes. This finding may indicate that crustacean melanization and cuticle sclerotization processes share some common features, which may be complex and require more than one PPO. Additionally, the finding that hindgut of insects use PPO as the last line of immune defense to clear pathogenic bacteria before the secretion of feces [110], supports the hypothesis that other tissues express PPOs.

1.5. Hematopoiesis and hemocytes

In coelomate animals, hemocytes are a fundamental component of the immunity system, which are usually found in the lumen of vascular cavities and coelom [111]. Frequently, a specialized tissue named as hematopoietic tissue (HPT) is the source of different hemocytes. HPT contains the stem cells which divide and differentiate into mature hemocytes. In most invertebrates, HPT is a mesenchymal, or gland-like structure, usually attached to the lining of either blood vessels or celomic cavities [111–113]. In decapod crustaceans, this tissue is described as a series of ovoid lobules which form a thin layer on the top part of the foregut [114–116]. The lobules contain the hemocytes stem cells (HSC), frequently called hemocyte progenitors in invertebrates [102,111,116,117], which advance in the prohemocytes. Later, prohemocytes will differentiate into mature hemocytes that will be released to the hemolymph [82,102,115,116,118].

The HPT of *Pacifastacus leniusculus*, a crayfish, has been well-studied and is used as a reference for all the decapod crustaceans. Five different types of prohemocytes are found within the lobules of this model [116]. Usually, crustacean hemocytes are considered to divide in the hemolymph [39,119], and as a result, the crustacean HPT is the primary source for all the types of hemocyte during the crustacean lifespan.

Hemocytes of all the arthropod groups have been investigated. However, there is much controversy over the hemocyte cell types and terminology because of the classification criteria [117,120]. The major hemocyte types in invertebrates, especially arthropods, have been classified in four principal groups: prohemocytes,

hyaline cells, granulocytes, and eleocytes [111,120]. Prohemocytes are small, round cells with a low cytoplasm/nucleus ratio, and correspond to the majority of immature hemocyte cells in the HPT [111]. Hyaline cells also called plasmatocytes or glassy hemocytes. They are differentiated cells either without or with few granular inclusions. Hyaline cells are typically phagocytic cells (macrophage-like) [82,101,103,104,111,115,120–122].

Granulocytes contain a dense number of granules in the cytoplasm [82,101,103,104,111,115,120–122] which correspond to enzyme-filled lysosomes [111]. These cells are involved in metabolic and developmental functions, but their principal role is in the innate immunity system as a source of cytotoxic and opsonic proteins and enzymes [116,119,123–126]. Eleocytes (or oenocytoids) are large oval cells that lack normal organelles, but with oleo inclusion and crystalline microtubules which agglomerate in the cytoplasm. As a result, they are also called crystal cells and are mainly found in insects [111,120,122,127].

In decapod crustaceans, the morphological classification of three main types of hemocytes; granulocytes, semigranulocytes, and hyaline cells is accepted [37,82,96–98]. The differentiation of each of these hemocytes occurs in the HPT before being released into the hemolymph stream. In crayfish, two lineages of cell-differentiation take place to form the three main types of crustacean hemocytes [102,115,116,123]. One of the lineages of prohemocytes produces the semigranulocytes and granulocytes, while hyaline cells have been shown to flow from an independent cell lineage [115,116,123]. The development and differentiation of the different groups of hemocytes are a result of the expression and interaction of

cytokines (astakines) and transcription factors (GATA-like), which modulate the HSC in the HPT [102,115,116].

Crustacean hemocytes have shown different functions in immunity. Cell cooperation and cell communication seem to be necessary to perform defensive reactions that allow detection, recognition and control of pathogens and parasites [39,40,80,81,89,128–134]. In the immunity system, while granulocytes are mentioned as the storage and release source of the PPO system, they are also involved in cytotoxicity [39,40,133]. Semigranulocytes are involved in encapsulation, limited phagocytosis, storage and release of the PPO and cytotoxicity as well [39,40,133]. Hyaline cells have the function of phagocytosis [39,40,133]. In a typical fungal infection, crustacean glycan-binding proteins (βGBP) are activated in the plasma, which recognize and bind to the cell wall of the fungal intruder. Afterwards, semigranulocytes and granulocytes respond to the βGBP, releasing the PPO system in the place of the infection by degranulation. The degranulation of these two types of hemocytes includes the secretion of opsonic and cell-adhesion factors (i.e. peroxinectin) [135]. The immune reaction is completed by hyaline cells, which respond to the opsonic factors performing the phagocytosis of the encapsulated pathogens [119,135] and dead cells.

1.6. Hormonal control of the shell-hardening process

In insects, the ecdysis and the cuticle sclerotization is a result of interactions among many hormones such as ecdysteroids, eclosion hormone, ecdysis triggering hormone, crustacean cardioactive peptide and bursicon [136–138]. Insect

glycoprotein neurohormone bursicon (Burs) initiates the tanning pathways of the cuticle in the last step of ecdysis and postmolt [56,137,139]. This hormone is a heterodimer protein encoded, in insects, by the *burs* and *pburs* genes. While *burs* encoded burs or alpha (α) subunit, *pburs* encoded the pburs or beta (β) subunit [56,60,140]. The mechanism of action of Burs is through its intracellular second messenger, cAMP [141].

Critical roles in the post-ecdysis events are mediated through regulation of the hydroxylation of tyrosine in the phenol cascade of melanin synthesis and cuticle sclerotization. In *Drosophila*, flies with a mutated *burs* gene have shown imperfect tanning [60] as a consequence of inactivation of TH, one of the enzymes that catalyze tyrosine into DOPA (see section 1.3). In the case of the fly, the phosphorylation of TH was disrupted, but this did not represent a deficiency in its gene expression. The injection of cAMP, the second messenger of Burs, reversed this situation [61], suggesting that this hormone upregulates the activity of TH and not its transcription. Arakane *et al* [28] showed that laccase 2 is the key PO in the tanning process of *Tribolium castaneum*. The enzyme laccase is essential for NAD and NBAD oxidation to synthesize the final quinones that sclerotize the new cuticle. In *T. castaneum,* the injection of *burs*(α+β)-*dsRNA* into pharate pupae did not affect its cuticle tanning or survival after adult ecdysis; however, the capacity to expand the wing was disrupted. This phenotype indicates that laccase activity may not be targeted by bursicon in this insect species [138]. Honegger *et al* [56] have suggested that it is possible that bursicon may act differently in different species of insects.

The hormonal mechanism that regulates the sclerotization in crustaceans is poorly understood. Recently, the cDNA of Burs has been isolated from the thoracic ganglion complex (TGC) of several crustacean species [62,64,65] including *Daphnia arenata*, *Carcinus maenas, Homarus gammarus*, *H. americanus*, and *C. sapidus*. However, its specific role in crustacean development is still unclear (Wilcockson & Webster, 2008; Sharp *et al.*, 2010; Chung et al., 2012). In *C. sapidus*, the co-release of CCAP and Burs was measured in the hemolymph at the highest levels of $8x10^{-11}$M of concentration at the completion of ecdysis (Chung et al., 2012). Even more, the presence of bursicon (hetero and homodimer) in pericardial organs (release site of Burs), as well as transcripts and proteins in TGC (expression site of *Burs*), remain constant throughout the molt cycle, suggesting that bursicon may have functions that are not associated molting. Insect bursicon homodimers (homo-Burs) have shown a different function and receptor than the heterodimer bursicon, the active hormone [56,58,60]. While the heterodimer bursicon participates in cuticle tanning and wing expansion, the homo-Burs have been shown to promote the transcription of genes involved in innate immunity during ecdysis [142].

1.7. Cuticle proteins and cuticle mineralization

The cuticle synthesis and the hardening process require protein-supplies that will largely be the target of the sclerotizing components. The principal hemolymph proteins (PPO, hemocyanin and cryptocyanin) have been mentioned above (section 1.4). The physiological roles of these proteins during molting likely involve supporting protein synthesis at different tissues, transporting other proteins for

possible incorporation to the new cuticle, and supplying oxygen to tissues and to reactive components that will be sclerotized in the formed cuticle [84].

Furthermore, a direct relationship between the amounts of cuticle proteins and the degree of calcification has been suggested. Since the deposition of carbonate crystals seems to depend on the soluble organic matrix that is cross-linked in the cuticle [78,143], the insoluble components such as chitin and chitosan inhibit the carbonate crystallization when the matrix contains little or no proteins [143]. Moreover, Coblentz et al. (1998) have proposed a working model that suggests that the changes in the cuticle protein composition are responsible for the different levels or/and timing of mineralization after ecdysis. This protein mediation may be responsible for the differences in calcification among the different parts of a crustacean body (external and internal cuticles). It is evident that there is a differential biomineralization between the dorsal carapace and chelipeds (heavily calcified) versus the suture line zone (thinly calcified) [76]. Therefore, the cuticle of the joints, gills, and walls of the brachial chamber can remain flexible and uncalcified.

Observations on the arthrodial membranes, which remain flexible and uncalcified after ecdysis, provide some supporting evidence. Comparing the arthrodial membranes with the hard cuticle of the carapace, no differences in their layers or lamellae structure is found. Both are also secreted at the same time during the molt cycle. However, their organic matrix composition is different (Hepburn and Chandler, 1976 in Shafer et al., 2006; Williams et al., 2003). On the other hand, changes at the level of glycosylation of proteins (pre- and postecdysis), glycoprotein

mobility (early postecdysial period), and glycosidase activity are found in the cuticle in different areas and molt stages [74,77]. Several biochemical changes by the recruitment of proteins before ecdysis together with conformational changes of the proteins after ecdysis, seem to mediate the sclerotization and mineralization. The regulatory process of these changes which could be under hormonal control, has not been studied yet.

1.8. Significance of the research

The shell-hardening process in crustaceans begins with the cuticle sclerotization. The information available in insects suggests that several POs act in concert for tanning the new cuticle. To date, only PPOs have been reported from the hemocyte of crustaceans, except a recently report that describes a PPO expressed in the hepatopancreas, and not in hemocytes [47]. It is not well-defined in crustaceans which type of hemocyte expresses PPO; if the cellular source of PPO may vary during the molt cycle, specifically at ecdysis and postmolt; and if this PPO is directly involved in the initial shell-hardening process.

Additionally, bursicon in most insects is the hormone that address the cuticle tanning and controls the activation of TH. In crustaceans it is known the hormone is present throughout the molt cycle. While its concentration increases in the hemolymph during ecdysis its role in cuticle sclerotization is still undetermined.

Previous findings suggest that crustaceans may have an intricate shell-hardening system, which may involve components from the immunity and endocrine systems. Contributions toward a better understanding of the shell-hardening process

of crustaceans may provide information on arthropod hard exoskeleton formation and evolution. It is possible that this knowledge may also contribute to crustacean fishery and aquaculture industry, where the period of softness after the ecdysis affects survival and growth. Soft shell crab production is of particular interest due to the seasonality and availability of peelers. More importantly, the production of soft shell crabs also depends on a short duration of the shell-softness at postmolt. Moreover, a better understanding of the crustacean shell-hardening process may contribute to generate biotechnological solutions on invasive and parasite crustacean species. An example is the sea louse (*Calligus spp*), a copepod group of parasite crustaceans that cost millions of dollar to the worldwide salmon industry.

1.9. Objective of the study

To understand the molecular mechanisms that regulate the initial shell-hardening process of the blue crab *C. sapidus*, the involvements of hemocyte PPO and the hormone bursicon are investigated. This study proposes the hypothesis that the shell-hardening process of crustaceans may rely on the presence of hemocyte PPO that may be differential expressed during the molt cycle. And this process may be regulated by bursicon.

To test the hypothesis, three specific objectives are carried out:

1. Objective 1: Identify a hemocyte PPO from the blue crab, *C. sapidus* and determine its enzyme activity during the molt cycle.

The objective 1 includes the molecular isolation and identification of *PPO* cDNA sequence (*CasPPO-hemo*) cloned from hemocytes of *C. sapidus*. The *CasPPO-hemo* expression during the molt cycle and the characterization of hemocyte PO activity is included. It is also incorporated the quantification of total hemocyte number and their derived PO activity during the molting cycle.

2. Objective 2: Examine the inherent changes in hemocyte PO activity during the molt cycle and its role in the initial shell-hardening process of the blue crab *C. sapidus*.

   The objective 2 includes the differentiation of main hemocyte types in the hemolymph of the blue crab during the molt cycle. It also states the identification of the specific hemocyte types that are the source of CasPPO-hemo during the molt cycle, using immunostaining and flow-cytometry. Moreover, the role of PPO and hemocytes during the shell-hardening process is determined using the multiple injections of specific *CasPPO-hemo-dsRNA*. Specifically, the effect of *dsRNA* on the shell-hardness is measured.

3. Objective 3: Investigate if bursicon regulate the activity of PPO during the shell-hardening process of the blue crab *C. sapidus*.

   The objective 3 includes the expression analysis of *CasPPO-hemo* in hemocytes and its PO activity after the post transcriptional knockdown of *Callinectes* bursicon using multiple injection of *CasBurs-dsRNA*, together with *CasBurs*-α or –β-*dsRNA* alone. The effects of the *dsRNA* injections on the shell-hardening are

also examined. More specifically, the levels of CasPPO-hemo hemocyte expression and its enzyme activity are determined together with total hemocytes number and differential hemocyte population. The levels of *CasPPAE* expression in hemocytes are also measured using qPCR assay.

Chapter 2: Cloning of prophenoloxidase from hemocytes of the blue crab, *Callinectes sapidus* and its expression and enzyme activity during the molt cycle

2.1. Abstract

The arthropods' cuticle undergoes dramatic morphological and biochemical changes from being soft to hardness through each molting process. Prophenoloxidase (PPO) known as a key enzyme in the arthropod innate immune system involved in the melanization reaction, has been related to the initial shell-hardening process, specifically in the sclerotization of the protein matrix in the new cuticle. Since hemocytes have been reported as the main PPO source in arthropods, the transport of hemocyte PPO into the newly laid, soft cuticle has been proposed for shell-hardening occurring during and immediately after ecdysis. In order to define the role of hemocyte PPO in the shell-hardening of crustaceans, the full-length cDNA sequence (2,806 nt) of hemocytes *PPO* of the blue crab *Callinectes sapidus* (*CasPPO-hemo*) is isolated using degenerate PCR and 5' - 3' RACE. *CasPPO-hemo* encodes a putative PPO (672 aa) showing three hemocyanin domains: N, M, and C in order and two copper binding sites (CuA & CuB). The sequence analysis identifies the putative CasPPO-hemo as zymogen which requires the cleavage at the *N*-terminus for its activation. Hemocyte extract (CasHLS) contains the PO, the activity of which depends on the *in vitro* activation of trypsin. The expression levels of *CasPPO-hemo* are kept constant during the molt cycle. The increase in the number of hemocytes at early premolt correlates with the elevated PO activity, while at late premolt, the reduced hemocytes in numbers reflect the decreased PO activity. The functional

importance of the changes in the levels of CasHLS-PO activity during molt cycle is discussed in relation to the cuticle hardening process.

2.2. Introduction

As one of most important immune systems in invertebrates, prophenoloxidase (PPO) cascade plays a central role in melanization in response to the invasion of pathogens and wound healings [17,38,44,80]. PPO is produced as an inactive form of zymogen that requires a serine protease, prophenoloxidase activating enzyme (PPAE) for its activation. Hemocytes are recognized as the principal source of PPO in arthropods including crustaceans [23,38,80,81,83,94].

Together with the changes in the total number of hemocytes, the levels of *PPO* expression and enzyme phenoloxidase (PO) activity in hemocytes are often associated with physiological conditions of the crustaceans including stress by environmental factors, harvest, microbial and fungal infections [83,145–151]. Specifically, the molt cycle appears to influence not only the number but also the type of hemocytes together with PO activity, especially with respect to a molt stage dependent susceptibility to disease [98,149,152]. In these species, animals at post-molt stage had hemocyte numbers lower than those at intermolt, which also corresponded to levels of PO activity relevant to each of these molt stages.

Interestingly, the PO activity present in hemocytes may participate in the sclerotization of the initial shell-hardening process of the new cuticle of crustaceans [37,38]. This is due to the finding that the hemocyte aggregation occurs underneath the hypodermis of crustaceans at ecdysis and early postmolt [37,38,62]. Furthermore,

bursicon, an arthropod tanning hormone, has been recently found to induce the recruitment and granulation of hemocytes beneath the hypodermis of the blue crab, *Callinectes sapidus* at ecdysis [62].

PO activities have also been measured in the cuticle of insects and crustaceans [14,153–156]. The cuticles undergo dramatic changes during molt cycle from softness during and after ecdysis to hardness most of the molt cycle and provide the first defensive barrier against invasive microorganisms [23,157]. More specifically, two insect studies show that PPOs present in the cuticle have been transported from hemolymph [25,30], while a laccase of *Tribolium castaneum*, a PO regulating the tanning of the new cuticle after ecdysis is not originated from insect hemocytes [31]. These reports suggest that POs either in hemocytes or cuticles may play a critical role in initial shell-hardening process of arthropods, the process of which resembles melanization reaction. We hypothesized that if a PO is involved in shell-hardening of crustaceans, hemocytes may be one of the sources.

To better understand the molecular mechanism(s) underlying the initial shell-hardening process after ecdysis of *C. sapidus*, we first isolated the cDNA sequence of *PPO* from hemocytes and characterized the PO activity. Herein, this paper reports the isolation of the full-length cDNA sequence of *PPO* from hemocytes of *C. sapidus* (*CasPPO-hemo*) using PCR with degenerate primers and 5' - 3' RACE and the levels of its expression during the molt cycle using a qPCR assay and of PO activity. With the changes in the levels of *CasPPO-hemo* expression, the PO activity in hemocytes and their numbers during the molt cycle, a functional role of PPO present in hemocytes is discussed in relation to the sclerotization of the new cuticle.

2.3. Materials and methods

2.3.1. Animals

Juvenile blue crabs, *C. sapidus* (15 – 30 mm carapace width, CW), were received from the blue crab hatchery [Aquaculture Research Center, the Institute of Marine and Environmental Technology (IMET), Baltimore, MD]. The animals were maintained in tanks with individual cages in aerated and recirculated artificial seawater at 22ºC and 20 ppt as described [62,158,159]. They were fed daily with a piece of squid and monitored their growth. The crabs with 75-85 mm CW were molt-staged as described [160].

2.3.2. Molecular cloning

The total RNA was isolated from hemocytes obtained from animals at various molt stages as describe Chung *et al.* and Chung & Zmora [158,159,161], using a QIAzol Lysis Reagent (Qiagen) following the manufacturer's protocol. The total RNA concentration and purity was estimated using a NanoDrop spectrophotometer (FisherSci). All RNA samples treated with DNase I (Fermentas) were subjected to syntheses of cDNAs using MMLV transcriptase (Fermentas) or 5'- 3' RACE cDNAs using a SMART™ cDNA synthesis kit (BD Biosciences).

Degenerate primers (Table 1) for *C. sapidus PPO* from hemocytes (*CasPPO-hemo*) were designed with the conserved regions based on the multiple sequence alignment of known crustacean PPOs listed in GenBank. A two-step PCR amplification was employed for degenerate PCR and 5'- 3' RACE as described [158,159,162–164]. In brief, the first touch-down PCR was performed using

*CasPPO*d3F1 primer and universal primer(UPM, BD Biosciences) at the following PCR conditions: 94ºC, 2.5 min; 94ºC, 30 sec, annealing temperature decreasing 1ºC/cycle starting at 47ºC and ending at 40ºC, 30 sec (8 cycles), extension at 72ºC for 2 min; 27 cycles at 94ºC, 30 sec, 48ºC, 30 sec, 2 min extension, and final extension at 72ºC for 7 min. The first PCR product was used as the template for the nested PCR after being diluted 20 fold in sterilized water with a combination of *CasPPO*d3F2 and *CasPPO*d5R2 (Table 1) at PCR conditions: 94ºC, 2.5 min; 40 cycles at 94ºC, 30 sec, 57ºC, 30 sec, 72ºC, 1 min; final extension at 72ºC for 7 min. The nested product was electrophoresed on a 1.5% agarose gel. The band at the expected size of ~1,100 bp was excised and used for cloning and sequencing as described [159]. Based on this initial sequence of *CasPPO-hemo,* gene specific primers (Table 1) for the cloning of the full-length cDNA sequence of *CasPPO-hemo* were designed.

Table 1. List of primer sequences that were used for the full-length cloning of *CasPPO-hemo* and qPCR assays

Primers	5' to 3' nucleotide sequence
*CasPPO*dF1	TAYTGGMGNGARGAYTAYGG
*CasPPO*dF2	CAYCAYTGGCAYTGGCA
*CasPPO*dR1	CKRTCRAANGGRAANCCCAT
*CasPPO*dR2	GGCCANCCRCANCCRCA
*CasPPO*3F1	TCTTGTCTACCCTGTGGACCTTAGT
*CasPPO*5R1	TTCAGGAGCACCTGGGTCATCTGG
*CasPPO*3F2	AGCAGTGGCACAATAGGATCATGGA
*CasPPO*5R2	AGAAGGTCAATCCCACGCTTCTCAG
*CasPPO*3F3	ATTCTGCAGTCCTCCACCTCTTCATCC
*CasPPO*5R3	TTCTCCACCCTGTTCAGCCCGACACT
*CasPPO*3F4	ATGGAGAGCGAACAGAAGCAAGTG
*CasPPO*5R4	CACTTCACTGGGTGATCTGAGCAT
*CasPPO*5R5	GGTACCCACTTCAAACACAATGG
*CasPPO*3F6-QF	CACCTCTTCATCCATCACAAACTC
*CasPPO*5R6-QR	CAACCACACCCACAGAAGTTAAAG
CasAK-LF	GACTTCGGCGATGTCCACCA
CasAK-LR	CCACACCAGGAAGGTCTTGT
CasAK-QF	CTACCACAACGACAACAAGACCTTC
CasAK-QR	ACGCGCTTCTCAATCTCGTTA

QF and QR primers used for qPCR assays
"d" indicates the degenerate primers.

For 5' and 3' RACE, a similar two-step PCR method was employed as above. Briefly, for 5' RACE, 5'RACE cDNA (50 ng total RNA equiv.) was amplified with *CasPPO*5R1 and UPM at the following PCR conditions: first denaturation 94ºC for 2.5 min; 94ºC, 30 sec, annealing temperature decreasing 1ºC/cycle starting at 57ºC and ending at 49ºC, 30 sec (8 cycles), extension at 72ºC for 2 min; 27 cycles at 94ºC, 30 sec, 58ºC, 30 sec, 2 min extension, and final extension at 72ºC for 7 min. This PCR product diluted 20 fold with sterilized water served as the template for the nested PCR with *CasPPO*5R2 primer and nested universal primer (NUP, BD Biosciences). The nested PCR products were treated similarly as above for cloning and sequencing. For 3' RACE, the same procedures were carried out with 3' RACE

cDNA and specific primers for the touch-down PCR with *CasPPO*3F1 and UPM and for the nested PCR with *CasPPO*3F2 primer and NUP (Table 1).

The ORF sequence of *CasPPO-hemo* was confirmed with the amplification of cDNAs of three different crabs using a High Fidelity Tag polymerase (Bioline) and *CasPPO*3F4 and *CasPPO*5R4 (Table 1). The phylogenetic analysis was carried out with the putative amino acid sequence of 19 number of crustacean PPOs including CasPPO-hemo (MEGA 5.05) [165].

2.3.3. Expression analysis

The expression levels of *CasPPO-hemo* at different molt stages were estimated using a qPCR assay with Fast SYBR Green Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). The standards of *CasPPO-hemo* were prepared similarly as described in Chung *et al.* [164]. The primers for the qPCR assay were selected in the region specific to *CasPPO-hemo* that is located between the hemocyanin domain M and C. The data were normalized with the levels of arginine kinase (*CasAK*) expression in the same sample cDNAs [166]. The expression levels are presented as mean ± SE copies/μg total hemocyte RNA.

The spatial expression of *CasPPO-hemo* was examined using an end point PCR assay with 10 different tissue cDNAs prepared from an animal at intermolt stage and CasPPO3F4 and -5R4 (Table 1) that amplify its entire ORF (2,019 nt).

2.3.4. Sequence analysis

The *CasPPO-hemo* cDNA sequence was analyzed using an ORF finder (www.ncbi.nlm.nih.gov/gorf/gorf.html) and RegRNA (regrna.mbc.nctu.edu.tw) [167]. The signal peptide was examined using Signal P 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The prediction of the potential N-glycosylation and phosphorylation sites was performed with NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc) and NetPhos 2. 0 Server (http://www.cbs.dtu.dk/services/NetPhos), respectively. The copper binding regions were located using SIB-MyHit motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and the identification of motif sequences was analyzed using NCBI Blast (http://blast.ncbi.nlm.nih.gov). Potential kinase specific phosphorylation was predicted using Net- PhosK 1.0 Server (www.cbs.dtu.dk/services/NetPhosK/). The identification of motif sequences and the sequence homology were done using the BLAST network server (http://blast.ncbi.nlm.nih.gov). A phylogenetic tree using the deduced amino acid sequences of *CasPPO-hemo* including 19 PPOs obtained from crustacean species (*Daphnia magna* was considered as out-group) and seven insects was constructed using the Maximum Likelihood method by MEGA 5.05 [165]

2.3.5. Total hemocyte counting (THC).

The THC was estimated using a modified procedure as described in Chung *et al.* [56]. In brief, hemolymph samples (150 µl) were withdrawn directly into a fixative solution (paraformaldehyde 4% in 10mM cacodylate buffer, pH. 7.1) in a ratio of 1:1. The fixed hemolymph samples were then centrifuged at 800 g for 10 min at 4ºC. The hemocyte pellets were gently re-suspended in 300 µl of PBS and placed on a

hemocytometer (Reichert Bright-Line) for counting under a compound light microscope. Each sample was counted twice and the data were presented as number of hemocytes/ml hemolymph (n=4-12).

2.3.6. Hemocyte Lysate Supernatant (HLS)

The hemolymph (500 µl) from crabs at various molt stages were collected directly into a syringe containing a crab anticoagulant solution (450mM NaCl, 10mM KCl, 100mM Glucose, 10mM $NaHCO_3$, 10mM EDTA, 10mM HEPES, pH 7.3) at a ratio 1:1 (v:v). The samples were kept on ice and centrifuged at 800g for 10 min at 4ºC for hemocyte separation. Each of these animals was used only once for just a single hemolymph collection. The hemocytes were washed twice with 200 µl of sodium cacodylate (10 mM) by centrifugation as above. The hemocytes were sonicated in 25 µl of working buffer (10 mM sodium cacodylate, 5 mM calcium chloride, pH 7.1) and then centrifuged at 15,000 g for 20 min at 4ºC. The protein concentration of the supernatant as HLS was estimated using a Pierce 660 nm kit.

2.3.7. Characterization of *Callinectes*-HLS (CasHLS) PO activity

The CasHLS-PO activity was determined by measuring the formation of dopachrome from L- 3,4 dihydrophenylalanine (L-dopa) using a modified assay as described [46,168,169]. Briefly, HLS samples (45 µg HLS in 50 µl working buffer) were preincubated with 50 µl of trypsin (1 mg/ml working buffer) for 15 min at 37ºC. Subsequently, 100 µl L-dopa (3 mg/ml working buffer) were added and further incubated for 20 min at 37ºC. At the end of 20 min incubation, the samples, diluted

with 150 µl of working buffer, were placed into a 96-well plate in triplicate (100 µl/well), and the absorbance was measured at 490 nm.

Substrate specificity of CasHLS was determined by evaluating the PO activity with four different phenol substrates at 3 mg/ml concentration: L-dopa, dopamine, catechol and L-tyrosine. The PO activity was measured at 490 nm, except for catechol at 410 nm. The proteolytic conversion of PPO into PO by trypsin was determined by subtracting the OD value obtained with cacodylate buffer alone.

Ca^{2+} dependent PO activity was evaluated by adding $CaCl_2$ to 10mM sodium cacodylate solution at the final concentrations of 0, 5, 10, and 20mM, while EDTA was added at 20mM final concentration. L-dopa was used as a substrate, unless described otherwise.

To further define the property of PO activity, sodium azide and thiourea known as PO inhibitors, were added into the substrate solution at 10mM final concentration.

The PO activity was calculated as $OD_{490\ nm}$/mg HLS and presented as mean ± SE (n = 5-15, molt cycle analysis).

2.3.8. Statistical analysis.

For each experimental analysis the parametric assumption of normality (Kolmogorov-Smirnov test) and homogeneity of variances (Cochran C test) were evaluated on the data. When the ANOVA test indicated significant differences at $P < 0.05$, a post-hoc Tukey test was adopted to distinguish the source of variation. The data that did not meet the parametric analysis, were analyzed with the non-parametric test, Kruskal-Wallis ANOVA and Median test were used to evaluate the statistical

difference (Statistica 7.0, Stat Soft., Inc). The data are presented as mean ± SE and (n) as the number of animals.

>*CasPPO-hemo* (JX047321)

```
   5′ cactctcgtctggctccgctctacctcacgaccataacagcaaacgagctttatctcagtcacacc
      accgagtacccgttggtcacc

  88 atggagagcgaacagaagcaagtgttggacatgctccagcgacctttttcggcagcaaactcaaaga
      M  E  S♦ E  Q  K  Q  V  L  D  M  L  Q  R  P  F  R  Q  Q  T♦ Q  R   22

 154 gaggagggtcatcccattgtgtttgaagtgggtacccgagtggcaggaagtgaaccacctcctcag
      E  E  G  H  P  I  V  F  E  V  G  T♦ R+ V  A  G  S♦ E  P  P  P  Q   44

 220 ccgttggctcttgctcgatccattcccaaaggcactgtcttctccatctttgttaaaaaccatcgc
      P  L  A  L  A  R+ S♦ I  P  K  G  T♦ V  F  S♦ I  F  V  K  N♦ H  R   66

 286 ctggctgccaaggaactctgcgactacttcatggaggcaagcagtgtgacagagctcaaggaaagg
      L  A  A  K  E  L  C  D  Y  F  M  E  A  S  S  V  T♦ E  L  K  E  R   88

 352 gtggaggaagtacgaagattcatcaacgagaagcttcttatctttgccctatcctttgtaatcatc
      V  E  E  V  R  R  F  I  N♦ E  K  L  L  I  F  A  L  S♦ F  V  I  I  110

 418 aggaagccagaaatgagacatctgcgcctgcctagcattgtggaaatcttcccaagcatgtttgta
      R  K  P  E  M  R  H  L  R  L  P  S  I  V  E  I  F  P  S  M  F  V  132

 484 ccagtcaccactgtatcaaagatggaacaggaggccaagaaatccacccctgaccaagagattgta
      P  V  T♦ T♦ V  S  K  M  E  Q  E  A  K  K  S♦ T♦ P  D  Q  E  I  V  154

 550 ataacagaatatggaccagagttctccagcactcatcttaagcctgaacatcgagtagcttactgg
      I  T♦ E  Y  G  P  E  F  S♦ S♦ T  H  L  K  P  E  H  R  V  A  Y  W  176

 616 cgtgaggactttggcattaatacccaccactggcactggcatcttgtctaccctgtggaccttagt
      R  E  D  F  G  I  N♦ T  H  H  W  H◄ W  H◄ L  V  Y  P  V  D  L  S  198

 682 gtgagtagggaccgcaagggagagctattctactacatgcatcaacaaatacttgctcgttatgac
      V  S  R  D  R  K  G  E  L  F  Y  Y  M  H◄ Q  Q  I  L  A  R  Y  D  220

 748 atggagcgcctcagtgtcgggctgaacagggtggagaaactgagcaactggcgcatccccattcct
      M  E  R  L  S  V  G  L  N  R  V  E  K  L  S♦ N  W  R  I  P  I  P  242

 814 gacggctactttcccaagctgaccatcagtaactctggccagacatggggctcccgtcaagacaat
      D  G  Y  F  P  K  L  T  I  S♦ N  S♦ G  Q  T♦ W  G  S♦ R  Q  D  N♦ 264

 880 acattaatgcaggactataagcgtgatgactttgggctgttgccatttgatgtctctgatcttgag
      T  L  M  Q  D  Y  K  R  D  D  F  G  L  L  P  F  D  V  S  D  L  E  286

 946 cagtggcacaataggatcatggatgccattcatctgggctgtcttgttgaccgtgacaataaccag
      Q  W  H  N♦ R  I  M  D  A  I  H  L  G  C  L  V  D  R  D  N♦ N♦ Q  308

1012 acaaggctcacagacaacactaaaccacctgagaagcgtgggattgaccttcttggtgacgcagtg
      T  R  L  T  D  N  T♦ K  P  P  E  K  R  G  I  D  L  L  G  D  A  V  330

1078 gaggcagacagcactatgagcctcaacagtctcttctatggtgacctgcacaacatggggcatgtt
      E  A  D  S♦ T  M  S  L  N♦ S  L  F  Y  G  D  L  H◄ N  M  G  H◄ V  352

1144 gtcatttcagcttcccatgacccagacaatgctcatcgggaaaaccttggggtcatgtcagacaca
      V  I  S♦ A  S♦ H  D  P  D  N♦ A  H  R  E  N  L  G  V  M  S  D  T  374

1210 gccacagccatgagggaccctgtgttctaccgctggcacaagtacattgacaacatcttccagcag
      A  T♦ A  M  R  D  P  V  F  Y  R  W  H◄ K  Y  I  D  N  I  F  Q  Q  396

1276 tacaaactgaaacagccaccttatactgctgaggaactgtccctctcatccgttgaggtggtgtca
      Y  K  L  K  Q  P  P  Y  T  A  E  E  L  S♦ L  S♦ S♦ V  E  V  V  S  418

1342 gtggccgtagagagccggggccagaagaaccagctgatcactggctggagtactcgggactttgag
      V  A  V  E  S  R  G  Q  K  N♦ Q  L  I  T  G  W  S♦ T♦ R  D  F  E  440

1408 gcgagtcgtggactggacttcaacgcagataagccagtgattgtgcgactcacccatctcaaccac
      A  S  R  G  L  D  F  N  A  D  K  P  V  I  V  R  L  T  H  L  N♦ H  462
```

Figure 3. The full-length cDNA sequence of *CasPPO-hemo* (GenBank JX047321) and its deduced amino acid sequence. The start codon of the ORF (atg) and the stop codon (*) are made in bold. The putative copper binding regions are identified by arrows in the H_{188}, H_{190}, H_{212} (CuA) and H_{347}, H_{351}, H_{387} (CuB). The predicted putative phosphorylation and glycosylation sites are shown in □ and ♦, respectively. The putative cleavage sites of PPO converting to PO are marked with '+'. The thiol-ester like motif (GCGWPQHM) is underlined within the ORF sequence. The regulatory elements in the 5' UTR (TOP) and 3' UTR (GAIT) are underline by double lines and bold dashed lines respectively.

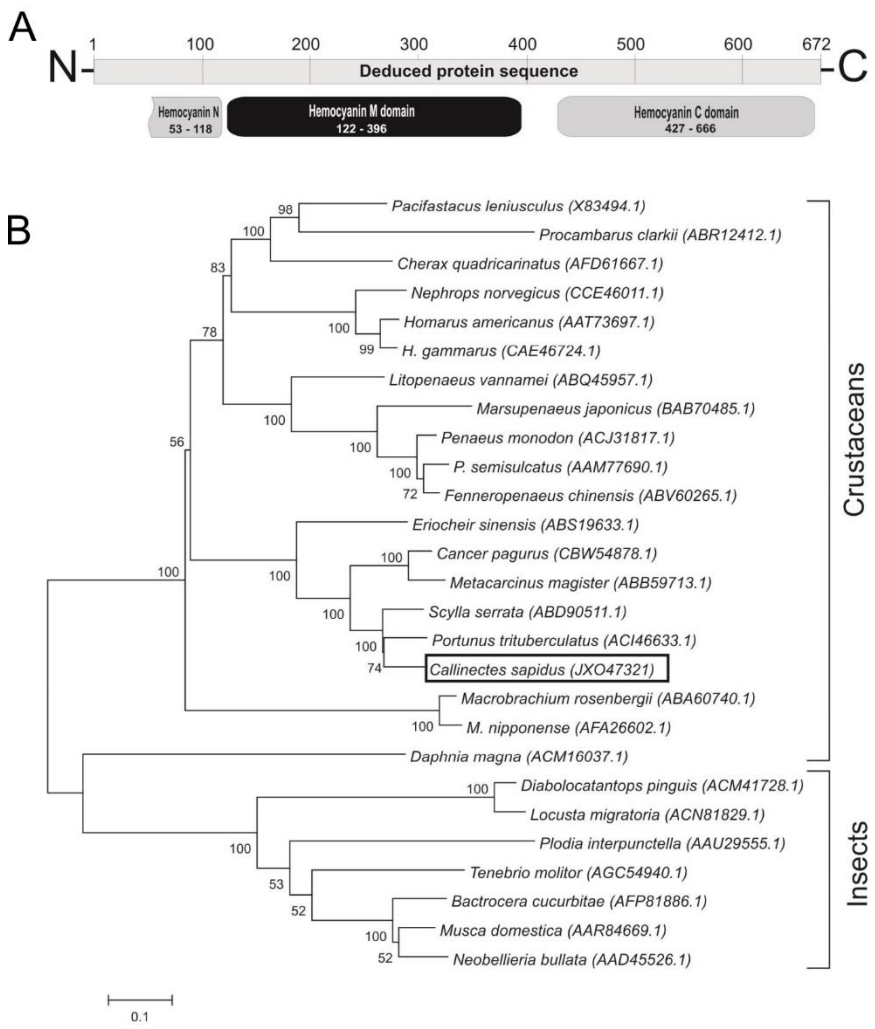


Figure 4. A) A schematic representation of the deduced sequence of CasPPO containing the three conserved hemocyanin N, M and C domains identified using the conserved domain search (http://blast.ncbi.nlm.nih.gov). The numbers below each domain indicate the location in the protein sequence. B) A phylogram generated with 19 PPOs of crustaceans and 7 insect species using the Maximum Likelihood method with 100 bootstrap replicates *C. sapidus* PPO (CasPPO-hemo) is marked in square. The scale bar represents fixed mutation per amino acid position.

2.4. Results

2.4.1. Cloning and sequence analysis of *CasPPO*-hemo

The full-length cDNA of *CasPPO-hemo* (2,806 nucleotides, GenBank accession JX047321) consists of a 5' UTR of 87 nt, a coding region (ORF) of 2,019 nt, and a 3' UTR of 700 nt (Fig. 3). The open reading frame encodes a putative 672 amino acid sequence of CasPPO-hemo (ORF finder, http://www.ncbi.nlm.nih.gov/projects/gorf/). The 5' UTR contains two terminal oligopyrimidine tract (TOP) regulatory RNA motifs (http://regrna.mbc.nctu.edu.tw). Two GAIT elements (Gamma interferon activated inhibitor of Ceruloplasmin mRNA translation) are found in the 3' UTR (http://regrna.mbc.nctu.edu.tw).

No signal peptide was predicted in CasPPO-hemo (http://www.cbs.dtu.dk/services/SignalP/) in which putative copper binding regions are identified in the His-188, His-190, His-212 (CuA) and His-347, His-351, His-387 (CuB) (http://myhits.isb-sib.ch/cgi-bin/motif_scan). A total of 22 putative phosphorylation sites were predicted (http://www.cbs.dtu.dk/services/NetPhos/) including 15 at S ($S_{39, 81, 122, 138, 147, 198, 225, 235, 260, 410, 412, 435, 474, 532, 606}$), six at T ($T_{20, 136, 148, 482, 514, 647}$), and one at Y_{389}. Sixty four putative glycosylation sites were predicted: 26 at S ($S_{3, 39, 51, 59, 106, 147, 163, 164, 235, 252, 254, 260, 334, 355, 357, 410, 412, 413, 435, 468, 474, 476, 527, 528, 612, 648}$), 21 at T ($T_{20, 34, 56, 83, 135, 136, 148, 156, 257, 315, 376, 436, 482, 529, 534, 540, 601, 638, 651, 662, 664, 671}$) and 17 at N ($N_{64, 97, 183, 264, 290, 306, 307, 339, 362, 428, 461, 535, 537, 641, 655, 667}$).

The Motif Scan web site MyHits (http://myhits.isb-sib.ch/cgi-bin/motif_scan) predicted one putative cAMP and cGMP-dependent protein kinase phosphorylation site (position 145-148), 11 casein kinase II phosphorylation sites (position 20-23; 81-

84; 122-125; 138-141; 147-150; 260-263; 283-286; 405-408; 412-415; 435-438; 540-543), 6 protein kinase C phosphorylation sites (position 20-22; 435-437; 468-470; 482-484; 514-516; 540-542), and one tyrosine kinase phosphorylation site (position 203-210).

Three conserved domains are found in the putative amino acid sequence of CasPPO-hemo (Fig. 4A): truncated hemocyanin N and the full hemocyanin M, and C domains (http://blast.ncbi.nlm.nih.gov). A thiol-ester complement motif (GCGWPQHM, underlined in Fig. 1A) was also detected at the C-terminus of the protein sequence. The putative cleavage sites for converting PPO to PO are found at R_{35}-V_{36}, R_{50}-S_{51} and R_{94}-F_{95} in the N-terminus of the putative CasPPO-hemo (http://web.expasy.org/cgi-bin/peptide_cutter/peptidecutter.pl).

The CasPPO shows 70-87% sequence identity to other crustacean PPOs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree that was generated with 19 putative PPOs of other crustacean species including *Daphnia magna* PPO (Fig. 4B) contains three groups: 1) Portunidae and Cancridae including *C. sapidus*, 2) Penaeidae and Astacidae, and 3) Palaemonidae group (*Macrobrachium sp.*). CasPPO-hemo is most closely related to the PPOs found in species belonging to the family Portunidae: *Portunus trituberculatus* and *Scylla serrata,* compared to those belonging to the family Cancridae: *Cancer pagurus* and *Metacarcinus magister.*

2.4.2. Tissue distribution of the *CasPPO*-hemo

The expression of *CasPPO-hemo* is exclusive in hemocytes, while nine other tissue cDNAs tested do not show *PPO* expression: eyestalk, thoracic ganglia

complex, brain, heart, gill, antennal gland, white muscle, Y-organs, and hypodermis (Fig. 5).

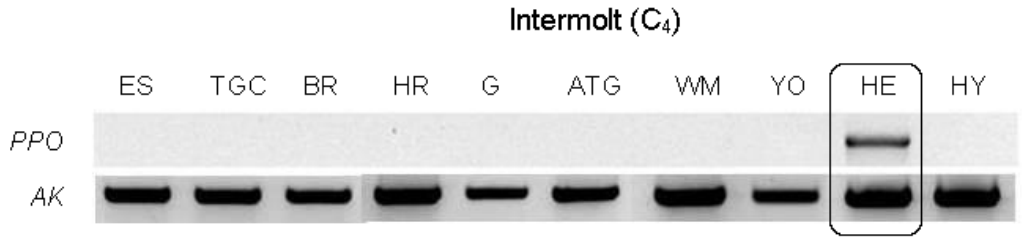


Figure 5. Expression of *CasPPO-hemo* in the tissues obtained from a juvenile crab at intermolt stage. Each cDNA sample (equivalent of 12.5 ng total RNA) was amplified for the entire ORF (2,019 nt) using an end-point PCR assay with Myfi High-Fidelity taq (Bioline) at annealing at 58ºC; extension at 72ºC for 1 min; 35 cycles). *CasAK* as a reference gene was amplified with the same sample cDNAs. ES = eyestalk; TGC = thoracic ganglia complex; BR = brain; HT = heart; G= gill; ATG = antennal gland; WM = white muscle (located at the base of the fifth walking leg); YO = Y-organs; HE = hemocytes; HYP = hypodermis.

2.4.3. The levels of *CasPPO*-hemo expression in hemocytes during molt cycle

The levels of *CasPPO-hemo* expression remain constant in all molt stages tested (ranging 1.8 x 10^5 copies/µg total RNA to 6.0 x 10^5 copies/µg total RNA), except in the ecdysis (Fig. 6A). The expression levels of *CasPPO-hemo* at ecdysis show a large variation from 5.9 x 10^4 to 6.3 x 10^6 copies/µg total RNA. The levels of arginine kinase (*CasAK*) measured as a reference gene in the same sample cDNAs, are constant throughout the molt cycle (Fig. 6B), except at premolt stage when the levels are slightly, but not significantly lower than those at the other stages.

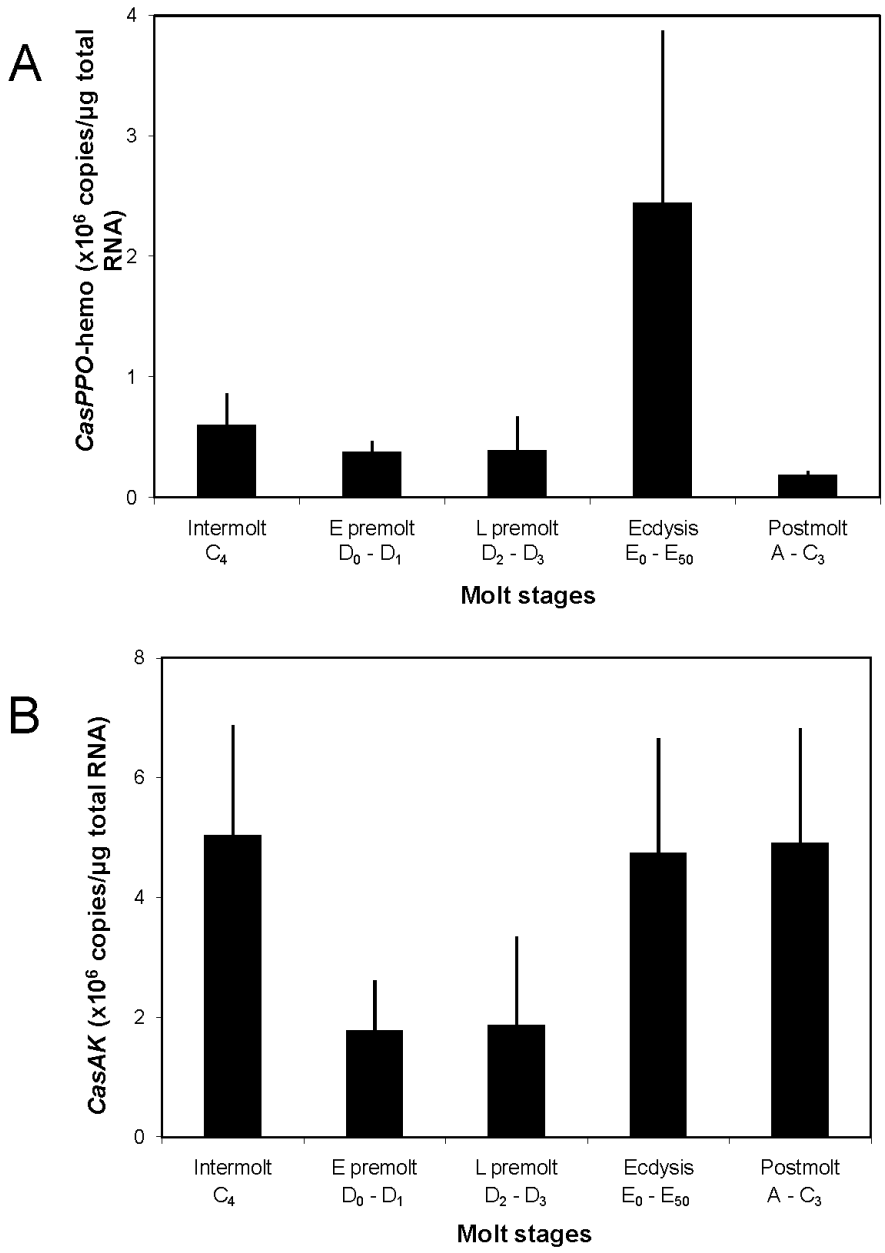


Figure 6. Expression levels of *CasPPO-hemo* (A) and *CasAK* (B) in hemocytes during the molt cycle. No statistical differences (Kruskal-Wallis ANOVA) are found in *CasPPO-hemo* or *CasAK*. The data are shown as mean ± SE (copies/µg total RNA, n=5-6).

2.4.4. Changes in CasHLS-PO activity and the number of circulating hemocytes during the molt cycle

The number of total hemocytes and PO activity of hemocytes vary during the molt cycle (Fig.7). The number of hemocytes at intermolt stage is 6.1 ± 0.8 $x10^6$ cells/ml hemolymph, which is gradually increased to the highest levels of 12.1 ± 3.0 $x10^6$ cells/ml hemolymph at late premolt. The numbers are then reduced to the lowest density at ecdysis (4.8 ± 0.7 $x10^6$ cells/ml hemolymph), but at post molt stage (9.5 ± 1.9 $x10^6$ cells/ml hemolymph), they are gradually elevated to the similar levels of those at the premolt stage. During the molt cycle, the levels of the CasHLS-PO activity change ~ three and a half folds with the highest at early premolt stage (2.9 ± 0.4 OD/THC protein/ml of hemolymph) and the lowest at ecdysis (0.8 ± 0.3 OD/THC protein/ml of hemolymph). These levels correspond to those of hemocyte numbers, except at late premolt.

2.4.5. Substrate specificity of CasHLS

CasHLS-PO activity requires the presence of trypsin in the reaction, showing a significant threefold increase in PO activity with diphenols (L-dopa, dopamine and catechol) as substrates. CasHLS also contains a small amount of PO activity that is not dependent on the addition of trypsin.

CasHLS-PO shows its substrate specificity in order of L-dopa, dopamine, catechol and L-tyrosine. The greatest PO activity is noted with L-dopa (3.6 ± 0.7 OD/mg HLS) followed by dopamine (1.2 ± 0.3 OD/mg HLS) and catechol (1.1 ± 0.2 OD/mg HLS). L-tyrosine yielded the lowest activity ($P < 0.05$) in about 0.1 OD/mg HLS (Fig. 8A).

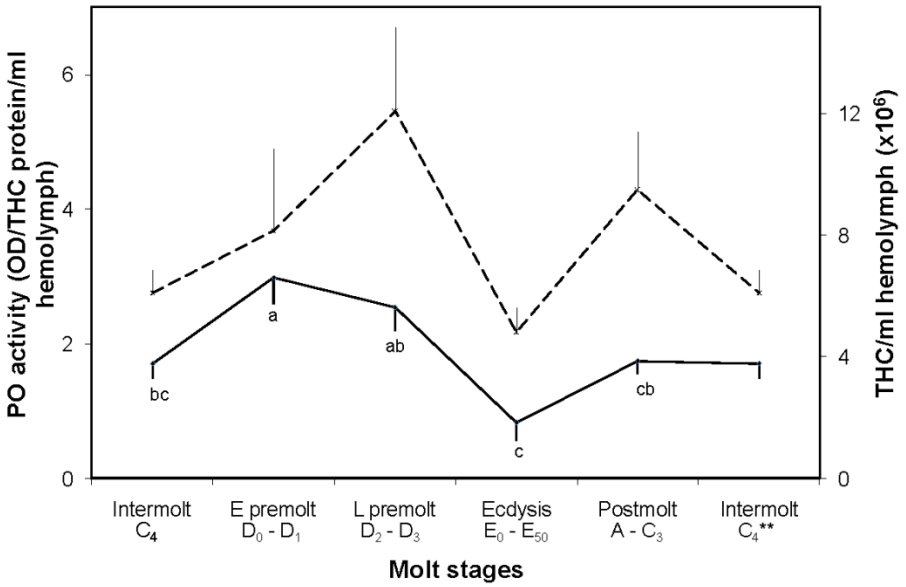


Figure 7. A) Changes in the number of hemocytes in the hemolymph (dashed line) and CasHLS-PO activity during the molt cycle (solid line). Different letters indicate significant differences at $P < 0.05$. The data are shown as mean ± SE (the number of hemocytes/ml hemolymph, n=4-12) for THC and (OD/mg HLS, n=5-15) for PO activity.

2.4.6. The effect of Ca2+ on CasHLS-PO activity

The addition of EDTA, a divalent chelator, at 20mM final concentration significantly reduces PO activity present in CasHLS (Fig. 8B). No additional Ca^{2+} to CasHLS yields the PO activity of 1.8 ± 0.7 OD/mg HLS that is ~ threefold higher than those with the addition of EDTA (0.5 ± 0.1 OD/mg HLS). Additional Ca^{2+} at the final concentration of 5, 10, 20mM does not affect the PO activity, compared to no Ca^{+2} addition.

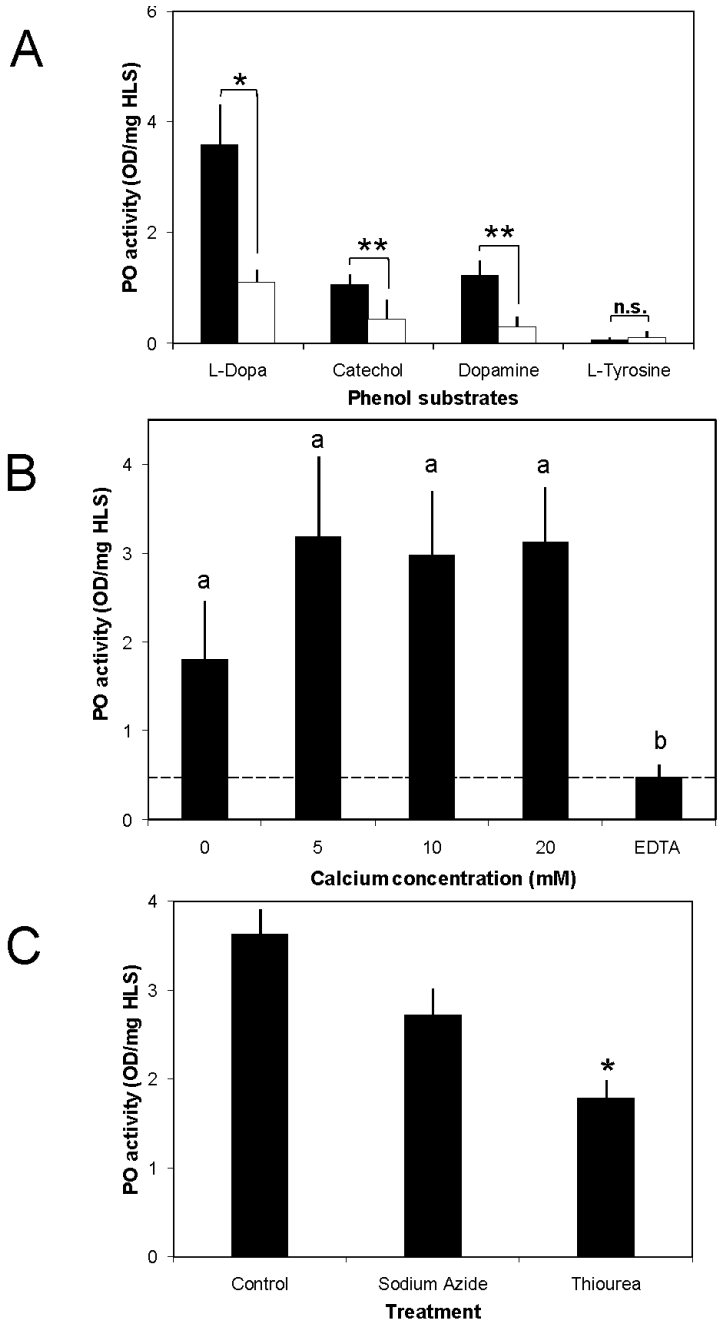


Figure 8. Enzyme properties of PO activity located in CasHLS prepared from the crabs at intermolt. A) Substrate specificity: Open bars represent the activity without trypsin; closed bars, with trypsin (n=6). B) Ca^{2+} dependent PO activity (n=6). C) Inhibitors of PO activity (n=3). The data are shown as the mean ± SE (OD/mg HLS). Different letters and '*' indicate statistical significance at $P < 0.05$.

2.4.7. Inhibitors of CasHLS-PO activity

Thiourea and sodium azide known as the inhibitor of tyrosinase and laccase, respectively, were tested to determine if they suppressed the CasHLS-PO activity. As shown in Fig. 8C, thiourea suppresses the CasHLS-PO activity by 50% (1.7 ± 0.2 OD/mg HLS), compared to controls ($P = 0.02$), which shows an activity of 3.6 ± 0.3 OD/mg HLS. Sodium azide, on the other hand, decreases CasHLS-PO activity, compared to controls, but no statistical difference is noted.

2.5. Discussion

This manuscript describes the isolation of the full-length cDNA sequence of *CasPPO-hemo* (2,806 nt) and the levels of its expression and PO activity during the molt cycle. The properties of CasHLS-PO activity are further defined in terms of substrate specificity and a specific inhibitor. Our results suggest that CaHLS-PO activity is likely to be a tyrosinase.

Crustacean PPOs as the members of crustacean hemocyanin superfamily [20,38,85,170] consist of three putative structural hemocyanin domains: truncated N domain and two complete full M and C domains, and the signatures of PPOs with highly conserved two copper binding motifs. The *CasPPO* ORF (2,019 bp) encoding a 672 putative aa sequence also display the two copper binding motifs and three putative hemocyanin domains, as those reported in *P. trituberculatus*, *S. serrata*, and *L. vannamei* [171–173]. With > 70% sequence identity among crustacean PPOs, CasPPO is related most closely to those found in other Portunidae crab species: S. *serrata* and *P. trituberculatus*.

It is widely known that the activation of zymogen PPO to PO depends on a trypsin-like proteinase known as PPAE. Three putative sites cleaved by PPAE were predicted in CasPPO-hemo: R_{35}-V_{36}, R_{50}-S_{51} and R_{94}-F_{95}. In insects, a conserved R-F residue, usually located within the first 50 aa residues is known as the cleavage site for PPO activation [89]. Similarly, a PPAE cleaves crustacean PPOs at R-X residues (X= S in *P. trituberculatus* [173] and *Eriocheir sinensis* [45], L in *L. vannamei* [94] and V in *S. serrata* [172]. Hence, it is likely that the cleavage site of CasPPO may be either at R_{35}-V_{36} or R_{50}-S_{51}.

Twenty- two phosphorylation sites were predicted in CasPPO-hemo similar to *Armigeres subalbatus* [174]. CasPPO-hemo contains 64 putative glycosylation sites: 47 *O*-glycosylation and 17 *N*-glycosylation. Particularly, the number of putative *N*-glycosylation sites predicted in CasPPO is much greater than 4-7 putative sites found in other PPOs including *Pascifastacus leniusculus* [44], *P. trituberculatus* [173], *A. subalbatus* [174], *S. serrata* [172], *L. vannamei* [171], *Cortesia glomerata* [175] and *Macrobrachium rosenbergii* [176].

The distribution of *PPO* expression varies in crustacean and insect tissues. In crustaceans like *L. vannamei*, *E. sinensis, P. trituberculatus* and *Marsupenaeus japonicus*, tissues such as hemocyte, hepatopancreas, the stomach epithelium, midgut, and nerve cord cells have *PPO* [45,47,94,173]. Insects (*A. subalbatus*, *Anopheles stephensi*, *Ostrinia furnacalis*, *Pimpla hypochondriaca*, *Apis mellifera*) also have *PPO* in multiple tissues including hemocytes, serum, venom-producing cells, integument, epidermis, fat body and midgut [174,177–180]. In this study, *PPO* expression occurs in hemocytes among the various tissues of *C. sapidus*, indicating

that hemocytes are likely the main source of PPO, as in other crustaceans (*L. vannamei*, *S. serrata*, and *Penaeus monodon*) [93,171,172]. However, since we examined only 10 tissues, it is likely that blue crabs may also have PPO expression in other tissues that have PPO in other crustacean species.

Both insect and crustacean PPOs show stronger PO activity with diphenols as substrates than monophenols [25,35,181]. As expected, the *Cas*HLS-PO activity prefers to diphenols: L-dopa, dopamine and catechol to monophenol: L-tyrosine. The highest PO activity is measured with L-dopa, together to dopamine. Hence, the most crustacean PO activity has been evaluated with L-dopa as a substrate [45,46,149,151,182,183]. The CasHLS-PO activity is dependent on Ca^{2+}, as the presence of EDTA (20mM) significantly reduces its activity. This result agrees with the POs found in other crustaceans which are known as Ca^{2+} dependent enzymes [46,168,169]. However, the required concentration of Ca^{2+} for PO activity seems to be negligible in *C. sapidus*, as the activity measured without additional Ca^{2+} is still significantly higher than those measured with EDTA. It is known that Ca^{2+}activates PPAE for transformation into PO [169,184,185] and is required for trypsin reaction [186]. Thus, we suggest that Ca^{2+} in the reaction medium acts on trypsin activity but not on CasHLS-PO activity. CasHLS-PO activity in the presence of PO inhibitors: thiourea, specific to POs [46]; and sodium azide for laccases [21] indicates that CasHLS-PO is likely to be a tyrosinase.

Our data show that the molt stage does not significantly affect the number of hemocytes, in contrast to the previous reports in penaeid shrimps [97,98]. However, the levels of CasHLS-PO activity vary in that only early postmolt show a significant

increase in the activity. At late premolt, on the other hand, the CasHLS-PO activity is inverted with the THC, indicating that there may be some changes in the structure of hemocyte populations. Crustacean hemolymph carries three types of hemocytes; hyaline, semigranular and granular hemocytes [37,82,96–98,115]. Yet, the hemocyte for PPO source has been conflicting [37,82,97,98] and the granules within semigranular and granular hemocytes contain the PPO [82,100,104,105].

The levels of CasHLS-PO activity can be affected by the following factors: 1) levels of PPO expression and translation, 2) the number of PPO expressing hemocytes, 3) a relationship between the levels of PPO expression vs its translation, and 4) the levels of PPAE activity, in addition to the health status of animals. In this study, we used the animals raised in the blue crab hatchery (Baltimore, MD) which were offspring of the brood females that had been pre-screened for typical diseases like *Hematodinium* [187] and a reo-like virus [188]. Our experimental crabs are considered healthy. Thus, the changes in the CasHLS-PO activity during the molt cycle are considered intrinsic, naturally occurring physiological facts, not caused by infection(s) or disease(s).

It has not been known in *C. sapidus* which type(s) of hemocytes express PPO, or if all the hemocytes have the same levels of *PPO* expression and PO activity during the molt cycle. Furthermore, little is known for the translation rate of *CasPPO-hemo* and the half-life of CasPPO-hemo. If all the hemocytes contain the same amounts of *PPO* expression and PPO, the levels of PO activity directly correspond to those of THC. Instead, our results show in Fig. 7 reveal that this is not the case, particularly at early and late premolt and postmolt stages and indicates that

the hemocytes do not contain equal amounts of *PPO* expression and PPO. Indeed, a specific type or types of hemocytes are responsible for CasPPO-hemo.

Importantly, our data indicate that a specific type of hemocyte recruitment into the hemolymph may occur in a molt-stage dependent manner. The molt cycle of crustaceans is controlled intimately by interactions between crustacean hyperglycemic hormone family and molting hormone, ecdysteroids [4,6,7,66]. It is a well-established fact in arthropods that elevated hemolymphatic ecdysteroids during premolt stage stimulate cell divisions in many tissues for their somatic growth and layering the new cuticle [6,189]. We infer that the increase in the numbers of THC may be driven by rising hemolymphatic ecdysteroid concentrations during premolt [190] and that ecdysteroids may trigger the proliferation of specific hemocytes.

The different molt stages: early and late premolt and postmolt, have shown a slight increase in the THC, compared to those of preceding stages: intermolt, early premolt, and ecdysis. The modest increase in the THC of the animals at early premolt showing elevated ecdysteroids [4,190] appears to be directly accounted for by the significantly elevated levels of PO activity. This may be due to the fact that there may be a typical cell type containing the PO activity and increase in the translation rate of *CasPPO-hemo*. The levels of *CasPPO-hemo* expression at these stages remain constant, compared to the other stages. At the late premolt and postmolt stages, on the contrary, the amounts of elevated THC have no effect on the levels of PO activity as they are kept constant. Thus, we suggest that these cells are not the same type as those seen at early premolt stage.

Interestingly, the reduced THC at the ecdysis stage, compared to that at premolt reflects the decreased levels of PO activity. And, a great degree of variation in the expression of *CasPPO-hemo* at this stage indicates a probable upregulation of *PPO* expression in these *CasPPO* expressing hemocytes. This does not immediately affect PO activity, indicating that there is a differential regulation(s) between the transcription of *CasPPO* and its translation.

Arthropod cuticles undergo dramatic, morphological, biochemical changes from softness to hardness during the molt cycle. The newly laid cuticle-hardening process in crustaceans is generally known to occur during and immediately after ecdysis. More specifically, however, it involves a two-sequential sclerotization process. Firstly, the epicuticle being layered by the hypodermis at early premolt is completed its sclerotization at the late premolt [18]. And secondly, the deposition of exocuticle begins at premolt and is completely formed prior to ecdysis, the hardening of which takes place at the postmolt. Thus, the hardening process of epicuticle and exocuticle clearly occurs at two different molt stages [2,18]. This allows the animals to stretch the soft cuticle of the body through iso-osmotic water uptake during and immediately after ecdysis [8], resulting in somatic growth known as molt increment.

Cuticle-hardening process that has been well documented in insects [11,17,21], is known to be hormonally regulated and to involve hemocytes and PPO. In *C. sapidus*, bursicon is known to stimulate the recruitment and granulation of hemocytes beneath the hypodermis at ecdysis stage [62], while the PPO transport from hemocytes to cuticle via the migration of hemocytes are reported in insects [25,30] and crustaceans [37]. Specifically, the aggregation of hemocytes is molt-

stage dependent and is found during the postmolt [37] and early and late premolt stages [38]. The low CasHLS-PO activity and THC at ecdysis might be a consequence of the recruitment of a specific type of hemocyte beneath the hypodermis for transport and allocation of PPO in the new cuticle as reported [37,62]. The initial shell-hardening being a time-sensitive process, usually completes within 1-1.5 hrs after ecdysis of *C. sapidus*. We consider that the levels of PO activity provided by hemocytes might not be sufficient for the process. Thus, it is plausible to suggest that there may be another tissue source of PPO, possibly hypodermis in which is also known to express *PPAE* during premolt [191].

2.6. Conclusion

CasPPO-hemo with 2,806 nt encodes the putative 672 aa residue protein containing in the order of three conserved hemocyanin N, M, and C domains. The levels of *CasPPO-hemo* remain constant during the molt cycle. However, changes in the number of hemocytes and the PO activity residing in hemocytes during the molt cycle may implicate their involvement in the shell-hardening process of *C. sapidus*.

Chapter 3: The involvement of hemocyte prophenoloxidase in the shell hardening of the new cuticle of the postmolt blue crab, *Callinectes sapidus*

3.1 Abstract

At each molt, cuticular structures of arthropods undergo dramatic changes from being soft to becoming hard. The shell-hardening process of decapod crustaceans includes sclerotization and mineralization. Hemocyte PPO plays a central role in the melanization and sclerotization particularly in wound healing in crustaceans. However, its role in the crustacean initial shell-hardening process is much less investigated. Based on earlier studies where heavily granulated hemocytes aggregated beneath the hypodermis during ecdysis, it was suggested they are involved in the shell-hardening process. In order to determine if hemocytes and PPO have a role in the shell-hardening of crustaceans, a knockdown experiment using specific *CasPPO-hemo-dsRNA* was carried out using juvenile blue crabs, *Callinectes sapidus*. Multiple injections of *CasPPO-hemo-dsRNA* reduce specifically the levels of *CasPPO-hemo* expression by 57% and PO activity by 54% in CasHLS at the postmolt, with no changes in the total hemocyte numbers. Immunocytocemistry and flow cytometric analysis with a specific antiserum generated against CasPPO show granulocytes, semigranulocytes and hyaline cells as the cellular sources for PPO at the postmolt. Interestingly, the cellular sources of PPO vary by molt stage, together with the composition of hemocyte populations. Throughout the molt cycle, granulocytes always contain PPO. Semigranulocytes and hyaline cells become CasPPO immune-positive only at early premolt and postmolt, especially hyaline cells

during postmolt, indicating that PPO expression in these cells may be involved in the shell-hardening process of the blue crab.

3.2 Introduction

Like all ecdysozoans, crustaceans undergo the molting process repeatedly during their life span in order to complete their growth, sexual maturation, and reproduction [1,2]. Successful molting is precisely coordinated through interactions of multiple hormones and neurohormones [4–8]. The newly laid cuticle that is synthesized by the hypodermis at premolt is soft and pliable and allows the expansion of the body through the uptake of isosmotic water at ecdysis [1,2,144,192,193].

The hardening process of the crustacean new cuticle is complex and primarily involves two sequential processes: sclerotization and mineralization [11,12,14,15,18]. The sclerotization occurs in the new soft and pliable cuticle immediately after ecdysis [70,73,194]. Mineralization as the second step involves the incorporation of calcium carbonate into the protein matrices of the cuticle 2.5 hrs after ecdysis [15,16,18,69,70,75,195–197].

In insects, sclerotization involves both prophenoloxidases (PPOs) and laccases that cross-link chitinous to protein fibers in the matrix of the cuticle through the oxidation of phenol substrates [11,12,17,21,198]. In decapod crustaceans, there is no report on the presence of laccase, whereas PPO present in hemocytes is primarily known for immunity responses [39,41,43,80,93,132]. However, earlier studies suggest that hemocytes may be involved in the shell-hardening process in crustaceans [37,38,95]. Heavily granulated hemocytes aggregate and are found

beneath the hypodermis during ecdysis [37,38]. These findings may suggest that crustacean PPO may be transported into the new cuticle for sclerotization [37,38], similar to that found in insects [25,30].

Three types of hemocytes are found in the hemolymph of decapod crustaceans [82,100,104]: granulocytes (G); semigranulocytes (SG); and hyaline cells (H). With the major function of PPO in the immune response, its cellular source varies by species and molt stage. It is reported that Gs and SGs are the exclusive PPO sources at the intermolt of *Homarus americanus*, *Panulirus interruptus*, *Loxorhynchus grandis*, *Pacifastacus leniusculus*, *Penaeus japonicus*, *Procambarus clarkii*, and *Scyonia ingentis* [82,98,100,105,115]. The Hs are the source of PPO during the ecdysis of *Uca pugilator* [37]. Nevertheless, Gs contain the PO activity in *P. interruptus* and *L. grandis*, a small group of Hs was found also showed PO activity [82]. With the differences in the cellular sources, PPO may be produced by a specific cell type at different molt stages.

Interestingly, the total hemocyte numbers and their PO activity vary during the molt cycle of *Callinectes sapidus* [95]. This suggests that PO activity may be derived from different types of hemocytes. Specifically, the initial shell-hardening process may be regulated by the PPO that is expressed in the hemocytes only at postmolt.

Herein, we report that during the molt cycle, there are changes in the types of hemocytes that are responsible for PPO expression. The role of hemocyte PPO in the shell-hardening process is defined with RNAi experiments, specifically using a multiple administration of *CasPPO-hemo-dsRNA* injections. To this end, we have

determined the effects of *dsRNA* injections on the transcript levels of *CasPPO-hemo* by a qPCR assay and protein levels in hemocytes using immunocytochemistry (ICC) and flow cytometry. More importantly, the cuticle hardness of these animals has been measured.

3.3 Materials and Methods

3.3.1 Animals

Juvenile *C. sapidus* crabs (15-30 mm carapace width, CW) were obtained from the blue crab hatchery [Aquaculture Research Center, the Institute of Marine and Environmental Technology (IMET), Baltimore, MD]. The animals were reared in individual compartments in recirculated, aerated artificial seawater (25 ppt; 22ºC) as described [62,158,159]. Juveniles with ~80-90 mm CW were molt-staged for experiments by following the criteria as described [160]. All animals were at intermolt stages, unless stated otherwise. Male and female crabs were used randomly.

3.3.2 Identification of hemocyte types in the hemolymph of *C. sapidus*

First, in order to identify hemocyte types, hemolymph of the animals at various molt stages were collected in a fixative (4% PFA in 10 mM cacodylate buffer) as described [95] at 1:1 ratio (v:v). Hemolymph smears were prepared as described [100] and stained with hematoxylin (1 min) and eosin (4 sec). Then they were observed immediately and photographed under a compound microscope (National Microscopes). The images were analyzed using AmScope MT software for

the cellular diameters (mean ± SE μm), with an assumption that cells are round. The hemocyte types were identified by following the criteria as stated [100,104]. In brief, the properties of these cells were detailed as listed in Table 2.

The types of hemocytes present in juvenile hemolymphs at different molt stages (n=6-18) were also identified using flow-cytometry. Fixed hemocytes were stained with SYBR-Green I (2X) nucleic acid staining (FMC BioProducts) as described [199] at 1:100 ratio (v:v = SYBR-Green I:sample). After 10 min incubation at RT, 50 μl of each sample was analyzed using a C6 CFlow flow-cytometer (Accuri Cytometers), with setting at a media fluidic rate (35 μl/min) and a core size of 16 μm at FL1-H and SSC-H. The types of hemocytes were characterized by their sizes (FSC-H) and cytosolic complexities and granularities (SSC-H).

3.3.3 Production of a rabbit-anti-CasPPO-hemo serum

Two peptides that were modified by adding 'C' at C-terminus: V_{421}ESRGQKNQL$_{430}$C and V_{199}SRDRKGELF$_{208}$C of the putative CasPPO-hemo (GenBank AGE48302.1) were synthesized (Peptide 2.0 Inc). These two regions were selected to increase the antiserum specificity by excluding the hemocyanin domains and to enhance the immunogenecity by including the surface of PPO (Phyre^2; http://www.sbg.bio.ic.ac.uk/phyre2/). Two different conjugation methods were carried out: m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) conjugating the petides to bovine thyroglobulin (bTG, Sigma) at a molar ratio of 50:1:15 (peptide: bTG: MBS) and 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) at a molar ratio of 50:1:560 (peptide:bTG: EDC) [200]. At the end of conjugation reactions (overnight at 4ºC), 90% of each conjugated material was mixed

and lyophilized for the antiserum production (ProteinTech). The remaining 10% was dried in a SpeedVac (Jouan) and used for pre-absorption controls. The rabbit antiserum (α-CasPPO-hemo) was further characterized in terms of a dilution factor, specificity and cross-reactivity using western blot analysis with hemocytes lysate supernatant (HLS) (Suppl. Figs. S1-S2; Suppl. Protocol S1) [201].

3.3.4 Determining CasPPO-hemo function in the initial shell-hardening process using dsRNA

3.3.4.1 In vitro transcription of *CasPPO-hemo-dsRNA*

The template for *dsRNA* was generated by amplification of hemocyte cDNA with double-strand (ds) *CasPPO-hemo* primers (Table 3). The template *dsRNA* amplification region of *CasPPO-hemo* was selected based on its specificity by being free of hemocyanin domains. After purification using these PCR products (243 bp), the template was *in vitro* transcribed using a TranscriptAid™ T7 High Yield Transcription kit (Fermentas) as described [202]. Prior to injections, *CasPPO-hemo-dsRNA* were dissolved in 0.2 μm filtered crustacean saline solution containing phenol red at 0.001% [203] to give a final concentration at 0.1 μg/μl Phenol red was added to monitor the delivery of injection materials.

Table 2. Properties of the different hemocyte types of *C. sapidus* and other decapod crustaceans

Hemocyte type	Characteristics under a light microscope	Reference
Granulocytes	Contain many large and a few small granules. Spindle, around or oval form	Wenli and Shield, 2007 (*C. sapidus*)
	Large eosinophilic granules. Low nuclear/cytoplasmic ratio. Mainly spherical shapes. The granules present in various degree of density that were compacted or fused into a homogeneous matrix	Lanz et al., 1993 (*P. clarkii*)
Semigranulocytes	Contain many small and a few large granules. Morphology varies: spindle, oval or round shape. Cell sizes vary from 12 to 20 um.	Wenli and Shield, 2007 (*C. sapidus*)
	Presence of small, round or slightly oval, eosinophilic granules. Ovoid and fusiform cells. Low nuclear/cytoplasmic ratio. Round or spindle shape, but most often oval.	Lanz et al., 1993 (*P. clarkii*)
Hyaline cells	Contain a few or no granules in the cytoplasm. Cells have a spindle or round form. Cell sizes with 9 -18 um diameter.	Wenli and Shield, 2007 (*C. sapidus*)
	Pleomorphic: flat pseudopodia normally extending from the hemocyte surface. Cytoplasm without granules and high to low nuclear/cytoplasmic ratio. Nucleus with well-defined heterochromatin.	Lanz et al., 1993 (*P. clarkii*)

3.3.4.2 Effects of multiple injections of *CasPPO-home-dsRNA* on shell-hardness at postmolt animals

3.3.4.2.1 Levels of *CasPPO-hemo* gene expression

The role of *CasPPO-hemo* in shell-hardening was examined using multiple injections of *CasPPO-hemo-dsRNA*. The crabs at intermolt stage (85.5 ± 2.3 mm CW, n=7) were injected with 10 µg *CasPPO-hemo-dsRNA* every other day until ecdysis (20-28 total injections). Control animals (88.0 ± 2.1 mm CW, n=7) were injected with 100 µl of crustacean saline. In order to determine the levels of *PPO* and PO activity at postmolt, hemolymph samples were collected after crabs completed the expansion of the body, at the half of early postmolt [194] (~24 hrs after ecdysis). The hemolymph samples (400 µl) were collected at 4^{o}C for RNA extraction [95] and hemocyte lysate supernatant (HLS) as described [95] (see below). The expression levels of *CasPPO-hemo* were estimated using qPCR assay with Fast SYBR Green Master Mix (Applied Biosystem) on a 7500 Fast Real-Time PCR System (Applied Biosystem). The procedures for qPCR standards were as described in [95]. The qPCR primers are listed in Table 3.

Table 3. List of primers used for *dsRNA* template amplification and qPCR assay

Primers	5' to 3' nucleotide sequence
*CasPPO*3F6-QF	CACCTCTTCATCCATCACAAACTC
*CasPPO*5R6-QR	CAACCACACCCACAGAAGTTAAAG
CasPPO-ds3F9	*TAATACGACTCACTATAGGG*CTTCGTCTACAGCATCAAGGTG
CasPPO-ds5R9	*TAATACGACTCACTATAGGG*TCCAAGTCACGGAAGGTAAAGT

Q = qPCR primers; ds = *dsRNA* primer with T7 promotor sequence italicized

3.3.4.2.2 Levels of CasHLS-PO activity and CasPPO-hemo protein expression

The PO activity of *Callinectes* HLS (CasHLS) was determined as described [95]. The data are shown as mean ± SE (n=6-7) of PO activity (OD/mg HLS).

For determining levels of CasPPO-hemo protein in hemocytes, hemolymph of the experimental animals at 24 hrs after ecdysis were withdrawn (100 µl) directly into a 1 ml syringe containing a fixative (4% PFA in 10 mM cacodylate buffer) [95] at 1:1 ratio (v:v). The contents of CasPPO-hemo in hemocytes were analyzed by the fluorescent intensity of the positive signal by α-CasPPO-hemo through an ICC analysis (described below) using ImageJ [204]. The ICC sample images were taken using the same settings. The hemocytes were also analyzed for cytosolic complexity using a C6 CFlow flow-cytometer (Accuri Cytometers). Additionally, CasPPO-hemo was detected in CasHLS using a western blot analysis (Suppl. Protocol S1).

3.3.4.2.3 Hardness of postmolt cuticle

After ecdysis, the cuticle hardness of each crab was measured using a hand-held Shore-type durometer (00 gauge, Instron) as described [62,195] for 48 hrs after ecdysis the period which includes all early postmolt stages as described [194]. The hardness of the carapace was measured in the mesogastric area (Suppl. Fig. S2). Because the rates of hardening vary depending on the area of cuticle [195], the hardness was measured at 6-8 different areas from each animal and averaged. The data are shown as mean ± SE (n=5-7) of durometer units (DU) as described [195].

3.3.5 Differential hemocyte counting during the molt cycle

The differential hemocyte type counting (DHC) and total hemocyte counting (THC) were obtained by flow cytometric analysis. The hemocytes (as above) from juvenile blue crabs at different molt stages (n=6-18) were stained with SYBR-Green I (2X) nucleic acid staining (FMC BioProducts) as described previously [199]. The types of hemocytes were characterized by their sizes (FSC-H) and cytosolic complexities (SSC-H). The data are presented as mean ± SE (number of cells/ml hemolymph).

3.3.6 Identification of CasPPO-hemo in hemocytes during the molt cycle

3.3.6.1 Immunocytochemistry (ICC)

Hemolymph (50 µl) was sampled (n=5-6) as described above and centrifuged for hemocyte isolation [95]. The pelleted hemocytes were resuspended in 50 µl of fixative solution (4% PFA in cacodylate buffer 10mM, pH 7.1). A drop of the samples (~5-8 µl) was smeared on a slide glass and dried for 20-30 min at RT [205]. These slides were immersed in methanol for 30s, rinsed in milliQ water and incubated for 10 min in PTX1 solution (PBS containing 0.25% Triton X-100 and 5% dimethyl sulfoxide, DMSO) [205,206]). After rinsing with milliQ water three times (5 min each), the slides were blocked with blocking buffer (PTX 1 containing 2% BSA) overnight at 4ºC. Then, the slides were incubated with α-CasPPO-hemo serum (1:200 dilutions in the blocking buffer) for 30 min at RT and were immediately washed 5 times in PBS (5 min each). The incubation with Alexa Flour goat anti-rabbit IgG (Life Technologies) at 1:100 dilution was carried out for 30 min at RT under

darkness. After washing 5 times in PBS, the slides were briefly post-fixed in the fixative solution and washed twice with PBS. Finally, the slides were mounted with a ProLong Gold antifade reagent with DAPI staining (Life Technology). The hemocytes were examined for their subtypes under a fluorescent microscope (Axioplan, Zeiss) and images were captured with a digital camera (AxioCam MRc, Zeiss). Staining intensity were further analyzed and calculated using Zen 2012 and ImageJ [204].

As for the pre-absorption control, synthetic CasPPO peptides (10 nmol each) were incubated with α-CasPPO-hemo serum (a final dilution at 1:10) overnight at 4ºC through gentle mixing on a magnetic stirrer. It was then diluted to a final dilution of 1:200, the same as the α-CasPPO-hemo serum.

3.4.6.2 Flow cytometry analysis

The hemocytes collected from the crabs at intermolt (n=8) and postmolt (n=6) stages were fixed and washed in 1 ml PTX2 (0.1M phosphate buffer containing 0.5% Triton-X and 2% BSA; DMSO is removed from the buffer solution improving cell suspension in the samples for flow-cytometry) and incubated for 15 min at RT for blocking. Then, the hemocytes were centrifigued (800 g for 10 min, 4ºC) and incubated in α-CasPPO-hemo solution similar to that of ICC (1:200 dilutions, using 200 µl PTX2 + 2% BSA) for 30 min at RT [207]. Afterwards, the samples were washed twice in 1 ml Dulbecco's Phosphate Buffer Saline, PBSG as described in [207], gently pipetting up-down and living in a rotational shaker for 5 min. The incubation with the secondary antibody, Alexa Fluor goat anti-rabbit IgG (Life Technology) was carried out under dark for 30 min at RT (1:100 dilutions in 200 µl

PTX2). The samples were then washed once in 1 ml PBSG, and finally resuspended in 100 µl PBSG. Forty µl of each sample were read in a C6 CFlow flow-cytometer (Accuri Cytometers) under the conditions described above.

In each case, fixed hemocytes were washed once and resuspended in 100 µl PBSG as negative controls. The positive staining of hemocytes with α-CasPPO-hemo serum were established and confirmed with negative controls and validation beads that have been provided by the manufacturer (Spherotech 8-Peak Validation Beads FL1 - FL3, BD Accuri).

3.4.7 Statistical analysis

All data were subjected to normality test using Kolmogorov-Smirnov test and the homogeneity of variances by Cochran C test (Statistica 7.0, StatSoft., Inc). Statistical significance of one way ANOVA was accepted at $P < 0.05$ and post-hoc Tukey test was adopted to distinguish the source of variation among the molt stages. The data are presented as mean ± SE (n), where n is the number of crabs. For shell-hardness data, analysis of covariance (ANCOVA) was used.

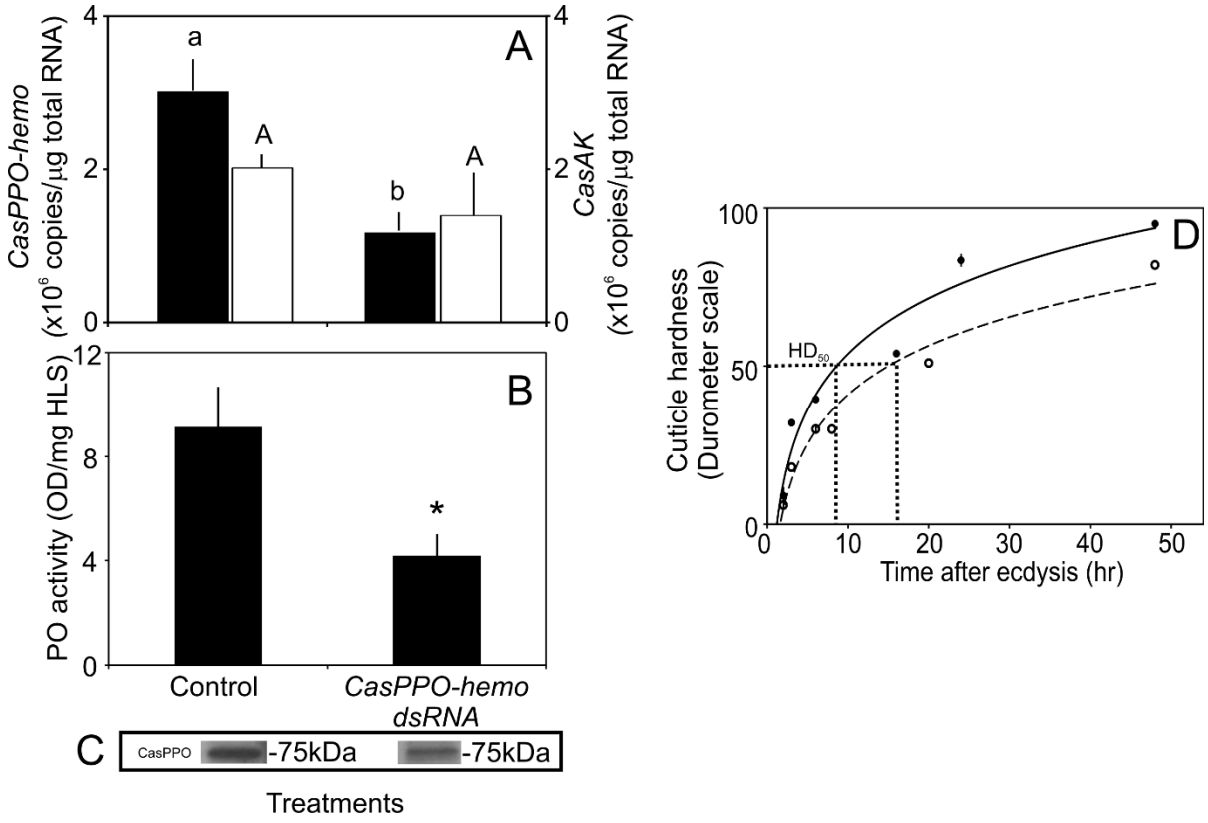


Figure 9. Effects of multiple *CasPPO-hemo-dsRNA* injections (20-28 injections) on the shell-hardening process. (A) Levels of *CasPPO-hemo* expression in hemocytes and (B) CasHLS-PO activity during postmolt (24 hrs after ecdysis). *CasAK* is used as reference gene in the expression analysis. Different letters and asterisk denote significant differences at $P < 0.05$ (n=7). C) The western blot analysis on the experimental samples identifies CasPPO-hemo in CasHLS. D) Shell-hardness of the new cuticle during 48 hrs after ecdysis. The hardness of the postmolt cuticle was measured by a hand-held durometer (durometer scale = 0-100 DU). Dashed line = *CasPPO-hemo-dsRNA* injected group ($R^2 = 0.92$) and solid line = control group ($R^2 = 0.90$). ANCOVA analysis show significant differences ($P < 0.05$; n=7). HD_{50} was calculated as the time after ecdysis when the cuticle reaches 50% of hardness.

3.4 Results

3.4.1 Identification of hemocyte types in the hemolymph of *C. sapidus*

Three types of hemocytes are identified in *C. sapidus* hemolymph: granulocytes (G), semigranulocytes (SG) and hyaline cells (H) (Suppl. Figs. S3A-B). Gs contain cytoplasmic granules with a nucleus in their center and the average diameters of cells with 12.0 ± 1.5 μm (n=35). SGs are smaller than Gs, with diameters of 9.2 ± 1.1 μm (n=35). They also show their nuclei in the center of the cytoplasm and contain fewer cytoplasmic granules, compared to those present in Gs. Hs are the smallest hemocytes with diameters of 8.5 ± 1.1 μm (n=35) but show a prominent nucleus in the center of the cytoplasm but without cytoplasmic granules

3.4.2 Determining *CasPPO-hemo* function in the initial shell-hardening process using *dsRNA*

Both *CasPPO-hemo-dsRNA* and control group do not show significant differences in the size and number of the hemocytes and molt increment and intervals (Suppl. Fig. S4; Suppl. Table S1).

The multiple injections of *CasPPO-hemo-dsRNA* reduce both its transcription in hemocytes by ~57% (Fig. 9A; Fig. S5) and protein levels with ~54% of the CasHLS-PO activity (Fig. 9B-C), compared to controls.

During 48 hrs after ecdysis, the animals that received the *CasPPO-hemo-dsRNA* show the shell-hardening process significantly slower than the controls (Fig. 9D). During the first ~2 hrs after ecdysis, the cuticle of *CasPPO-hemo-dsRNA* injected crabs is 18 ± 1 DU (n=7), while the controls show a hardness of 32 ± 3 DU

(n=7). At 24 hrs after ecdysis, the *CasPPO-hemo-dsRNA* injected crabs increase the hardness to 51 ± 2 DU (n=7) , while the controls have 84 ± 5 DU (n=7). At 48 hrs after ecdysis, the hardness of the carapace is 82 ± 2 (n=7) and 95 ± 2 DU (n=7) for the *CasPPO-hemo-dsRNA* and control groups, respectively.

As shown in Fig. 9D, the values of HD_{50} (time after ecdysis when the cuticle reach the 50% of hardness) significantly differ with 18.9 ± 1.4 hrs (n=7, $P < 0.05$) for *CasPPO-hemo-dsRNA* group and 8.7 ± 0.9 hrs (n=7) for control group.

In *CasPPO-hemo-dsRNA* injected animals, the hemocytes collected at 24 hrs after ecdysis display significantly reduced staining intensities with α-CasPPO-hemo (Figs. 10A-B), specifically in SGs and Hs, compared to those of controls. The flow-cytometry analysis (Fig. 10C) of hemocytes signifies the presence of three different cell types. In *CasPPO-hemo-dsRNA*, the cytosolic complexity and granularity do not have significant differences respect to the control group (Fig. 10C).

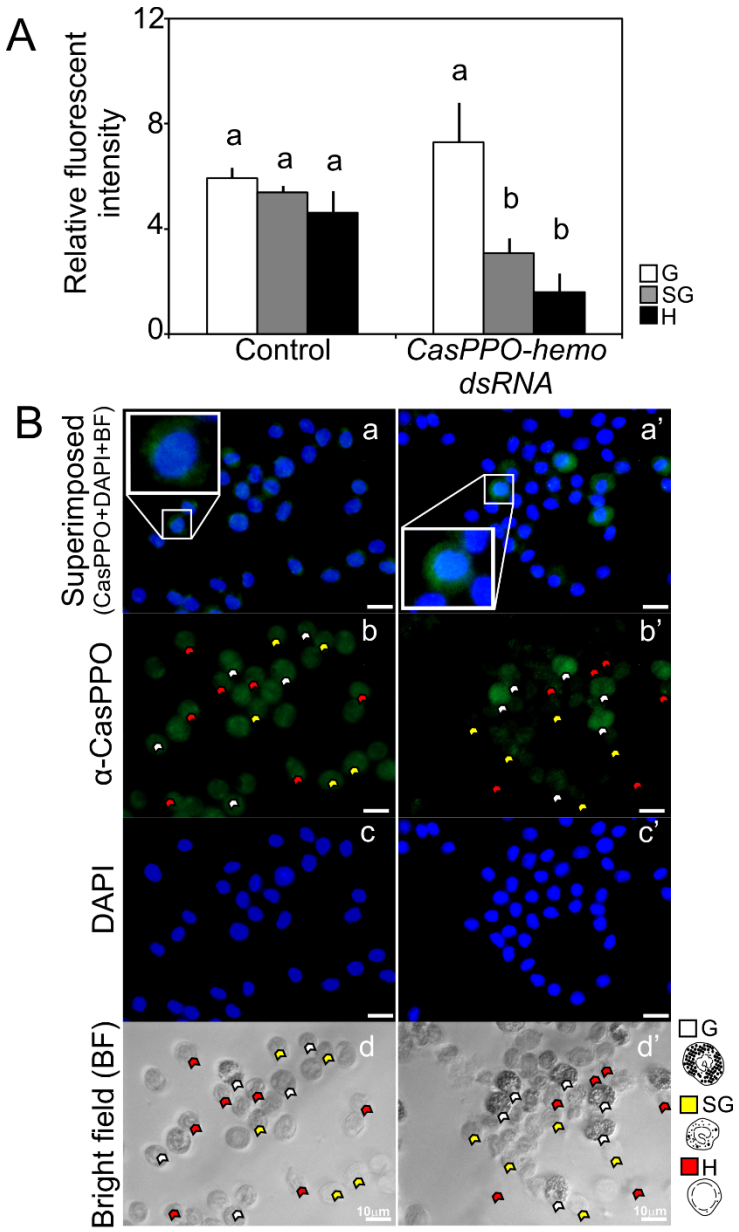
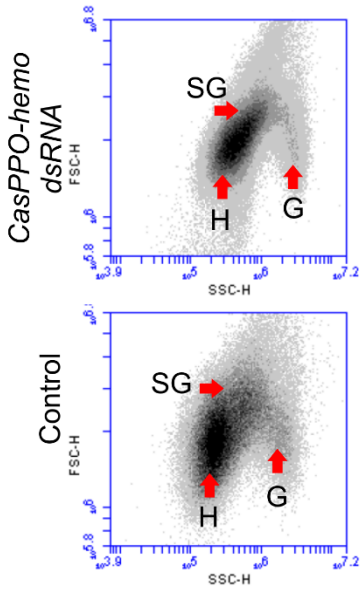


Figure 10. Levels of CasPPO-hemo in hemocytes after multiple injections of *CasPPO-hemo-dsRNA*. (A) Fluorescent intensity analysis, (B) ICC and (C) flow-cytometric analysis of hemocytes from control and *CasPPO-hemo-dsRNA* group. The fluorescent intensity analysis was calculated by ImageJ using the positive staining by α-CasPPO-hemo serum in each type of hemocyte 24 hrs after ecdysis, as a reference for PPO content. The insertion in the ICC pictures shows positive H (a) and G (a') (Continues in next page).

C



SSC-H light (cytosolic complexity and granularity)

Hemocytes	*dsRNA* (n=5)	Control (n=4)	Significance
G	$2.5 \pm 0.2 \times 10^6$	$2.9 \pm 0.2 \times 10^6$	$P > 0.05$
SG	$7.9 \pm 1.0 \times 10^5$	$8.1 \pm 0.7 \times 10^5$	$P > 0.05$
H	$3.2 \pm 0.3 \times 10^5$	$3.7 \pm 0.6 \times 10^5$	$P > 0.05$

Figure 10. (From the anterior page) The flow-cytometry indicates no changes in the cytosolic complexity and granularity of hemocytes in the *CasPPO-hemo-dsRNA* injected group respect to the control. Cytosolic complexity (SSC-H) is shown as mean ± SE (n=4-5). Different letters indicate significant differences at $P < 0.05$, two way ANOVA (B) and Student's t test (C). Bar scale = 10 µm. G= granulocytes, SG= semigranulocytes, H= hyaline cells.

3.4.3 Differential hemocyte counting (DHC) during the molt cycle

Callinectes hemolymph always carries three types of hemocytes throughout the molt cycle. The total hemocyte counting (THC) does not show significant differences (one way ANOVA; $P > 0.05$) but the number of each cell types (DHC) vary significantly during molt cycle. At intermolt, the THC ranges $6.2 \pm 1.9 \times 10^6$ cells/ml hemolymph (n=6) and increases up to $14.1 \pm 3.0 \times 10^6$ cells/ml hemolymph (n=6) at late premolt. At intermolt, the majority of hemocytes (Fig. 11 and Table 4)

are Hs with 4.3 ± 1.4 x10^6 cells/ml hemolymph. SGs are the second major type in this stage, with 1.6 ± 0.5 x10^6 cells/ml hemolymph and Gs are 0.3 ± 0.1 x10^6 cell/ml hemolymph.

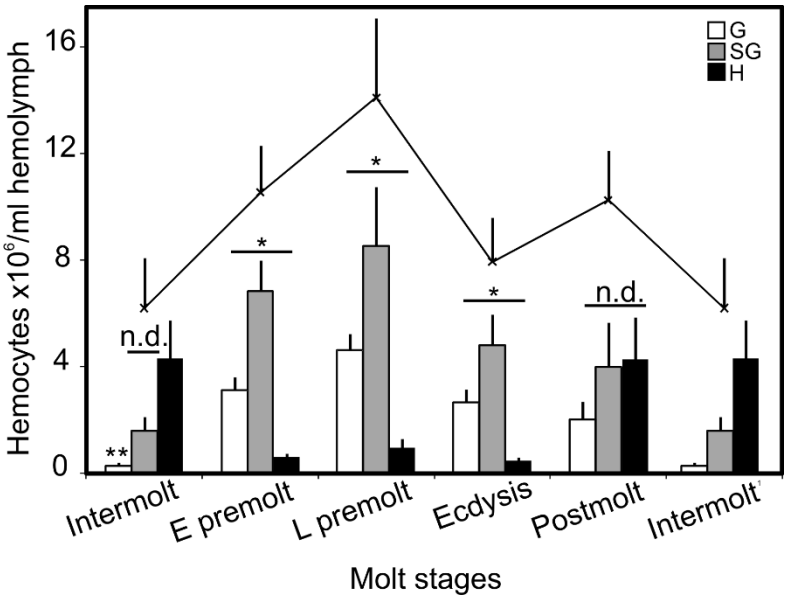


Figure 11. Differential counting of hemocytes during the molt cycle of the blue crab using the flow-cytometry analysis. The THC (solid line) does not show significant differences among the molt stages. n.d. = no significant difference; the "*" denotes significant difference at $P < 0.05$, two way ANOVA.

From early premolt and late premolt, THC in the hemolymph increases to 11.0 ± 1.7 x10^6 cells/ml hemolymph and 14.0 ± 3.0 x10^6 cells/ml hemolymph, respectively. The composition of hemocyte types throughout premolt (early and late premolt) in the hemolymph changes. SGs (~65% of THC) and Gs (~30% of THC) are the majority in hemocytes and Hs represent ~6% of THC. Compared to intermolt and postmolt, a significant reduction in numbers of Hs is accounted for only 0.6 ± 0.1 x10^6 cells/ml hemolymph at early premolt and 1.0 ± 0.3 x10^6 cells/ml hemolymph at late premolt. At early premolt, SGs and Gs significantly increase in numbers with 6.8

± 1.2 x10^6 cells/ml hemolymph and 3.1 ± 0.5 x10^6 cells/ml hemolymph respectively. Both hemocyte types increase between 1.3-1.5 fold at late premolt (Fig. 11 and Table 4). At ecdysis, THC is reduced to 7.9 ± 1.7 x10^6 cells/ml hemolymph but the composition of hemocyte types remains the same as both premolt stages.

At postmolt, THC consists of all three hemocyte types at close contribution (Fig. 11). Compared to ecdysis, the number of Hs significantly (P <0.05) increases to 4.2 ± 1.6 x10^6 cells/ml hemolymph (~41% of THC). SGs are constant in their numbers, although significantly (P <0.05) low compared to premolt. Gs represent ~20% of hemocyte with 2.0 ± 0.7 x10^6 cells/ml hemolymph.

Table 4. Changes in different of cell types in hemocyte populations of the hemolymph of *C. sapidus* during the molt cycle. Significant differences by ANOVA (P < 0.05). Different letter represent significant differences at each molt stage.

	Intermolt	E. premolt	L. premolt	Ecdysis	Postmolt
H	69.9 ± 22.6 a	5.9 ± 1.2 a	6.8 ± 2.3 a	6.0 ± 1.3 a	41.4 ± 15.5 a
SG	25.8 ± 7.9 a	64.7 ± 10.9 b	60.5 ± 15.8 b	60.7 ± 14.4 b	39.0 ± 15.0 a
G	4.2 ± 2.0 b	29.4 ± 4.9 c	32.8 ± 4.4 c	33.4 ± 6.3 c	19.6 ± 6.7 a

Relative abundance (Mean ± SD %, Intermolt, late premolt and postmolt: n=6; Early premolt: n=18, Ecdysis: n=7). H = hyaline cells; SG = semigranulocytes; G = granulocytes.

3.4.4 Immnunostaining of CasPPO-hemo in each type of hemocytes during the molt cycle

The presence of CasPPO-hemo protein in hemocytes is examined using ICC and flow cytometry. ICC results show that Gs displays the strongest immunostaining with α-CasPPO-hemo serum through molt stages (Figs. 12A-B; n=5-6). Hs have immunostaining only at postmolt (Figs. 12A-B; n=5-6) while SGs are immunoreactive only at early premolt and postmolt.

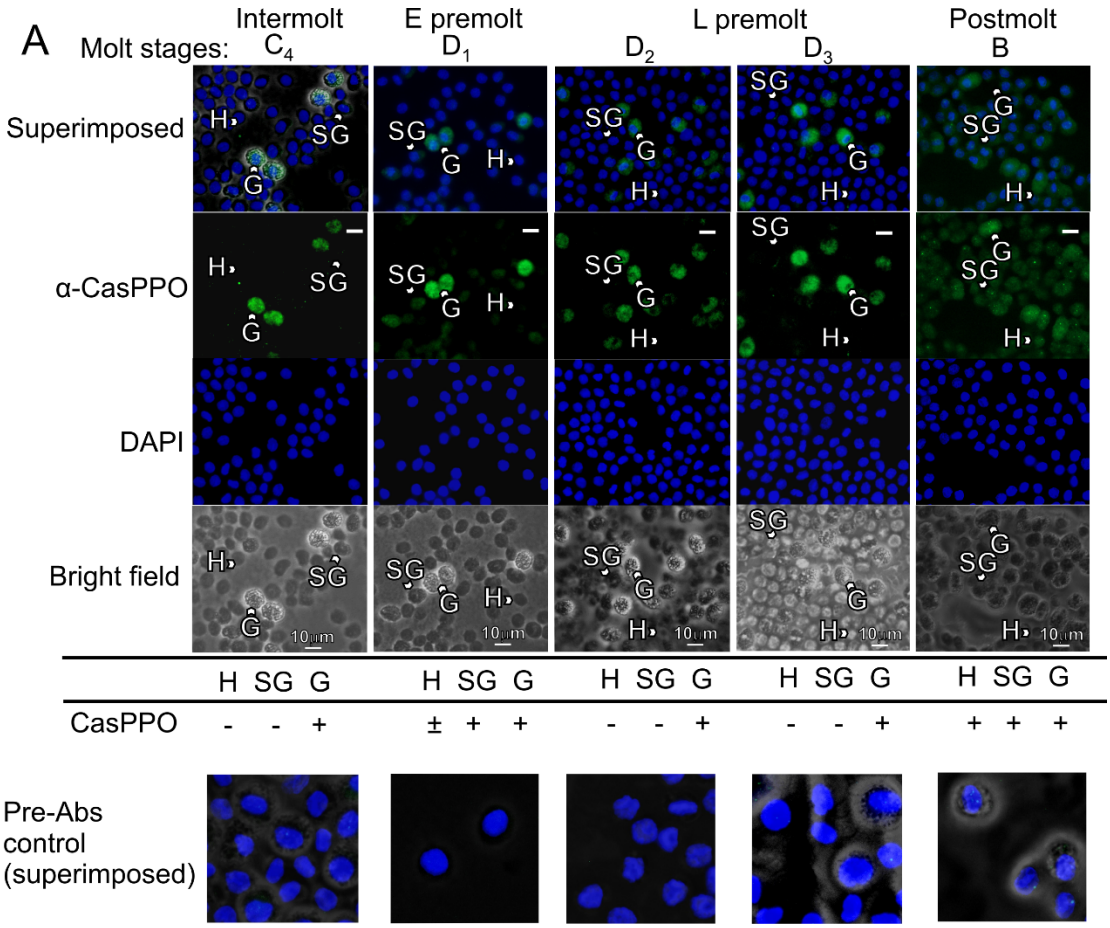
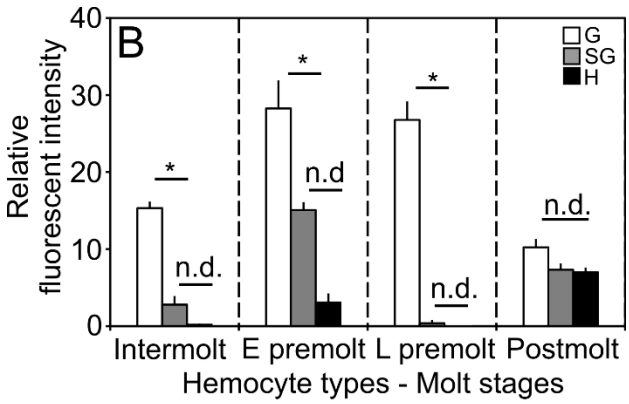
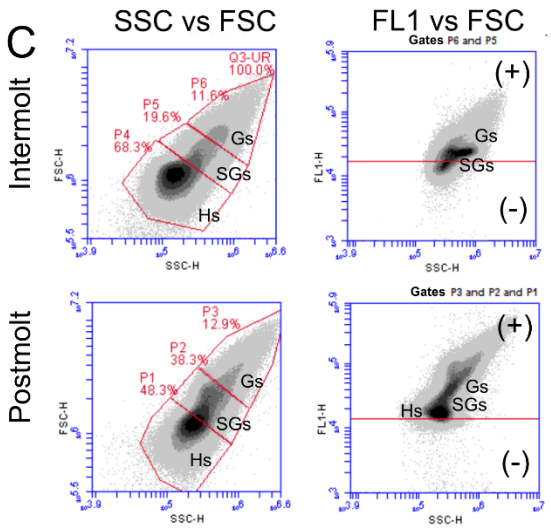


Figure 12. Identification of CasPPO-hemo in hemocytes during the molt cycle of the blue crab. (Continues in next page).





Hemocytes	Intermolt	Postmolt
α-CasPPO-hemo positive (%)	13.5 ± 1.5 n = 8	92.1 ± 2.5 n = 6

Figure 12. (From the previous page) Identification of CasPPO-hemo in hemocytes during the molt cycle of the blue crab. Immunostaining of hemocytes at different molt stages using a specific α-CasPPO-hemo serum. Gs contain PPO during all the molt cycle, while SGs and Hs show a PPO protein expression which is molt stage dependent. Signals: + PPO and – PPO. A) ICC (n=5–6) and B) fluorescent intensity of the positive signal of α-CasPPO-hemo staining, using ImageJ. C) Immunostaining of CasPPO-hemo by flow-cytometry analysis: At intermolt stage, ~14% of hemocytes are PPO positive while at postmolt, the PPO positive hemocytes are over 90% (n=5-8). Scale bar = 10 µm. G= granulocytes; SG= semigranulocytes; H= hyaline cells.

The flow-cytometry analysis (Fig.12C; n=6-8) with immunostained hemocytes is in agreement with ICC. At intermolt ~14% of the cells are positive staining by α-CasPPO-hemo, while during postmolt over the 90% of the hemocytes contain PPO.

3.5 Discussion

This study provides evidence that PPO present in hemocytes is an essential component in the shell-hardening process of *C sapidus*. Further, PPO is expressed in different hemocyte types during the molting cycle of *C. sapidus*. At postmolt, SGs and Hs are the source of PPO, while at intermolt, Gs are the major type of cells that produce PPO.

Our data show during the molt cycle that changes in the number of cell types are consistent to the levels of hemocyte population [95]. The hemolymph of *C. sapidus* always carries three types of hemocytes throughout the molt cycle, as similar to that found in other crustacean species including *H. americanus*, *P. interruptus*, *L. grandis*, *P. leniusculus*, *P. japonicus*, *P. clarkii*, *U. pugilator* and *S. ingentis* [37,82,98,100,105,115].

To define the direct role of CasPPO-hemo in the shell-hardening process in *C. sapidus*, the animals have been injected with *CasPPO-hemo-dsRNA*. Multiple injections of *dsRNA* have been employed to define the functions of a gene of interest in several crustacean species [53,58, Techa and Chung, in press]. The *dsRNA* injected crabs show significantly reduced levels of *CasPPO-hemo* transcripts and CasPPO-hemo protein, subsequently resulting in low PPO activities. More

importantly, the animals treated with *CasPPO-hemo-dsRNA* have significantly slower shell-hardening process than controls.

Since the hemolymph of *C. sapidus* contains three types of hemocytes [96,104], the following question was asked: Which hemocyte type(s) that produce PPO is/are affected by *dsRNA* injections at postmolt? Isolating each cell type may result in the loss of cells [209]. In this study, the values of THC that have been re-examined using flow cytometry are in agreement with the levels obtained by cell counting using a hemocytometer [95]. Hence, the cell population present in the hemolymph are determined using flow-cytometric analysis, combined with the ICC with a specific α-CasPPO-hemo serum and the three cell types, showing distinctive properties as previously stated [104].

PPOs contain three homocyanin domains: N, M and C [95]. In order to increase antiserum specificity, CasPPO antiserum has been generated against two regions located between the hemocyanin N and M domains and between hemocyanin M and C domains of CasPPO [95]. α-CasPPO-hemo serum recognizes ~0.6 µg HLS (= ~900 hemocytes equivalent) at a final dilution of 1:2000 (Suppl. Fig. S2). The pre-absorption controls show no staining in the cells.

Multiple injections of *CasPPO-hemo-dsRNA* affect the cell types but differ in the staining intensities at postmolt. In both SGs and Hs, the most significantly reduced CasPPO-hemo immunostaining intensities are found. In the Gs, the staining intensities remain similar to those of controls and no significant differences in its granularity are found with respect to the control.

Our data suggest that specific hemocyte types may be recruited into the hemolymph of a specific molt stage. This indicates that hematopoietic activity may inherently differ by a molt stage. Interestingly, a molt stage-dependent pattern of hemocyte production has been demonstrated in the crustacean hematopoietic organs, together with increments changes of its mitotic rates during premolt [97] resulting primarily in the release of Gs. It is unknown what triggers such differences in hematopoietic activities during the molt cycle. When considered that the elevated concentrations of ecdysteroids in the hemolymph stimulate cellular mitotic activity in the tissues [6,189,210,211], they may also affect the activity of hematopoietic organs. On the other hand, the role of small cell signaling molecules in hemocyte proliferation is known [116,212]. Astakines 1, an invertebrate cytokine expressed in the hematopoietic organs and hemocytes, promotes the proliferation and differentiation of hemocyte in *P. leniusculus* [116,212].

Our earlier study shows that hemocytes are aggregated beneath the hypodermis immediately after ecdysis [62]. The fact that the transport of PPO from hemocytes into the cuticle has been suggested in insects [38] indicates that these hemocyte PPOs may play a similar role in shell-hardening in crustaceans, as reported in insects.

At postmolt, all types of hemocytes are a rabbit anti-CasPPO-serum positive. Hs, which are positively immunostained only at postmolt suggest that there may be a particular function for these hemocytes after ecdysis. This suggestion is closely related to the observation made in *U. pugilator* [37], reporting that Hs were PPO positive during the ecdysis. And, as a result, these authors proposed Hs are the

hemocytes that mediate the shell-hardening in the fiddler crab. It is notable that Hs and SGs show reduced PPO contents when the expression of *CasPPO-hemo* is knocked-down, by the injection of *dsRNA*.

The hemocytes may undergo inherent changes in their functions during the molt cycle (Fig. 4B), although the life span of each cell is unknown. The staining intensities of PPO noted in the Gs and hemocyte types vary with molt stage, suggesting that PPO activities are contributed by different cell types at a given molt stage. In the shell-hardening process at postmolt, when crabs are injected with *CasPPO-hemo-dsRNA*, SGs and Hs reduce their total PPO contribution to ~19% and ~65% (respectively) lower than the total PPO that may be contributed by Gs. In contrast to the *dsRNA* injected animals, in the control, Hs and SGs represent the major contribution of PPO.

However, SGs may also be PPO positives during the intermolt stage and increasing its PPO contribution. The flow-cytometry shows ~14% of hemocytes are PPO positive at intermolt. At this stage, SGs and Gs are equivalent ~30 ± 8% of THC. Nonetheless, the differentiation of hemocyte types by flow-cytometry is based on internal complexity (SSC-H) and size (FSC-H) of the cells, therefore, Gs and SGs might have been under- or overestimated. SGs have been found PPO positive during the intermolt of other crustacean species [82,98,100,105,115]. On the other hand, the ICC shows Gs as the PPO positive hemocytes at the intermolt stage, implying that Gs could be under-estimated by the flow-cytometric analysis.

Immediately after ecdysis the final expansion of the body is completed and a rapid hardening is required. The transport and delivery of PPO into the new cuticle should not be delayed and happened during and after completion of ecdysis.

The contribution of each type of hemocytes toward total PPO is different by a molt stage, which indicates that the hematopoiesis involves a molt stage-specific hemocyte differentiation. Patterns of hematopoietic production and release of hemocytes vary during the molt cycle [97]. Within the hematopoietic tubules, the hemocytes are differentiated to a specific type of mature cells [102,117,123,213]. However, functional differentiation of these hemocytes by expressing PPO is completed by their release into the hemolymph [102,115]. Similar upregulation of PPO occurs when the animals respond to immune challenges [39,80,214–216]. One may argue that our animals might have been infected. Specifically, the immunity role of Hs has been reported as phagocytosis, while Gs are the reservoir of the humoral defense as the PPO cascade system [115,125,209,217]. However, we used hatchery-raised animals that are considered healthy as they have been reared in controlled conditions and the broodstock have been screened from known diseases [187,188].

This study clearly shows that hemocytes respond to chemical cues or pathogens that are present in the hemolymph including ecdysteroids, cytokines [119,218] and hormones in the hemolymph upregulating PPO. Further study is warranted, specifically isolating the naïve differentiated hemocytes from the hematopoietic tubules and recognizing newly released hemocytes (for example by bromodeoxyuridine, BrdU, [102]) may provide information to answer if hemocytes undergo functional PPO differentiations during the molt cycle.

The levels of *CasPPO-hemo* transcripts remain constant during the molt cycle [95], but little is known about the translation rate of *CasPPO-hemo* or the half-life of the protein. Differences in levels of immunostaining, detected in a same type of hemocytes during the molt cycle, may suggest differential translation rates. Using Gs as an example, ~2.5 x 10^5 Gs/ml hemolymph represent ~3.0 x 10^5 transcripts of *CasPPO*-hemo at intermolt. At postmolt, 2.0 x 10^6 Gs are equivalent to 1.7 x 10^4 transcript copies. The expression per each G is 1.2 and 0.009 (transcripts/cell/ml hemolymph) at intermolt and postmolt respectively. If all hemocytes express CasPPO during ecdysis, the equivalent transcripts per type of hemocyte correspond to: Hs = 0.7 x 10^5, SG = 7.4 x 10^5, and Gs = 4.1 x 10^5 *CasPPO-hemo* copies. Surprisingly, the expression per cell, in each hemocyte type, is the same ~0.15 (transcripts/cell/ml hemolymph). Moreover, if there is a constant transcription-translation for the amounts of immunoreactive PPO in the stained hemocytes, a value for translation can be extrapolated. At ecdysis, even though Hs represent 6% of the THC, they show the highest level of intensity per transcripts (0.1 intensity units/*CasPPO-hemo* transcripts), while SG and Gs show 0.01 and 0.03 % respectively.

Hs may pass easily through the hypodermis to rapidly distribute the PPO into the new cuticle and quickly initiate the shell-hardening during postmolt. Hemocyte tissue infiltration is a process that has previously been reported in crustaceans [80,133].

Taken all together, a model is proposed integrating hemocytes and CasPPO-hemo in the shell-hardening process is presented in Fig. 13. SGs and Gs are

estimated as the main hemocyte types during premolt stages. Premolt is the preparation for ecdysis through the synthesis of the pre-ecydsial layers of the new soft cuticle [2,18], which is induced by the increase in the levels of hemolymphatic ecdysteoids [4,6]. The positive detection of PPO in SGs and Gs at early premolt may be associated with the preparation for ecdysis, which may include the PPO transport that has been suggested [38]. The sclerotization occurring during the first hours after ecdysis involve the hemocyte characteristics observed during postmolt, when all hemocytes are PPO positive. It appears that Hs and SGs may likely be involved in the shell-hardening process at the postmolt, while Gs, as the constant PPO reservoir throughout the molt cycle, may be involved with supporting the immunity system [132].

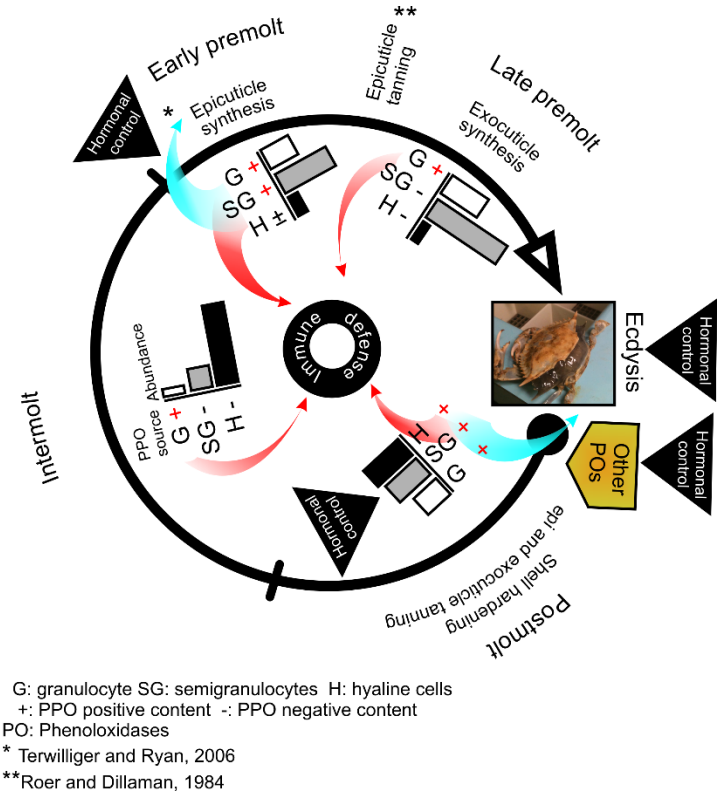


Figure 13. A proposed model for the functional involvement of hemocytes and its PPO (CasPPO-hemo) in the shell-hardening process of the blue crab *C. sapidus*. The diagram summarizes the results obtained from the current study. Changes in hemocyte numbers are shown with bar plots. The positive (+) and negative (-) symbols indicate the presence or absence of PPO expression in each type of hemocytes, respectively. Blue arrows indicate the potential transport of PPO from hemocytes into the cuticle, as described [38]. Red arrows indicate the suggested hemocytes and PPO role in the immunity system [39,40]. Asterisks denote that the previous reports contributed by others are incorporated in the model.

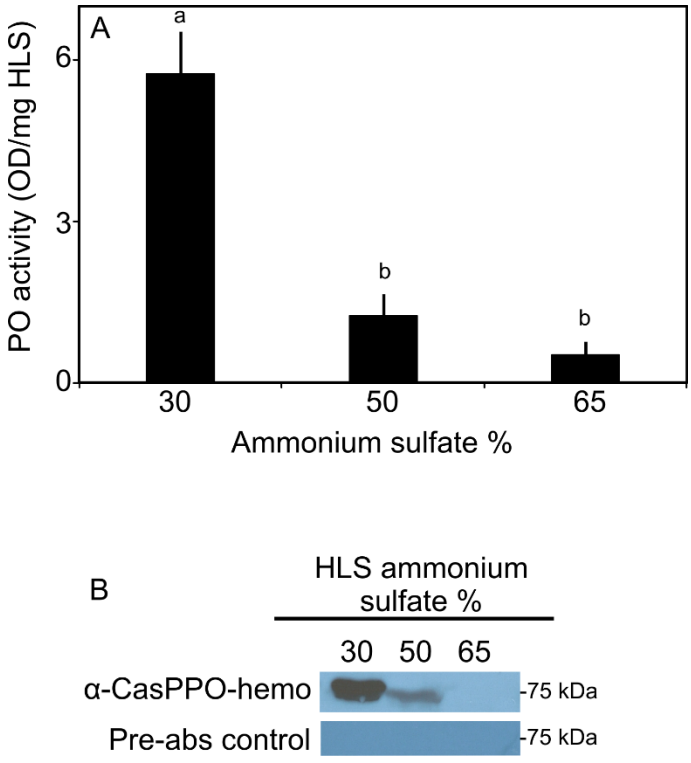
3.6 Supplemental Tables and Figures

3.6.1 Supplemental Tables

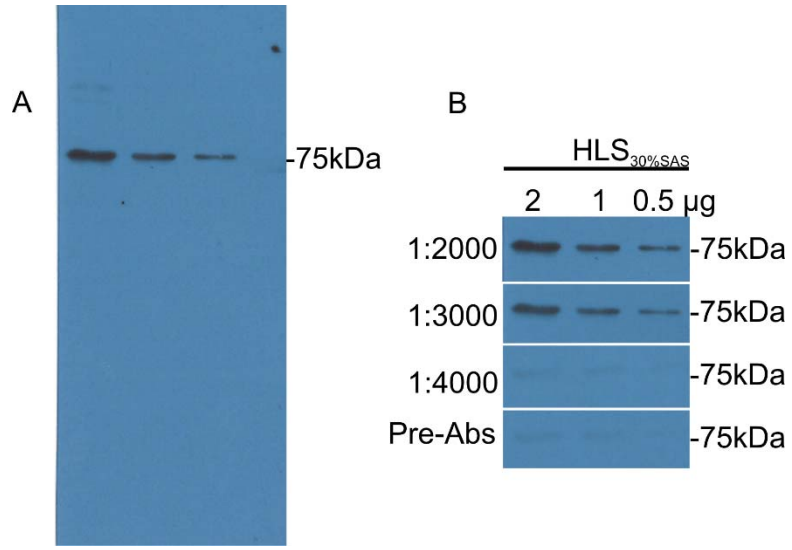
Supplemental Table S1. Hemocyte abundance (cells/ml hemolymph) 24 hrs after ecdysis in *CasPPO-hemo-dsRNA* injected crabs and control group. No significant differences ($P > 0.05$) are observed by two way ANOVA.

Hemocytes	*dsRNA* (n=5)	Control (n=4)	Significance
Granulocytes	$8.8 \pm 3.1 \times 10^5$	$6.6 \pm 2.3 \times 10^5$	$P > 0.05$
Semigranulocytes	$1.7 \pm 0.6 \times 10^6$	$2.1 \pm 0.5 \times 10^6$	$P > 0.05$
Hyaline cells	$1.4 \pm 0.7 \times 10^6$	$1.8 \pm 0.6 \times 10^6$	$P > 0.05$
Total	$5.5 \pm 1.9 \times 10^6$	$5.2 \pm 1.7 \times 10^6$	$P > 0.05$

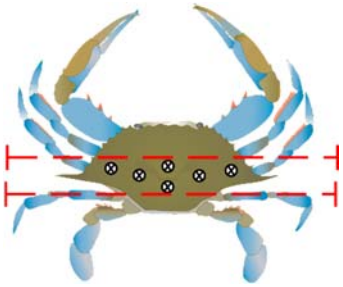
3.6.2 Supplemental Figures



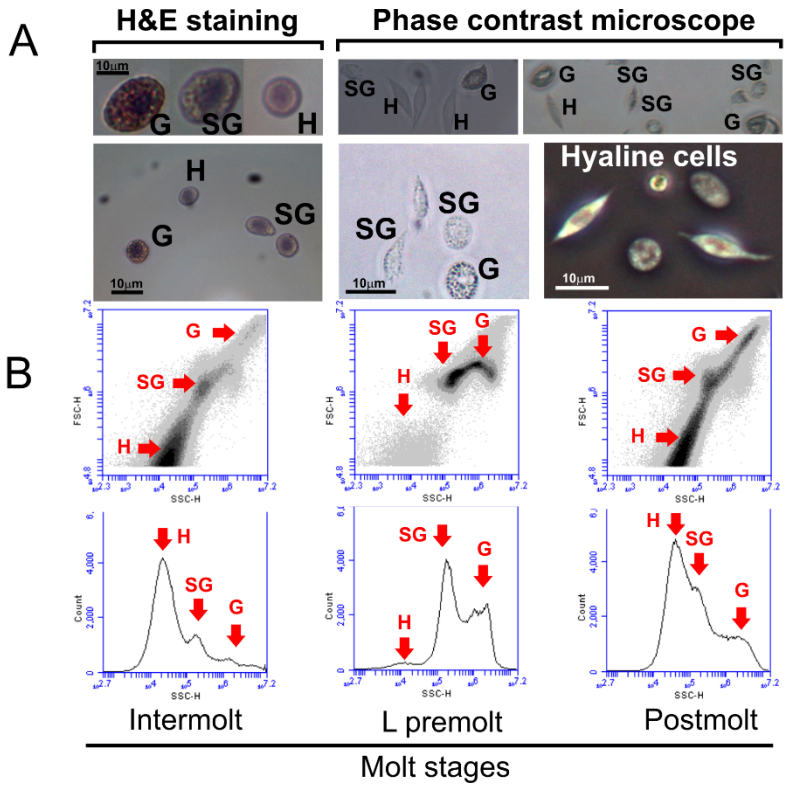
Supplemental Figure S1. Saturated ammonium sulfate (SAS) precipitations of proteins present in hemocytes (HLS) and its PO activity isolation. A) The PO activity was recovered in % saturation of ammonium sulfate. (B) The Identification of CasPPO-hemo in the SAS fractions was performed by western blot using a rabbit CasPPO-hemo antiserum (α-CasPPO-hemo).



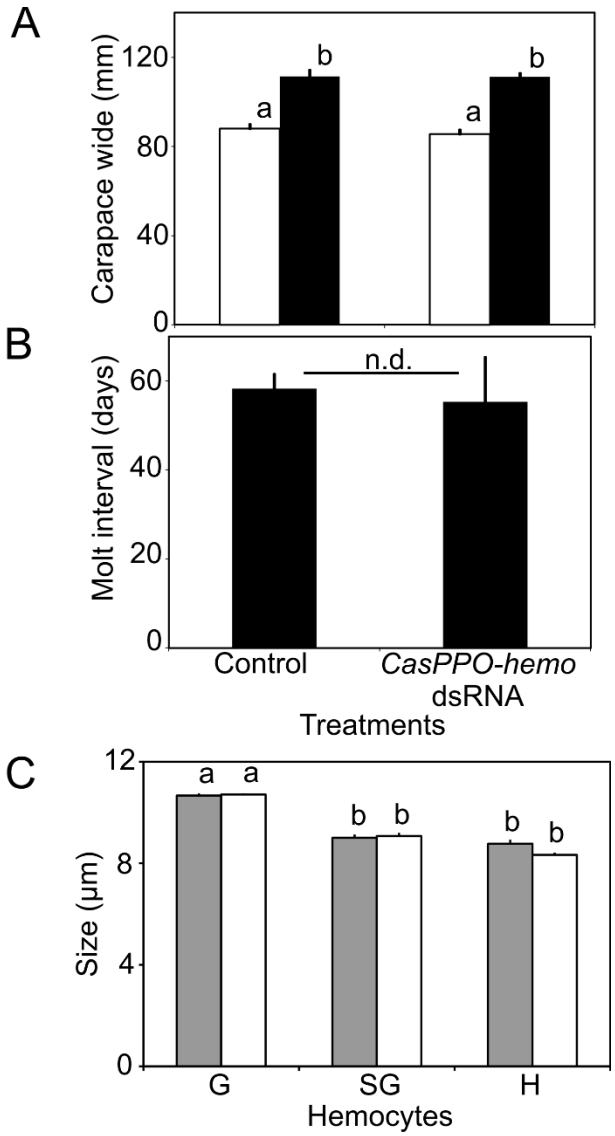
Supplemental Figure S2. The properties (sensitivity and a dilution factor) of α-CasPPO-hemo antiserum are determined using western blot analysis with $HLS_{30\%SAS}$. A) Western blot using the antiserum at 1:2000 dilution. B) Differential α-CasPPO-hemo dilutions. Pre-Abs = pre-absorption control. For Pre-Abs, synthetic CasPPO peptides (10 nmol each) were incubated with α-CasPPO-hemo serum (a final dilution at 1:10) for overnight at 4ºC through gentle mixing on a magnetic stir. It was then diluted at a final dilution of 1:2000, same as α-CasPPO-hemo serum.



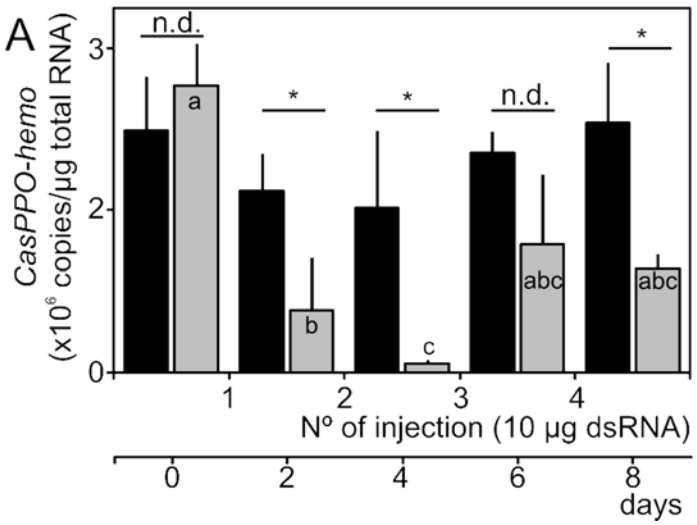
Supplemental Figure S3. Sampling areas of the shell hardness at postmolt. The hardness is measured on 6 spots (white dots) located of dorsal carapace mesogastric area of the blue crab (between dashed lines) using a durometer. The blue crab image is used with permit (http://ian.umces.edu/symbols/).



Supplemental Figure S4. Hemocyte types differentiation. Morphological differentiation of the blue crab hemocyte by A) microscopy (H&E = hematoxylin and eosin staining) and B) flow cytometry. The differentiation is made, based on the criteria as described [100,104]. Three main types of hemocytes are recognized during the molt cycle: Granulocytes (G), semigranulocytes (SG) and hyaline cells (H). Hemolymph is collected in a fixative and read by flow cytometry.



Supplemental Figure S5. Effects of *CasPPO-hemo* knockdown experiment in the body size (molt increment), molt interval, and size of hemocytes in control and experimental crabs. Injections of 10 µg of *CasPPO-hemo-dsRNA* during the experiment do not affect A) molt increments: White bars = time zero; black bars = at the end of the experiment (~48 hrs after ecdysis); B) The molt intervals; and C) the size of hemocytes in the *dsRNA* injected crabs and controls. Solid bars = control group; open bars = *dsRNA* injected crabs. G = granulocytes, SG = semigranulocytes, H = hyaline cells.



Supplemental Figure S6. The knockdown-efficiency of *CasPPO-hemo-dsRNA*. The intermolt crabs received 10 µg of *CasPPO-hemo-dsRNA* every other day. *CasPPO-hemo* transcripts are reduced by ~90% after the second injection, compared to controls that received crustacean saline. The overall final knockdown efficiency after 4 injections (8 days) is ~60%.

3.6.3 Supplemental Protocol

Protocol S1. Isolation of HLS-PO activity and characterization of a rabbit anti-CasPPO-hemo serum (α-CasPPO-hemo)

HLS was prepared with the hemocytes of juvenile crabs at intermolt stage, as described [95] and further precipitated using saturated ammonium sulfate (SAS) [183,184,219,220]: SAS was added to HLS at 30% final concentration (v/v) and mixed on ice for 20 min by constant stirring (~1000 rpm), centrifuged at 15,000 g at 4ºC for 20 min and then, the protein pellet was retrieved and resuspended in 50 or 100 µl of ice-cold working buffer. The supernatant was retrieved and was used as the source for subsequent ammonium sulfate precipitation at 50 and 65%. The protein concentration of each SAS fraction was assayed for PO activity after the protein estimation as described [95].

HLSs (2 μg) were separated on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad) and further processed using the procedure as described [94]. In brief, the membrane was blocked with 7% nonfat milk (NFM) in PBST (1X PBS, 0.05% Tween 20) at RT for 2 hrs. The primary antibody (α-CasPPO-hemo; 1: 2,000 dilutions in NFM+PBST) was incubated overnight at 4ºC. After washing with PBST (three times per 20 min each), the membrane was then incubated with a secondary antibody, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Jackson Immuno-Research) at 1:10,000 dilutions for 1 hr at RT. The signals on the autoradiography film (HyBlot CL, Denville Scientific Inc) were detected by chemiluminescence using luminol as a substrate.

Chapter 4: Does the role of bursicon in the shell-hardening process of crustaceans involve hemocytes and PPO cascade system?

4.1 Abstract

The heterodimer neurohormone bursicon mediates the cuticle tanning of insects (*Drosophila* and *Manduca)* after ecdysis. Recently bursicon has been found also in crustaceans and its molecular structure is highly conserved as in insects. Moreover, crustacean bursicon titers increase in the hemolymph peaks at the completion of ecdysis, suggesting its putative function in shell-hardening, similar to insects. The mechanisms by which shell-hardening of crustaceans occurs are not yet understood. However, the involvement of hemocytes and prophenoloxidase (PPO) in the sclerotization of new cuticle has been reported in several crustacean species. Our earlier study shows that bursicon induces hemocyte granulation, aggregation, and recruitment beneath the new cuticle. We hypothesized that hemocytes may be one of the target tissues of bursicon in shell-hardening and may modulate the PPO cascade system, possibly through regulation of hemocyte *PPO* and its activating enzyme expression. Multiple injections of *CasBurs*-α and *CasBurs*-β *dsRNA* in the blue crab, *Callinectes sapidus* show the heterodimer bursicon (the active neurohormone) does not regulate the *PPO* expression of PO activity in hemocytes. However, the injection of either *CasBurs*-α or -β *dsRNA* delays the shell-hardening of injected crabs. The co-injections of *CasBurs*-α+β *dsRNA* do not change the normal progress of the shell-hardening at postmolt. However, bursicon homodimers may be implicated in the

shell-hardening process. The exact mechanism of hormonal actions of CasBurs in shell-hardening process needs further studies.

4.2 Introduction

The molting process in crustaceans and all arthropods is controlled by a battery of hormones and ends with ecdysis, the stage when the animals shed the hard exoskeleton [4,6,221] Immediately after ecdysis in crustaceans, a soft external cuticle is rapidly transformed in a hard exoskeleton [2] by two consecutive processes: sclerotization and mineralization [11,13,15,16,18,70,196]. Cuticle sclerotization, one of the last hormonal regulated ecdysial process, has been well studied in insects [11,12,17,21,219]. Bursicon (Burs) as a heterodimer protein (α and β at 1:1 ratio) that is produced as a ~30 kDa central nervous system hormone, controls the cuticle tanning and the wing expansion of many insects after ecdysis [54,56,58–61,138–140,222]. Recently homodimers (α+α and β+β) are also found in *Manduca* [58,60,142]. While the insect heterodimer molecule is the active hormone responsible for insect tanning, the homodimer forms mediate the expression of antimicrobial peptides (AMP) during ecdysis [142].

The neuroendocrine regulatory mechanisms by which cuticular sclerotization at the postmolt stage of crustaceans occur have not been much studied. Recently, bursicon has been found in several crustacean species including *Callinectes sapidus*, *Daphnia arenata*, *Carcinus maenas*, *Homarus gammarus*, *H. americanus* [62,64,65]. The bursicon titers in the hemolymph of *C. sapidus peaks* at the completion of ecdysis [62], indicating that the functions of crustacean bursicon may be similar to

those of insects. An *in vitro* incubation of the late premolt cuticle with recombinant Burs indicates that hemocytes and hypodermis may be candidates of its target tissues [62].

The pattern of expression of *Burs-α* and -β in crustaceans seems to be different from that of insects [54,62,64]. In *Callinectes*, a constant expression of *CasBurs* during the molt cycle can be explained by little variation of *CasBurs*-α. On the other hand, the skewed expression of *CasBurs-β* is described in the ratio *CasBurs-β* vs -α. As a result, a *CasBurs-β* homodimer in addition to heterodimer has been suggested [62].

Hemocytes that play key roles in the innate immunity system of arthropods [96,115,119,147,223–225] are now thought to have an additional role in shell-hardening in decapod crustaceans [37, Alvarez and Chung in preparation]. *C. sapidus* carries three main types of hemocytes as described [82,104,105,115,123]: granulocytes (Gs), semigranulocytes (SGs) and hyaline cells (Hs). We reported that CasPPO-hemo [95], the hemocyte prophenoloxidase (PPO) of *C. sapidus* participates in the sclerotization of the cuticle during postmolt (Alvarez and Chung, in preparation). Together with our earlier data and other reports, we hypothesized that CasBurs, the *C. sapidus* bursicon, may be involved in the shell-hardening process via modulating the PPO expression in hemocytes.

In this study, we used multiple injections of *CasBurs-α* and *-β dsRNA* in *C. sapidus* juveniles that were raised in the blue crab hatchery (Aquaculture Research Center, IMET, Baltimore, MD). We analyzed the levels of expression of *CasBurs*-α and -β transcripts in thoracic ganglion complex (TGC) and CasPPO-*hemo* in

hemocytes, together with immunostaining of CasPPO-hemo and flow-cytometry of hemocytes. The results show that multiple injections of *CasBurs*-α or -β *dsRNA* alone slowed the shell-hardening, although they had no effect on the levels of PPO expression, implicating the role of bursicon homodimer in the shell-hardening process. The co-injections of *CasBurs*-α+β *dsRNAs* (1:1 ratio) do not interfere the shell-hardening process.

4.3 Materials and Methods

4.3.1 Animals

Juvenile blue crabs *C. sapidus* (15-30 mm carapace wide, CW) were obtained from the blue crab hatchery [Aquaculture Research Center, the Institute of Marine and Environmental Technology (IMET), Baltimore, MD]. The animals were grown in tanks with individuals cages in recirculated aerated artificial seawater (25 ppt; 22ºC) as described [62,158,159]. The water quality was monitored daily for The Clinical Laboratory Analysis of National Aquarium, Baltimore, MD (NAIB). Juvenile females and males animals with 76.7 ± 1.7 mm CW, at intermolt stage were used as experimental animals unless described otherwise.

4.3.2 Effects of multiple injections of *CasBurs*-α and -β *dsRNAs* on the cuticle hardness and *CasPPO-hemo* expression in hemocytes

4.3.2 1 *In vitro* transcription of *CasBurs*-α and -β *dsRNAs*

The templates for *CasBurs*-α and -β *dsRNAs* were generated by amplification of TGC cDNA (α=363 bp; β=348 bp) using specific *dsRNA* primers, listed in Table 5.

Purification of templates and *in vitro* transcription were made as described [202] using a TranscriptAid™ T7 High Yield Transcription kit (Fermentas). Ten µg of each *dsRNA* were dissolved in crustacean saline solution (100 µl) contained 0.001% phenol red [203]. Similarly, the co-injection of *CasBurs*-α and -β *dsRNAs* (α+β) was prepared in a ratio 1:1 (10 µg each) [138] in 100 µl of crustacean saline and 0.001% phenol red.

Table 5. List of primers used for *dsRNA* template amplification and qPCR assay

Primers	5' to 3' nucleotide sequence
*CasPPO*3F6-QF	CACCTCTTCATCCATCACAAACTC
*CasPPO*5R6-QR	CAACCACACCCACAGAAGTTAAAG
CasBurs-Qb3F	AGTTCTCTCCTAACCTTCCCCA
CasBurs-Qb5R	CTTCAGGCAACACCGCCAAC
CasBurs-Qa3F	CGAGCTCTATCCGTGCACCACTAC
CasBurs-Qa5R	TAGCCCAGGTCAGATTGCTGTTCA
*CasBurs-ds3F*b	*TAATACGACTCACTATAGGTACT*AGAACGTACGGTGTTGAATGCGC
*CasBurs-ds5R*b	*TAATACGACTCACTATAGGTACT*TTACCGGGTCGAGTCGCCACACTTG
*CasBurs-ds3F*a	*TAATACGACTCACTATAGGTACT*GACGAGTGTTCCCTGCGGCCTGT
*CasBurs-ds5R*a	*TAATACGACTCACTATAGGTACT*CTCAGAAAGGGAACGCTGTCCATTG
*CasPPAE*3F	ATTGATGGAGAAGACGCTCCACTGCTCGCCT
*CasPPAE*5R	TGGATTCTCCAGACCTTGCGGCCTTAAA
*CasPPAE*3F-QF	CCTGAGTATGAATCGCCTTGCAAGGAGTGC
*CasPPAE*5R-QR	CCCAACCAGCAGCATAAGCAAACTTCCCTT

Q = qPCR primers ds = *dsRNA* α=alpha; β=beta;
T7 promoter sequence is italicized.

4.3.2.2 Multiple injections of *CasBurs*-α and -β *dsRNAs*

Juvenile intermolt crabs (76.7 ± 1.7 mm CW) were injected with 100 µl of *dsRNA* three times a week until ecdysis. During this period, animals received in total of 22-30 injections. Three experimental groups (n=6-7) and one control (n=7) were as follows: i) crabs injected with co-injection of *CasBurs*-α+β *dsRNA* (dsRNAαβ group); ii) crabs injected with *CasBurs*-α *dsRNA* (dsRNAα group); and iii) crabs injected with

CasBurs-β *dsRNA* (dsRNAβ group). The control group was injected with 100 µl of crustacean saline alone. Phenol red was added to monitor the delivery on injection materials [203].

4.3.2.3 Quantification of cuticle hardness during postmolt

After ecdysis, the cuticle hardness of each crab was measured for 48 hrs by a hand-held Shore-type durometer (00 gauge, Instron, USA) as described [27,46]. Regulations of *PPO* expression affect the whole hardening of the cuticle through the initial sclerotization process. Because the rates of hardness vary depending on the area of cuticle [195], the hardness of the cuticle was evaluated on the mesogastric area of the carapace by sampling 6-8 points (Suppl. Fig S6) as described [27,46, Alvarez and Chung, in preparation]. The data are shown as mean ± SE of durometer unit (DU) as described [195].

4.3.2.4 Levels of *CasBurs*-α and -β expression in TGC

At the end of the experiments (48 hrs after ecdysis), crabs were placed on ice for 5-10 min and dissected for thoracic ganglion complex (TGC) and pericardial organs. Immediately after dissection, the tissues were kept on dry ice and kept in -80ºC. TGC and PO are known as the expression and storage tissues of bursicon, respectively [62]. Then, TGC was used as source of RNA for cDNA synthesis and pericardial organs (POs) were the source for proteins.

The cDNA was synthesized as described [62]. Each sample cDNA was assayed in duplicated for expression levels of *CasBurs-α* and –β, using qPCR (n=6-7) with a Fast SYBR Green Master Mix (Applied Biosystems) on a 7500 Fast Real-

Time PCR System (Applied Biosystem). qPCR primers are listed in Table 1. The standards preparation and qPCR conditions are as described [62,164]. The data are shown as mean ± SE copies/μg total RNA.

4.3.2.5 Levels of CasBurs proteins

The protein contents of pericardial organs were prepared as described [62]. Briefly, a single pericardial organ were placed in 25 μl of lysing buffer, sonicated (Branson Sonifier 450) twice for 5 sec each in cold condition, and centrifuged at 15,000 g for 20 min at 4ºC. The supernatants were retrieved into fresh tubes and kept at -20ºC until further use. Twenty five percent of a pericardial organ were loaded and separated by a 15% SDS-PAGE, for 4 different crabs in each treatment, and stained using coomassie brilliant blue staining. For western blot, the proteins were transferred onto a nitrocellulose membrane (Bio-Rad) for 1 hr at constant 100 v. Then, the membrane was blocked with 7% nonfat milk (NFM) in PBST (1X PBS, 0.05% Tween 20) at RT for 2 hrs. The primary antibody (anti-CasBurs; 1:3,000 dilutions in NFM+PBST) [62] was incubated overnight at 4ºC. After washing with PBST (three times per 20 min each), the membrane was then incubated with a secondary antibody, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Jackson Immuno-Research) at 1:10,000 dilutions for 1 hr at RT. The signals on the autoradiography film (HyBlot CL, Denville Scientific Inc.) were detected by chemiluminescence using luminol as a substrate. Western blot membranes were analyzed using ImageJ [204].

4.3.2.6 Levels of *CasPPO-hemo* expression in hemocytes

Twenty four hrs after ecdysis, 400 µl of hemolymph were collected in an anticoagulant solution [95] at 1:1 ratio (v:v) and kept on ice for RNA extraction and hemocyte lysate supernatants (HLS) as described [41, Alvarez and Chung, in preparation]. One µg total RNA was subjected to cDNA synthesis using a PrimeScript RT Reagent Kit with gDNA Eraser for Real Time PCR (Takara Biotechnology). Using qPCR assays, the levels of *CasPPO-hemo* expression in samples were assayed in duplicate, along with the standards that were prepared the same as described as described [95]. Primers for qPCR assay were listed in Table 5. The data are shown as mean ± SE copies/µg total RNA.

4.3.2.7 Levels of CasHLS-PO activity 24 hrs after ecdysis

Callinectes HLS (CasHLS) was obtained as described in [95]. The PO activity of CasHLS was evaluated by estimating the formation of dopachrome from L-3,4-dihydroxyphenylalanine (L-DOPA) using 15 µg of HLS (~100,000 hemocytes or ~11 µl of hemolymph equivalent) as described [95]. The data are shown as mean ± SE (n=5-7) of PO activity (OD/mg HLS).

4.3.2.8 Immunocytochemistry (ICC) of hemocytes 24 hrs after ecdysis

Twenty four hrs after ecdysis, 100 µl of hemolymph were collected in a fixative (4% PFA in 10 mM cacodylate buffer) as described [95]. The levels of CasPPO-hemo in hemocytes were analyzed by the fluorescent intensity of the positive signal using a rabbit CasPPO-hemo antiserum (α-CasPPO-hemo; Alvarez and Chung, in preparation) through an ICC analysis (each sample in duplicate; n=5)

as described by Alvarez and Chung (in preparation). The ICC samples were pictured using a binocular fluorescent microscope Axioplan, Zeiss with a digital camera AxioCam MRc, Zeiss and the software Zen 2012 (Zeiss). The relative staining intensities were analyzed using the ImageJ [204] and presented as mean ± SE of fluorescent intensity.

4.3.2.9 Levels of *CasPPAE* expression in hemocytes

Using qPCR assays, the levels of *CasPPAE* (*Callinectes* PPO activating enzyme, GenBank accession No DQ667138.1) expression in hemocytes cDNA samples were assayed in duplicate, along with the standards that were prepared as described [95]. Primers for standard preparation and qPCR assay were listed in Table 5. The data are shown as mean ± SE copies/µg total RNA.

4.3.2.10 Hemocyte granularity using flow-cytometry

Fixed hemocyte sample (as above) were analyzed by flow-cytometry. Hemocytes were stained with SYBR-Green I nucleic acid (FMC Bioproducts) staining (2X) at 1:100 ratio (v:v = SYBR-Green I:sample). After 10 min incubation at RT, 40 µl of each sample were analyzed using a C6 CFlow flow-cytometer (Accuri Cytometers), with setting at a media fluidic rate (35 µl/min) and a core size of 16 µm at FSC-H, SSC-H and FL1-H. Hemocytes types were characterized by their size (FSC-H) and cytosolic complexity and granularity (SSC-H). The data are presented as mean ± SE of FSC-H or SSC-H.

4.3.3 Statistical analysis

The data were subjected to normality test using Kolmogorov-Smirnov test (K-S) and the homogeneity of variances by Cochran C test (Statistica 7.0, Stat Soft., Inc). Statistical significance of ANOVA was accepted at $P < 0.05$ and post-hoc Tukey test was adopted to distinguish the source of variation among the molt stages. When a nonparametric test was employed, a Kruskal-Wallis ANOVA with a Multiple Comparison of Mean Ranks was used. The data are presented as mean ± SE (n), where n is the number of crabs per each experimental group. ANCOVA analysis was used for shell-hardness of postmolt crabs. The ANCOVA assumptions were evaluated by K-S and Cochran C test.

4.4 Results

4.4.1 Effects of multiple injections of *CasBurs*-α and -β *dsRNAs* on *CasPPO-hemo* expression in hemocytes and the cuticle hardness

4.4.1.1 Levels of *CasBurs*-α and -β expression and CasBrus proteins

The multiple injection of *CasBurs*-α *dsRNA*, *CasBurs*-β *dsRNA*, and *CasBurs*-α+β *dsRNA* reduce the levels of *CasBurs-α and -β* transcripts in the TGC obtained at 48 hrs after ecdysis (Fig. 14A and S7). The control group (n=7) shows an expression level of 1.1 ± 0.3 x 10^6 copies/µg total RNA for α and 7.3 ± 1.7 x 10^6 copies/µg total RNA for β. In the experimental dsRNAαβ group, a significant knockdown of both α and β is observed ($P < 0.05$; n=7): α = 4.8 ± 2.8 x 10^3 copies/µg total RNA (~99% knockdown) and β = 9.2 ± 4.4 x 10^5 copies/µg total RNA (~90% knockdown). The

dsRNAα group (n=6) has reduced levels of α transcripts, estimated with 2.0 ± 1.2 x 10^5 copies/µg total RNA (81% knockdown) and β expression of 2.0 ± 0.4 x 10^7 copies/µg total RNA (175% over the control expression). On the other hand, dsRNAβ group (n=5) shows levels of α, with 9.7 ± 1.1 x 10^6 copies/µg total RNA (811% over the control expression) and of β expression with1.6 ± 0.6 x 10^5 copies/µg total RNA (98% knockdown).

4.4.1.2 The effects of *CasBurs*-α and -β *dsRNA* injections in the hardness of postmolt cuticle

The cuticle hardness in each treatment is shown as in Figs. 15A-C. Control group cuticle hardness increases from 25 ± 6.8 DU at 2 hrs after ecdysis to 42.9 ± 0.9 DU at 8 hrs. After, control cuticle reaches 61.7 ± 2.6 and 88.6 ± 0.4 at 24 and 48 hrs after ecdysis, respectively (n=7). The dsRNAαβ group does not show differences in the hardness of the cuticle compared to controls (n=7). The hardness of the cuticle of dsRNAαβ group is quantified in 25.0 ± 3.0 DU at 2 hrs after ecdysis, while dsRNAα and dsRNAβ groups show 18.0 ± 1.0 and 10.0 ± 4.5 DU respectively. At 24 hrs after ecdysis the cuticle hardness for each experimental group is from 63.8 ± 3.2 DU (dsRNAαβ, n=7), 52.1 ± 3.6 DU (dsRNAα, n=6), and 53.5 ± 7.4 DU (dsRNAβ, n=5) increasing to 78.4 ± 4.1 (dsRNAαβ, n=7), 71.1 ± 4.5 (dsRNAα, n=6), and 69.5 ± 1.5 (dsRNAβ, n=5) at 48 hrs after ecdysis.

The values of HD_{50}, the time to reach the 50% of cuticle hardening, are estimated at 8.3 ± 0.9 hrs for the control group (n=7). Similar to the control, dsRNAαβ shows a HD_{50} of 7.7 ± 0.9 hrs (n=7). On the other hand, dsRNAα shows HD_{50} estimated in 14.3 ± 1.3 hrs (n=6) and dsRNAβ an HD_{50} of 12.8 ± 1.5 hrs (n=4).

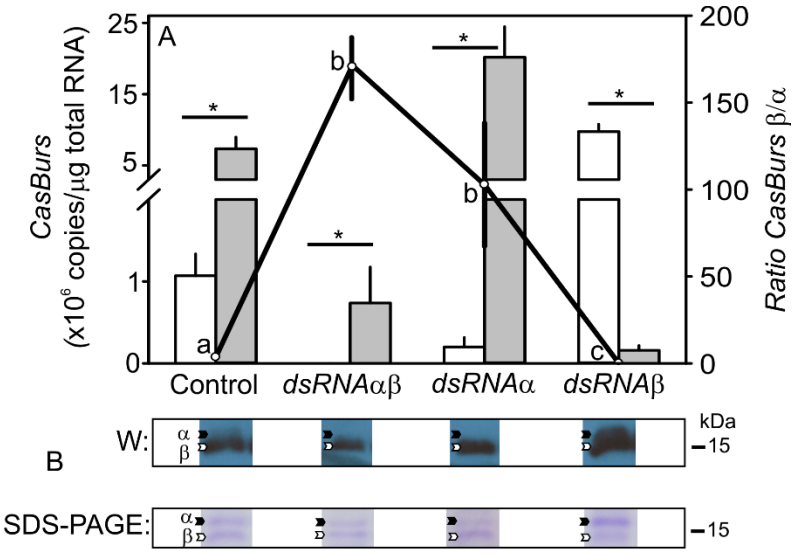


Figure 14. *CasBurs*-α and -β expression in the TGC at 48 hrs after ecdysis. A) A significant knockdown by the multiple injections of *CasBurs*-α and *CasBurs*-β *dsRNAs* is observed respect to the control (n = 6-7). Open bars=*Burs*-α and close bars=*Burs*-β. Asterisk represents significant differences ($P < 0.05$). Solid line represents the ratio of *CasBurs*-β/α. B) CasBurs detected in pericardial organ, 48 hrs after ecdysis; W= western blot analysis; 15% SDS-PAGE.

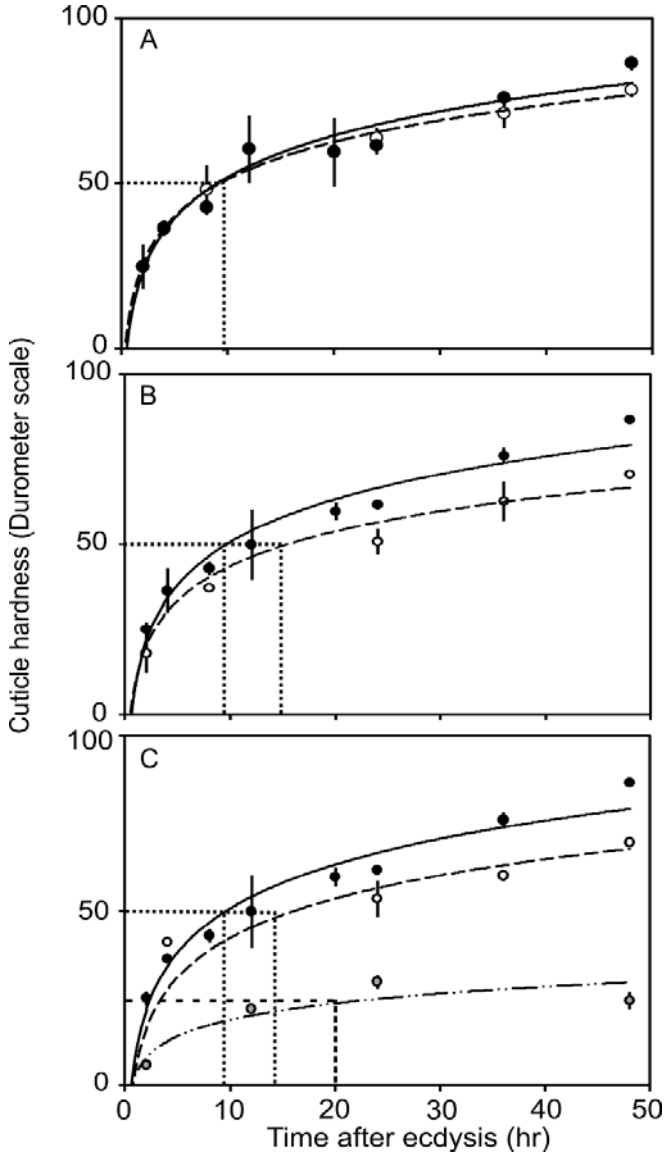


Figure 15. Effects of *CasBurs*-α and –β *dsRNA* injections on the cuticle hardness after ecdysis (48 hrs). The hardness of the cuticle was evaluated using a hand-held Shore-type durometer. Solid lines=control; dashed lines=*dsRNA* injected crabs; dot lines=HD_{50}; short dashed line at C=HD_{25}. A) The dsRNAαβ group and control show similar hardening models ($P > 0.05$). B) dsRNAα group shows a significant slowed shell-hardening of the cuticle ($P < 0.05$). Similar, C) dsRNAβ group significantly slows the shell-hardening during postmolt ($P < 0.05$). At "C" the dashed-double dot line (-··-) represents the *CasBurs*-β-*dsRNA* injected crab which shows extended cuticle softness. The significant differences were estimated by ANCOVA at $P < 0.05$. R^2 means correlation indexes for the models and the values are between 0.87 – 0.99.

4.4.1.3 Effects of *CasBurs*-α and -β *dsRNA* injections on hemocyte *CasPPO-hemo* expression and CasHLS-PO activity

The expression of *CasPPO-hemo* in hemocytes (24 hrs after ecdysis) does not show significant differences between control and *CasBurs*-α and –β dsRNA experimental groups (Fig. 16A; $P > 0.05$). In the hemocytes of control animals, levels of *CasPPO-hemo* expression are measured as $2.5 \pm 0.4 \times 10^6$ copies/µg total RNA (n=7). Hemocytes *CasPPO-hemo* expression in dsRNAαβ group shows transcript levels of $2.2 \pm 0.6 \times 10^6$ copies/µg total RNA (n=7), while dsRNAα and dsRNAβ have $3.2 \pm 0.9 \times 10^6$ (n=6) and $3.3 \pm 1.0 \times 10^6$ copies/µg total RNA (n=5) respectively (Fig. 16A).

No differences were found in CasHLS-PO activities of all experimental groups and control (n=5-7; Fig. 16B). The CasHLS-PO activities in each experimental group are 12.5 ± 1.5 OD/mg HLS (control; n=7) and 12.7 ± 3.5 OD/mg HLS (dsRNAαβ; n=7), 11.5 ± 2.6 (dsRNAα; n=6) and 10.0 ± 2.4 OD/mg HLS (dsRNAβ; n=5).

4.4.1.4 The effects of *CasBurs-α and -β dsRNA* injections on CasPPO-hemo in hemocytes

During postmolt, all the hemocytes show a positive staining with a rabbit-CasPPO-hemo antiserum in all the experimental groups and control (Figs. 16C and 17A). The intensity of CasPPO-hemo staining is significantly reduced in the hemocytes of dsRNAα and dsRNAβ groups ($P < 0.05$; Fig. 16C). However, the hemocytes injected from dsRNAαβ group have a staining intensity similar to of controls.

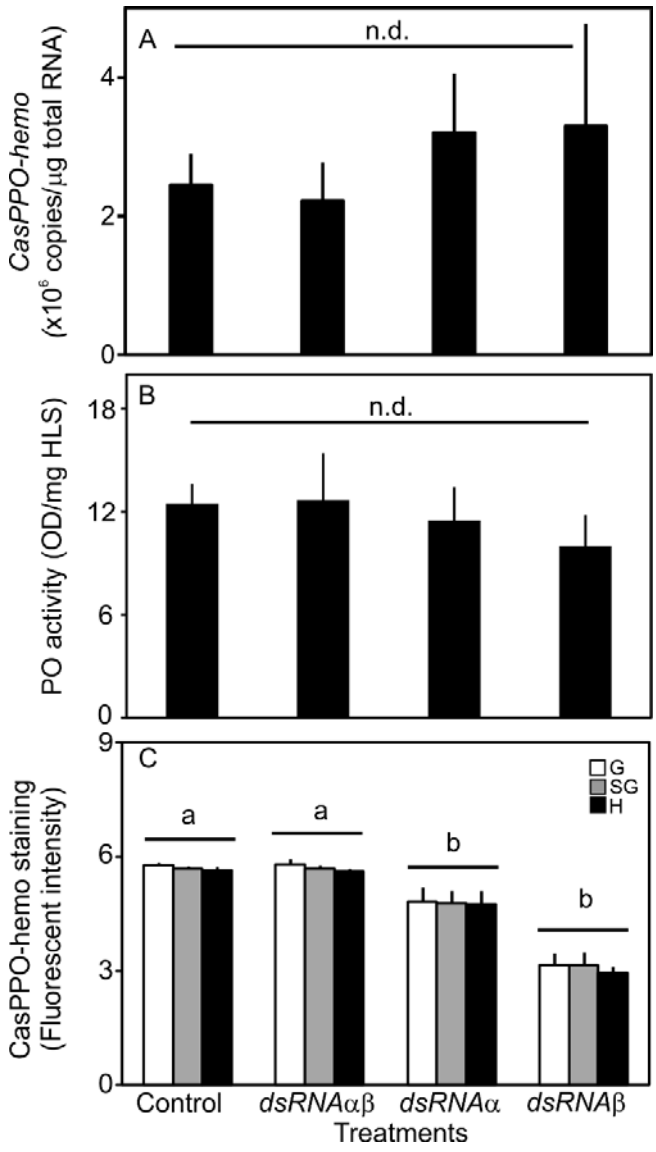


Figure 16. Levels of Prophenoloxidase (*CasPPO-hemo*) transcripts and PO activity from hemocytes at 24 hrs after ecdysis. A) Expression analysis by qPCR. No significant differences (n.d.) are detected between control and experimental groups by one way ANOVA (n=5-7). B) CasHLS-PO activity, no significant differences are detected by one way ANOVA (n=5-7). C) Intensity of the positive signal of α-CasPPO-hemo serum staining in hemocytes. Different letters represent significant differences ($P < 0.05$; n=5-6).

4.4.1.5 The effects of *CasBurs*-α and –β *dsRNA* injections on cytosolic complexity and granularity of hemocytes

Hemocytes are grouped into three type by their size (Fig. 18A and B, FSC-H) and into three by cytosolic complexity and granularity (Fig. 18A and C, SSC-H). Three hemocyte types are distinguished based on FSC-H vs SSC-H values (Fig. 18A): Gs with the highest SSC-H values, SGs with intermedia SSC-H values, and Hs with lowest SSC-H values. The distribution of the hemocyte sizes (Suppl. Table S2) in all the experimental groups are similar to those of control (Figs 18A-A'; Fig. 18B). The granularity observed in Gs in the dsRNAαβ group is significant reduced than the control ($P < 0.05$). For each differentiated hemocyte type in control the granularity values are: Gs = 22.7 ± 0.7 x 10^5 SSC-H, SGs = 57 .6± 0.8 x 10^4 SSC-H, and Hs = 24.4 ± 0.5 x 10^4 SSC-H. The granularity values in the dsRNAαβ group are: Gs = 19.6 ± 0.6 x 10^5 SSC-H, SGs = 61.6 ± 6.2 x 10^4 SSC-H, and Hs = 26.1 ± 1.8 x 10^4 SSC-H.

Granularity of hemocytes in dsRNAα and dsRNAβ groups (Figs. 18C) is not significant different ($P > 0.05$) respect to control. The detected values of granularity in Gs, SGs, and Hs with dsRNAα and dsRNAβ groups are: Gs = ~24.1 ± 0.1 x10^5 SSC-H, SGs = ~58.1 ± 0.1 x 10^4 SSC-H. Hs increased their SSC-H to ~29.0 ± 0.2 x 10^4.

There are no differences in total and differential counting of hemocytes (THC and DHC; $P < 0.05$) between control and experimental groups (Fig. 19A-B).

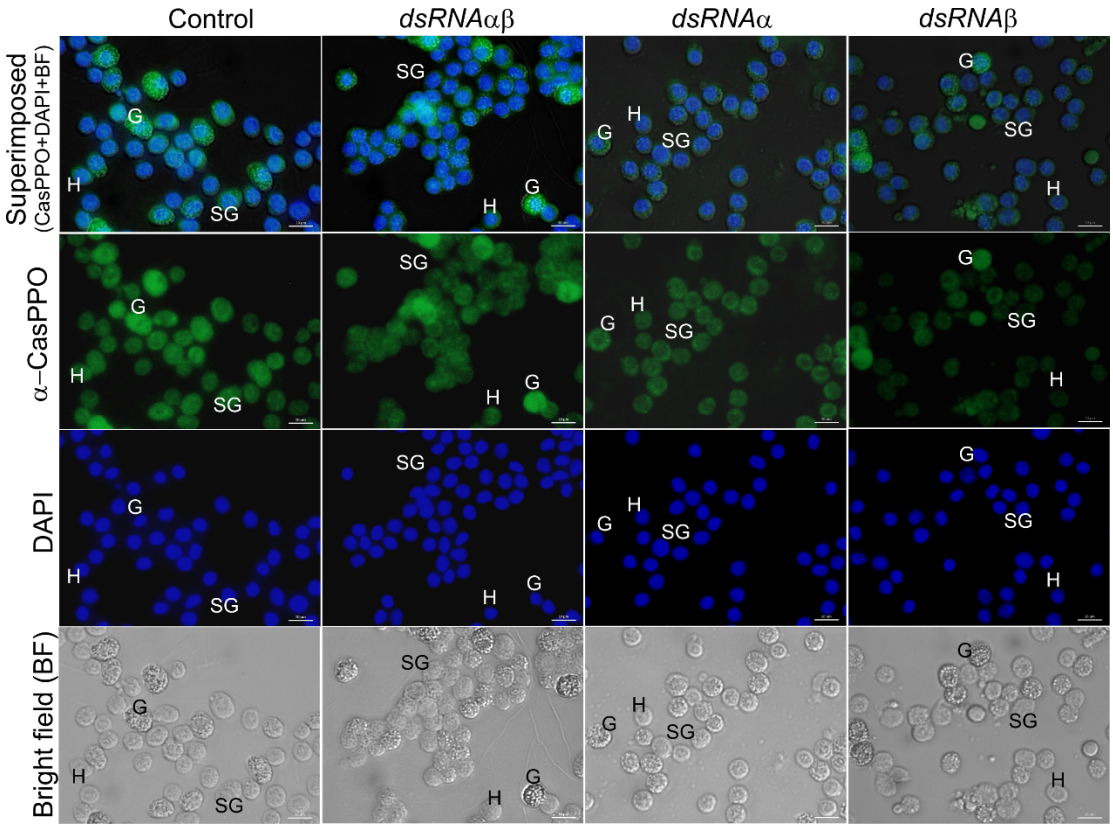


Figure 17. CasPPO-hemo immunostaining in hemocytes collected 24 hrs after ecdysis using α-CasPPO-hemo serum. Representative images for each experimental group (n=5-6). G=granulocytes; SG=semigranulocytes; H=hyaline cells.

4.4.1.6 Effects of *CasBurs*-α and -β *dsRNA* injections on hemocyte *CasPPAE* expression

The expression of *CasPPAE* in hemocytes (24 hrs after ecdysis) is reduced in *CasBurs*-α and –β *dsRNA* injected crabs respect to the control. Specifically, a significantly reduced expression is observed (Fig. 20; $P < 0.05$) in *CasBurs*-α *dsRNA* injected group. In the hemocytes of control animals, levels of *CasPPAE*

expression are measured as 4.4 ± 0.9 x 10^6 copies/µg total RNA (n=7). Hemocytes *CasPPAE* expression in dsRNAαβ group shows transcript levels of 4.1 ± 1.1 x 106 copies/µg total RNA (n=7), while dsRNAα and dsRNAβ have 2.0 ± 0.3 x 10^6 (n=6) and 2.8 ± 0.6 x 10^6 copies/µg total RNA (n=5), respectively (Fig. 20).

4.5 Discussion

We report in this paper that CasBurs plays a role in the shell-hardening process of the blue crab, specifically involving hemocytes. Hemocytes are one of the target tissues of CasBurs or a putative homodimer of CasBurs-α or -β. However, our data indicate that the mode of action of CasBurs, homodimer of α or β does not involve *PPO* expressed in hemocytes.

Multiple injections of *dsRNA* have been used in crustaceans to define functions of a gene of interest [45,56; Techa and Chung, in press]. Moreover, *dsRNA* injections have been used to study the bursicon roles in insects by the co-injection of α+β and α *dsRNA* [31,138,139,226]. We executed multiple injections (22-30 injections) of 10 ug *dsRNA* of *CasBurs-α* and *-β* into crabs starting at intermolt and continuing to ecdysis. Since a skewed ratio of Burs-α and -β is common in most arthropod species [58,62,64,65,227] and in *C. sapidus*, there may be a CasBurs-β homodimer [62], we also injected *CasBurs*-α or -β *dsRNA* alone.

Multiple injections of *CasBurs*, *Burs*-α, or -β *dsRNA* reduced the expression of *Burs*-α and -β in the TGC of all the experimental animals by ~80-90%, compared to controls. In addition, remaining encoded Burs-α and -β were detected in the pericardial organ [62,64,65,228] during postmolt. The injections of either α or β

dsRNA lengthen the duration of the hardening of the cuticle at postmolt, while no differences were found on *CasPPO-hemo* expression and PO activity in hemocytes.

Multiple injections of *CasBurs dsRNA* do not affect shell-hardness as measured by a durometer, while *CasBurs*-α or -β alone slows the process, compared to controls [27,46, Alvarez and Chung, in preparation]. The rates of hardness vary depending on the area of cuticle [195] and the hardness of the cuticle was evaluated on several point of the mesogastric area and for 48 hrs.

However, in dsRNAα and dsRNAβ experimental groups, all three hemocytes exhibit α-CasPPO-hemo immunostaining intensities lower than those of controls. While Gs in the dsRNAαβ group show a reduced cytosolic granularity compared to those of controls. Hemocyte granules are described as enzyme-filled lysosomes [111] and represent an important cytoplasmic storage machinery for many proteins [111,209] in which enzymes and other proteins are associated with the PPO activating cascade system [209]. The degranulation of hemocytes, particularly Gs, is known as a prototypical immune reaction in response to pathogenic bacterial infection, indicating the exocytosis of the granule contents and cell lysis [209,217]. Low granularity may indicate the amounts of proteins stored in the cytosol. All experimental groups injected with *dsRNA* show no differences in *PPO* expression and PO activity measured using an *in vitro* assay, implying there may be no changes in the content of PPO in cytoplasmic granules of the hemocytes [209,229]. However, the endogenous PO activity depends on the proteolytic activation of PPO by PPO activating enzymes (PPAEs) [26,36,230,231]. PPAEs are known components of hemocyte granules [229]. We tested the expression of a known *Callinectes PPAE*

(*CasPPAE*, GenBank accession No DQ667138.1) in hemocytes in all the experimental treatments using a qPCR (Fig. 7). Interestingly, a reduced level of *CasPPAE* transcripts are observed in α and β *dsRNA* injected animals with respect to the control, although these values do not statistically differ. Particularly, the dsRNAα group has significantly lower expression levels of *CasPPAE* than the other treatments.

The reduced granularity detected in Gs in *CasBurs*-α+β *dsRNA* injected crabs, may indicate that Gs respond to bursicon knockdown releasing its PPO cascade system to support the cuticle hardening in this experimental group. Previously, it has been observed SGs and Hs may have a more specific role in shell-hardening of the blue crab (Alvarez and Chung, in preparation). However, Gs may support the cuticle hardening system with the PPO cascade system stored in its granules [111,209]. Bursicon may mediate specific pathways during shell-hardening that are affected by the co-injection of α and β *dsRNA* resulting in the degranulation of Gs as happened in wound healing and cuticle repairs [232].

The injections of *CasBur*-α *dsRNA* are effective and reduce the levels of the target transcripts in experimental crabs. However, the efficiency of knockdown appears to be different: control expression of α = 1.1 ± 0.3 x 10^6 copies/µg total RNA and β = 7.3 ± 1.7 x 10^6 copies/µg total RNA; *CasBurs*-α+β *dsRNA* 99% and 90% knockdown of α and β; *CasBurs*-α *dsRNA* 81% knockdown of α; *CasBurs*-β *dsRNA* 98% knockdown of β. The co-injection of α+β increases the knockdown efficiency of α unlike α *dsRNA* injection alone. When the expression of α is reduced by 81% the

expression of β is enhanced ~2 folds respect to the control group. Similarly, the knockdown of β affects positively the transcription of α in ~8 folds.

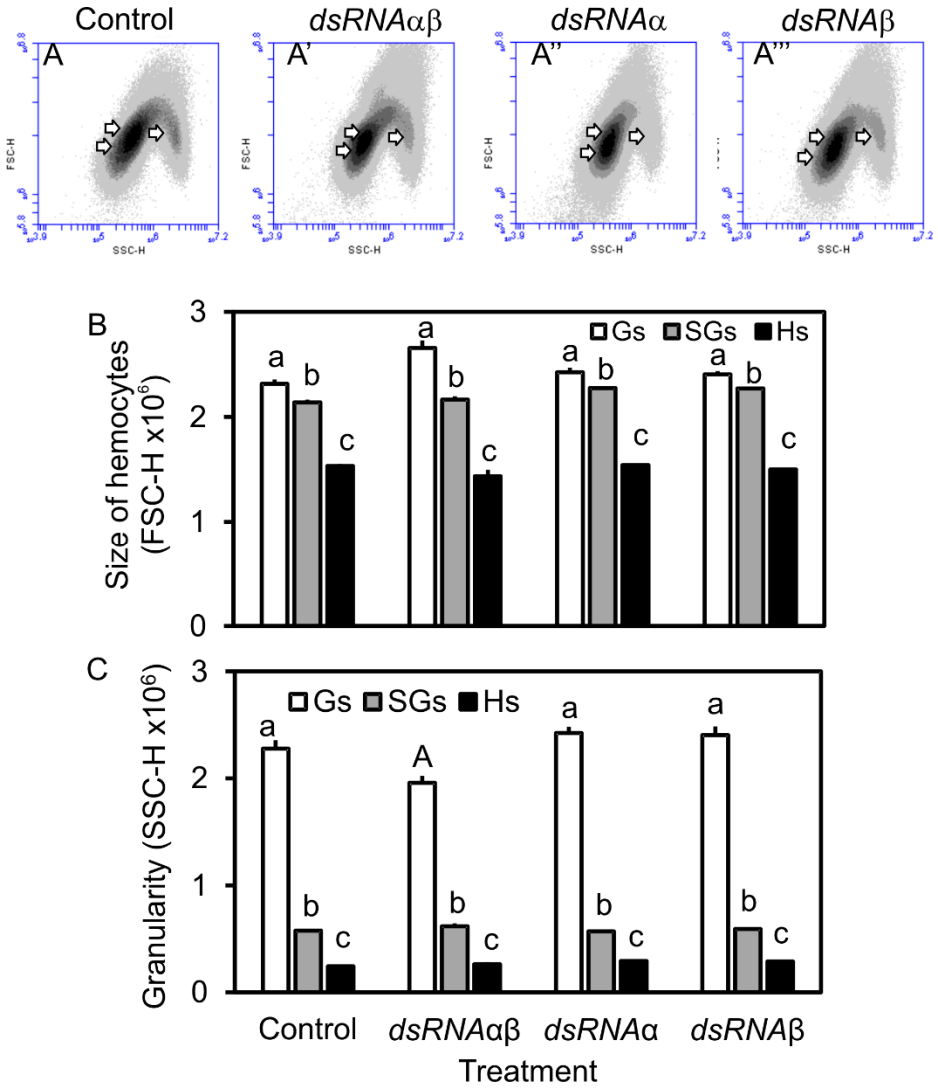


Figure 18. Flow-cytometric analysis of hemocytes, 24 hrs after ecdysis in *dsRNA* injected crabs and control group (n=5-7). Control and experimental dsRNA groups show similar patterns. A-A''') Three hemocyte types are differentiated. B) The values of FSC-H, associated to cell sizes do not show significant differences between control and experimental groups. C) Cell granularity: Significant differences between control and dsRNAαβ are detected in the granularity of Gs ($P < 0.05$; n=7). The hemocytes granularity of dsRNAα and dsRNAβ does not show significant differences respect to the control (two way ANOVA, n=5-7) .

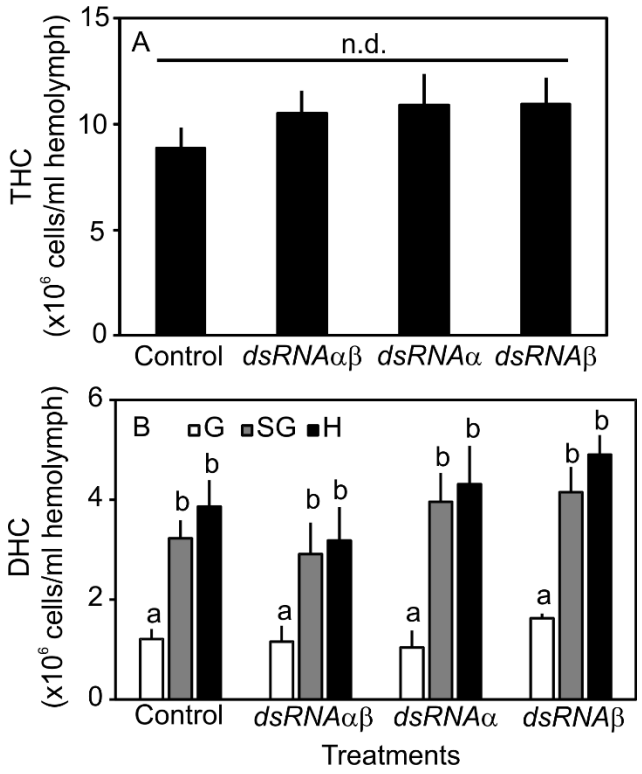


Figure 19. Total (THC) and differential (DHC) hemocyte counting using flow-cytometry. No significant differences are detected between control and experimental groups (n=5-7).

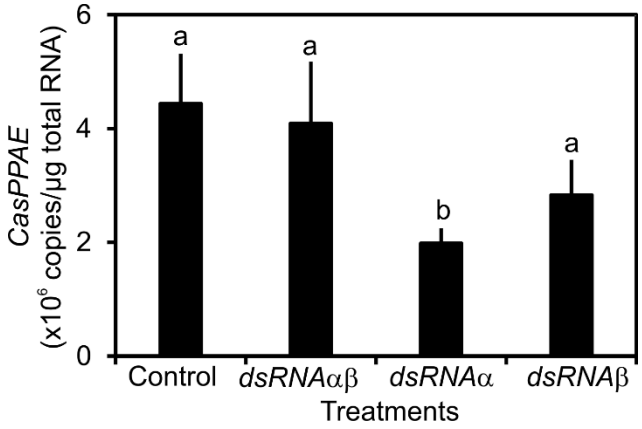


Figure 20. *Callinectes sapidus* prophenoloxidase activating enzyme, *CasPPAE* (GenBank accession No DQ667138.1) expression levels in hemocytes (n=5-7). The expression analysis was performed by a qPCR assay using a Fast SYBR Green Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystem). The data are expressed in mean ± SE of copies/µg total RNA. The significant differences were analyzed by one way ANOVA at $P < 0.05$.

Skewed ratios between α and β expression levels are commonly noted in arthropods [64,65,227]: some species exhibit higher expression of α than β [58,64] and others higher expression of β than α [62,65,227]. The skewed ratios between α and β also suggest the presence of putative homodimers [18,25,26]. In *C. sapidus*, our previous study shows that there may be a putative CasBurs-β homodimer present in POs. The function of a homodimer has not been well-defined in arthropods: recently in *Drosophila*, both α-α and β-β homodimers were found to induce the expression of antimicrobial peptides genes via the upregulation of NF-kappaB Relish transcription factor during and immediately after the ecdysis [142]. It appears that maintaining the inherent physiological ratio between α and β may be of great importance in Burs functions. The over expression of one bursicon transcript may increase its translation rate and induce the formation of homodimers.

Not all insect tanning appears to depend on the presence of bursicon [138,226]. In *D. melanogaster*, bursicon binds to LGR transduces its signal via cAMP [58,140,222]. Moreover, it induces the expression of antimicrobial peptides during the insects ecdysis, via the upregulation of transcription factors [142]. The presence of homodimer receptors are not known but these homodimers may have their own separate receptors [58,140,142,222]. In some insects including *Drosophila*, bursicon acts on the phosphorylation of tyrosine hydroxylase (TH) and increases its enzymatic activity [52,91,233]. Specifically, the monophenol activity of TH allows the hydroxylation of tyrosine to DOPA, a required substrate for sclerotization and melanization of the cuticle [51,61,91]. Downstream in the sclerotization pathway, POs oxidize diphenol substrates such as DOPA to crosslink

the protein and chitin fibers in the cuticle [17,21]. In *Tribolium castaneum* and *Bombyx mori,* the tanning process of these species does not depend on bursicon [138,226]. *Tribolium* and *Bombyx* may have a sclerotization system that is independent of TH [138,226]. Additionally, the monophenol activity of PPO has been reported and proposed in the earlier step of the sclerotization system [11,17,21,88,234,235].

In *Callinectes*, the cuticle sclerotization system may depend on the monophenol activity of PPO. The results demonstrate that the co-injections of α+β *dsRNAs* do not affect the levels of *CasPPO-hemo* in the experimental crabs. Moreover, hemocyte CasPPO-hemo that has monophenol activity [95], involves the shell-hardening of this species (Alvarez and Chung, in preparation).

Additionally, the synthesis of (a) diphenol (or s) precursor for sclerotization is not limited to the activity of POs. The synthesis of NAD and NBAD, precursors of quinones in the insect tanning process are synthesized by hypodermal cells being the phenol precursors for cuticle tanning in many insects [11,12].

The endogenous concentrations of bursicon in the hemolymph of *C. sapidus* [62], strongly support that this hormone have a physiologically important role in ecdysis. Bursicon titers increase ~5 folds at the completion of ecdysis and decline immediately at 2 hrs after ecdysis [62]. Similar to bursicon, the crustacean cardioactive peptide (CCAP) and crustacean hyperglycemic hormone (CHH) increase at ecdysis [62]. CCAP is essential in the ecdysis behavior and timing of insect and crustaceans [10,161]. While CHH mediates the hyperglycemic activity, ion transport, and water uptake in the process of body expansion [9,66,236]. The disruption of

these endocrine control pathways during the ecdysis have shown to be lethal for the animals [59,237]. We have also observed that only the animals injected with the β *dsRNA* show some mortality during the ecdysis. This implies that the injection of β *dsRNA* may have disrupted a coordinated hormonal cascade that is required for successful ecdysis.

In our laboratory, we have identified several putative target tissues of CasBurs by isolating partial cDNA sequences of LGR like molecule (unpublished data). By using RNAi-mediated suppression of genes encoding Burs receptor, the resulting phenotypes may unravel the role of bursicon during ecdysis and postmolt of crustaceans.

4.6 Supplemental Tables and Figures

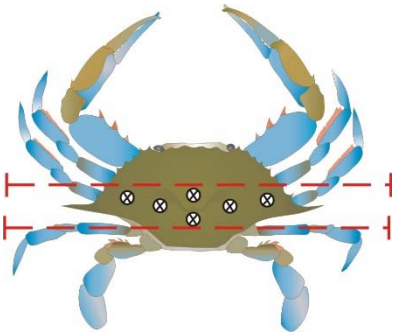
4.6.1 Supplemental Tables

Supplemental Table S2. Differentiated cell sizes of hemocytes using flow-cytometry. The values are expressed as mean ± SE FSC-H x 10^5. No significant differences are observed between control and experimental groups ($P > 0.05$). G= Granulocytes, SG= Semigranulocytes, H= Hyaline cells.

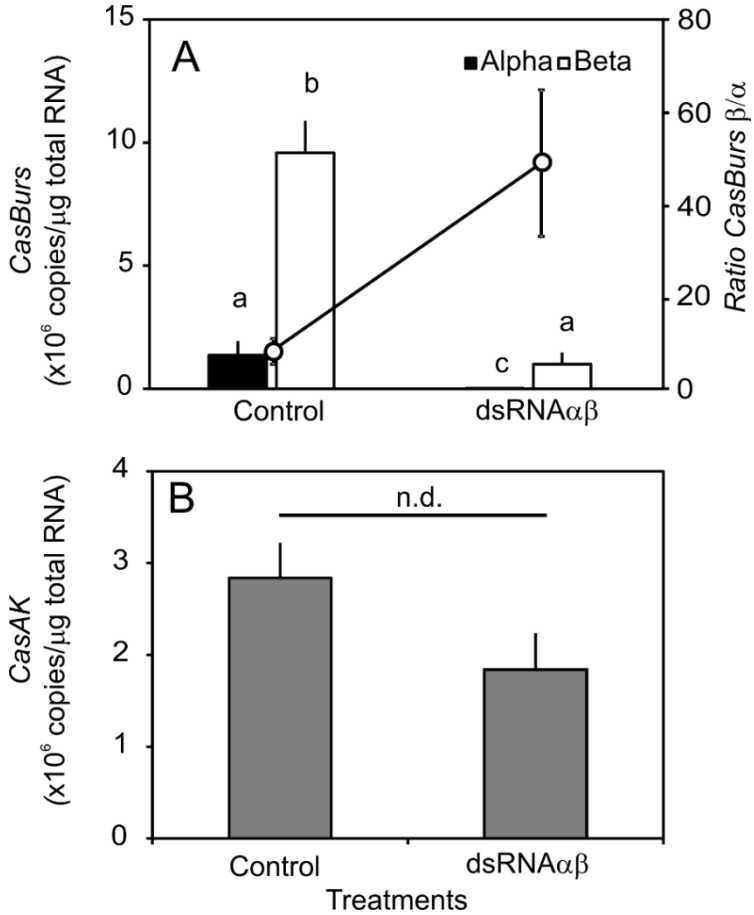
Experimental groups	G	SG	H
Control	23.1 ± 0.4	21.4 ± 0.2	15.3 ± 0.2
*dsRNA*αβ	26.5 ± 0.7	21.6 ± 0.3	14.3 ± 0.5
*dsRNA*α	24.3 ± 0.4	22.7 ± 0.1	15.4 ± 0.0
*dsRNA*β	24.0 ± 0.3	22.7 ± 0.8	14.9 ± 0.0

SE values as 0.0 denote SE<10^4

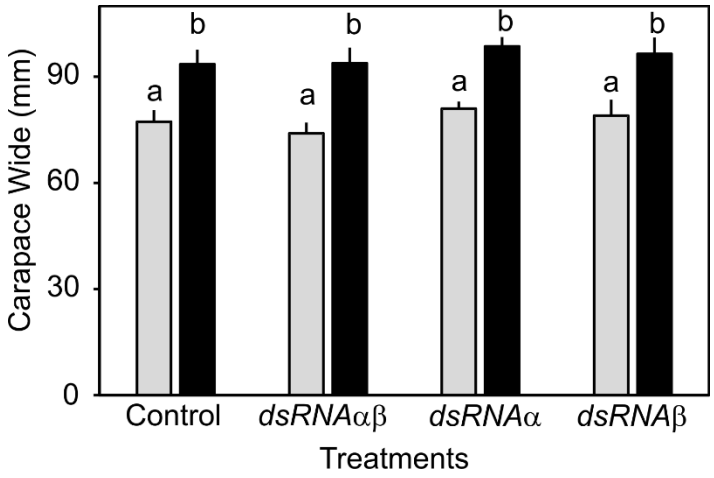
4.6.2 Supplemental Figures



Supplemental Figure S7. Dorsal carapace mesogastric area of the blue crab (between dashed lines) and the durometer sampling points (white dots).



Supplemental Figure S8. Preliminary data. Co-injections *CasBurs*-α and *CasBurs*-β *dsRNA* (10 µg each) in juvenile crabs (n=4). A knockdown effect on α and β transcripts is observed after 4 injections. Crabs were injected every other day during a week. A) Beta transcripts are significant ($P < 0.05$) knocked-down by ~90% and α by ~98 %. B) *CasAK* in the TGC was used as referent gene.



Supplemental Figure S9. Molt related growth. Carapace wide (mean ± SE CW) of crabs after ecdysis (n=6-7). Grey bars = intermolt crabs (time zero). Black bars = 48 hrs after ecdysis. With a similar initial size the control and experimental crabs do not show significant differences in body size after ecdysis.

Chapter 5: General Discussion and future work

Molting is the key process in the life history of arthropods and is essential for their growth, development, and reproduction. Crustaceans undergo the first molting processes during embryogenesis, then embryonic molts direct the preparation of embryos for hatching [238–242]. After hatching, the swimming larvae undergo several stages, each stage is mediated by a molt that includes changes in larval physiology and morphology [243,244]. Next, in juvenile crustaceans the molting cycle permits growth and transition into adulthood. Additionally, in many crustaceans the adult females only mate in the softest condition that occurs after the pubertal molt [1,2].

This dissertation focuses on the hypothesis that hemocyte PPO and bursicon hormone are involved in the shell-hardening of crustaceans. The results include a complete description about the molecular cloning and characterization of *PPO* cDNA sequence (*CasPPO-hemo*, JX047321, GenBank). Also, it included *PPO* expression and PO activity analyses during the molting cycle and RNAi experiments. In addition, hemocyte differentiation and its endogenous distributions in the hemolymph help to define the role of hemocytes and PPO in the shell-hardening process of the blue crab. Moreover, it is reported that bursicon, as a hormone candidate to involve the shell-hardening of crustaceans, does not upregulate *PPO* expression and PO activity. So, the role of bursicon during the shell-hardening process of the blue crab is discussed. Furthermore, it is suggested that bursicon homodimers may be involved in the sclerotization in crustaceans, perhaps through the upregulation of PPAEs, the serine-like enzymes which activate PPO in the sclerotization process.

Every molt involves synthesis of a new soft cuticle, exuviation, and hardening of the new soft cuticle by the shell-hardening process. The shell-hardening process is an essential part of the molting cycle, because the crustacean exoskeleton protects against predators, offers a barrier against pathogens and body support by muscle attachments [1,2,245]. As a consequence, the exoskeleton is a requirement for mobility, defense, and feeding. Hence, the investment in energy that each crustacean implements for growing throughout the molting cycle is capitalized in survival after ecdysis, and a hard exoskeleton maximizes the survival probabilities. Therefore, a hard exoskeleton soon after ecdysis is a priority during postmolt.

The shell-hardening process of insects has been well studied and is a reference for other arthropods [11,12,14,17,21]. The tanning process, which involves the sclerotization of cuticle proteins and melanin synthesis deposited in the cuticle, facilitates insect hardening. Melanization also develops the different coloring observed in insects [11,12,235,246,247]. Unlike crustaceans, most insects do not incorporate calcium in the tanned cuticle. However, biomineralization (i.e. deposition of calcium carbonate and calcium phosphate) of crustacean cuticles depends on the previous sclerotization, proteins in the cuticle, and chitin concentration [74–79].

In this study is reported *Callinectes* hemocytes are recruited into the hemolymph in a molt stage dependent manner and so is their derived PO activity. The hemocyte source of PPO seems to vary in different molt stages and crustacean species [37,82,105], this dissertation began with the assumption that all hemocytes may be a potential source of PPO. If so, only a positive correlation between total hemocytes and its derived PO activity in the hemolymph should be expected during

the molting cycle. However, this research demonstrates that all hemocyte cells do not contain equal quantities of PPO, and specific types of hemocytes are the source of PPO [95].

Every differentiated type of hemocyte in the blood stream of the blue crab has a distinct distribution during the molting cycle. Hence, the prevalence of a specific group of hemocytes during one molt stage may correlate with functions of those hemocytes at particular period of the molting cycle. As an example, a particular role is suggested for SGs and Gs during premolt, in preparation for ecdysis. On the other hand, a particular role of Hs is suggested during postmolt. These suggestions are based on three particular findings: i) Hs are PPO positive during postmolt, ii) They increase in number specifically in this stage, and iii) injections of *CasPPO-hemo-dsRNA* reduce their detected PPO at postmolt.

Using a molecular approach, the use of RNAi by the injection of specific *CasPPO-hemo-dsRNA* was found to endanger the cuticle hardening of the experimental crabs by the knockdown of *CasPPO-hemo*. This discovery demonstrates that i) CasPPO is part of the sclerotization system in the blue crab and ii) infers the transport of the CasPPO from hemocyte into the new cuticle. Interestingly, the experimental results also suggest that hemocytes may have distinct roles during the shell-hardening process, supporting the hypothesis that Hs and SGs may be involved in the shell-hardening process of the blue crab.

In decapod crustaceans, multiple injections of *dsRNA* have been employed to define the functions of a gene of interest [29,30, Techa and Chung, in press]. Additionally, *dsRNA* injections in crustaceans have been used to investigate the

immune role of PPO [132,248,249]. The suppression of PPO results in higher host susceptibility to pathogens [248]. The use of short-term injections of *PPO-dsRNA* (1 or 2 injections) have shown the upregulation of PPO, when the animals are challenged by bacteria [249]. Here, apparently, the use of multiple injections of *dsRNA* to knock down *CasPPO-hemo* does not have an adverse on the experimental crabs. Perhaps, the repeated puncturing of the experimental crabs with needles may positively upregulate *PPO* expression in some hemocytes, but not those involved in the shell-hardening process that contain PPO only at specific molt stages.

It is evident that the sclerotization of the cuticle and the immune system are intimately related during the shell-hardening process of crustaceans. So, a relative comprehensive model of the shell-hardening process may consider this relationship in the role of hemocytes and PPO during the progression of the cuticle hardening. The sequential events included in this progression start with ecdysis, which is the result of interactions between a set of hormones and neurohormones [4–8].

Ecdysis in crustaceans begins with the increase in body volume by a regulated isosmotic intake of water, which provides the necessary expansion of body to split the old exoskeleton [1,250]. Once the exoskeleton is opened, the animals execute corporal movements. Helped by corporal hydrostatic support, crustaceans release their limbs and body from the exuvia [1,2,251]. The pressurization of corporal cavities with fluid permits the development of muscle while the cuticle is soft and pliable [1,2,251].

Ecdysis may result in the mortality of many cells, as a consequence of the stress of the exuviation [73,250,252], changes in hemolymph osmotic conditions,

and/or ongoing growth of internal organs [1,2]. Ecdysis in insects includes apoptosis of cells from different tissues such as wing discs, hypodermis, and prothoracic gland, especially when the adult emerges [141,253,254].

Apoptosis activates mechanisms to clear dead cells. These mechanisms depend on phagocytes [255]. In crustaceans, the immune system relies on the innate responses of hemocytes and AMPs [39,40]. The principal immune function of Hs is phagocytosis [39]. It is widely known, the role of phagocytic cells is the digestion of dead cells [141,253–255]. Overall, the dead cells resulting from apoptosis events in insect molting are phagocytized by the plasmatocytes [141,253,254], the insect blood cells which are the equivalent to the crustacean Hs. Moreover, the increase of *Callinectes* Hs during postmolt and the accompanying reduction of SGs and Gs fit to similar patterns observed in some insect species during the final ecdysis, when the adults emerge [256,257]. In the blue crab Gs are the main cells that express PPO during the entire molt cycle. The data support that SGs contain PPO at different molt stages. During premolt these two types of hemocytes represent the maximal number of cells in the hemolymph, in a characteristic pattern suggesting preparation for ecdysis. At ecdysis, the total number of these two hemocyte types declines. This decline could be attributed to an increase in volume of the hemolymph by the isosmotic intake of water which allows to perform the shedding of the exoskeleton [1,250]. However, the abundance of Hs stays constant from premolt to ecdysis, suggesting the increment in plasma volume may not be the only cause that brings out the depletion in numbers of SGs and Gs during ecdysis. Causes such as hemocyte mortality may also be involved.

It is a fact that crustacean hemocyte PPOs lack a signal peptide [38,44–46,88,94,95,171–173,176]. Therefore, its release from hemocytes does not include the cellular secretory pathway. Exocytosis and cell autolysis are the mechanisms of release [125,209,217,229]. For instance, when the immune system is threatened, Gs and SGs are lysed to release immune components (PPO activating system, cytokines, adhesive and opsonic factors) that perform the encapsulation and cytotoxic attack to parasites and pathogens. Responding to the opsonic factors, Hs phagocytize the pathogens [119,218]. Moreover, Hs perform the clearance of dead hemocytes as well, during an immune reaction [119,218].

Arthropod immune responses are coordinated reactions that are based on cooperative work between the hemocyte types [40,217,258–260]. It is notable that insect plasmatocytes induce apoptosis of Gs in the moth *Pseudoplusia includens* [259] during encapsulation reactions. In the coordinated and subsequent steps the encapsulation of parasites in the moth, a monolayer of Gs attaches to the parasites triggering the release of opsonic and adhesive factors. The arrival of plasmatocytes, responding to these factors, contributes to the encapsulation process by layering over Gs and inducing their apoptosis. As a result, the PPO activating system is released. Moreover, plasmatocytes release one or more factors that induce apoptosis of Gs and phagocytize dead Gs [259].

So far, there is no evidence that Hs induce apoptosis of Gs or SGs in crustaceans. However, the crustacean encapsulation process has been studied in the past [103,261]. One of the main reports, in the crayfish *Astacus leptodactylus*, describes the role of hemocytes during the encapsulation process using *in vitro*

assays [261]. The results contradict the findings in insects and report that Hs do not participate in the encapsulation. Even though plasmatocytes do not form the capsule without Gs primary layer [262], plasmatocyte participation seems essential for encapsulation. Crustacean hemocyte types do show cooperating during immune reactions [40,217,258], and more information about hemocyte interactions at specific molt stages may require future investigations, especially during the shell-hardening of the cuticle. For example, investigation into peroxinectin levels during ecdysis may contribute to define interaction among Hs, SGs and Gs. Peroxinectin, a known opsonic factor of crustacean, is released by Gs and stimulates phagocytosis by Hs [42].

The distributions of hemocytes during ecdysis and postmolt of the blue crab suggest hemocyte autolysis may occur. This mechanism may facilitate the release of PPO and other components from Gs and SGs during ecdysis as during an immune reaction. On the other hand, the aggregation of hemocytes beneath the hypodermis during ecdysis has been associated with cuticle sclerotization [37,38], and may resemble how hemocytes aggregate around parasites in the encapsulation reaction [40,217,258–260]. Whereas, the specific mechanisms that allow crustacean hemocytes to aggregate under the hypodermis have not been investigated, and it should be the target of future investigation. However, PPO is positively detected in Hs during postmolt, and this may explain the role of Hs in the shell-hardening process. In a previous chapter it was discussed that Hs infiltrate easily through the hypodermis to transport and deliver PPO into the new cuticle, initiating the shell-hardening during postmolt. This discussion is based on hemocyte tissue infiltration, a

process that has previously been reported [80,133]. The detection of PPO in Hs may certainly represent a dedicated role of this group of hemocytes during the shell-hardening. The total PPO contribution that Hs provide during postmolt exceeds ~45% the contribution of Gs, similar to SGs, which total contribution during postmolt is ~42% over that of Gs contribution. It is likely that these values of PPO contributions may represent the high demand for PPO during the shell-hardening necessary for the sclerotization of whole cuticle.

The role of PPO in the shell-hardening process involves the mechanisms of enzyme activation that allow the catalytic activity of PO. The zymogen PPO is converted to PO by the proteolytic action of PPAEs. PPAEs are serine proteases that have been isolated from cuticles, hemolymph, hypodermis and other tissues [36,191,231,263–265] PPAEs and hemocytes are coupled to the immune system, in response to immune challenges presented by pathogens and injuries [39,132,134]. Hypodermal expression of PPAEs have been associated with the shell-hardening process, and function to activate PPO for sclerotization during postmolt in the new cuticle [263].

The PPAEs are trigged for PPO activation as an immune response in a cascade system called the PPO activating system [39,132,134]. The activation of this cascade is a result of the recognition of foreign molecules that are components of bacteria, fungi and parasites [39,132,134]. The activation of PPO into PO is precisely regulated, perhaps due to sclerotization and melanization involved in the production of cytotoxic quinones [80]. Many proteins are involved in the control of this cascade, including both PO and PPAE inhibitors [266]. The PPO cascade system activates

PPO for sclerotization. For example, sclerotization is necessary for the process of wound healing together with melanization [232,267–269]. Therefore, similar triggering mechanisms of PPAEs in the activation of PPO during the shell-hardening at postmolt may be expected.

Serine protease inhibitors modulate the action of PPAEs [89], pacifastin, a protease inhibitor isolated from the hemolymph of the crayfish *Pacifastacus*, specifically inhibits PPAEs [270–272]. Reduced levels of pacifastin results in elevated melanization capacity [273]. Examination of the pacifastin levels during the molt cycle may provide valuable information correlated to PPO activation during the hardening of the cuticle. On the other hand, the ubiquitous expression of PPAEs [Chung, personal communication] may suggest that they are not limited elements in the process of control and modulation of PPO activities. Thus, the control on the PO activity may include the upregulation of *PPO* expression and translation. Constant levels of *CasPPO-hemo* transcripts have been observed during the molt cycle of *Callinectes* [95], but little is known about the translation rate and half-life of the protein.

PO inhibitors have also been identified in arthropods and downregulate the activity of POs by the formation of a protein complex [230]. Not much is known about these molecules in crustaceans.

Cuticle sclerotization is a complex system that includes many POs [11,21]. Hemocytes are the main known source of PPO [46,93–95,182,274]. Even though there is limited information in crustaceans, the expression of PPOs in other tissues such as hepatopancreas has been reported [47]. Additionally, the expression of *PPO*

in the hypodermal cells has been found in insects [87,180]. In crustaceans, it is possible that many PPOs participate in the sclerotization process of the cuticle. Evidence that the *Callinectes* hypodermis may express a *PPO* during a specific and short period of premolt (data not shown) supports the view that the *Callinectes* shell-hardening process may also include PPOs that are not expressed in hemocytes.

All events during postmolt describe a synchronized and progressive sequence that suggests the shell-hardening process may be hormonally regulated. Testing the hypothesis that the bursicon hormone involves the endogenous changes observed in hemocytes and its PO activity, contributes to define the unknown regulatory elements of the shell-hardening of crustaceans.

Bursicon is a neurohormone that mediates the events of tanning, wing expansion, and apoptosis of wing epidermis and abdominal ganglion during ecdysis and postmolt in insects such as *D. melanogaster* and *M. sexta* [58,59,61,65,141,275]. Moreover, the pupal ecdysial behavior in *Drosophila* is controlled by bursicon [139,276,277]. Even though the presence of the hormone has been described in crustacean species such as *D. arenata*, *C. maenas*, *H. gammarus*, *H. americanus*, and *C. sapidus* [62,64,65], little is known about its functions in this group of arthropods. Crustacean bursicon is maintained in low titers in the hemolymph during the majority of the molting cycle and increases during ecdysis [62]. Therefore, it is likely that bursicon has a role during crustacean ecdysis and postmolt.

This dissertation reports the use of *dsRNA* injections to define whether bursicon upregulates PPO in hemocytes and the shell-hardening process in the blue crab. The co-injection of *CasBurs*-α+β *dsRNA* show normal progression of shell-

hardening and ecdysis. In *Drosophila* and *Manduca*, the cuticle tanning depends on bursicon upregulation. However, in some insects the cuticle tanning process may be independent of bursicon. Studies on the red flour beetle, *T. castaneum*, and the silkworm, *B. mori*, show that bursicon is not required for cuticle tanning, but is for wing expansion [138,226]. It is possible that bursicon may involve different functions in diverse group of insects [56], and other arthropods as crustaceans.

Bursicon mediates the phosphorylation of TH, which is necessary for the activation of this enzyme, in *Drosophila* and *Manduca* [61,222,278]. The monophenol activity of TH allows the hydroxylation of tyrosine to DOPA, a required substrate for downstream activity in the sclerotization and melanization of the cuticle [51,61,91]. Perhaps in *Callinectes*, the cuticle sclerotization system is based on the monophenol activity of PPO and does not rely on TH activity. CasPPO-hemo has shown monophenol activity in *in vitro* assay [95]. It would be interesting to determine whether TH also participates in the shell-hardening process of crustaceans.

It is peculiar that the injection of either *CasBurs*-α or *CasBurs*-β *dsRNA* does delay the shell-hardening process in *Callinectes*. When one *Burs* subunit is knocked-down, the other enhances its expression and it is suggested that the bursicon homodimer is formed during these experiments. Bursicon homodimers (α-α and β-β) are described in insects [54,140,142]. Moreover, α recombinant bursicon has shown to freely form a homodimer [62]. The results suggest bursicon homodimers may be involved the shell-hardening of *C. sapidus*. It is necessary that further investigations determine the role of bursicon homodimers in crustaceans. However, it is suggested by the experimental results, that bursicon homodimers may regulate PPAEs. It would

be interesting to know the effects that injections of recombinant bursicon homodimers have on the shell-hardening process of crustaceans. Additionally, further approaches to investigate the role of bursicon in crustaceans should include the bursicon receptor, which is encoded in insects by the *rickets* gene (*rk*) [58,140].

Perhaps the motor-control of the shell-hardening process in some arthropods is based on mechanisms such as controlling the supply of the reactive components necessary for sclerotization. This mechanism has been suggested previously in insects [11] and may include the control of transporting or restraining the synthesis of phenolic precursors for sclerotization. It is well-known that the color (and likely the hardness) of the insect cuticle depends on the reactive phenol precursor incorporated into the cuticle for sclerotization [11,12]. NADA and NBAD are phenol substrates in almost all insect cuticles. Insect cuticles rich in NADA are straw colored or colorless, while the cuticle with a dominant proportion of NBAD results in a dark brown color [11,235,246]. A selective allocation and distribution of the phenol substrates into the cuticle for sclerotization occurs in insects and it possible in crustaceans.

The insect hypodermal cells can synthesize some of these reactive precursors. For example, the hypodermal cells of *Locusta migratoria* and *Schistocera gregaria* synthesize NADA [12]. However, it is possible other cells may participate in this synthesis, such as hemocytes and fat body cells (equivalent to hepatopancreas in crustaceans) [11,247]. It is likely that the hypodermal cells may possess a system of transporters that control the quantity of reactive precursors allocated into the cuticle for sclerotization [11]. This type of mechanism may apply to the initial pre-ecdysis sclerotization of the epicuticle as well. A limited sclerotization of the epicuticle

occurs before ecdysis in *C. maenas* [18]. Furthermore, the observation that hemocytes aggregate beneath the hypodermis at early premolt implies an active transport of PPO [38], and the transport of reactive precursors is likely occurred during this period of the molting cycle.

Is it possible that the transport or synthesis of phenol substrates in the hypodermis is hormonally controlled? Indeed, it may be hormonally regulated. An example is the balance between ecdysteroids and molt-inhibiting hormone addresses the preparation for the ecdysis in crustaceans [4–8,10,279]. As a result, the mitotic rates in hypodermal cells increase and these cells are initiated to secrete the new cuticle, and the activation of many genes occurs [6,189,280–282].

In summary, hemocytes show endogenous changes in their number and content of PPO at different molt stages. The knockdown of *CasPPO-hemo* supports that hemocyte PPO is involved in the shell-hardening of the blue crab at postmolt. Future research is needed to further define the transport of PPO through the hypodermis to the cuticle. The role of bursicon in crustacean cuticle hardening still remains to be defined; it is suggested that this hormone does not directly modulate *PPO* expression and PO activity that are required in the shell-hardening process. However it may involve in other components of the PPO cascade system. Additionally, it is necessary to investigate the role of bursicon homodimers in shell-hardening process of crustaceans.

This dissertation is a complement and a contribution to knowledge and the understanding of the shell-hardening process of crustaceans. It opens new hypotheses and questions that encourage new investigations. It provides information that

components of innate immunity are involved in the shell-hardening process of *C sapidus*. The links between the shell-hardening and the immune system allow us to explore the evolutionary origin of the hard exoskeleton of arthropods. An early development of PPOs is considered in the evolution of the superfamily of hemocyanin proteins [20,283,284]. It has been suggested the development of PPOs could have a protective role in capturing the dioxygen molecule and its highly reactive derivatives, after the atmospheric levels of oxygen rose due to the increase in photosynthesis [20]. This new PO activity was surely an advantage to fix oxidative radicals and used as immune defense by developing biological pigments such as melanin [20,283,284]. As a consequence a hard exoskeleton could have originated through the construction of a cross-linked protein network that later could allow the deposition of mineral materials from the seawater. This hard structure may provide better protection and mobility by muscle attachment to earlier arthropods. However, the fact that a hard exoskeleton limits continuous growth, is resolved through the repetitive molts during the life cycle of arthropods.

Appendix

1. Preliminary experiments for the evaluation of PO activity in hemocytes, plasma and hypodermis

1.1 Source of PO activity in the hemolymph of the blue crab

Hemolymph (500 µl) from crabs at intermolt stage was collected directly into a syringe containing a crab anticoagulant solution (450mM NaCl, 10mM KCl, 100mM Glucose, 10mM $NaHCO_3$, 10mM EDTA, 10mM HEPES, pH 7.3) in a ratio 1:1 (v:v). The samples were kept on ice and centrifuged at 800g for 10 min at 4ºC for hemocyte and plasma separation. The hemocytes were washed twice in 200 µl of sodium cacodylate (10 mM) by centrifugation as above. Plasma was retrieved in a new tube and kept on ice. The hemocytes were sonicated in 25 µl of working buffer (10 mM sodium cacodylate, 5 mM calcium chloride, pH 7.1) and then centrifuged at 15,000g for 20 min at 4ºC. The protein concentration of the supernatant as HLS and plasma were estimated using a Pierce 660 nm kit (Bio-Rad).

The CasHLS-PO and plasma-PO activities were determined by measuring the formation of dopachrome from L- 3,4 dihydrophenylalanine (L-DOPA) using a modified assay as described [46,168,169]. Briefly, HLS and plasma samples (45 µg HLS or plasma in 50 µl working buffer) were preincubated with 50 µl of trypsin (1 mg/ml working buffer) for 15 min at 37ºC. Subsequently, 100 µl L-DOPA (3 mg/ml working buffer) were added and further incubated for 20 min at 37ºC. At the end of 20 min incubation, the samples were diluted with 150 µl of working buffer and placed into a 96-well plate in triplicate (100 µl/well), and the absorbance was measured at

490 nm. The experiment also included the estimation of PO activity using the hemolymph (plasma + hemocytes; 45 µg hemolymph in 50 µl working buffer).

The PO activity was calculated as OD/mg protein/ml of hemolymph and presented as mean ± SE (n = 3).

Hemocytes are the main source of PO activity in the hemolymph of intermolt blue crabs. A significant ($P < 0.05$; one way NOVA) higher activity has detected in HLS than the plasma and whole hemolymph.

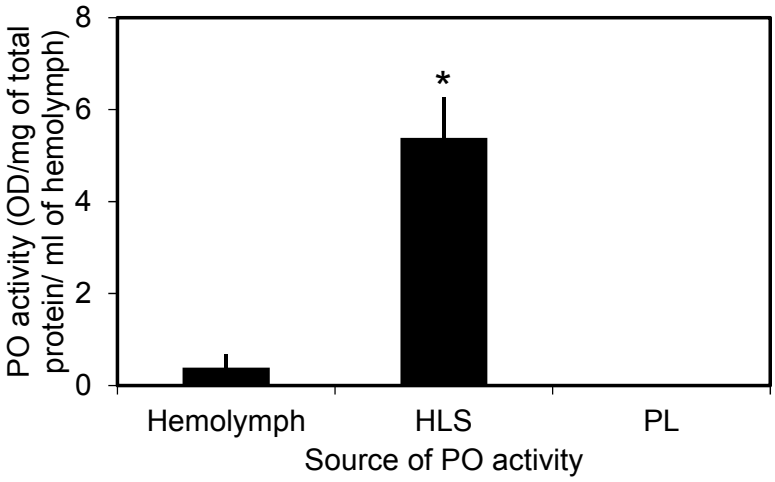


Figure 21. Source of PO activity in the hemolymph of the blue crab at intermolt stage. HLS is the main source of PO activity in the hemolymph of the blue crab (one way ANOVA, $P < 0.05$). HLS = hemocyte lysate supernatant; PL = plasma

1.2 Kinetic characterization of CasHLS-PO activity

The values of Km and Vmax of CasHLS-PO activity were determined with L-DOPA as a substrate with final concentrations at 0.57, 1.125, 2.25, 4.50, and 9.0mM. The values of Km and Vmax were estimated using a nonlinear regression of the Michaelis-Menten plot using CurveExpert 1.4.

The changes in CasHLS-PO activity are noted in the values of Vmax and Km during the molt cycle (Fig. 6). The lowest values of Vmax is observed with hemocytes at ecdysis (101.9 ± 17.5 OD/min/mg HLS), while the highest is observed at late premolt (174.1 ± 29.8 OD/min/mg HLS) followed by intermolt (147.5 ± 25.3 OD/min/mg HLS). However, they show no statistical differences. On the other hand, a significant variation of the values of Km is noted with hemocytes obtained at ecdysis, compared to those measured at late premolt and postmolt (Table 2, $P = 0.03$). The values of Km of CasHLS-PO activity remain constant at intermolt, early premolt, late premolt, and postmolt molt stages: 3.6 ± 0.8, 2.5 ± 0.4, 2.1 ± 0.7, and 1.3 ± 0.3 (mM) respectively. The lowest Km value is found at ecdysis with 13.7 ± 5.9mM.

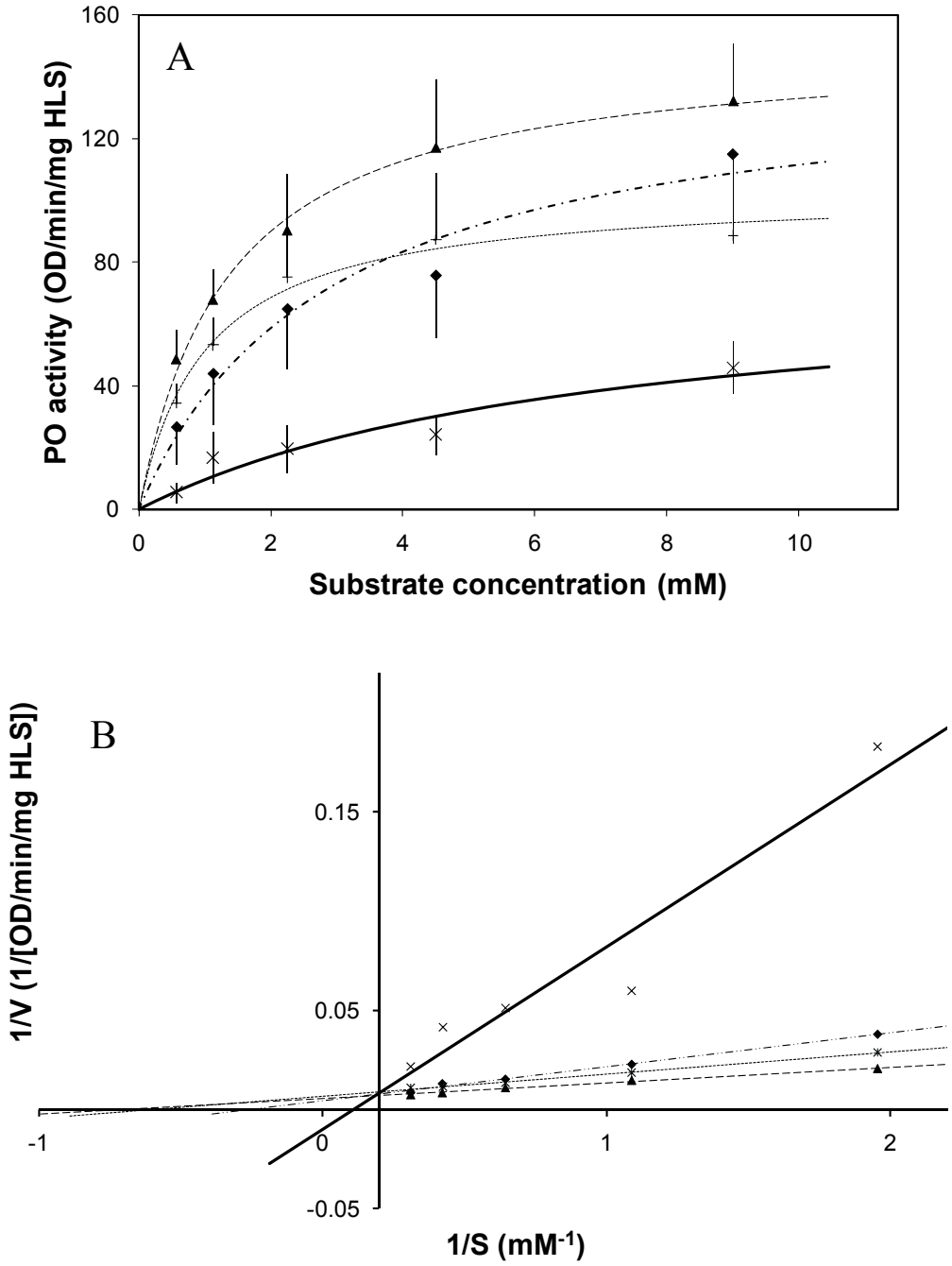


Figure 22. A) Michaelis-Menten plot and the changes in the values of Km and Vmax of PO activity of CasHLS during the molt cycle. B) Lineweaver–Burk plot of enzyme kinetic. Kinetics of PO activity was measured using L-dopa as a substrate. The reaction rate is expressed as delta ΔOD/min/ mg of HLS protein. x = ecdysis (n = 3); ▲= late premolt (n = 8); ♦ = intermolt (n = 3); + = postmolt (n = 8)

1.3. Hypodermis PO activity during the molt cycle

The hypodermis-PO activity was determined by measuring the formation of dopachrome from L- 3,4 dihydrophenylalanine (L-DOPA) using a modified assay as described [46,168,169]. Briefly, hypodermis sections of 1.28 ± 0.66 mg were collected from the dorsal carapace of crabs at different molt stages. Next, 20 µg of hypodermis total proteins in 50 µl working buffer were preincubated with 50 µl of trypsin (1 mg/ml working buffer) for 15 min at 37ºC. Subsequently, 100 µl L-DOPA (3 mg/ml working buffer) were added and further incubated for 20 min at 37ºC. At the end of 20 min incubation, the samples were diluted in 150 µl of working buffer, and placed into a 96-well plate in triplicate (100 µl/well). The absorbance was measured at 490 nm. The PO activity was calculated as OD/mg protein and presented as mean ± SE (n = 12).

The maximal PO activities in the hypodermis were detected at late premolt (0.31 ± 0.07 OD/mg of protein) and ecdysis (0.22 ± 0.09 OD/mg of protein). The lower PO activity was detected at postmolt (0.10 ± 0.03 OD/mg of protein). No significant differences in hypodermis PO activity during the molt cycle were detected by one way ANOVA ($P > 0.05$).

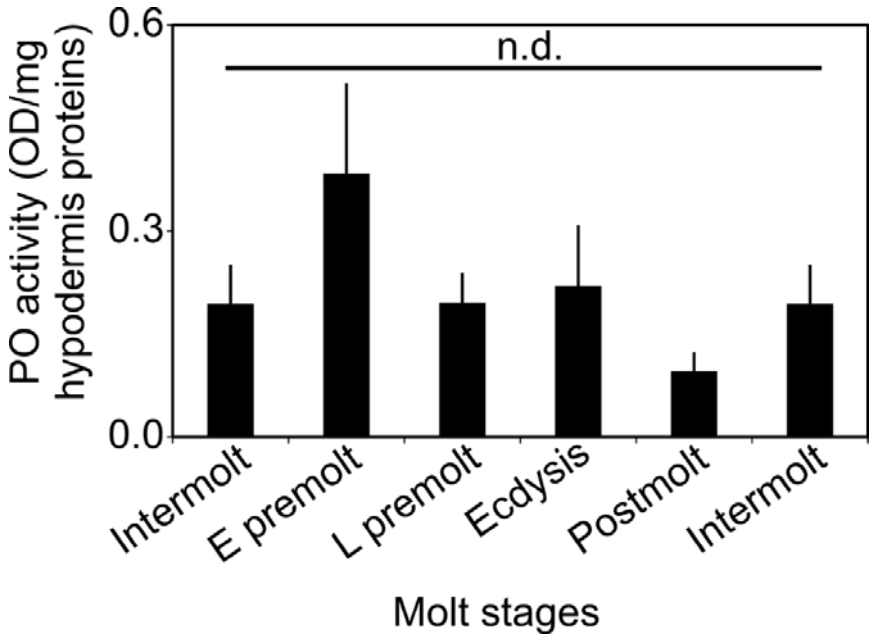


Figure 23. Hypodermis PO activity during the molt cycle of the blue crab. No significant differences of the hypodermis PO activity are detected during the molt cycle using L-DOPA as substrate (one way ANOVA, $P > 0.05$). Protein were isolated from hypodermis sections of 1.28 ± 0.66 mg collected at different molt stages (n=12)

Bibliography

[1] Passano L. Molting and its control. In: Waterman T, editor. Physiol. Crustac. Vol. I. Metab. growth, New York: Academic Press Inc, New York and London; 1960, p. 473–536.

[2] Smith SG, Chang E. Molting and growth. In: Kennedy VS, Cronin LE, editors. blue crab *Callinectes sapidus*, vol. 2. 1st ed., College Park: Maryland Sea Grant; 2007, p. 197–294.

[3] Skinner D. Interacting factors in the control of the crustacean molt cycle. Am Zool 1985;25:275–84.

[4] Chang E, Mykles DL. Regulation of crustacean molting: a review and our perspectives. Gen Comp Endocrinol 2011;172:323–30.

[5] Chang E, Bruce M, Tamone S. Regulation of crustacean molting: a multi-hormonal system. Am Zool 1993;33:324–9.

[6] Chang E, Chang S, Mulder E. Hormones in the lives of crustaceans: an overview. Am Zool 2001;41:1090–7.

[7] Chung JS, Webster SG. Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *Carcinus maenas*. Endocrinology 2005;146:5545–51.

[8] Chung JS, Dircksen H, Webster SG. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. Proc Natl Acad Sci U S A 1999;96:13103–7.

[9] Chung JS, Zmora N, Katayama H, Tsutsui N. Crustacean hyperglycemic hormone (CHH) neuropeptides family: Functions, titer, and binding to target tissues. Gen Comp Endocrinol 2010;166:447–54.

[10] Katayama H, Ohira T, Nagasawa H. Crustacean peptide hormones: structure, gene expression and function. Aqua-BioScience Monogr 2013;6:49–90.

[11] Andersen SO. Cuticular sclerotization and tanning. In: Gilbert LI, editor. Insect Mol. Biol. First edit, Academic Press, Elsevier; 2012, p. 178–203.

[12] Andersen SO, Peter M, Roepstorff P. Cuticular sclerotization in insects. Comp Biochem Physiol 1996;113:689–705.

[13] Dennell R. The occurrence and significance of phenolic hardening in the newly formed cuticle of crustacea Decapoda. Proc R Soc Med 1947;134:485–503.

[14] Hopkins TL, Kramer KJ. Insect cuticle sclerotization. Annu Rev Entomol 1992;37:273–302.

[15] Sakamoto K, Honto W, Iguchi M, Ogawa N, Ura K, Takagi Y. Post-molt processes of cuticle formation and calcification in the Japanese mitten crab *Eriocheir japonicus*. Fish Sci 2009;75:91–8.

[16] Summers NM. Cuticle sclerotization and blood phenol oxidase in the fiddler crab, *Uca pugnax*. Comp Biochem Physiol 1967;23:129–38.

[17] Sugumaran M. Unified mechanism for sclerotization of insect cuticle. Adv Insect Physiol 1998;27:229–334.

[18] Roer RD, Dillaman RM. The structure and calcification of the crustacean cuticle. Am Zool 1984;24:893–909.

[19] Terwilliger N. Hemolymph proteins and molting in crustaceans and insects. Am Zool 1999;39:589–99.

[20] Decker H, Terwilliger N. Review cops and robbers: Putative evolution of copper oxygen-binding proteins. J Exp Biol 2000;1782:1777–82.

[21] Andersen SO. Insect cuticular sclerotization: A review. Insect Biochem Mol Biol 2010;40:166–78.

[22] Kim MS, Baek MJ, Lee MH, Park JW, Lee SY, Söderhäll K, et al. A new easter-type serine protease cleaves a masquerade-like protein during prophenoloxidase activation in *Holotrichia diomphalia* larvae. J Biol Chem 2002;277:39999–40004.

[23] Ashida M, Brey PT. Role of the integument in insect defense: pro-phenol oxidase cascade in the cuticular matrix. Proc Natl Acad Sci U S A 1995;92:10698–702.

[24] Shiao SH, Higgs S, Adelman Z, Christensen BM, Liu SH, Chen CC. Effect of prophenoloxidase expression knockout on the melanization of microfilariae in the mosquito *Armigeres subalbatus*. Insect Mol Biol 2001;10:315–21.

[25] Asano T, Ashida M. Cuticular pro-phenoloxidase of the silkworm, *Bombyx mori*. Purification and demonstration of its transport from hemolymph. J Biol Chem 2001;276:11100–12.

[26] Satoh D, Horii A, Ochiai M. Prophenoloxidase-activating enzyme of the silkworm, *Bombyx mori*. J Biol Chem 1999;274:7441–53.

[27] Sugumaran M, Nellaiappan K. Characterization of a new phenoloxidase inhibitor from the cuticle of *Manduca sexta*. Biochem Biophys Res Commun 2000;268:379–83. doi:10.1006/bbrc.2000.2138.

[28] Jiang H, Wang Y, Kanost MR. Pro-phenol oxidase activating proteinase from an insect, Manduca sexta: a bacteria-inducible protein similar to *Drosophila easter*. Proc Natl Acad Sci U S A 1998;95:12220–5.

[29] Cui L, Luckhart S, Rosenberg R. Molecular characterization of a prophenoloxidase cDNA from the malaria mosquito *Anopheles stephensi*. Insect Mol Biol 2000;9:127–37.

[30] Asano T, Ashida M. Transepithelially transported pro-phenoloxidase in the cuticle of the silkworm, *Bombyx mori*. Identification of its methionyl residues oxidized to methionine sulfoxides. J Biol Chem 2001;276:11113–25.

[31] Arakane Y, Muthukrishnan S, Beeman RW, Kanost MR, Kramer KJ. Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proc Natl Acad Sci USA 2005;102:11337–42.

[32] Asada N, Yokoyama G, Kawamoto N, Norioka S, Hatta T. Prophenol oxidase A3 in *Drosophila melanogaster*: activation and the PCR-based cDNA sequence. Biochem Genet 2003;41:151–63.

[33] Andersen SO. Sclerotization of insect cuticles. Sci Top 2008;4:1–2.

[34] Hattori M, Konishi H, Tamura Y, Konno K, Sogawa K. Laccase-type phenoloxidase in salivary glands and watery saliva of the green rice leafhopper, *Nephotettix cincticeps*. J Insect Physiol 2005;51:1359–65.

[35] Barrett FM, Andersen SO. Phenoloxidases in larval cuticle of the blowfly, *Calliphora vicina*. Insect Biochem 1981;11:17–23.

[36] Aspán A, Sturtevant J, Smith VJ, Söderhäll K. Purification and characterization of a prophenoloxidase activationg enzyme from crayfish blood cells. Insect Biochem 1990;20:709–18.

[37] Vacca L, Fingerman M. The roles of hemocytes in tanning during the molting cycle: A histochemical study of the fiddler crab, *Uca pugilator*. Biol Bull 1983;165:758–77.

[38] Terwilliger N, Ryan MC. Functional and phylogenetic analyses of phenoloxidases from Brachyuran (*Cancer magister*) and Branchiopod (*Artemia franciscana*, *Triops longicaudatus*) crustaceans. Biol Bull 2006;210:38–50.

[39] Cerenius L, Jiravanichpaisal P, Liu H, Söderhäll I. Crustaean immunity. In: Söderhäll K, editor. Invertebr. Immun. First, New York, USA: SpringerLink; 2010, p. 239–59.

[40] Johansson MW, Söderhäll K. Cellular immunity in crustaceans and the proPO system. Parasitol Today 1989;5:171–6.

[41] Söderhäll K, Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr Opin Immunol 1998;10:23–8.

[42] Thörnqvist P, Johansson MW, Söderhäll K. Opsonic activity of cell adhesion proteins and β-1,3-glucan binding proteins from two crustaceans. Dev Comp Immunol 1994;18:3–18.

[43] Sritunyalucksana K, Söderhäll K. The proPO and clotting system in crustaceans. Aquaculture 2000;191:53–69.

[44] Aspán A, Huang TS, Cerenius L, Söderhäll K. cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. Proc Natl Acad Sci U S A 1995;92:939–43.

[45] Gai Y, Zhao J, Song L, Li C, Zheng P, Qiu L, et al. A prophenoloxidase from the Chinese mitten crab *Eriocheir sinensis*: gene cloning, expression and activity analysis. Fish Shellfish Immunol 2008;24:156–67.

[46] Gollas-Galván T, Hernández-López J, Vargas-Albores F. Prophenoloxidase from brown shrimp (*Penaeus californiensis*) hemocytes. Comp Biochem Physiol B Biochem Mol Biol 1999;122:77–82.

[47] Masuda T, Otomo R, Kuyama H, Momoji K, Tonomoto M, Sakai S, et al. A novel type of prophenoloxidase from the kuruma prawn *Marsupenaeus japonicus* contributes to the melanization of plasma in crustaceans. Fish Shellfish Immunol 2012;32:61–8.

[48] Birman S, Morgan B, Anzivino M, Hirsh J. A novel and major isoform of tyrosine hydroxylase in *Drosophila* is generated by alternative RNA processing. J Biol Chem 1994;269:26559–67.

[49] Friggi-Grelin F, Coulom H, Meller M, Gomez D, Hirsh J, Birman S. Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. J Neurobiol 2003;54:618–27.

[50] Gorman M, An C, Kanost M. Characterization of tyrosine hydroxylase from *Manduca sexta*. Insect Biochem Mol Biol 2007;37:1327–37.

[51] Gorman M, Arakane Y. Tyrosine hydroxylase is required for cuticle sclerotization and pigmentation in *Tribolium castaneum*. Insect Biochem Mol Biol 2010;40:267–73.

[52] Laxmyr L. Tyrosine hydroxylase activity in the central nervous system of the crayfish, *Pacifastacus leniusculus* (Crustacea, Decapoda). J Comp Physiol B 1985;155:603–9.

[53] Tierney AJ, Kim T, Abrams R. Dopamine in crayfish and other crustaceans: distribution in the central nervous system and physiological functions. Microsc Res Tech 2003;60:325–35.

[54] Dai L, Dewey E, Zitnan D. Identification, developmental expression, and functions of bursicon in the tobacco hawkmoth, *Manduca sexta*. J Comp Neurol 2008;506:759–74.

[55] Fraenkel G, Hsiao C. Bursicon, a hormone which mediates tanning of the cuticle in the adult fly and other insects. J Insect Physiol 1965;11:513–56.

[56] Honegger H-W, Dewey EM, Ewer J. Bursicon, the tanning hormone of insects: recent advances following the discovery of its molecular identity. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 2008;194:989–1005.

[57] Kostron B, Marquardt K, Kaltenhauser U, Honegger H. Bursicon, the cuticle sclerotizing hormone - comparison of its molecular mass in different insects. J Insect Physiol 1995;41:1045–53.

[58] Luo C-W, Dewey EM, Sudo S, Ewer J, Hsu SY, Honegger H-W, et al. Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2. Proc Natl Acad Sci USA 2005;102:2820–5.

[59] Peabody NC, Diao F, Luan H, Wang H, Dewey EM, Honegger H-W, et al. Bursicon functions within the *Drosophila* CNS to modulate wing expansion behavior, hormone secretion, and cell death. J Neurosci 2008;28:14379–91.

[60] Dewey EM, Mcnabb SL, Ewer J, Kuo GR, Takanishi CL, Truman JW, et al. Identification of the gene encoding Bursicon, an insect neuropeptide responsible for cuticle sclerotization and wing spreading. Curr Biol 2004;14:1208–13.

[61] Davis MM, O'Keefe SL, Primrose D a, Hodgetts RB. A neuropeptide hormone cascade controls the precise onset of post-eclosion cuticular tanning in *Drosophila melanogaster*. Development 2007;134:4395–404.

[62] Chung JS, Katayama H, Dircksen H. New functions of arthropod bursicon: Inducing deposition and thickening of new cuticle and hemocyte granulation in the blue crab, *Callinectes sapidus*. PLoS One 2012;7:e46299.

[63] Van Loy T, Van Hiel MB, Vandersmissen HP, Poels J, Mendive F, Vassart G, et al. Evolutionary conservation of bursicon in the animal kingdom. Gen Comp Endocrinol 2007;153:59–63.

[64] Wilcockson DC, Webster SG. Identification and developmental expression of mRNAs encoding putative insect cuticle hardening hormone, bursicon in the green shore crab *Carcinus maenas*. Gen Comp Endocrinol 2008;156:113–25.

[65] Sharp JH, Wilcockson DC, Webster SG. Identification and expression of mRNAs encoding bursicon in the plesiomorphic central nervous system of *Homarus gammarus*. Gen Comp Endocrinol 2010;169:65–74.

[66] Webster SG, Keller R, Dircksen H. The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. Gen Comp Endocrinol 2012;175:217–33.

[67] Skinner D. The structure and metabolism of a crustacean integumentary tissue during a molt cycle. Biol Bull 1962;123:635–47.

[68] Bucholz F. Drach's molt staging system adapted for Euphausiids. Mar Biol 1982;66:301–5.

[69] Cameron JN. Post-moult calcification in the blue crab (*Callinectes sapidus*): Relationships between apparent net H+ excretion, calcium and bicarbonate. J Exp Biol 1985;119:275–85.

[70] Cameron JN. Post-moult calcification in the blue crab *Callinectes sapidus*: Timing and mechanism. J Exp Biol 1989;143:285–304.

[71] Perry H, Trigg C, Larsen K, Freeman J, Erickson M, Henry R. Calcium concentration in seawater and exoskeletal calcification in the blue crab, *Callinectes sapidus*. Aquaculture 2001;198:197–208.

[72] Pierce DC, K.D.Butler, Roer RD. Effects of exogenous N-acetylhexosaminidase on the structure and mineralization of the post-ecdysial exoskeleton of the blue crab, *Callinectes sapidus*. Comp Biochem Physiol Part B Biochem Mol Biol 2001;128:691–700.

[73] Neufeld DS, Cameron JN. Mechanism of the net uptake of water in moulting blue crabs (*Callinectes sapidus*) acclimated to high and low salinities. J Exp Biol 1994;188:11–23.

[74] Shafer TH, Roer RD, Miller CG, Dillaman RM. Postecdysial changes in the protein and glycoprotein composition of the cuticle of the blue crab *Callinectes sapidus*. J Crustac Biol 1994;14:210–9.

[75] Shafer TH, Roer RD, Midgette-Luther C, Brookins TA. Postecdysial cuticle alteration in the blue crab, *Callinectes sapidus*: Synchronous changes in glycoproteins and mineral nucleation. J Exp Zool 1995;271:171–82.

[76] Shafer TH, McCartney M a, Faircloth LM. Identifying exoskeleton proteins in the blue crab from an expressed sequence tag (EST) library. Integr Comp Biol 2006;46:978–90.

[77] Roer RD, Halbrook KE, Shafer TH. Glycosidase activity in the post-ecdysial cuticle of the blue crab, *Callinectes sapidus*. Comp Biochem Physiol B Biochem Mol Biol 2001;128:683–90.

[78] Coblentz FE, Shafer TH, Roer RD. Cuticular proteins from the blue crab alter in vitro calcium carbonate mineralization. Comp Biochem Physiol Part B Biochem Mol Biol 1998;121:349–60.

[79] Tweedie EP, Coblentz FE, Shafer TH. Purification of a soluble glycoprotein from the uncalcified ecdysial cuticle of the blue crab *Callinectes sapidus* and its possible role in initial mineralization. J Exp Biol 2004;207:2589–98.

[80] Cerenius L, Söderhäll K. The prophenoloxidase-activating system in invertebrates. Immunol Rev 2004;198:116–26.

[81] Nagai T, Kawabata S. A link between blood coagulation and prophenol oxidase activation in arthropod host defense. J Biol Chem 2000;275:29264–7.

[82] Hose J, Martin G, Gerard AS. A decapod hemocyte classification scheme integrating morphology, cytochemistry, and function. Biol Bull 1990;178:33–45.

[83] Moullac G, Haffner P. Environmental factors affecting immune responses in Crustacea. Aquaculture 2000;191:121–31.

[84] Terwilliger N, Dangott L, Ryan M. Cryptocyanin, a crustacean molting protein: evolutionary link with arthropod hemocyanins and insect hexamerins. Proc Natl Acad Sci USA 1999;96:2013–8.

[85] Terwilliger N. Hemocyanins and the immune response: defense against the dark arts. Integr Comp Biol 2007;47:662–5.

[86] Decker H, Rimke T. Tarantula hemocyanin shows phenoloxidase activity. J Biol Chem 1998;273:25889–92.

[87] Dittmer NT, Kanost MR. Insect multicopper oxidases: diversity, properties, and physiological roles. Insect Biochem Mol Biol 2010;40:179–88.

[88] Jaenicke E, Decker H. Tyrosinases from crustaceans form hexamers. Biochem J 2003;371:515–23.

[89] Kanost M, Gorman M. Phenoloxidases in insect immunity. In: Beckage NE, editor. Insect Immunol. First Edit, San Diego California: Academic Press, Elsevier; 2008, p. 69–109.

[90] Wevers R a, de Rijk-van Andel JF, Bräutigam C, Geurtz B, van den Heuvel LP, Steenbergen-Spanjers GC, et al. A review of biochemical and molecular genetic aspects of tyrosine hydroxylase deficiency including a novel mutation (291delC). J Inherit Metab Dis 1999;22:364–73.

[91] Dunkley PR, Bobrovskaya L, Graham ME, von Nagy-Felsobuki EI, Dickson PW. Tyrosine hydroxylase phosphorylation: regulation and consequences. J Neurochem 2004;91:1025–43.

[92] Locke M. The localization of a peroxidase associated with hard cuticle formation in an insect, *Calpodes ethlius stoll*, Lepidoptera, Hesperiidae. Tissue Cell 1969;1:555–74.

[93] Sritunyalucksana K, Cerenius L, Söderhäll K. Molecular cloning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*. Dev Comp Immunol 1999;23:179–86.

[94] Ai H-S, Liao J-X, Huang X-D, Yin Z-X, Weng S-P, Zhao Z-Y, et al. A novel prophenoloxidase 2 exists in shrimp hemocytes. Dev Comp Immunol 2009;33:59–68.

[95] Alvarez J V, Chung JS. Cloning of prophenoloxidase from hemocytes of the blue crab, Callinectes sapidus and its expression and enzyme activity during the molt cycle. Fish Shellfish Immunol 2013;35:1349–58.

[96] Bodammer J. Cytological observations on the blood and hemopoietic tissue in the crab, *Callinectes sapidus*. Cell Tissue Res 1978;187:79–96.

[97] Hose J, Martin G, Tiu S. Patterns of hemocyte production and release throughout the molt cycle in the penaeid shrimp *Sicyonia ingentis*. Biol Bull 1992;183:185–99.

[98] Sequeira T, Vilanova M, Lobo-Da-Cunha A, Baldaia L, Arala-Chaves M. Flow cytometric analysis of molt-related changes in hemocyte type in male and female *Penaeus japonicus*. Biol Bull 1995;189:376–80.

[99] Cerenius L, Jiravanichpaisal P, Liu H, Söderhäll I. Crustacean immunity. In: Söderhäll K, editor. Invertebr. Immun. 1 st, New York: Springer Science; 2010, p. 239–59.

[100] Lanz H, Tsutsumi V, Aréchiga H. Morphological and biochemical characterization of *Procambarus clarkii* blood cells. Dev Comp Immunol 1993;17:389–97.

[101] Mix MC, Sparks AK. Hemocyte classification and differential counts in the dungeness crab, *Cancer magister*. J Invertebr Pathol 1980;35:134–43.

[102] Söderhäll I, Bangyeekhun E, Mayo S, Söderhäll K. Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of *Pacifastacus leniusculus*. Dev Comp Immunol 2003;27:661–72.

[103] Vargas-Albores F, Gollas-Galvan T, Hernández-López J. Functional characterization of *Farfantepenaeus californiensis*, *Litopenaeus vannamei* and *L. stylirostris* haemocyte separated using density gradient centrifugation. Aquac Res 2005;36:352–60.

[104] Wenli C, Shields JD. Characterization and primary culture of hemocytes from the blue crab, *Callinectes sapidus*. Trans Chinese Crustac Soc 2007;5:25–35.

[105] Hose J, Martin G, Nguyen V, Lucas J, Rosenstein T. Cytochemical features of shrimp hemocytes. Biol Bull 1987;173:178–87.

[106] Adachi K, Endo H, Watanabe T, Nishioka T, Hirata T. Hemocyanin in the exoskeleton of crustaceans: enzymatic properties and immunolocalization. Pigment Cell Res 2005;18:136–43.

[107] Adachi K, Hirata T, Nishioka T, Sakaguchi M. Hemocyte components in crustaceans convert hemocyanin into a phenoloxidase-like enzyme. Comp Biochem Physiol B Biochem Mol Biol 2003;134:135–41.

[108] Nellaiappan K, Sugumaran M. On the presence of prophenoloxidase in the hemolymph of the horseshoe crab, *Limulus*. Comp Biochem Physiol Part B Biochem Mol Biol 1996;113:163–8.

[109] Terwilliger N, Ryan MC, Towle D. Evolution of novel functions: cryptocyanin helps build new exoskeleton in *Cancer magister*. J Exp Biol 2005;208:2467–74.

[110] Shao Q, Yang B, Xu Q, Li X, Lu Z, Wang C, et al. Hindgut innate immunity and regulation of fecal microbiota through melanization in insects. J Biol Chem 2012;287:14270–9.

[111] Hartenstein V. Blood cells and blood cell development in the animal kingdom. Annu Rev Cell Dev Biol 2006;22:677–712.

[112] Dales PR. The coelomic and peritoneal cell systems of some Sabellid Polychaetes. Microsc Sci 1961;102:327–46.

[113] Eckelbarger KJ. Origin and development of the amoebocytes of *Nicolea zostericola* (Polychaeta : Terebellidae) with a discussion of their possible role in oogenesis. Mar Biol 1976;182:169–82.

[114] Chaga O, Lignell M, Söderhäll K. The haemopoietic cells of the freswater cryfish, *Pacifastacus leniusculus*. Anim Biol 1995;4:59–70.

[115] Johansson MW, Keyser P, Sritunyalucksana K, Söderhäll K. Crustacean haemocytes and haematopoiesis. Aquaculture 2000;191:45–52.

[116] Lin X, Söderhäll I. Crustacean hematopoiesis and the astakine cytokines. Blood 2011;117:6417–24.

[117] Grigorian M, Hartenstein V. Hematopoiesis and hematopoietic organs in arthropods. Dev Genes Evol 2013;223:103–15.

[118] Wu C, Söderhäll I, Kim Y-A, Liu H, Söderhäll K. Hemocyte-lineage marker proteins in a crustacean, the freshwater crayfish, *Pacifastacus leniusculus*. Proteomics 2008;8:4226–35.

[119] Jiravanichpaisal P, Lee BL, Söderhäll K. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 2006;211:213–36.

[120] Ribeiro C, Brehélin M. Insect haemocytes: what type of cell is that? J Insect Physiol 2006;52:417–29.

[121] Evans C, Hartenstein V, Banerjee U. Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis. Dev Cell 2003;5:673–90.

[122] Meister M, Lagueux M. *Drosophila* blood cells. Cell Microbiol 2003;5:573–80.

[123] Söderhäll K, Wu C, Lin X, Jiravanichpaisal P, Söderhäll I. Hemocyte marker proteins and regulation of the proPO system in a crustacean , *Pacifastacus leniusculus*. In: Bondad-Reantaso M, Jones J, Corsin F, Aoki T, editors. Deseases Aquac. VII, Selangor, Malaysia: Asian Fishery Society; 2011, p. 177–94.

[124] Benton J, Kery R, Li J, Noonin C, Söderhäll I, Beltz B. Cells from the immune system generate adult-born neurons in crayfish. Dev Cell 2014;30:322–33.

[125] Johansson MW, Söderhäll K. Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. J Comp Physiol B Biochem Syst Environ Physiol 1985;156:175–81.

[126] Smith VJ, Söderhäll K. B-l,3 glucan activation of crustacean hemocyte in vitro and in vivo. Biol Bull 1983;164:299–314.

[127] Krzemien J, Crozatier M, Vincent A. Ontogeny of the *Drosophila* larval hematopoietic organ, hemocyte homeostasis and the dedicated cellular immune response to parasitism. Int J Dev Biol 2010;54:1117–25.

[128] Bachère E. Shrimp immunity and disease control. Aquaculture 2000;191:3–11.

[129] Anggraeni MS, Owens L. The haemocytic origin of lymphoid organ spheroid cells in the penaeid prawn *Penaeus monodon*. Dis Aquat Organ 2000;40:85–92.

[130] Arala-Chaves M, Sequeira T. Is there any kind of adaptive immunity in invertebrates? Aquaculture 2000;191:247–58.

[131] Bayne C. Phagocytosis and non-self recognition in invertebrates. Bioscience 1990;40:723–31.

[132] Cerenius L, Lee BL, Söderhäll K. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol 2008;29:263–71.

[133] Fontaine CT, Lightner D V. Cellular response to injury in Penaeid shrimp. Mar Fish Rev 1974;37:4–10.

[134] Vazquez L, Alpuche J, Maldonado G, Agundis C, Pereyra-Morales A, Zenteno E. Review: Immunity mechanisms in crustaceans. Innate Immun 2009;15:179–88.

[135] Johansson MW, Söderhäll K. Isolation and purification of a cell adhesion factor from crayfish blood cells. J Cell Biol 1988;106:1795–803.

[136] Truman JW. Interaction between ecdysteroid, eclosion hormone, and bursicon titers in *Manduca sexta*. Am Zool 1981;21:655–61.

[137] Fraenkel G, Hsiao C. Hormonal and nervous control of tanning in the fly. Science (80)1962;138:27–9.

[138] Arakane Y, Li B, Muthukrishnan S, Beeman RW, Kramer KJ, Park Y. Functional analysis of four neuropeptides, EH, ETH, CCAP and bursicon, and their receptors in adult ecdysis behavior of the red flour beetle, *Tribolium castaneum*. Mech Dev 2008;125:984–95.

[139] Loveall BJ, Deitcher DL. The essential role of bursicon during *Drosophila* development. BMC Dev Biol 2010;10:92–9.

[140] Mendive FM, Van Loy T, Claeysen S, Poels J, Williamson M, Hauser F, et al. *Drosophila* molting neurohormone bursicon is a heterodimer and the natural agonist of the orphan receptor DLGR2. FEBS 2005;579:2171–6.

[141] Kimura K, Kodama A, Hayasaka Y, Ohta T. Activation of the cAMP/PKA signaling pathway is required for post-ecdysial cell death in wing epidermal cells of *Drosophila melanogaster*. Development 2004;131:1597–606.

[142] An S, Dong S, Wang Q, Li S, Gilbert LI, Stanley D, et al. Insect neuropeptide bursicon homodimers induce innate immune and stress genes during molting by activating the NF-κB transcription factor Relish. PLoS One 2012;7:e34510.

[143] Gunthorpe M, Sikes C, Wheeler A. Promotion and inhibition of calcium carbonate crystallization in vitro by matrix protein from blue crab exoskeleton. Biol Bull 1990;179:191–200.

[144] Williams CL, Dillaman RM, Elliott E, Gay DM. Formation of the arthrodial membrane in the blue crab, *Callinectes sapidus*. J Morphol 2003;256:260–9.

[145] Jussila J, Jago J, Tsvetnenko E, Dunstan B, Evans LH. Total and differential haemocyte counts in western rock lobsters (*Panulirus cygnus* George) under post-harvest stress. Mar Freshw Res 1997;48:863–7.

[146] Ravindranath MH. Changes in the Population of Circulating Hemocytes during Molt Cycle Phases of the Millipe, *Thyropygus poseidon*. Physiol Zool 1974;47:252–60.

[147] Cain T, Morado JF. Changes in total hemocyte and differential counts in dungeness crabs infected with *Mesanophrys pugettensis*, a marine facultative parasitic ciliate. J Aquat Anim Health 2001;13:310–9.

[148] Fotedar S, Tsvetnenko E, Evans L. Effect of air exposure on the immune system of the rock lobster *Panulirus cygnus*. Mar Freshw Res 2001;52:1351–5.

[149] Moullac G, Groumellec M, Ansquer D, Froissard S, Levy P, Aquacop. Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against vibriosis. Fish Shellfish Immunol 1997;7:227–34.

[150] Tanner C a, Burnett LE, Burnett KG. The effects of hypoxia and pH on phenoloxidase activity in the Atlantic blue crab, *Callinectes sapidus*. Comp Biochem Physiol A Mol Integr Physiol 2006;144:218–23.

[151] Vargas-Albores F, Hinojosa-Baltazar P. Influence of temperature and salinity on the yellowleg shrimp, *Penaeus californieinsis* Holmes, prophenoloxidase system. Aquaculture 1998;29:549–53.

[152] Ferrer O, Koburger J, Simpson B, Gleeson R, Marshall M. Phenoloxidase levels in Florida spiny lobster (*Panulirus argus*): relationship to season and molting stage. Comp Biochem Physiol Part B … 1989;93:595–9.

[153] Krishnan G. Phenolic tanning and pigmentation of the cuticle in *Carcinus maenas*. Q J Microsc Sci 1951;92:333–42.

[154] Nellaiappan K, Thangaraj T, Ramalingam K. Phenoloxidase Activity and Its Role in Cuticular Sclerotization in a mole crab *Emerita asiatica* Milne Edwards. J Exp Mar Bio Ecol 1982;61:75–83.

[155] Stevenson J, Adomako T. Diphenol oxidase in the crayfish cuticle. Localization and changes in activity during the moulting cycle. J Insect Physiol 1967;13:1803–11.

[156] Stevenson J. Polyphenol oxidase in the tegumental glands in relation to the molting cycle of the isopod crustacean *Armadillidium vulgare*. Biol Bull 1961;3:554–60.

[157] Kunkel JG, Jercinovic MJ. Carbonate apatite formulation in cuticle structure adds resistance to microbial attack for American lobster. Mar Biol Res 2013;9:27–34.

[158] Chung JS, Bachvaroff TR, Trant J, Place A. A second copper zinc superoxide dismutase (CuZnSOD) in the blue crab *Callinectes sapidus*: Cloning and up-

regulated expression in the hemocytes after immune challenge. Fish Shellfish Immunol 2012;32:16–25.

[159] Chung JS, Zmora N. Functional studies of crustacean hyperglycemic hormones (CHHs) of the blue crab, *Callinectes sapidus* - the expression and release of CHH in eyestalk and pericardial organ in response to environmental stress. FEBS J 2008;275:693–704.

[160] Drach P, Tchernigovtzeff. Sur la méthode de détermination des stades d'intermue et son application générale aux crustacés. Vie Milieu 1967;18:595–610.

[161] Chung JS, Wilcockson DC, Zmora N, Zohar Y, Dircksen H, Webster SG. Identification and developmental expression of mRNAs encoding crustacean cardioactive peptide (CCAP) in decapod crustaceans. J Exp Biol 2006;209:3862–72.

[162] Chung JS. A trehalose 6-phosphate synthase gene of the hemocytes of the blue crab, *Callinectes sapidus*: cloning, the expression, its enzyme activity and relationship to hemolymph trehalose levels. Saline Systems 2008;4:18.

[163] Chung JS, Bembe S, Tamone S, Andrews E, Thomas H. Molecular cloning of the crustacean hyperglycemic hormone (CHH) precursor from the X-organ and the identification of the neuropeptide from sinus gland of the Alaskan Tanner crab, *Chionoecetes bairdi*. Gen Comp Endocrinol 2009;162:129–33.

[164] Chung JS, Manor R, Sagi A. Cloning of an insulin-like androgenic gland factor (IAG) from the blue crab, *Callinectes sapidus*: implications for eyestalk regulation of IAG expression. Gen Comp Endocrinol 2011;173:4–10.

[165] Kumar S, Nei M, Dudley J, Tamura K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 2008;9:299–306.

[166] Zmora N, Trant J, Chan S-M, Chung JS. Vitellogenin and its messenger RNA during ovarian development in the female blue crab, *Callinectes sapidus*: gene expression, synthesis, transport, and cleavage. Biol Reprod 2007;77:138–46.

[167] Huang H-Y, Chien C-H, Jen K-H, Huang H-D. RegRNA: an integrated web server for identifying regulatory RNA motifs and elements. Nucleic Acids Res 2006;34:429–34.

[168] Hernández-López J, Gollas-Galván T, Vargas-Albores F. Activation of the Propheoloxidase System of Brown Shrimp (*Penaeus californiensis* Holmes). Comp Biochem Physiol B Biochem Mol Biol 1996;113C:61–6.

[169] Gollas-Galván T, Hernández-López J, Vargas-Albores F. Effect of calcium on the prophenoloxidase system activation of the brown shrimp (*Penaeus californiensis*, Holmes). Comp Biochem Physiol Part A Physiol 1997;117:419–25.

[170] Decker H, Tuczek F. Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. Trends Biochem Sci 2000;25:392–7.

[171] Lai C-Y, Cheng W, Kuo C-M. Molecular cloning and characterisation of prophenoloxidase from haemocytes of the white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 2005;18:417–30.

[172] Ko C-F, Chiou T-T, Vaseeharan B, Lu J-K, Chen J-C. Cloning and characterisation of a prophenoloxidase from the haemocytes of mud crab *Scylla serrata*. Dev Comp Immunol 2007;31:12–22.

[173] Chen P, Li J, Li J, Liu P, Gao B, Wang Q. Molecular cloning and characterization of prophenoloxidase gene in swimming crab *Portunus trituberculatus*. Fish Shellfish Immunol 2010;28:106–12.

[174] Cho W, Liu H, Lee C, Kuo C. Cloning, characterization and tissue expression of prophenoloxidase cDNA from the mosquito *Armigeres subalbatus* inoculated with *Dirofilaria immitis* microfilariae. Insect Mol Biol 1998;7:31–40.

[175] Zhu J, Yang P, Wu G. Prophenoloxidase from Pieris rapae: gene cloning, activity, and transcription in response to venom/calyx fluid from the endoparasitoid wasp *Cotesia glomerata*. J Zhejiang Univ Sci B 2011;12:103–15.

[176] Liu C-H, Tseng D-Y, Lai C-Y, Cheng W, Kuo C-M. Molecular cloning and characterisation of prophenoloxidase cDNA from haemocytes of the giant freshwater prawn, *Macrobrachium rosenbergii*, and its transcription in relation with the moult stage. Fish Shellfish Immunol 2006;21:60–9.

[177] Sidjanski S, Mathews G, Vanderberg J. Electrophoretic separation and identification of phenoloxidases in hemolymph and midgut of adult *Anopheles stephensi* mosquitoes. J Parasitol 1997;83:686–91.

[178] Feng C, Fu W. Tissue distribution and purification of prophenoloxidase in larvae of Asian Corn Borer, *Ostrinia furnacalis* Guenee (Lepidoptera: Pyralidae). Acta Biochim Biophys Sin (Shanghai) 2004;36:360–4.

[179] Parkinson N, Smith I, Weaver R, Edwards JP. A new form of arthropod phenoloxidase is abundant in venom of the parasitoid wasp *Pimpla hypochondriaca*. Insect Biochem Mol Biol 2001;31:57–63.

[180] Lourenço AP, Zufelato MS, Bitondi MMG, Simões ZLP. Molecular characterization of a cDNA encoding prophenoloxidase and its expression in *Apis mellifera*. Insect Biochem Mol Biol 2005;35:541–52.

[181] Clark KD, Strand MR. Hemolymph melanization in the Silkmoth *Bombyx mori* involves formation of a high molecular mass complex that metabolizes Tyrosine. J Biol Chem 2013;288:14476–87.

[182] Sung HH, Chang HJ, Her CH, Chang JC, Song YL. Phenoloxidase activity of hemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. J Invertebr Pathol 1998;71:26–33.

[183] Martin E. Purification of enzyme phenoloxidase from freshwater crab, *Barytelphusa cunicularis* and its kinetic study. World J Zool 2011;6:357–9.

[184] Ashida M, Söderhäll K. The prophenoloxidase activating system in crayfish. Comp Biochem Physiol Part B Comp Biochem 1984;77:21–6.

[185] Ashida M, Ishizaki Y, Iwahana H. Activation of pro-phenoloxidase by bacterial cell walls or beta-1,3-glucans in plasma of the silkworm, *Bombyx mori*. Biochem Biophys Res Commun 1983;113:562–8.

[186] Nord F, Bier M. On the mechanism of enzyme action . LV . A study of the interaction between calcium and trypsin. Biochim Biophys Acta n.d.;12:56–67.

[187] Nagle L, Place a R, Schott EJ, Jagus R, Messick G, Pitula JS. Real-time PCR-based assay for quantitative detection of Hematodinium sp. in the blue crab *Callinectes sapidus*. Dis Aquat Organ 2009;84:79–87.

[188] Bowers H, Messick G, Hanif A, Jagus R, Carrion L, Zmora O, et al. Physicochemical properties of double-stranded RNA used to discover a reo-like virus from blue crab *Callinectes sapidus*. Dis Aquat Organ 2010;93:17–29.

[189] Brody M, Chang E. Development and utilization of crustacean long-term primary cell cultures: Ecdysteroid effects in vitro. Invertebr Reprod Dev 1989;16:141–7.

[190] Chung JS. Hemolymph ecdysteroids during the last three molt cycles of the blue crab, *Callinectes sapidus*: quantitative and qualitative analyses and regulation. Arch Insect Biochem Physiol 2010;73:1–13.

[191] Buda ES, Shafer TH. Expression of a serine proteinase homolog prophenoloxidase-activating factor from the blue crab, *Callinectes sapidus*. Comp Biochem Physiol B Biochem Mol Biol 2005;140:521–31.

[192] Chan S, Rankin S, Keeley L. Characterization of the molt stages in *Penaeus vannamei*: setogenesis and hemolymph levels of total protein, ecdysteroids, and glucose. Biol Bull 1988;175:185–92.

[193] Elliott E, Dillaman RM. Formation of the inner branchiostegal cuticle of the blue crab, *Callinectes sapidus*. J Morphol 1999;240:267–81.

[194] Dillaman R, Hequembourg S, Gay M. Early pattern of calcification in the dorsal carapace of the blue crab, *Callinectes sapidus*. J Morphol 2005;263:356–74.

[195] Stevens BG. Hardening of red king crab *Paralithodes camtschaticus* (Tilesius, 1815) shells after molting. J Crustac Biol 2009;29:157–60.

[196] Williams DL, Modla S, Roer RD, Dillaman RM. Post-ecdysial change in the permeability of the exoskeleton of the blue crab, *Callinectes sapidus*. J Crustac Biol 2009;29:550–5.

[197] Cameron JN, M. WC. Apparent H+ excretion and CO2 dynamics accompanying carapace mineralization in the blue crab (*Callinectes sapidus*) following moulting. J Exp Biol 1985;114:181–96.

[198] Sugumaran M, Semensi V, Kalyanaraman B, Bruce JM, Land EJ. Evidence for the formation of a quinone methide during the oxidation of the insect cuticular sclerotizing precursor 1, 2-dehydro-N-acetyldopamine. J Biol Chem 1992;267:10355–61.

[199] Hégaret H, Wikfors GH, Soudant P. Flow-cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation I. Haemocyte types and morphology. J Exp Mar Bio Ecol 2003;293:237–48.

[200] Skowsky W, Fisher D. The use of thyroglobulin to induce antigenicity to small molecules. J Lab Clin Med 1972;80:134–44.

[201] Gassmann M, Grenacher B, Rohde B, Vogel J. Quantifying western blots: Pitfalls of densitometry. Electrophoresis 2009;30:1845–55.

[202] Chung JS. An insulin-like growth factor found in hepatopancreas implicates carbohydrate metabolism of the blue crab *Callinectes sapidus*. Gen Comp Endocrinol 2014;199:56–64.

[203] Zmora N, Sook Chung J. A novel hormone is required for the development of reproductive phenotypes in adult female crabs. Endocrinology 2013;155:230–9.

[204] Collins T. ImageJ for microscopy. Biotechniques 2007;43:25–30.

[205] Bratthauer G. Processing of tissue culture cells. In: Oliver C, Jamur MC, editors. Immunocytochemical methods Protoc. Third, London: Springer; 2010, p. 85–92.

[206] Bratthauer G. Processing of cytological specimens. In: Oliver C, Jamur MC, editors. Immunocytochemical methods Protoc. Third Edit, New York, USA: Springer Protocols; 2010, p. 75–84.

[207] Cunningham R. Indirect immunofluorescent labelling of fixed cells. In: Oliver C, Jamur MC, editors. Immunocytochemical methods Protoc. Third Edit, New York, USA: Springer Protocols; 2010, p. 335–40.

[208] Pamuru RR, Rosen O, Manor R, Chung JS, Zmora N, Glazer L, et al. Stimulation of molt by RNA interference of the molt-inhibiting hormone in the crayfish *Cherax quadricarinatus*. Gen Comp Endocrinol 2012;178:227–36.

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

[210] Szopa T, Rousseaux J, Yuncker C, Happ G. Ecdysteroids accelerate mitoses in accessory glands of beetle pupae. Dev Biol 1985;107:325–36.

[211] Kato Y, Riddiford L. The role of 20-hydroxyecdysone in stimulating epidermal mitoses during the laval-pupal transformation of the tobacco hornworm, *Manduca sexta*. Development 1987;100:227–36.

[212] Lin X, Novotny M, Söderhäll K, Söderhäll I. Ancient cytokines, the role of astakines as hematopoietic growth factors. J Biol Chem 2010;285:28577–86.

[213] Loret SM. Hemocyte differentiation in the shore crab (*Carcinus maenas*) could be accompanied by a loss of glycogenosynthesis capability. J Exp Zool 1993;267:548–55.

[214] Destoumieux D, Munoz M, Bulet P, Bachère E. Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). Cell Mol Life Sci 2000;57:1260–71.

[215] Destoumieux-Garzón D, Saulnier D, Garnier J, Jouffrey C, Bulet P, Bachère E. Crustacean immunity. Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. J Biol Chem 2001;276:47070–7.

[216] Cheng W, Juang F-M, Li J-T, Lin M-C, Liu C-H, Chen J-C. The immune response of the giant freshwater prawn *Macrobrachium rosenbergii* and its susceptibility to *Lactococcus garvieae* in relation to the moult stage. Aquaculture 2003;218:33–45.

[217] Söderhäll K, Smith VJ, Johansson MW. Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: evidence for cellular co-operation in the defence reactions of arthropods. Cell Tissue Res 1986;245:43–9.

[218] Wang W, Zhang X. Comparison of antiviral efficiency of immune responses in shrimp. Fish Shellfish Immunol 2008;25:522–7.

[219] Chase MR, Raina K, Bruno J, Sugumaran M. Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*. Insect Biochem Mol Biol 2000;30:953–67.

[220] Ashida M, Kinoshita K, Brey PT. Studies on prophenoloxidase activation in the mosquito *Aedes aegypti* L. Eur J Biochem - FEBS 1990;188:507–15.

[221] Mykles DL, Adams ME, Gäde G, Lange AB, Marco HG, Orchard I. Neuropeptide action in insects and crustaceans. Physiol Biochem Zool 2010;83:836–46.

[222] Baker JD, Truman JW. Mutations in the *Drosophila* glycoprotein hormone receptor, rickets, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. J Exp Biol 2002;205:2555–65.

[223] Christensen BM, Huff BM, Miranpuri GS, Harris KL, Christensen LA. Hemocyte population changes during the immune response of *Aedes aegypti* to inoculated Microfilariae of *Dirofilaria immitis*. J Parasitol 2014;75:119–23.

[224] Inoue N, Hanada K, Tsuji N, Igarashi I, Nagasawa H, Mikami T, et al. Characterization of phagocytic hemocytes in *Ornithodoros moubata* (Acari: Ixodidae). J Med Entomol 2001;38:514–9.

[225] Jiang Y-S, Zhan W-B, Wang S-B, Xing J. Development of primary shrimp hemocyte cultures of *Penaeus chinensis* to study white spot syndrome virus (WSSV) infection. Aquaculture 2006;253:114–9.

[226] Huang J, Zhang Y, Li M, Wang S, Liu W, Couble P, et al. RNA interference-mediated silencing of the bursicon gene induces defects in wing expansion of silkworm. FEBS 2007;581:697–701.

[227] Honegger H-W, Estévez-Lao TY, Hillyer JF. Bursicon-expressing neurons undergo apoptosis after adult ecdysis in the mosquito *Anopheles gambiae*. J Insect Physiol 2011;57:1017–22.

[228] Kostron B, Kaltenhauser U, Seibel B, BrÄUnig P, Honegger H. Localization of bursicon in CCAP-immunoreactive cells in the thoracic ganglia of the cricket *Gryllus bimaculatus*. J Exp Biol 1996;199:367–77.

[229] Sricharoen S, Kim JJ, Tunkijjanukij S, Söderhäll I. Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish. Dev Comp Immunol 2005;29:1017–31.

[230] Lu Z. Initiation and regulation of prophenoloxidase activation in tobacco hornworm,. Oklahoma State University, 2007.

[231] Satoh D, Horii A, Ochiai M, Ashida M. Prophenoloxidase-activating enzyme of the Silkworm, *Bombyx mori*: Purification, characterization, and cDNA cloning. J Biol Chem 1999;274:7441–53.

[232] Vafopoulou X. Mechanisms of wound repair in crayfish. Invertebr Surviv J 2009;6:125–37.

[233] Seymour K. Tyrosine hydroxylase. Adv Enzymol Relat Areas Mol Biol 1995;70:103–220.

[234] Aso Y, Kramer KJ, Hopkins TL, Lookhart GL. Characterization of haemolymph protyrosinase and a cuticular activator from *Manduca sexta* (L.). Insect Biochem 1985;15:9–17.

[235] Hopkins TL, Morgan TD, Kramer KJ. Catecholamines in haemolymph and cuticle during larval, pupal, and adult development of *Manduca sexta* (L.). Insect Biochem 1984;14:533–40.

[236] Fanjul-Moles ML. Biochemical and functional aspects of crustacean hyperglycemic hormone in decapod crustaceans: review and update. Comp Bochemistry Physiol Toxicol Pharmacol CBP 2006;142:390–400.

[237] Luan H, Lemon WC, Peabody NC, Pohl JB, Zelensky PK, Wang D, et al. Functional dissection of a neuronal network required for cuticle tanning and wing expansion in *Drosophila*. J Neurosci 2006;26:573–84.

[238] Charmantier G, Charmantier-Daures M. Endocrine and neuroendocrine regulations in embryos and larvae of crustaceans. Invertebr Reprod Dev 1998;33:273–87.

[239] Helluy SM, Beltz BS. Embryonic Development of the American Lobster (*Homarus americanus*): Quantitative Staging and Characterization of an Embryonic Molt Cycle. Biol Bull 1991;180:355–71.

[240] Subramoniam T. Crustacean ecdysteriods in reproduction and embryogenesis. Comp Biochem Physiol Toxicol Pharmacol 2000;125:135–56.

[241] Chung JS, Webster SG. Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas*. Development 2004;131:4751–61.

[242] Goudeau M, Lachaise F, Carpentier G, Goxe B. High titers of ecdysteroids are associated with the secretory process of embryonic envelopes in the european lobster. Tissue Cell 1990;22:269–81.

[243] Costlow J, Bookhout C. The larval development of *Callinectes sapidus* Rathbun reared in the laboratory. Biol Bull 1959;116:373–96.

[244] Kennedy VS, Cronin LE. Anatomy of the post-larval blue crab. In: Kennedy VS, Cronin LE, editors. blue crab *Callinectes sapidus*. 1st ed., College Park: Maryland Sea Grant; 2007, p. 55–132.

[245] Znidaršič N, Mrak P, Tušek-Žnidarič M, Strus J. Exoskeleton anchoring to tendon cells and muscles in molting isopod crustaceans. Zookeys 2012;53:39–53.

[246] Brunet PCJ. The metabolism of the aromatic animo acids concerned in the cross-linking of insect cuticle. Insect Biochem 1980;10:467–500.

[247] Kramer KJ, Hopkins TL. Tyrosine metabolism for insect cuticle tanning. Arch Insect Biochem Physiol 1987;6:279–301.

[248] Eleftherianos I, Boundy S, Joyce S a, Aslam S, Marshall JW, Cox RJ, et al. An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. Proc Natl Acad Sci USA 2007;104:2419–24.

[249] Liu H, Jiravanichpaisal P, Cerenius L, Lee BL, Söderhäll I, Söderhäll K. Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* Infection in a crustacean, *Pacifastacus leniusculus*. J Biol Chem 2007;282:33593–8.

[250] Lewis EG, Haefner PA. Oxigen comsumption of the blue crab *Callinectes sapidus* Rathbun, from proecdysis to postecdysis. Comp Biochem Physiol Part A Physiol 1976;54:55–60.

[251] DeFur P, Mangum C, McMahon B. Cardiovascular and ventilatory changes during ecdysis in the blue crab *Callinectes sapidus* Rathbun. J Crustac Biol 1985;5:207–15.

[252] Mykles D. The mechanism of fluid absorption at ecdysis in the American lobster *Homarus americanus*. J Exp Biol 1980:89–101.

[253] Death PC, Glands P, Metamorphosis DP. Programmed cell death of the prothoracic metamorphosis. Insect Biochem Mol Biol 1997;27:69–78.

[254] Milán M, Campuzano S, García-Bellido a. Developmental parameters of cell death in the wing disc of *Drosophila*. Proc Natl Acad Sci USA 1997;94:5691–6.

[255] Hart SP, Dransfield I, Rossi AG. Phagocytosis of apoptotic cells. Methods 2008;44:280–5.

[256] Jones J. Current concepts concerning insect hemocytes. Am Zool 1962;2:209–46.

[257] Jones J. Normal differential counts of haemocytes in relation to ecdysis and feeding in *Rhodnius*. J Insect Physiol 1967;13:1133–41.

[258] Söderhäll K. Biochemical and molecular aspects of cellular communication in arthropods. Bolletino Di Zool 1992;59:141–51.

[259] Pech L, Strand M. Plasmatocytes from the moth *Pseudoplusia includens* induce apoptosis of granular cells. J Insect Physiol 2000;46:1565–73.

[260] Pech L, Trudeau D, Strand M. Effects of basement membranes on the behavior of hemocytes from *Pseuduphsia inchdens* (Lepidoptera; Noctuidae): Development of an in vitro encapsulation assay. J Insect Physiol 1995;41:801–7.

[261] Persson M, Vey A, Söderhäll K. Encapsulation of foreign particles in vitro by separated blood cells from crayfish, *Astacus leptodactylus*. Cell Tissue Res 1987;247:409–15.

[262] Pech L, Strand M. Granular cells are required for encapsulation of foreign targets by insect haemocytes. J Cell Sci 1996;2060:2053–60.

[263] Buda ES. Prophenoloxidase activating factor from the blue crab, *Callinectes sapidus*. University of North Carolina, 2004.

[264] Vaseeharan B, Valli . J S. In silico homology modeling of prophenoloxidase activating factor serine proteinase gene from the haemocytes of *Fenneropenaeus indicus*. J Proteomics Bioinform 2011;04:53–7. doi:10.4172/jpb.1000166.

[265] Simonet G, Claeys I, Broeck J Vanden. Structural and functional properties of a novel serine protease inhibiting peptide family in arthropods. Comp. Biochem. Physiol. - B Biochem. Mol. Biol., vol. 132, 2002, p. 247–55.

[266] Sugumaran M. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res 2002;15:2–9.

[267] Theopold U, Schmidt O, Söderhäll K, Dushay MS. Coagulation in arthropods: defence, wound closure and healing. Trends Immunol 2004;25:289–94.

[268] Maningas MBB, Kondo H, Hirono I. Molecular mechanisms of the shrimp clotting system. Fish Shellfish Immunol 2013;34:968–72.

[269] Nagai T, Kawabata S. A link between blood coagulation and prophenol oxidase activation in arthropod host defense. J Biol Chem 2000;275:29264–7.

[270] Liang Z, Sottrup-Jensen L, Aspán A, Hall M, Söderhäll K. Pacifastin, a novel 155-kDa heterodimeric proteinase inhibitor containing a unique transferrin chain. Proc Natl Acad Sci USA 1997;94:6682–7.

[271] Breugelmans B, Simonet G, van Hoef V, Van Soest S, Vanden Broeck J. Pacifastin-related peptides: structural and functional characteristics of a family of serine peptidase inhibitors. Peptides 2009;30:622–32.

[272] Breugelmans B, van Hoef V, Simonet G, Van Soest S, Smagghe G, Vanden Broeck J. In vitro activity of pacifastin-like inhibitors in relation to their structural characteristics. Peptides 2011;32:539–44.

[273] Cerenius L, Babu R, Söderhäll K, Jiravanichpaisal P. In vitro effects on bacterial growth of phenoloxidase reaction products. J Invertebr Pathol 2010;103:21–3.

[274] Gai Y, Zhao J, Song L, Li C, Zheng P, Qiu L, et al. A prophenoloxidase from the Chinese mitten crab *Eriocheir sinensis*: gene cloning, expression and activity analysis. Fish Shellfish Immunol 2008;24:156–67.

[275] An S, Wang S, Gilbert L, Beerntsen B. Global identification of bursicon-regulated genes in *Drosophila melanogaster*. BMC Genomic 2008;9:424–38.

[276] Veverytsa L, Allan DW. Retrograde BMP signaling controls *Drosophila* behavior through regulation of a peptide hormone battery. Development 2011;138:3147–57.

[277] Lahr EC, Dean D, Ewer J. Genetic analysis of ecdysis behavior in *Drosophila* reveals partially overlapping functions of two unrelated neuropeptides. J Neurosci 2012;32:6819–29.

[278] Ewer J, Gammie S, Truman J. Control of insect ecdysis by a positive-feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. J Exp Biol 1997;881:869–81.

[279] Chung JS, Ahn IS, Yu OH, Kim DS. Crustacean hyperglycemic hormones of two cold water crab species, *Chionoecetes opilio* and *C. japonicus*: Isolation of cDNA sequences and localization of CHH neuropeptide in eyestalk ganglia. Gen Comp Endocrinol 2014.

[280] Kuballa A V, Merritt DJ, Elizur A. Gene expression profiling of cuticular proteins across the moult cycle of the crab Portunus pelagicus. BMC Biol 2007;5:45.

[281] Kuballa A V. Novel molecular approach to study moulting in crustaceans. Bull Res 2007;20:53–7.

[282] Kuballa A V, Elizur A. Differential expression profiling of components associated with exoskeletal hardening in crustaceans. BMC Genomics 2008;9:575.

[283] Burmester T. Molecular evolution of the arthropod hemocyanin superfamily. Mol Biol Evol 2001;18:184–95.

[284] Beintema J, Stam W, Hazes B, Smidt M. Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). Mol Biol Evol 1994;11:493–503.