ABSTRACT

Title of dissertation: The functional importance and significance of ecdysteroids in molt-cycle regulation of the blue crab, *Callinectes sapidus*

Sirinart Techa, Doctorate of Philosophy, 2014

Dissertation directed by Associate Professor J. Sook Chung
Marine Estuarine Environmental Science

This study aims to expand our understanding of how ecdysteroids and neuropeptide hormones (MIH/CHH) regulate molting in crustaceans using the blue crab *Callinectes sapidus* as a model animal. The hypothesis of this study is that ecdysteroids have a stimulatory effect on MIH/CHH production in eyestalks while generating both positive and negative feedback on ecdysteroidogenesis in Y-organs. Since ecdysteroids exert their signals through an ecdysteroid receptor complex, composed of an ecdysone receptor (EcR) and its partner, the retinoid-X receptor (RXR), the functional activity of ecdysteroids on tissues of interest is examined through EcR expression. Endogenous levels of ecdysteroids as well as expression in *EcR, RXR* and *MIH/CHH* are assayed in both embryonic and juvenile molt cycles. Similar patterns of changes in these two cycles suggest that molting control is driven by the same mechanisms in each. During embryonic molt, the correlation of ecdysteroid levels and *MIH/CHH* expression suggests that ecdysteroids are involved in *MIH/CHH* production. Using an *in vitro* system, stimulation of *MIH* expression occurs in eyestalks incubated with ponasterone A (PoA) and 20-hydroxyecdysone (20-HE) in the ratio found endogenously (3:1)

and in ecdysteroid concentrations of 75 and 150 ng/ml. The *in vivo* response of *MIH* expression to depletion of ecdysteroid signals was examined by RNAi knockdown of an ecdysone receptor that was found to reduce the levels of *MIH* to those found by *MIH* knockdown, providing support for the hypothesis that ecdysteroids stimulate *MIH* expression through an ecdysone receptor. This study also found evidence suggesting that crustaceans have long-loop feedback for ecdysteroids in the neuroendocrine axis, which is similar to that found in insects.

THE FUNCTIONAL IMPORTANCE AND SIGNIFICANCE OF ECDYSTEROIDS IN MOLT-CYCLE REGULATION OF THE BLUE CRAB, *Callinectes sapidus*

By

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LIST OF ABBREVIATION

AF	Activation function
CasAK	*C. sapidus* Arginine Kinase
CasBTBDP	*C. sapidus* BTB domain protein
CasCHH	*C. sapidus* Crustacean Hyperglycemic Hormone
CasEcR	*C. sapidus* Ecdysone Receptor
CasMIH	*C. sapidus* Molt-Inhibiting Hormone
CasRXR	*C. sapidus* Retinoid-X receptor
DBD	DNA-binding domain
dsRNA	double-stranded RNA
Ecd-RIA	Ecdysteroid-radioimmunoassay
IRES	Internal ribosome entry site
LBD	Ligand-binding domain
LBP	Ligand-binding pocket
PoA	Ponasterone A
PTTH	Prothroracicotropic hormone
uORF	Upstream open reading frame
20-HE	20-hydroxyecdysone

CHAPTER 1. INTRODUCTION

1.1 Molting and its regulation

Growth and the developmental processes of all animals involves an increase in body size and changes in body-plan formation. However, these changes in arthropods are limited to their exoskeleton. In order to grow, the old shell must be shed and the new one synthesized. This process is called molting. Since molting is essential for growth and development in arthropods, the mechanisms that regulate these processes must be better understood.

It has been well documented that molting is stimulated by ecdysteroids and the circulating levels of ecdysteroids in all arthropods highly correlate with the molt stages [1-9]. As a comparison, the regulation of ecdysteroidogenesis in insects and crustaceans is different. In insects, prothoracicotropic hormone (PTTH) is produced in the brain and secreted from the corpora allata to stimulate prothoracic glands that then produce ecdysteroids [10-12]. Alternatively, neuropeptides, produced in the eyestalks of crustaceans, suppress ecdysteroidogenesis in the Y-organs, which is equivalent to the prothoracic gland [5,13-23]. It has been reported that ecdysteroids have stimulatory effects on the production of PTTH in *Manduca sexta* [24-27]. Moreover, it has been proposed that ecdysteroids may have a positive effect on neuropeptide production in the eyestalks of crustaceans [14,28].

1.1.1 Neuropeptide hormones

Insects

In insects, PTTH is produced in the brain and secreted by the corpora allata to stimulate the prothoracic gland. PTTH targets the prothroracic gland and triggers ecdysteroidogenesis through a signaling cascade involving Ca^{2+}, cAMP and a MAP kinase [11,29]. In addition to physiological and environmental cues such as weight gain and photoperiod, PTTH is positively regulated by ecdysteroids as reported in *M. sexta* [24-27]. As reported in *M. sexta* and *Pieris brassicae,* ecdysteroids also generate a short-loop feedback on the prothoracic gland. Lower levels stimulate, while higher titers inhibit the action of the prothoracic gland [30-32].

Crustaceans

The finding that the molting progression is induced by eyestalk ablation [33] has led to the discovery that molting in crustaceans is suppressed by eyestalk factors, which were later attributed to neuropeptides. Neuropeptide hormones belong to the crustacean hyperglycemic hormone (CHH) family and include CHH, molt-inhibiting hormone (MIH), gonad-stimulating and -inhibiting hormone (GS/IH), and mandibular organ-inhibiting hormone (MOIH) [34-38]. MIH and CHH are synthesized in neurosecretory cells in the X-organ located within the eyestalk ganglia. These neuropeptides are transported through the axonal tract, stored in the sinus gland, and secreted into the hemolymph to suppress ecdysteroidogenesis in Y-organs [18-21,37,39-43]. Inhibitory actions of

MIH and CHH on the Y-organs have been reported in several crustaceans including *Carcinus maenas*, *Callinectes sapidus*, *Gecarcinus lateralis*, *Procambarus clarkii,* and *Uca pugilator* through *in vitro* incubation studies [5,13-22,44,45]. In addition, only two *in vivo* studies confirm the intrinsic hormonal signaling of MIH and CHH on the activity of Y-organs [37,46].

To date, regulation in the levels of *MIH* expression during the molt cycle has not been well understood. A few reports indicate that ecdysteroids may have an effect on the levels of MIH in the eyestalks. Administering ecdysteroids in *Cancer anternarius* increased neuropeptide production in eyestalk ganglia [28]. Moreover, there is a report in *Uca pugilator* that a concentration of ecdysteroids in the eyestalk ganglia was higher than the hemolymph [14], implicating a possible accumulation of ecdysteroids in this tissue. Recent studies have shown that the upstream promoter regions of *MIH* genes, isolated from *C. pagurus* and *Charybdis feriatus,* contain the binding sites for ecdysteroid-responsive factors [45,47]. Furthermore, hemolymph titers of MIH and CHH during the molt cycle of *C. maenas* show that MIH levels are significantly higher during the late premolt compared to the other stages [48]. These lines of evidence suggest that ecdysteroids may have a positive effect on the expression of neuropeptides in the eyestalk ganglia.

1.1.2 Ecdysteroid hormones

Ecdysteroids are a class of steroid hormones that are derived from cholesterol and found in animals, plants and fungi [1,2]. Ecdysteroids play important roles in several processes in arthropods, most significantly in molting. Ecdysteroidogenesis in the prothroracic gland or Y-organ is similar, but the crustacean Y-organ produces a greater diversity of ecdysteroids [49-52]. According to the review by Mykles [52], the synthetic pathway of ecdysteroids can be divided into two stages: 1) conversion of cholesterol to 5-diketol, and 2) conversion of 5-diketol to secreted products. The former stage is common to all decapod species, while the latter one has four pathways that are named according to the secreted ecdysteroids by the Y-organ: 1) ecdysone, 2) 3-dehydroecdysone, 3) 25-deoxyecdysone, and 4) 3-dehydro-25-deoxyecdysone [52]. Among the four forms, crustacean ecdysteroids are usually secreted in three forms (ecdysone, 3-dehydroxyecdysone, 25-deoxyecdysone, and 3-dehydro-25-deoxyecdysone) depending on the species [50,52-55]. In peripheral tissues, these compounds are modified to an active form.

It is well known that molting is induced by ecdysteroids. Molt stages are defined by the levels of hemolymphatic ecdysteroids and setogenesis [3-9,56]. Within a molt cycle, ecdysteroid titers fluctuate: low during postmolt and intermolt, increasing at early premolt, peaking at midpremolt and returning to basal levels prior to ecdysis, as reported in *C. magister, C. maenas*, *C. sapidus, Daphnia magna, Hyas araneu, Metopograpsus messor*, and *P. clarkii* (Fig. 1-1) [3-9].

The clearance of ecdysteroids in crustaceans is reported in *Homarus americanus*, and the rate is molt-stage dependent: lowest at early premolt, followed by the mid premolt stage, and highest during the postmolt and intermolt stages [57]. Ecdysteroids were hydrolyzed into high polarity metabolites including 20-hydroxyecdysonoic acid and 20, 26-dihydroxyecdysone and were excreted in urine (95%), while the apolar metabolites were found in feces (5%) [58,59]. Therefore, it is suggested that titers of circulating ecdysteroids during each molt stage are determined by the difference between the rate of synthesis and clearance [60].

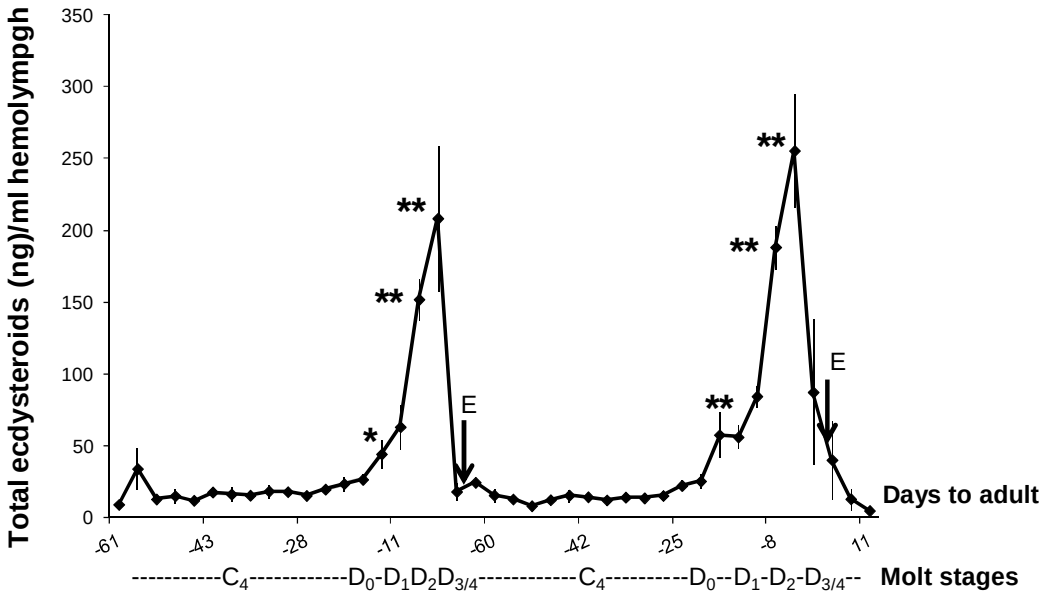


Figure 1-1. Circulating ecdysteroid from a *C. sapidus* female during the last two molts: pre-pubertal molt and adult molt assayed by ecdysteroid-RIA. Numbers on the X-axis represents days before each molt. C_4= intermolt, D_0= early molt, D_1-D_2= mid premolt, $D_{3/4}$=late premolt and E= ecdysis. The *p* value: *, $p < 0.05$; **, $p < 0.005$

Nuclear hormone receptor superfamily

Nuclear receptors are phylogenetically related proteins clustered into a large superfamily, including receptors for hydrophobic molecules such as steroid hormones, retinoic acids (all *trans* and 9-*cis* isoforms), thyroid hormones, and fatty acids [61]. Nuclear receptors are commonly found in sponges and Metazoans including echinoderms, tunicates, arthropods and vertebrate [62].

The nuclear hormone receptor family is characterized by 5-independent functional domains (domains A-F as originally defined by Krust): 1) A/B or N-terminal domains or activation function-1 (AF1): exhibiting ligand-independent trans-activation; 2) C or DNA-binding domain (DBD): binding to regulatory elements of the target genes; 3) D or hinge region (HR) domain: providing flexibility within the molecule; 4) E or ligand-binding domain (LBD): serving as docking sites for its cognate ligands and transcriptional regulators (co-activator or co-repressor); and 5) F or C-terminal or AF2 domain: trans-activating with ligand-dependent manner [63-65] as shown in Fig. 1-2.

Since the DBD and AF reside within the same molecule, the nuclear hormone receptor can directly trans-activate the target genes when its cognate ligands bind to the LBD [66]. In general, binding of the ligands to the LBD stimulates conformational changes in the LBD and AF2 which strengthens partner dimerization or releases a co-repressor. Eventually, the pre-initiation complex is recruited to the promoter, and transcription initiation is started.

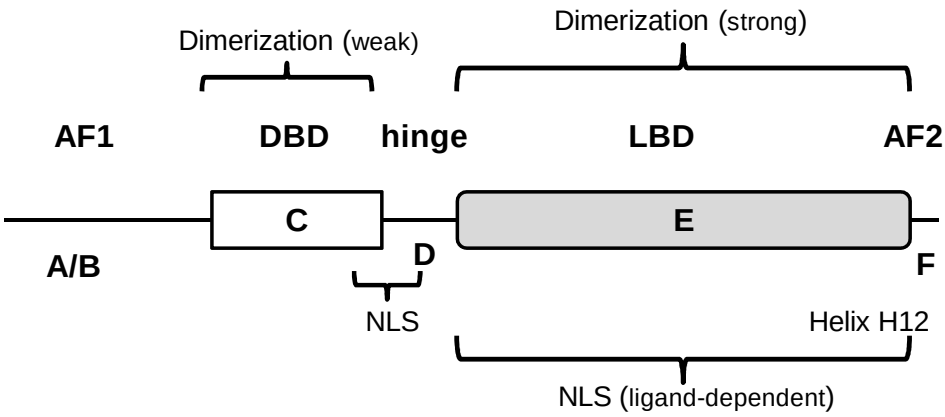


Figure 1-2. Schematic illustration of the structural and functional organization of a nuclear hormone receptor family. The evolutionarily conserved regions C and E are indicated as white and grey boxes, respectively, while the line represents the divergent regions A/B, D, and F. Two transcription activation functions (AFs) have been described: a constitutively active AF1 in the A/B region and a ligand-inducible AF2 in region E or F. DBD: DNA binding domain, LBD: ligand binding domain, and NLS: nuclear localization signal [67].

Ecdysteroid-responsive genes

After receiving the ecdysteroid signals, the EcR-RXR complex, a master transcription factor, will trigger the expression of downstream transcription factors including EcR-RXR itself. In *Drosophila*, the EcR/USP heterodimer induces the expression of early ecdysteroid-responsive genes such as *Broad complex*, *E74*, and *E75* which are required during development and metamorphosis [68-75]. The *Broad complex* gene is located in the 2B5 locus and encodes three alternate pairs of C_2H_2 zinc finger motifs for binding to regulatory elements of late ecdysteroid-responsive genes such as *E71* [70,72,76]. *E74* genes encode an ETS DNA-binding domain [73,77-80], while *E75* genes located in 75B locus encode a nuclear hormone receptor [72,81-83]. Although these transcriptional factors are downstream of ecdysteroid responses, their transcription is directly induced by 20-hydroxyecdysone [72,82,83]

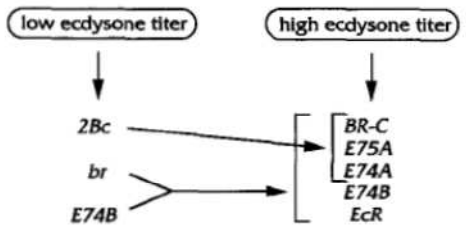


Figure 1-3. Schematic diagram illustrating the differential effects of ecdysteroids titers on the expressions of their responsive genes in *Drosophila*: higher levels of ecdysteroids trigger early ecdysteroid- responsive genes including of the *Broad complex* (*BR-C), E74A, E75A* and *EcR*. *2Bc* and *br* are alleles of *Broad complex* genes [73].

1.2. The blue crab, *Callinectes sapidus*

Blue crabs, *Callinectes sapidus,* are classified into the Phylum Arthropoda and Subphylum Crustacea. *C. sapidus* belongs to the family known as Portunidae and is distributed across two hemispheres from the north Atlantic coast of the United States to the coast of Uruguay in South America [84]. Blue crabs are important to the ecosystem and commercial fishery along the Chesapeake Bay where the brood stock density has drastically declined in the last few decades. Therefore, a better understanding of how their growth is regulated is necessary.

Similar to other arthropods, blue crabs undergo growth, development, and reproduction through molting. Molting in *C. sapidus* is repeated 27 to 29 times during their lifecycle starting from hatching and until adulthood (Fig.1-4) [60]. As a result, the body size increases 20-50% in each molt cycle, which is referred to as a molt increment. Females undergo a terminal-pubertal molt during the active reproductive period, while males continue to molt during their adulthood [85].

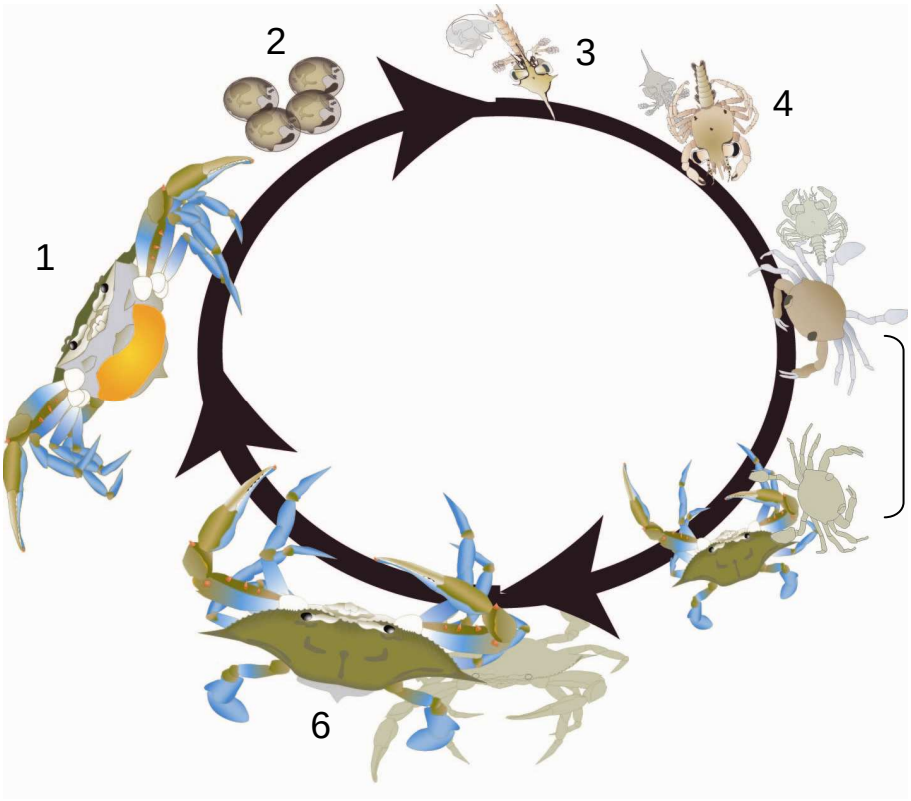


Figure 1-4. Lifecycle of *C. sapidus* starting from (1) a sponge female carrying the fertilized eggs, (2) developing embryo inside embryonic envelopes, (3) hatching larva, (4) megalops, (5) juvenile and (6) adult crab.

1.2.1. Ovarian development

Similar to other arthropods, reproduction in the adult female crab includes nuclear replication, oocyte differentiation, and yolk (or vitellin) deposition [86]. The early phase of oocyte development, called previtellogenesis, occurs during both sexual rest and reproductive activity. During this phase, the cellular organelles are synthesized and the glycoproteins are accumulated [87].

The latter phase is vitellogenesis and only occurs during reproduction. Vitellogenesis is involved in the synthesis of vitellogenin, a precursor yolk protein, in the fat body of insects or hepatopancreas of crustaceans [23,88,89]. After being processed from vitellogenin, vitellin, a lipoprotein complex, is circulated in

the hemolymph and endocytosed into the oocytes [87]. The differentiated oocytes continue accumulation of vitellin, maternal mRNAs, and other cytoplasmic proteins, resulting in a significant increase in oocyte diameter [23,87,90]. Moreover, vitellin is known as a carrier of the ecdysteroids that are required for early embryogenesis in insects and crustaceans [91-96].

1.2.2 Embryonic development

In *C. sapidus,* the fertilized eggs are extruded from the gonopores of the females and attached on the inner-branch hairs (ovigerous hairs) of swimmerets, and this egg mass is referred to as sponge [97]. The developmental stages of decapod embryos such as *C. maenas, C. sapidus, H. americanus* and *Palaemon serratus* are classified according to the size of remaining yolk, together with the levels of development of eyes, appendages, and heart [98-101].

In *C. sapidus*, embryogenesis is divided into five stages based on the criteria reported in *C. maenas*, as shown in Fig. 1-5 [98]. The yolk stage includes embryos from zygotic formation until the end of gastrulation. Organogenesis occurs at the limb-formation stage when the divided cells are differentiated and migrate to one pole of the egg, forming a clear cluster of these cells. The organogenesis of the embryos continues and can be observed by the presence of eye pigment at the eye-anlage stage. While the eyes continue to develop, the heart starts to function at the heart-beat stage. Eye development is completed at the developing-eye stage and the fully-developed embryos are referred to as hatching imminent. In *C. sapidus*, these processes are completed in 14-19 days

depending on water temperature [102] and the larvae are released as the first zoeae (Zoeal stage 1).

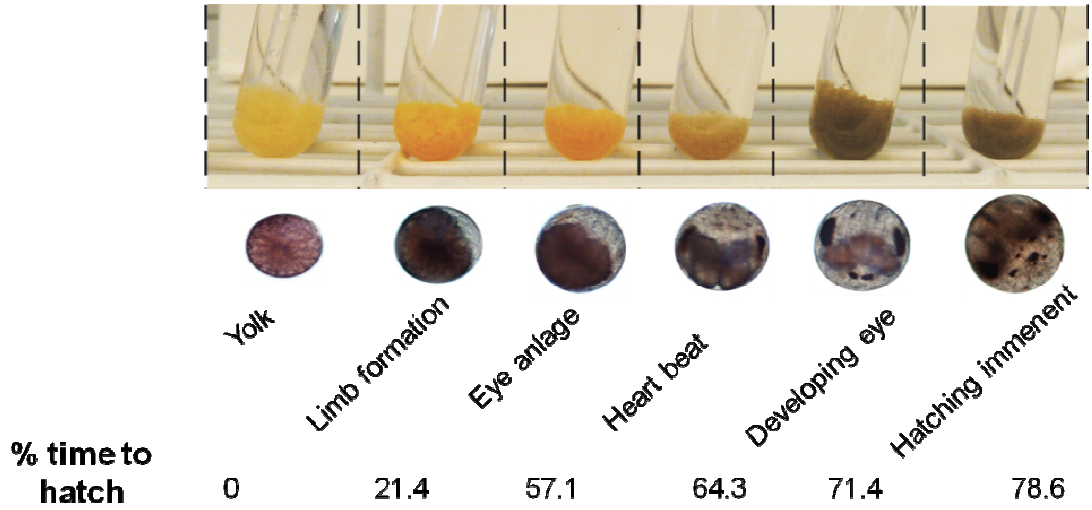


Figure 1-5. The developmental stages of *C. sapidus* embryos classified according to the criteria as stated [98].

During crustacean embryogenesis, vitellin is being continuously utilized which results in the release of ecdysteroids [101,103,104]. However, changes in the levels of ecdysteroids during embryogenesis of decapod crustaceans suggest that zygotic ecdysteroidogenesis may occur during this period [100,105].

1.2.3. Larval and post-larval development

After hatching, the released larva are called zoea and have a cylindrical shape, a long and sharp beak, two pairs of antennae, and four pairs of leg-like appendages [97,106]. The zoea undergoes 7 or 8 cycles of molting and finally becomes a post-larval form called a megalopa. The megalopa has a flattened body with a shorter and wider abdomen than that of the zoea. In *C. sapidus*, the megalopa metamorphoses into the first juvenile crab (crab 1) [97].

Development of zoeae and megalopae in the Chesapeake Bay might be affected by endocrine disruptors, a consideration due to the transport of larvae and megalopae to lower salinity in the upper bay. During this period, the bay watersheds peak in contamination with pesticides that are considered endocrine disruptors, such as atrazine, chlordane and endosulfan [107-111]. In particular, endosulfan sulphate promotes embryonic abnormalities, reduces molting frequency, and interferes with molting processes through blocking of the binding of ecdysteroids and EcR as reported in *D. magna* and *Macrobrachium malcolmsoii* [110-112]. Therefore, it is possible that the blue crab population might be affected by these endocrine disruptors that interfere with molting.

1.2.4. Molt stages and physiological changes

During the molt cycle, physiological and structural changes are closely related to molt stages, and crustacean molting is classified into four different stages: intermolt (C_4), premolt (D_{0-4}), ecdysis (E), and postmolt (A-C_3). Premolt is

further characterized by three stages: early premolt (D_0), mid premolt (D_1-D_2), and late premolt (D_3-D_4) [113,114], as shown in Fig.1-6.

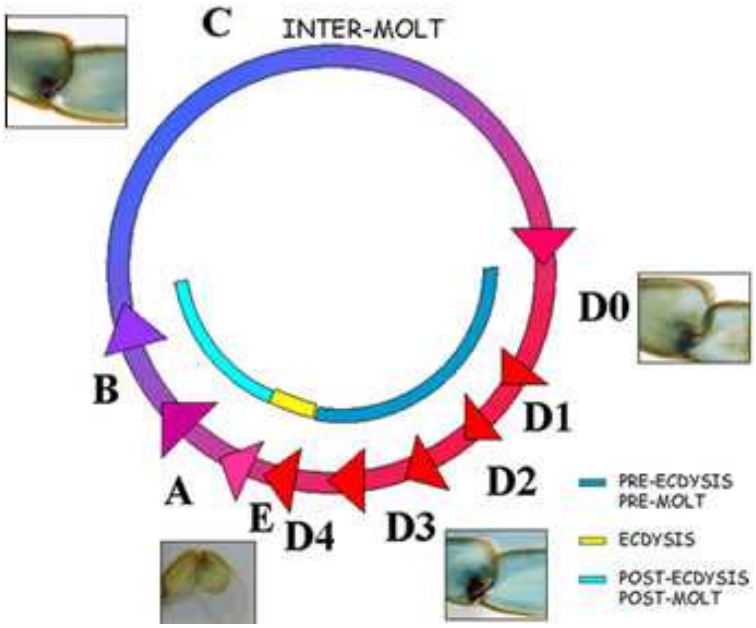


Figure 1-6. A schematic diagram of the molt cycle of *C. sapidus* with associated phenotypic changes of the swimming leg. Inner ring: blue=premolt, yellow=ecdysis, and light blue=postmolt; outer ring: color represents the progression of molting: blue=intermolt (C_4), magenta=premolt (D_0-D_4), red=ecdysis (E), purple=postmolt (A-B). (http://www.serc.si.edu/education/resources/bluecrab/molting.aspx)

Normal activities such as eating and swimming occur during intermolt when the exoskeleton is rigid, and the membranous tissue is attached to the hypodermis and the cuticle [115]. During premolt, the old cuticle is detached from the hypodermis by digestive enzymes (chitinases) secreted by epithelial cells [116], while the new cuticles composed of an organic matrix are laid. At the late midpremolt (D_2) stage, organic materials from the old shell are reabsorbed, resulting in a breakable shell. Ecdysis begins through the breakage of the ecdyseal line assisted by the hydrostatic pressure that is generated by the water

uptake induced by the release of gut-borne CHH [117,118]. After tanning and calicification during postmolt (A, B, C_1-C_3 stages), the animals resume normal activities.

1.2.5 Ecdysteroids during a molt cycle

In *C. sapidus*, a recent study reported that during the mid premolt (D_2) stage, Ponasterone A (PoA) is the major active form followed by 20-hydroxyecdysone (20-HE) as shown in Fig. 1-7, and they are circulated with a 3:1 ratio [60]. Interestingly, ratios of the two major forms in the last three molts seem variable when the ratios of 20-HE to PoA are increased when the animals become mature.

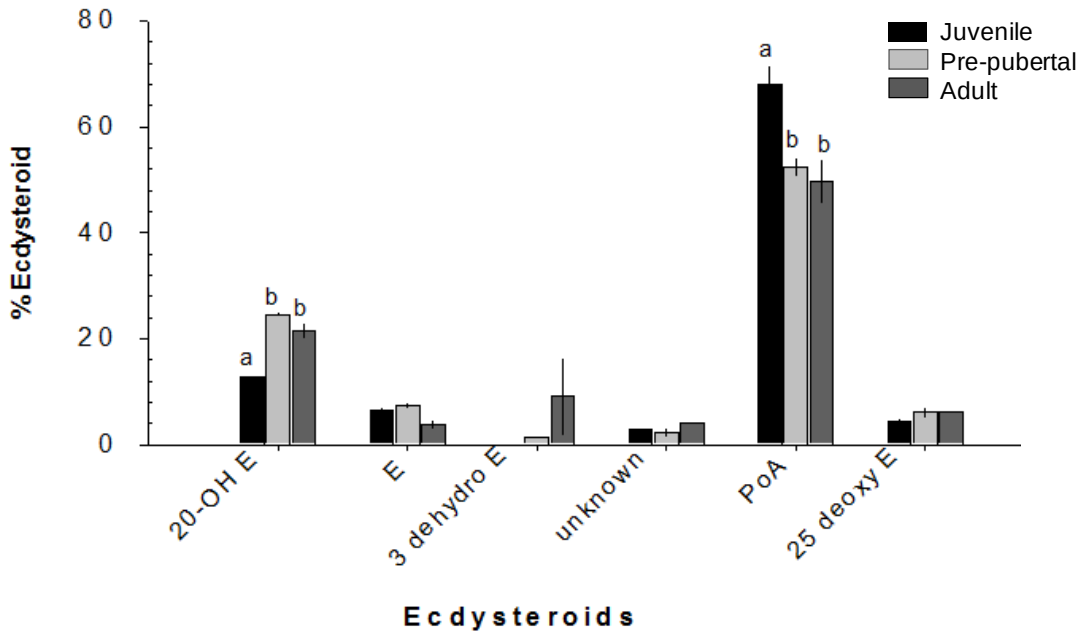


Figure 1-7. Different forms of ecdysteroids circulating during mid premolt (D_2) from juvenile (black bar), pre-pubertal (light-grey bar), and adult (grey bar) molts, assayed by HPLC-RIA. % Ecdysteroid represents percentages of 20-hydroxyecdysone (20-OH E), ecdysone (E), 3-dehydroxyecdysone (3 dehydro E), Ponasterone A (PoA), 25-deoxyecdysone (25 deoxy E), and unknown [60].

1.2.6 Ecdysteroid receptor complex in *C. sapidus* (CasEcR and CasRXR)

In a previous study, cDNA sequences encoding for *EcR* and *RXR* in *C. sapidus* (*CasEcR-CasRXR*) were isolated and characterized [119]. The 5' untranslated region (5'UTR) of both the *CasEcR* and *CasRXR* cDNA sequences contain translational regulatory sites: an upstream open reading frame (uORF) and an internal ribosome entry site (IRES).

Messenger RNAs containing uORFs are often up-regulated in their translations under stress or a starvation condition, while the translations of other mRNAs are down-regulated by phosphorylated Eukaryotic Initiation Factor 2 (eIF2) [120]. Reduction in the levels of ternary complexes (Met-$tRNA_i^{Met}$ and eIF2-GTP) will slow the reinitiation of uORFs and allow the 40S preinitiation complex to scan through the encoding ORF. On the contrary, IRES provides an alternative pathway for translation initiation. Specific mRNAs containing IRES can be continuously translated when the cap-dependent mechanism is impaired [121-123].

Four putative isoforms of both CasEcR and CasRXR are found in this species. CasEcR is especially important because it binds directly to ecdysteroids [124,125]. Two putative CasEcR isoforms (CasEcR1a/CasEcR2a) have an insert in the HR domain that lies between the LBD and the DBD. This insert (HR^i) contains four residues of Pro that may generate steric hindrance and interfere with conformational changes, resulting in an inefficiency inof ecdysteroid transduction. In addition, there are two types of ligand-binding pocket (LBP): type-1 (CasEcR1/CasEcR1a) and type-2 (CasEcR2/CasEcR2a) that differ in

hydrophobic fields (Fig. 1-8), suggesting that these differences may be responsible for binding with several forms of circulating ecdysteroids (Fig. 1-7). This notion seems to agree with the finding that during the mid premolt, two active ecdysteriods, PoA and 20-HE, are found in the hemolymph. PoA is more hydrophobic than the 20-HE [60].

Considered together, this suggests that type-1 CasEcR would be stronger for binding with 20-HE than PoA, whereas type-2 CasEcR would prefer to bind with the PoA. However, the specific binding to ecdysteroids and significant roles of four CasEcR isoforms in molt-related tissues (eyestalks and Y-organs) still remain unknown. CasRXR appears to have variations from inserted fragment(s) at the T-box within the DBD and/or at the anterior of the LBD. Both inserts are predicted to have effects on the dimerization of CasRXR to its CasEcR partner as suggested in *U. pugilator* [126-128].

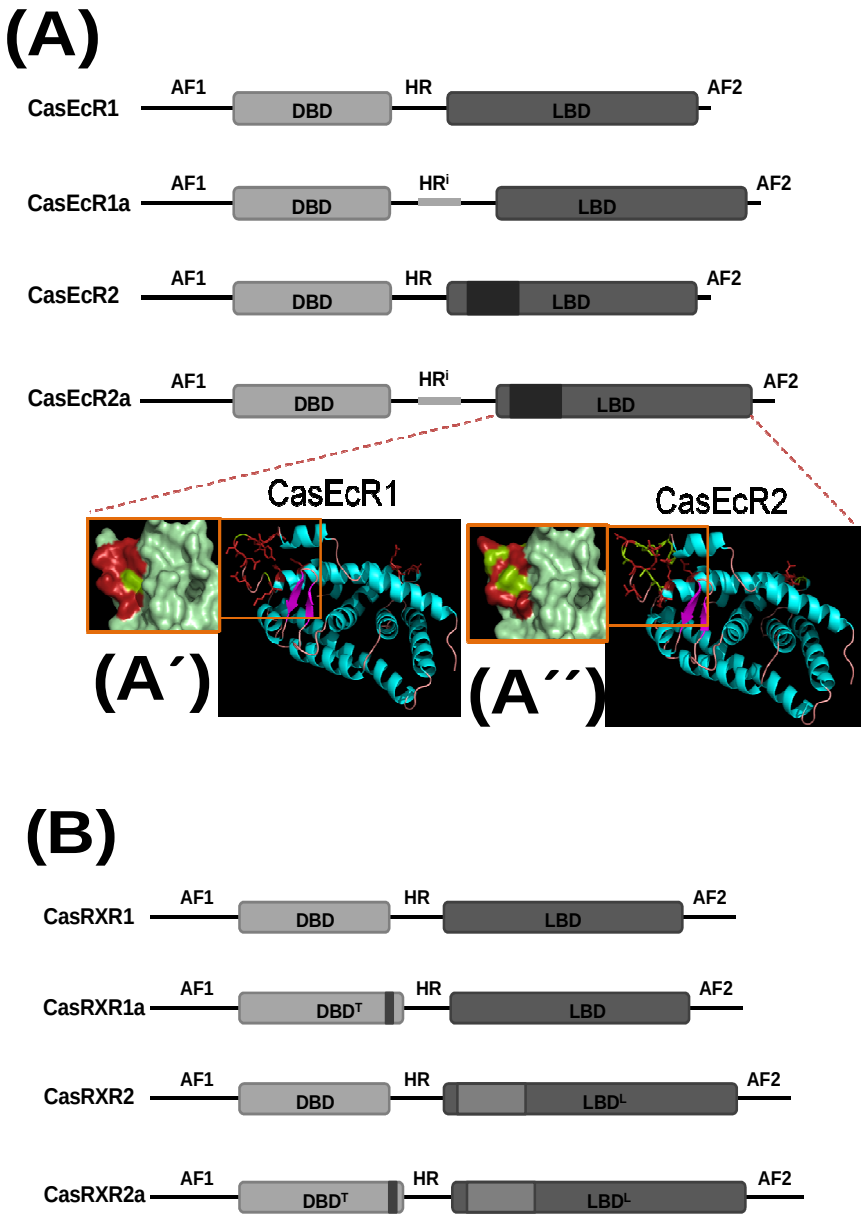


Figure 1-8. Isoform characterization of CasEcR (A) and CasRXR (B) from multiple alignment of putative encoding protein. Putative protein structures of CasEcR1 (A´) and CasEcR2 (A´´) analyzed with PyMol program based on the X-ray structure of *Heliothis virescens* EcR-USP complex bound to Ponasterone A. Variations in CasEcR1 and CasEcR2 are labeled in the ball-and-stick model: green and red representing hydrophobic and hydrophilic residues, respectively. Small legends represent surface model differences between *CasEcR1* and *CasEcR2*, respectively [119].

1.3. Hypothesis

This study aims to expand the knowledge of how ecdysteroids and neuropeptide hormones regulate molting in crustaceans, using blue crab *C. sapidus* as a model animal. The hypothesis of this study is that ecdysteroids have a stimulatory effect on neuropeptide production in eyestalks while generating both positive and negative feedback on ecdysteroidogenesis in Y-organs. Since ecdysteroids exert their signals through the ecdysteroid receptor complex composed of EcR and RXR, the functional activity of ecdysteroids on tissues of interest can be examined through EcR expression. Two main objectives that guided the effort to determine whether ecdysteroids have a direct effect on activities of eyestalk ganglia included:

1) An aim to determine the expression levels of endogenous neuropeptides (*CHH* and *MIH*), ecdysteroid-responsive factors (*EcR*, *RXR*, and *BTBDP*) and ecdysteroid levels during *C. sapidus* embryogenesis. As proposed in research on arthropods, the first cycle of molting occurs during embryogenesis (referred to as embryonic molt). Due to the times and patterns of their expressions, it has been suggested that there is an interaction between ecdysteroids and neuropeptides. By obtaining these results, expressions of neuropeptides and ecdysteroids can be compared to determine whether the embryonic ecdysteroids are correlated with the newly produced neuropeptides.

2) An aim to assess the direct effects of ecdysteroids on *MIH* expression in eyestalk ganglia where neuropeptides are specifically produced. Endogenous expressions of *MIH* and *EcR1*, the major isoform in eyestalks, were examined

from eyestalk ganglia during the preceding juvenile molt. In addition, expression of three isoforms of *EcR* in Y-organs was compared to determine whether the expression patterns were similar with those of the eyestalks. Induction of ecdysteroids on *MIH* expression was performed by *in vitro* incubation of the intermolt eyestalks with different ecdysteroid concentrations and ratios that mimic endogenous ecdysteroids during the *C. sapidus* premolt. To confirm direct interactions between ecdysteroids and *MIH* expression, knockdown of *EcR1* using double-stranded RNA (dsRNA) was carried out to test its functional significance *in vivo*. It was expected that the dsRNA specific for *EcR1* and *MIH* would experience a reduction in *MIH* expression levels.

2. CHAPTER 2

Changes in ecdysteroid levels and expression patterns of ecdysteroids-responsive factors and neuropeptide hormones during the embryogenesis of the blue crab, *Callinectes sapidus*

Abstract

Embryogenesis requires the involvement and coordination of multiple networks of various genes, according to a timeline governing development. Crustacean embryogenesis usually includes the first molt, a process that is known to be positively controlled by ecdysteroids. We determined the amounts of ecdysteroids, as well as other related factors: the ecdysone receptor (*CasEcR*), the retinoid X receptor (*CasRXR*), the molt-inhibiting hormone (*CasMIH*), and crustacean hyperglycemic hormone (*CasCHH*) during the ovarian and embryonic developments of *Callinectes sapidus*. In summary, the ovaries at stages 1-4 have expression levels of maternal *CasEcR* and *CasRXR* 10-50 times higher than levels seen in embryos at the yolk stage. This large difference in the amount of these factors in *C. sapidus* ovaries suggests that these maternal ecdysteroid-responsive factors may be utilized at the initiation of embryogenesis. During embryogenesis, the changes in total ecdysteroids and levels of *CasEcR* and *CasRXR* expression are similar to those observed in juvenile molts. The full-length cDNA sequence of the *C. sapidus* BTB domain protein (CasBTBDP) initially isolated from Y-organ cDNA, contains only Broad-Complex, Tramtrack, and Bric a brac (BTB) domains. The levels of *CasBTBDP* are kept constant throughout embryogenesis. The expression profiles of *CasMIH* and *CasCHH* are similar to the titers of ecdysteroids. However, the timing of their appearance is

followed by increases in *CasEcRs* and *CasRXRs*, implying that the expressions of these neuropeptides may be influenced by ecdysteroids. Moreover, the ecdysteroid profile during embryogenesis may track directly with the timing of organogenesis of Y-organs and their activity. Our work reports, for first time, the observed expression and changes of ecdysteroid-responsive factors, along with *CasCHH* and *CasMIH,* during embryogenesis in the crustacean *C. sapidus*.

2.1 Introduction

Embryogenesis is a developmental process that entails a complex network of regulatory genes [129]. The embryo of many arthropod species starts with a large amount of yolk protein (vitellins= VT) at fertilization. Extensive research reports that for crustaceans, their embryos undergo molting within an embryonic envelope during a relatively long period of embryogenesis [95,99,130-134].

Molting is essential for growth and development that recapitulates throughout the life cycle of each given arthropod species. During embryogenesis of decapod crustaceans, such as *Carcinus maenas*, *Callinectes sapidus*, *Homarus americanus,* and *Palaemon serratus*, the first molt occurs within embryonic envelopes [98-101]. Hatching coincides with the true first molt accompanied by shedding of the exuvium. The crustacean molt is known to be regulated mainly through the interplay between ecdysteroids and neuropeptide hormones [34,35,37,38,98,135-137] However, molecular mechanism(s) of the endocrine system by which the growth of embryos occur, still remains less well understood.

The hormonal mechanism of hatching that accompanies shedding of the exuvium is equivalent to that of the ecdysis recapitulating events in juvenile and adult stages. Both of these processes are likely driven largely by hormonal cascades and characterized by ecdysial behavior and a significant amount of water uptake [98,118]. During embryogenesis, eyestalk-originated neuropeptides, including crustacean hyperglycemic hormone (CHH) and molt-

inhibiting hormone (MIH), are expressed early at the eye-anlage stage, with levels of these hormones continuing to increase until completion of embryogenesis [98].

Ecdysteroids conjugated to VT and transported into oocytes play a critical role in the early embryogenesis of arthropods [91-96]. During embryogenesis, the utilization of VT often coincides with the release of ecdysteroids. Therefore, embryogenesis is staged with the size of the remaining yolk, together with the development of eye, appendage, and heart tissues [98-101]. The continuous utilization of VT during crustacean embryogenesis may reflect the companion levels of ecdysteroids [101,103,104]. However, changes in the levels of ecdysteroids during embryogenesis of decapod crustaceans [100,105] suggest that the newly formed Y-organs are active for the production of zygotic ecdysteroids during this period.

The functional importance of ecdysteroids involves the presence of ecdysteroids receptors (EcRs) and other ecdysteroid-responsive transcriptional proteins such as Broad complex (BR-C) and Broad-Complex-Tramtrack-Bric-a brac (BTB) domain protein (BTBDP) [70,138]. In *C. sapidus*, the presence of four different cDNAs of EcRs and RXRs suggests that there may be putative structural isoforms of these receptors [119]. BR-C, an early ecdysteroid-responsive factor, is involved in reproduction, embryogenesis, and molting of both insects and crustaceans such as *Drosophila melanogaster*, *Tribolium castaneum*, and *Penaeus monodon* [139-144]. The functional role of BTBDP has not yet been examined during the embryogenesis of any crustaceans.

We aim to profile changes in eyestalk neurohormones and ecdysteroids during embryogenesis. Herein, we report for the first time in crustaceans that expression levels of ecdysteroid-responsive factors (including *CasEcR*, *CasRXR*, and *CasBTBDP*) and *CasMIH* and *CasCHH* during the embryogenesis of the blue crab *C. sapidus,* correspond to the ecdysteroid profiles.

2.2 Materials and methods

2.2.1 Animal and sample collection

Study batches of adult females (*C. sapidus*) were obtained from a Chesapeake Bay watershed spawning ground by the annual winter dredge survey of the Virginia Institute of Marine Science (VIMS, Virginia) and screened for diseases [145,146]. Disease-free animals were maintained in a closed-recirculatory system holding at 30 ppt artificial seawater at 22°-23℃ (8L:16D), fed daily with chopped squid and shrimp, and monitored daily for the extrusion of eggs (= spawning). Ovarian samples were collected from adult females and staged according to the criteria as described [147]. Upon spawning, embryos were biopsied at every two days until they hatched. These embryos were examined for developmental stages under a dissecting microscope following the criteria described [98], collected, and stored at -80^{o}C until analysis.

2.2.2 Degenerate primers for cloning the full-length cDNA of *C. sapidus BTBDP*

Degenerate primers of *CasBTBDP* (Table 2-1) were generated based on conserved regions identified from the multiple sequence alignment (www.genome.jp/ClustalW) of the putative BR-C isolated in insects including *A. aegypti* (AY499537.1, AY499538.1, AY499539.1, AY499540.1), *B. mori* (NM_001111334.1, NM_001111333.1, NM_001043511.), and *D. melanogaster* (NM_001144672.3, NM_166897.2). The identified conserved amino acid (aa) sequences were reverse translated (http://www.bioinformatics.org/sms2/rev_trans.html) using *C. sapidus* codon usage (http://www.kazusa.or.jp/codon/).

2.2.3 PCR with degenerate primers of *CasBTBDP* with 5´and 3´ rapid amplification of cDNA ends (RACE)

The cDNAs for 5´and 3´ rapid amplification of cDNA ends (RACE) were generated with total RNA extracted from Y-organs of a juvenile female at intermolt stage as described [119]. The initial *BTBDP* sequence was obtained from a two-step PCR assay with degenerate primers [119,148-153]. Touch-down (TD) PCR was carried out with 3´RACE cDNA of Y-organ and primers of BTBDP-dF1 and universal primers (UPM) using Advantage Taq Polymerase (BD Biosciences) under the PCR conditions as described [119,150-153]. A nested PCR was performed as described [119] with two primers: BTBDP-dF2 and Nested Universal Primer (NUP).

Table 2-1 The list of primers for *CasBTBDP* cloning and expression analysis

Forward	5´ to 3´	Reverse	5´ to 3´
dF1	ATHACNWSYGCYTTYGARAA	dR1	TGRTGNACRTTVACYTCDCC
dF2	TTYGTNGAYGTSACYYTSGCYTG	dR2	ACYTCNCCRTGRTARATRAA
F2	CACTCAAGTGGAACAATCACAAGTCC	R1	CATCCTTCATGAAGACAATGGGGTG
F3	GTCGCCTCATCACGAACCCAACAGCC	R2 or qR	GTAGTCGCTGCATGTTGACAGCACG
qF	CAGGAGGTATTCATTGACGTCACAC		

"d" represents degenerate primers. 'q' primers are for qRT-PCR assays. Forward primers (F1, F2, and F3) were used for 3' RACE and reverse for *CasBTBDP* (R1, and R2) were used for 5´RACE.

A band was identified with the expected size of 700 bp on a 1.5% agarose gel. The remaining procedures for cloning, sequencing, and sequence analysis were described [149-153]. This initial sequence was used for generating the gene-specific primers (BTBDP-5R1, -5R2, -3F2 and -3F3 listed in Table 2-1) that were used for obtaining the full-length *CasBTBDP* using 5´ and 3´RACE, respectively.

2.2.4 Sequence analyses of *CasBTBDP*

The open reading frame (ORF) of the full-length cDNA sequence of *CasBTBDP* was identified with ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The Conserved Domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd) remarked the conserved domains. The sequence similarity of the deduced aa sequences between *CasBTBDP* and other arthropod BR-C was analyzed using ClustalW (http://www.ebi.ac.uk) and Boxshade server (http://www.ch.embnet.org/software/BOX_form.html). The 5´ and 3´ UTR sequences of *CasBTBDP* were further analyzed to examine whether

or not they contained putative translational regulatory elements using RegRNA (http://regrna.mbc.nctu.edu.tw/html/prediction.html) [154].

2.2.5 Total ecdysteroids during embryogenesis using ecdysteroid-radioimmunoassay (Ecd-RIA)

Staged ovaries and embryos (~30 mg) were homogenized in ice-cold DEPC-treated PBS and dissolved in 0.1 M borate buffer (pH 8.0). Our preliminary data showed that the same amounts of total ecdysteroids were measured in both the crude extract and the samples treated with esterase and protease (unpublished results). Therefore, the samples homogenized in the borate buffer were estimated for the levels of ecdysteroids using the same protocol as described [60]. The amounts of ecdysteroids were calculated using AssayZap program (BIOSOFT) and presented in pg/oocyte or pg/embryo.

2.2.6 Expression levels of ecdysteroid-responsive factors (*CasEcRs*, *CasRXRs,* and *CasBTBDP*) and neuropeptide hormones (*CasMIH* and *CasCHH*) using qRT-PCR assays

Embryos at various developmental stages (10-20 mg wet weight/ sample) were homogenized in 150 µl of ice-cold DEPC-treated PBS. Two thirds of each of these homogenized samples was used for the total RNA by following the procedures described [153]. After treating with DNase I (Fermentas), the total RNAs (1.5 µg) were subjected to cDNA synthesis using RevertAid Reverse Transcriptase (Fermentas) and finally diluted to 12.5 ng total RNA/µl. The sample cDNAs (25 ng of total RNA eqv.) of ovaries, embryos, and zoeae were

estimated in duplicate for levels of *CasBTBDP, CasMIH,* and *CasCHH* expression, using Fast SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems 7500 instrument. In particular in this study, the expression of *CasCHH* only in the eyestalk, not in the pericardial organ was measured [153]. For measuring total levels of *CasEcRs* and *CasRXRs,* the primers were designed from the region that was conserved and shared in all four isoforms. *Arginine kinase* (*AK)* was measured in the same sample cDNAs as a reference gene. The standards for qRT-PCR assays were generated similarly as described [149,150,152,153]. The expression levels are noted as copies/µg total RNA.

2.2.7 Immunolocalization of CasMIH and CasCHH in *C. sapidus* embryos

Whole-mount immunohistochemistry was carried out for the localization of CasCHH and CasMIH in the embryos at heart-beat and developing-eye stages by following the procedures as described [98]. Embryos were fixed in a *Drosophila* fixative overnight [150] and their embryonic envelopes were removed for antisera penetration. After washing with PTX containing 0.05% sodium azide, the embryos were incubated with specific anti-rabbit-CasMIH serum [23] or -CasCHH serum that recognized CHH present only in the eyestalk ganglia [149] at a final dilution of 1:2,000 for 3 days at room temperature. After washing as above, the embryos were incubated with an Alexa 488 conjugated goat anti-rabbit IgG (Life Technologies) as described [150]. The embryo samples that were obtained from at least 3-4 adult females were examined using a confocal microscope (Zeiss), with the pictures collected using Z projection (BioRad). The

staining intensity and area of CasCHH and CasMIH were determined and compared using ImageJ (http://imagej.nih.gov/ij).

2.2.8 Statistical analysis

All results represent mean ± 1SE (n), in which n is the number of replicates. The data were subjected to the normality test using the Shapiro-Wilk test (SigmaPlot) prior to all statistical tests SigmaPlot (version 12.3). The data that did not show a normal distribution were analyzed using nonparametric ANOVA (Kruskal-Wallis Test) or unpaired *t* test. In all cases, statistical significance was accepted at $P<0.05$ and noted with letters or asterisks (* or **) .

2.3. Results

2.3.1 Changes in the levels of ecdysteroids during ovarian and embryonic development

Significant changes in amounts of total ecdysteroids are observed during ovarian development and embryogenesis (Fig. 2-1). The stage-1 ovary shows undetectable amounts of total ecdysteroids, while the stage-2 ovary contains 0.3 ± 0.1 pg/oocyte (n=7), the similar amount as observed in the stage-3 ovary. The levels of ecdysteroids are positively co-related with ovarian development and significantly ($P<0.001$) increased from 0.3 ± 0.1 pg/oocyte (n=5) at stage 3 to 0.9 ± 0.1 pg/oocyte (n=6) at stage 4 (Fig. 2-1, inset).

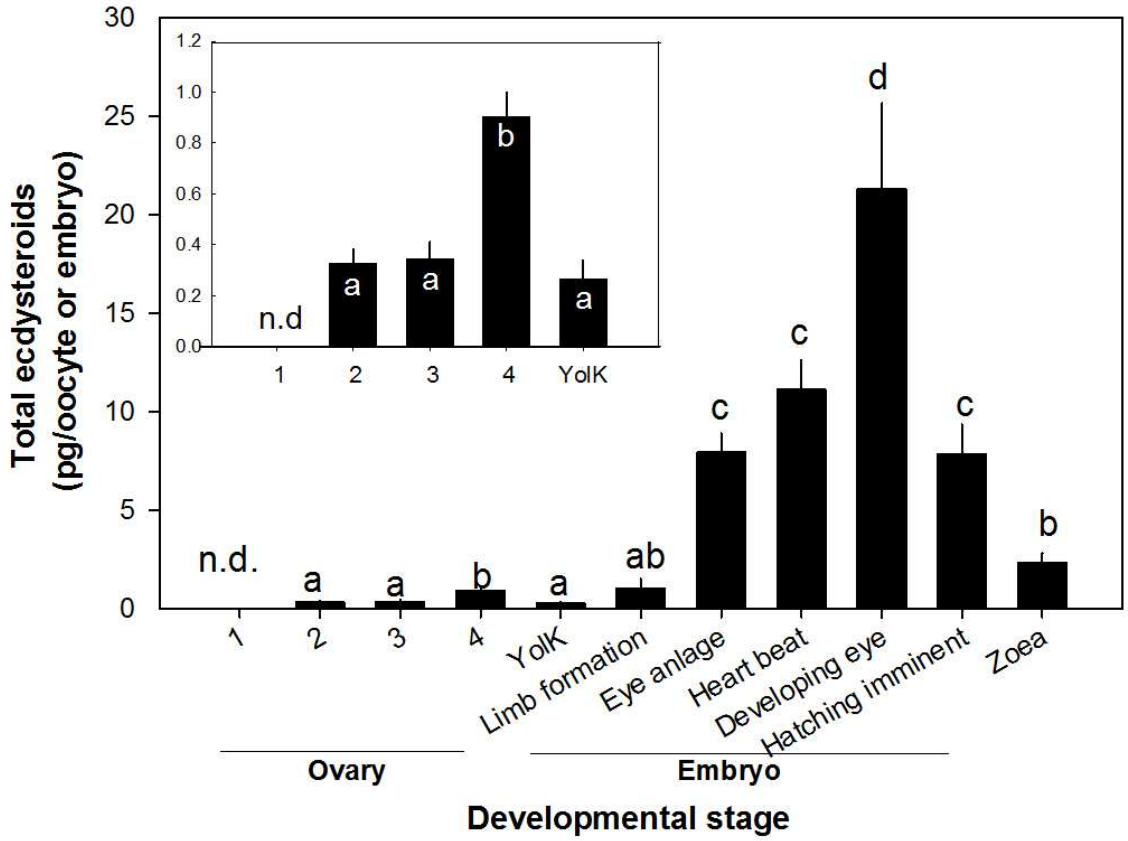


Figure 2-1. Levels of ecdysteroids by Ecd-RIA during ovarian (inset) and embryonic development The data are presented as mean ± SE pg/oocyte or embryo. All data were subjected to a normal distribution using the Shapiro-Wilk test (SigmaPlot). When the data did not show the normal distribution, a nonparametric test (Mann-Whitney Rank Sum test) was employed. Statistical significance was accepted at $P < 0.05$ and noted with letters at each developmental stage. n.d.= not detectable in

At the yolk stage, the ecdysteroid levels observed are statistically significant ($P<0.001$) reduced to 0.3 ± 0.1 pg/embryo (n=6) from 0.9 ± 0.1 pg/oocyte at ovarian stage 4. At the limb-formation stage, the ecdysteroid levels start to rise (1.0 ± 0.4 pg/embryo, n=12) and continue to increase significantly ($P<0.001$) to 7.9 ± 0.1 pg/embryo (n=9) at the eye-anlage stage and 11.1 ± 1.5 pg/embryo (n=6) at the heart-beat stage. Total ecdysteroid levels reach a peak at the developing-eye stage (21.3 ± 4.4 pg/embryo, n=9). With hatching-

imminent embryos, these levels then precipitously drop to 7.8 ± 1.5 pg/embryo (n=7), similar to the levels observed at the eye-anlage and heart-beat stages, and continue to decrease significantly (P<0.05) to 2.3 ± 0.5 pg/embryo (n=5) at zoeal stage (Fig. 2-1).

2.3.2 Expression levels of *CasEcRs* and *CasRXRs* during ovarian development and embryogenesis

a) Total *CasEcRs*

Ovary and embryo samples contain different levels of *CasEcRs* expression: ovary tissue shows 10-50 times higher levels than those of embryo tissue (Fig. 2-2). During the ovarian development from stage 1 to 4, there is no marked difference in the *CasEcRs* expression: 8.4 ± 0.5 x10^5 copies/µg total RNA (n=3) at stage 1; 10.9 ±1.8 x10^5 copies/µg total RNA (n=6) at stage 2; 7.1 ± 1.2 x10^5 copies/µg total RNA (n=7) at stage 3; and 6.2 ± 2.8 x 10^5 copies/µg total RNA (n=2) at stage 4.

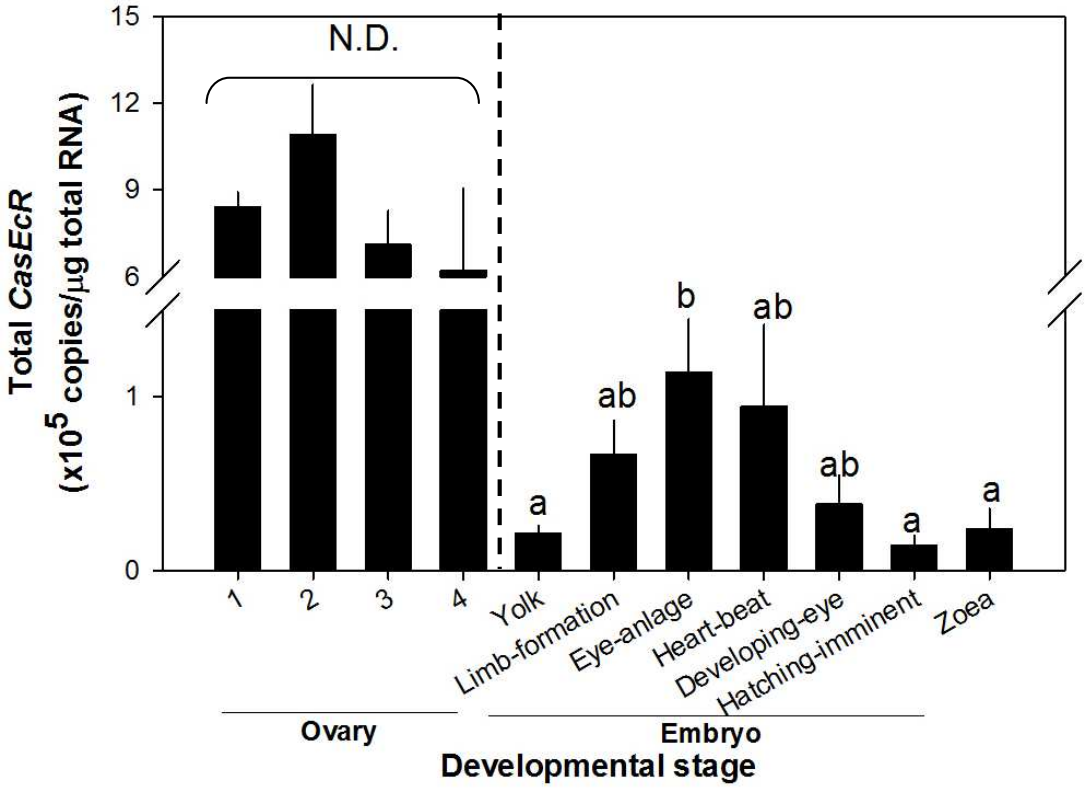


Figure 2-2. Levels of total *CasEcR* expression during ovarian and embryonic development of *C. sapidus* by qRT-PCR assays. Sample cDNAs (25 ng total RNA eqv.) of ovaries (n=4) and embryos (n= 5-10). The data are presented as mean ± SE copies/µg total RNA. All data were subjected to a normal distribution using the Shapiro-Wilk test (SigmaPlot). When the data did not show the normal distribution, a nonparametric test (Mann-Whitney Rank Sum test) was employed. Statistical significance was accepted at $P < 0.05$ and noted with letters at each developmental stage. N.D= no statistical difference

The levels of *CasEcRs* change during embryogenesis. At the yolk stage, levels of *CasEcRs* were measured at 2.1 ± 0.4 x10^4 copies/µg total RNA (n=11) and start to rise at the limb-formation stage (0.7 ± 0.2 x10^5 copies/µg total RNA, n=12). At the eye-anlage stage, the expression of *CasEcRs* is elevated to 1.1 ± 0.3 x10^5 copies/µg total RNA (n=9). However, these levels begin to decline to 0.9 ± 0.5 x10^5 copies/µg total RNA (n=7) at the heart-beat stage, continuing to

drop to 0.4 ± 0.2 x10^5 copies/µg total RNA (n=5) and 0.1 ± 0.1 x10^5 copies/µg total RNA (n=5) at both the developing-eye and hatching-imminent stage, respectively. In zoeae, the expression of *CasEcRs* is measured at 0.2 ± 0.1 x10^5 copies/µg total RNA (n=5).

b) Total *CasRXRs*

Similar to the expression of *CasEcRs*, the levels of *CasRXRs* in ovaries are ~10 times higher than the embryos (Fig. 2-3). The expression of *CasRXRs* during ovarian development remains constant, ranging from 6.3 ± 0.9 (x10^5 copies/µg total RNA, n=3) at stage 1; 6.9 ± 1.1 x10^5 copies/µg total RNA (n=6) at stage 2; 5.5 ± 0.7 x10^5 copies/µg total RNA (n=7) at stage 3; and 6.2 ± 1.1 x10^5 copies/µg total RNA (n=2) at stage 4.

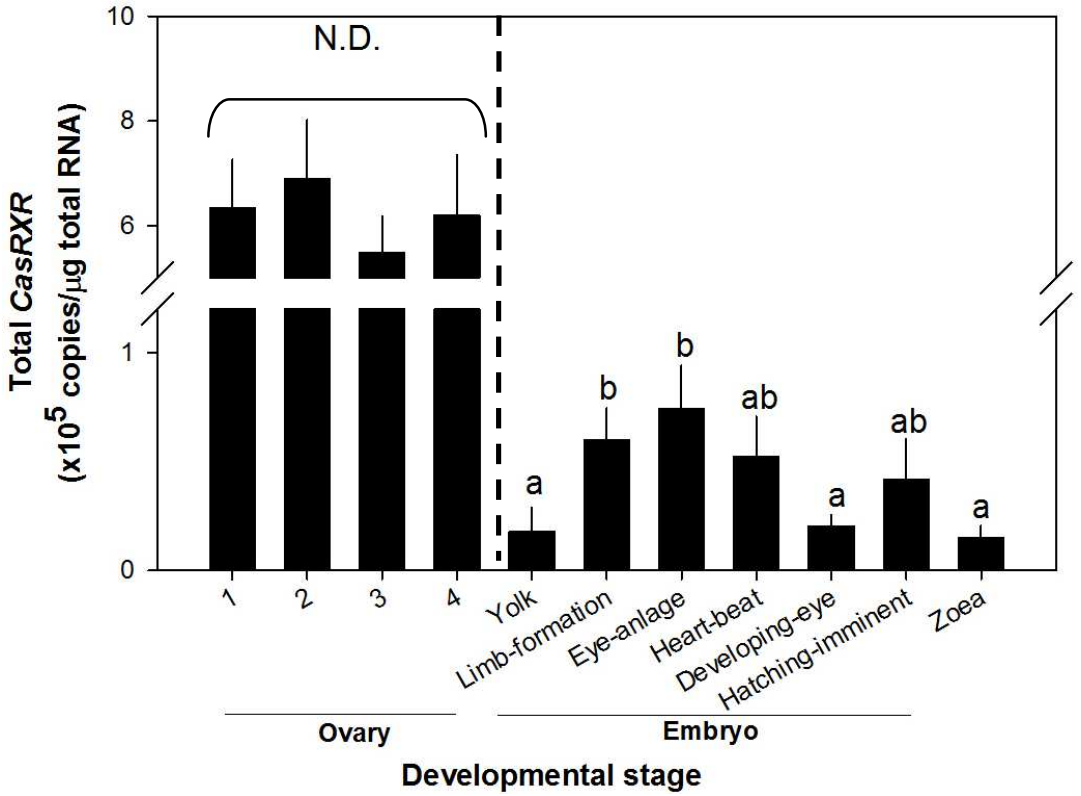


Figure 2-3. Levels of total *CasRXR* expression during ovarian and embryonic development of *C. sapidus* by qRT-PCR assays. Sample cDNAs (25 ng total RNA eqv.) of ovaries (n=4) and embryos (n= 5-10). The data are presented as mean ± SE copies/µg total RNA. All data were subjected to a normal distribution using the Shapiro-Wilk test (SigmaPlot). When the data did not show the normal distribution, a nonparametric test (Mann-Whitney Rank Sum test) was employed. Statistical significance was accepted at $P < 0.05$ and noted with letters at each developmental stage. N.D= no statistical difference

Similar to the expression pattern of *CasEcRs,* the levels of *CasRXRs* are as low as 0.2 ± 0.1 x10^5 copies/µg total RNA (n=8) at the yolk stage. At the limb-formation and eye-anlage stages, levels of *CasRXRs* are significantly (P<0.05) increased to 0.6 ± 0.1 x10^5 copies/µg total RNA (n=8) and peaked to 0.7 ± 0.1 x10^5 copies/µg total RNA (n=10), respectively. After the heart-beat stage, levels of *CasRXRs* are reduced from 0.5 ± 0.2 x 10^5 copies/µg total RNA (n=7), to 0.2 ±

0.1 x 10^5 copies/µg total RNA (n=5) at the developing-eye stage, and to 0.4 ± 0.2 x 10^5 copies/µg total RNA (n=5) at the hatching-imminent stage. Hatching zoeae contained levels of *CasRXRs* with 0.1 ± 0.1 x 10^5 copies/µg total RNA (n=4).

2.3.3 Full-length cDNA sequence of *BTB domain protein* of *C. sapidus* (*CasBTBDP*)

The full-length cDNA sequence (1,650 nt) of *CasBTBDP* (GenBank accession no. KC736588) includes 204 nt of 5´untranslated region (UTR), 948 nt of the open reading frame (ORF), and 495 nt of 3´UTR (Fig. 2-4).

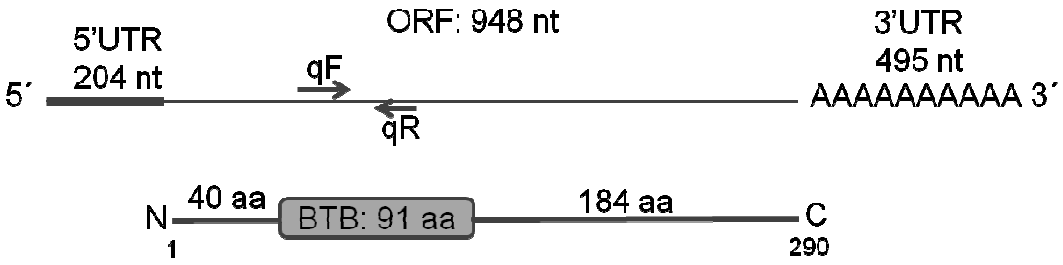


Figure 2-4. Schematic diagram of *BTB domain protein* cDNA isolated in *C. sapidus* (*CasBTBDP*: accession no. KC736588) and its putative regulatory elements analyzed using Regulatory RNA analysis program [155]. Post transcriptional control is predicted in 5´UTR.

The putative protein encoded in ORF contains BTB domain and is identified as a member of BTB superfamily. The putative *C. sapidus* BTB domain protein (CasBTBDP) shows the highest sequence identity (72%) with BR-C of a crustacean species *P. monodon,* and followed by those from insect species: *B. mori*, *Apis mellifera*, and *T. castaneum* with 51, 50, and 47%, respectively. The putative CasBTBDP does not contain a zinc-finger motif at the C-terminus (Figs.

2-4 and 2-5). As shown in Fig. 2-4, two putative translational, regulatory elements are predicted in the 5´UTR: internal ribosome entry site (IRES: C_4-G_{99} and G_{167}-G_{258}) and an upstream ORF (uORF: A_{54}-A_{146}).

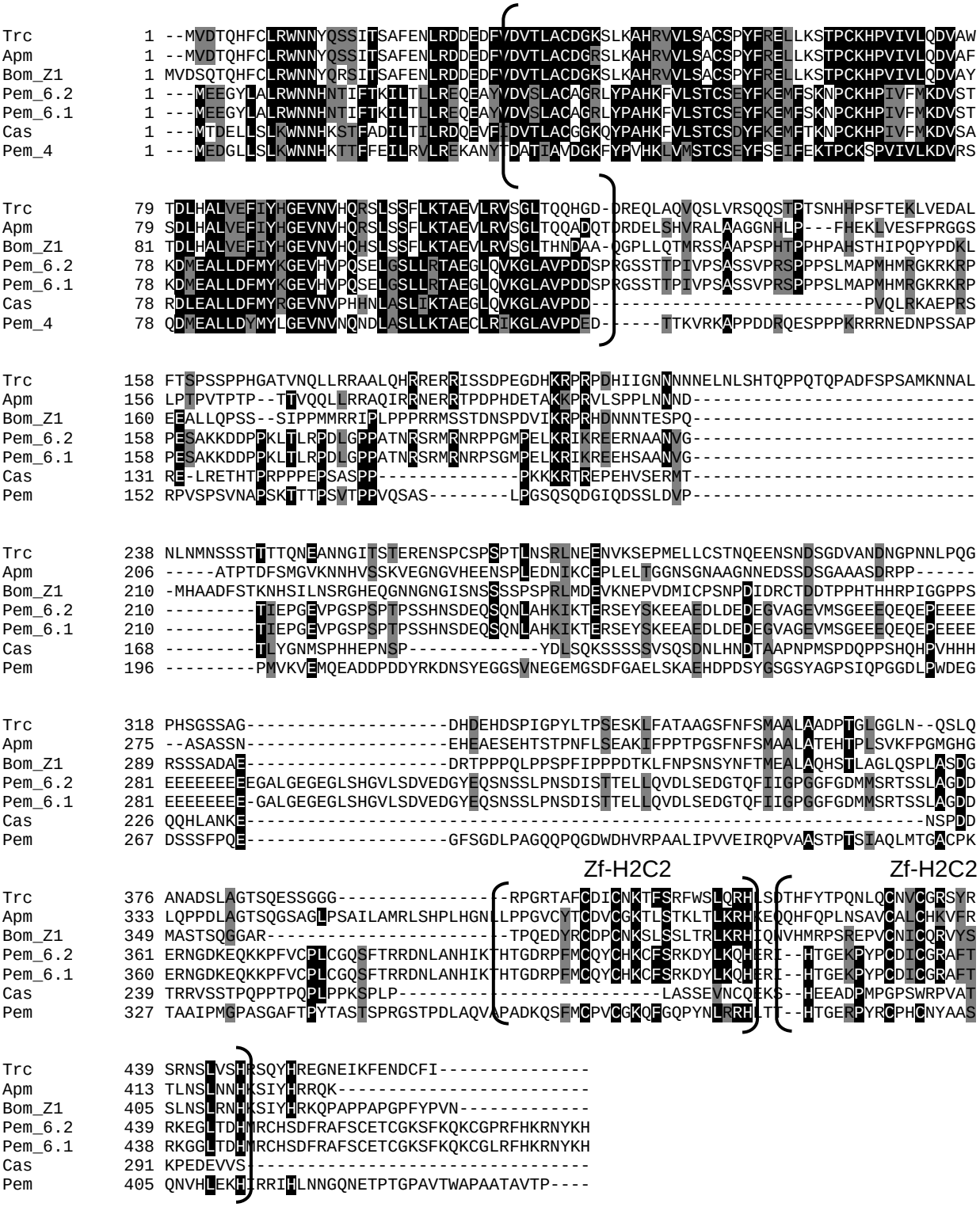


Figure 2-5. Multiple alignment of *BTBDP* in *C. sapidus* (*Cas*), and BTB domain protein from other arthropods: *A. mellifera* (*Apm*: NP_001035356.1), *B. mori* (*Bom _Z1*: NP_001104804.1), *P. monodon* (*PemBR-C*: *Pem_4*: JN638741.1; *Pem_6.1*: JN638739.1; *Pem_6.2*: JN638740.1), and *T. castanaum* (*Trc*: NP_001104734.1) using ClustalW (http://www.ebi.ac.uk) website and Boxshade server (http://www.ch.embnet.org/software/BOX_form.html). The gray and black shades

represent the highly conserved residues among the analyzed sequences. Putative zinc finger domain (Zf-H2C2) of PemBR-C isoforms and common BTB domain are bracketed.

2.3.4 Expression levels of *CasBTBDP* during ovarian development and embryogenesis

As shown in Fig. 2-6, during ovarian development, levels of *CasBTBDP* expression are high and remain constant ranging from $6.3 \pm 0.9 \times 10^5$ copies/µg total RNA (n=4) at stage 1; $11.7 \pm 5.3 \times 10^5$ copies/µg total RNA (n=4) at stage 2; $10.6 \pm 3.0 \times 10^5$ copies/µg total RNA (n=4) at stage 3; and to $11.2 \pm 2.7 \times 10^5$ copies/µg total RNA (n=2) at stage 4. During embryogenesis, these levels are low with $0.3 \pm 0.1 \times 10^5$ copies/µg total RNA at the yolk stage (n=7) but rise to 1.1 - 1.4×10^5 copies/µg total RNA (n=6-7) from the limb-formation to the hatching-imminent stages, without showing a statistically significant difference ($P<0.05$). Hatching zoeae show levels of *CasBTBDP* expression with $0.6 \pm 0.1 \times 10^5$ copies/µg total RNA.

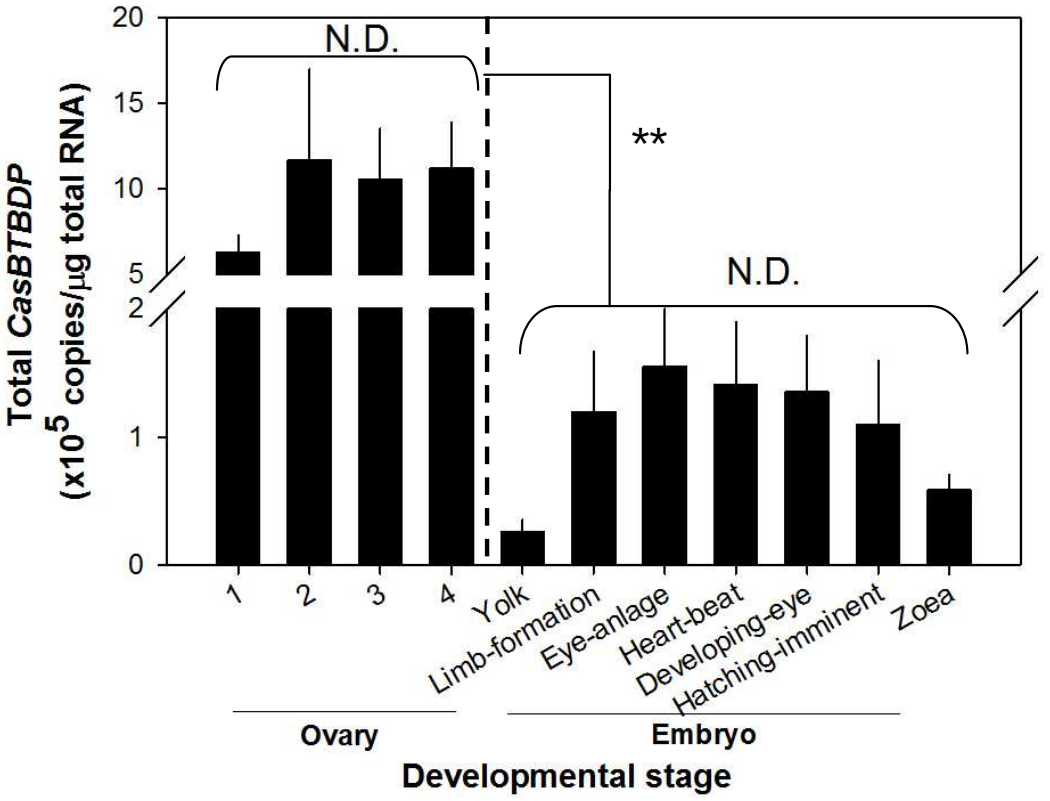


Figure 2-6. Expression levels of *CasBTBDP* by qRT-PCR assays during ovarian and embryo development of *C. sapidus*. Sample cDNAs (25 ng total RNA eqv.) of ovaries (n=4) and embryos (n= 5-10) at each particular stage. The data are presented as mean ± SE copies/µg total RNA. All data were subjected to a normal distribution using the Shapiro-Wilk test (SigmaPlot). When the data did not show the normal distribution, a nonparametric test (Mann-Whitney Rank Sum test) was employed. Statistical significance was accepted at $P < 0.05$ and noted with asterisk. N.D. = no statistical difference

2.3.5 *CasCHH* and *CasMIH* expression

The patterns of *CasCHH* and *CasMIH* expression in this study during embryogenesis are similar to the total ecdysteroids and ecdysteroid-responsive factors, especially in *CasCHH* (Fig. 2-7). At the early stages (yolk and limb-formation), *CasCHH* is detected with 610 ± 16 (n=8) and 710 ± 24 (n=10) copies/µg total RNA. These levels significantly increase to 1.4 ± 0.4 x10^4

copies/μg total RNA (n=10, P<0.05) at the eye-anlage stage and to 2.6 ± 0.4 x10^4 copies/μg total RNA (n=9, P<0.05) at the heart-beat stage. *CasCHH* levels peak at the developing-eye stage (5.0 ± 1.2 x10^4 copies/μg total RNA, n=6). The levels decline at the hatching-imminent stage: copies/μg total RNA 2.8 ± 0.6 (n=6) and remain low at the zoeal stage: 2.0 ± 0.4 x10^4 copies/μg total RNA (n=3).

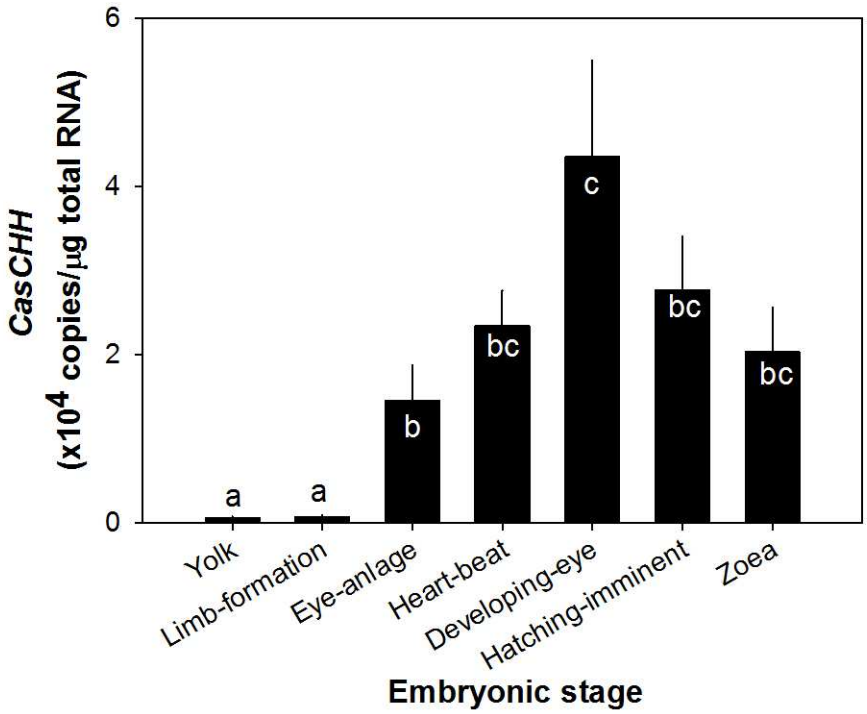


Figure 2-7. Expression levels of *CasCHH* during eyestalk organogenesis in *C. sapidus* embryos. Each cDNA sample containing 25 ng total RNA eqv. was assayed in duplicate. The expression levels are represented as copies/μg total RNA. The data are presented as mean ± SE (n). All data were subjected to a normality test using the Shapiro-Wilk test (SigmaPlot). When the data did not show the normal distribution, a nonparametric test (Mann-Whitney Rank Sum test) was employed. Statistical significance was accepted at P < 0.05 and noted with letters.

CasMIH levels do not fluctuate as we also found in *CasCHH* (Figs. 2-8). During yolk and eye-anlage stages, the levels of *CasMIH* are similar, ranging

between 4.3 - 7.3 x 10^3 copies/μg total RNA (n=5-6); however, the levels significantly increase to 2.3 ± 0.6 x10^4 copies/μg total RNA (n=6, P<0.05) at the heart-beat stage and 2.2 ± 0.6 x10^4 copies/μg total RNA (n=8) at the developing-eye stage, compared to those measured at earlier developmental stages. Then, the *CasMIH* levels drop to 1.2 ± 0.3 and 1.1 ± 0.3 x10^4 copies/μg total RNA (n=5-6) at the hatching-imminent and zoeal stages, respectively.

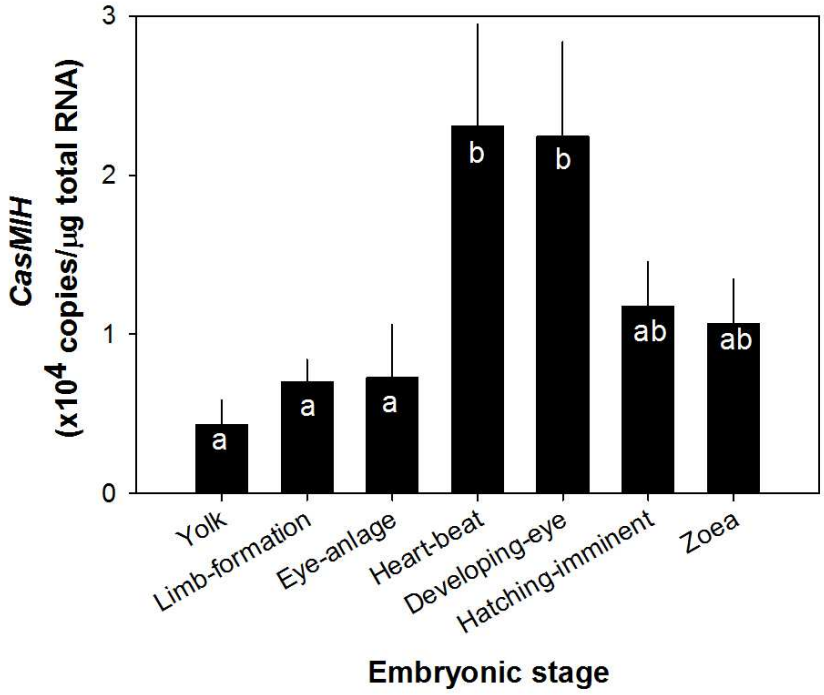
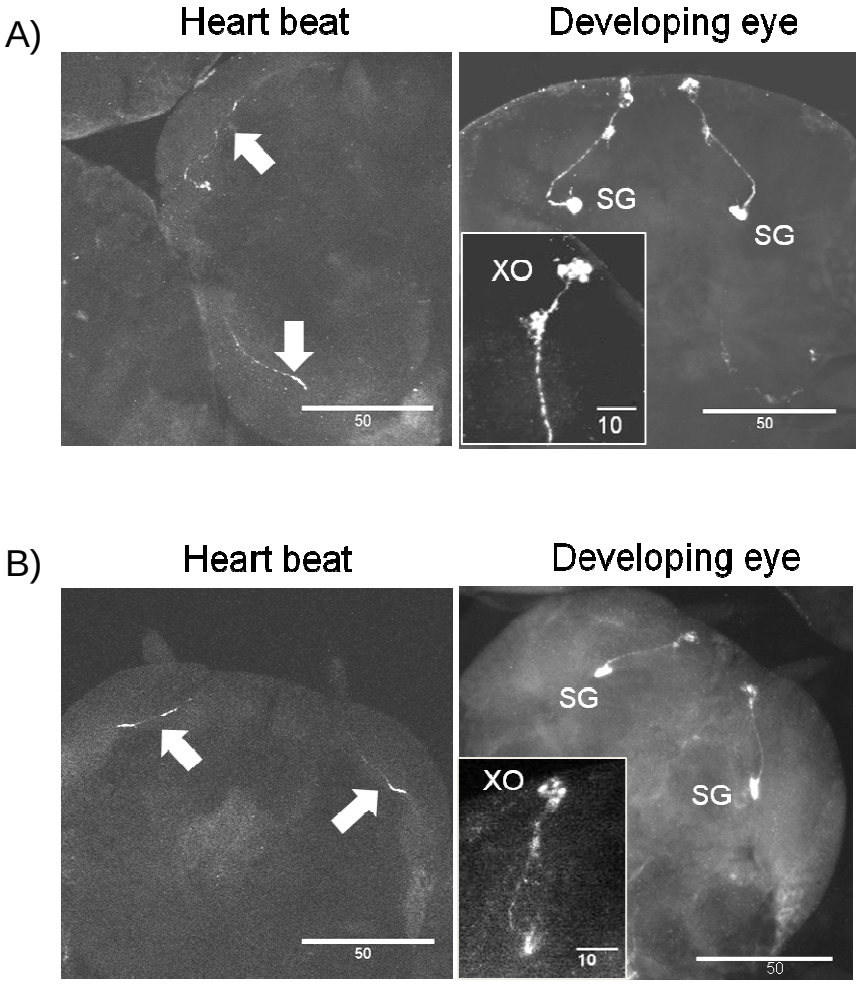


Figure 2-8. Expression levels of *CasMIH* during eyestalk organogenesis in *C. sapidus* embryos. Each cDNA sample containing 25 ng total RNA eqv. was assayed in duplicate. The expression levels are represented as copies/μg total RNA. The data are presented as mean ± SE (n). All data were subjected to a normality test using the Shapiro-Wilk test (SigmaPlot). When the data did not show the normal distribution, a nonparametric test (Mann-Whitney Rank Sum test) was employed. Statistical significance was accepted at $P < 0.05$ and noted with letters.

2.3.6 Localization of CasCHH and CasMIH during eyestalk formation

The presence of CasCHH and CasMIH is noted in embryos at the heart-beat stage of the *C. sapidus* embryos (arrow in Figs. 2-9A and B): staining intensity is stronger in the sinus gland than in the cells. Similar to the expression patterns as shown in Fig. 2-7 and 2-8, embryos at the developing-eye stage show greater staining intensity in the sinus gland than those at the heart-beat stage (Fig. 2-9C). However, there is no difference between the neuropeptides: CasCHH and CasMIH at the same embryonic stages.



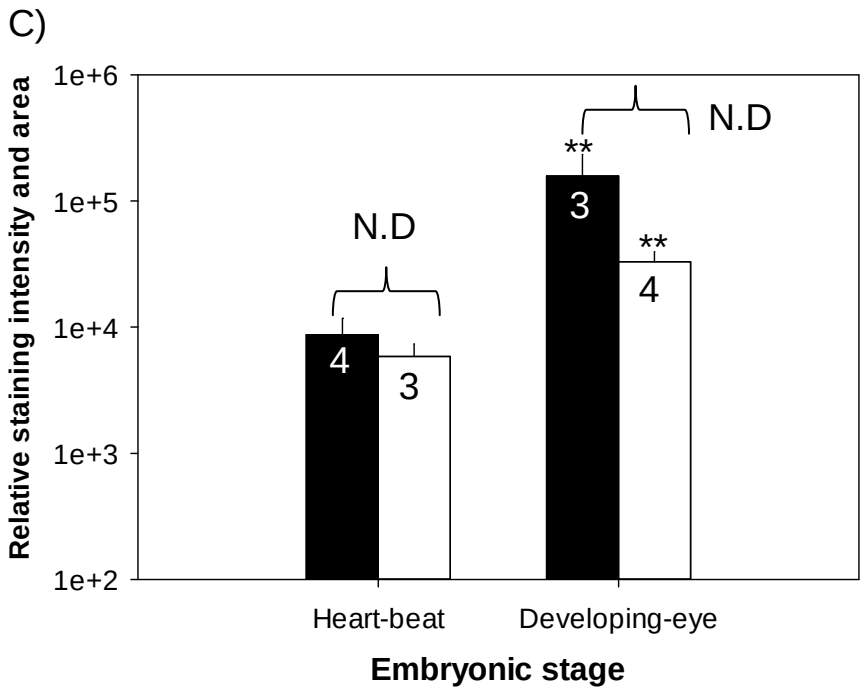


Figure 2-9. Immunolocalization of CasCHH (A) and CasMIH (B) in the embryos at heart-beat and developing-eye stages. The insets show the magnification of the X-organ (XO). C) The analysis of staining intensity and area of CasCHH and MIH of the sinus gland of embryos at heart-beat and developing-eye stages (ImageJ). Statistical significance (P<0.01) between the two stages was noted with ** for CasCHH (closed bar) and CasMIH (open bar). No difference was found between the neuropeptides: CasCHH and CasMIH at the same embryonic stages. The number of adult females are noted in the graph. Arrows indicate axonal tracts; SG= sinus gland. Scale bars are noted in µm.

2.4 Discussion

Our study shows the profiling of changes in the titers and transcriptional levels of major molt regulators: ecdysteroids and their receptor (*CasEcRs* and *CasRXRs*) and two neuropeptide hormones (*CasCHH* and *CasMIH*) during the ovarian development and embryogenesis of *C. sapidus.* In addition, we isolated the full-length cDNA sequence of *C. sapidus BTB domain protein* (*CasBTBDP*), as well as quantified for temporal changes along with these molt regulators.

The somatic growth of arthropods requires molting processes, which are coordinated by ecdysteroids and precisely-timed pulsatile release of neurohormones [35,38,60,135]. Ecdysteroids produced and secreted by the Y-organs in crustaceans or prothoracic glands in insects trigger these animals to enter and to prepare for the premolt as well as initiate embryogenesis. Prior to organogenesis of Y-organs or prothoracic glands, ecdysteroids are derived from the mother as conjugated to a large amount of vitellin (VT) [94,156,157]. From the phosphorylated-inactive forms conjugated to VT in the ovary, maternal ecdysteroids are being released via VT utilization at the initiation of embryogenesis [158].

The increase of ecdysteroid levels in the occytes is comparable to the VT contents that is reported in developing ovaries of *C. sapidus* [89]. Our current results are similar to the findings reported in several crustacean species including *C. maenas*, *M. rosenbergii, Orchestia gammarella* and *Lysmata seticaudata,* where the levels of ecdysteroids are parallele to those of vitellogenesis

[95,105,133,159-163]. As stated above, vitellogenin is suggested to carry ecdysteroids to the ovary of crustaceans. By contrast, some insect ovaries synthesize ecdysone [164,165], the function of which is known to stimulate vitellogenesis in fat body and follicle cells of the mosquito, *Aedes aegypti* [166,167]. The direct involvement of ecdysteroids in vitellogenesis itself has not yet been defined in decapod crustaceans. The maternal ecdysteroids that are accumulated in the oocytes during ovarian development of *C. sapidus* may trigger the initiation of embryogenesis, as in *D. melanogaster* [156].

Levels of ecdysteroids change during *C. sapidus* embryogenesis (Fig. 2-1), implying their functional importance in embryogenesis. The accumulation of ecdysteroids in the developing ovaries noticeably correlates with ovarian development, specifically with the amount of VT in the ovary [89], suggesting that CasVT serves as a carrier protein, similar to what is reported in insects such as *B. mori*, *Dacus cucurbitae* and *D. melanogaster* [94,156,157]. Hence, the maternal ecdysteroids released from VT through the utilization of CasVT may be responsible for the changes in ecdysteroid levels in early *C. sapidus* embryogenesis, particularly at the yolk stage, which is similar to what is reported in *B. mori* and *D. melanogaster* [94,156].

The levels of ecdysteroids in *C. sapidus* embryos are akin to those of other crustaceans and insects, ranging between 2-80 pg/embryo, which reflect the size of embryos [94,100,105,161,168-170]. Interestingly in this study, as the *C. sapidus* embryo begins to develop, ecdysteroid levels are reduced. This finding is similar to the earlier observation in the *B. mori* embryogenesis when the

ecdysteroid levels abruptly decline during cell differentiation [158]. The continuous utilization of VT during embryogenesis indicates the release and use of maternal ecdysteroids. Hence, this condition may result in the reduced levels of total ecdysteroids seen during early embryogenesis.

The overall patterns of the changes in embryonic ecdysteroids in later stages are similar to those seen in the juvenile molt [37,119]. Levels of ecdysteroids are low at early embryogenesis, then increased to peak, and returned to the low levels, before hatching of the embryos. The increase in ecdysteroid levels at the limb-formation stage of *C. sapidus* embryogenesis may reflect the organogenesis and activity of Y-organs. This notion is supported by which levels of cholesterol are gradually reduced during *C. sapidus* embryogenesis (Prof. R. Lee, personal communication). The presence of Y-organs that were observed at the limb-formation stage of *P. serratus* embryos coincides with the increase in the total ecdysteroids [100,137]. However, in *Machrobrachium rosenbergii*, increase in ecdysteroid levels occurs at the eye-anlage stage [105], indicating that the timing of organogenesis of Y-organs may somewhat differ in this species. Taken together, it is suggested that the increase in ecdysteroids during *C. sapidus* embryogenesis may be primarily contributed to *de novo* synthesis from the developing Y-organs.

Changes in ecdysteroid levels during ovarian development and embryogenesis may often forecast the differences in the amounts of EcRs and its responsive factors. However, ovaries contain levels of *CasEcR*, *CasRXR*, and *CasBTBDP* expression 10-50 times higher than embryos, contrary to the

ecdysteroid patterns. Such high expression levels of these genes in ovaries indicate that maternal *CasEcR*, *CasRXR*, and *CasBTBDP* may be immediately translated at early embryogenesis when transcription machinery may not be yet functional [171,172]. Subsequently, these results imply that the ecdysteroid receptor complex (CasEcR-CasRXR) and CasBTBDP may be involved in the early embryogenesis of *C. sapidus*.

Compared to the juvenile molt, the pattern changes of embryonic ecdysteroids, and the receptor complex (*CasEcR-CasRXR)* we observed are similar to those of the hemolymph and Y-organ, respectively [119]. In embryonic development, levels of both *CasEcR* and *CasRXR* are peaked earlier than that of the ecdysteroids. Up-regulation of *EcR* and *RXR* by ecdysteroids that occurs in prothoracic gland of *M. sexta* further modulates this gland's activity for ecdysteroid synthesis [10,173,174]. Therefore, we infer that ecdysteroids may be responsible for controlling the newly formed Y-organ in the embryos for ecdysteroidogenesis. Our data imply that ecdysteroids and their receptors in *C. sapidus* embryos may have similar functions in the juvenile molt.

BTBDPs, with BTB domain for a protein-protein interaction motif, share a function in protein degradation by ubiquination [175,176]. Particularly, in the embryos of *Caenorhabditis elegans* and *D. melanogaster*, ubiquitination through BTBDP is required for degradation of various cell cycle regulators such as CDC25 and cyclin D, and hedgehog morphogen [177]; Zhang, 2006 #494]. Thus, we infer that CasBTBDP may have a similar function in *C. sapidus* as in *C.*

elegans and *D. melanogaster*, because its expression levels are maintained high in oocytes and embryos.

During early developmental stages (yolk and limb-formation stages), transcriptional levels of *CasMIH* are higher than those of *CasCHH*. Our result is somewhat contradictory to earlier reports that *MIH* levels are much lower than *CHH* in embryo and adult *C. maenas* as well as in the eyestalk ganglia of juvenile and adult *C. sapidus* [23,44,178]. In *C. sapidus*, *CasMIH* expression is detected in the ovary and even prior to the eye anlage stage when *CasCHH* expression is also seen. In *C. maenas*, the expression of *CHH* and *MIH* is seen only at the eye-anlage stage but not in mature oocytes [98].

These observed differences in the timing of occurrence and levels of *MIH* suggest that the expression pattern of *MIH* may be species-specific, due to their different life histories and ecological conditions. In particular, *C. sapidus* inhabits relatively warmer water temperature than *C. maenas,* which affects the overall duration of embryogenesis, particularly initiation at the yolk stage. Therefore, we suggest that the regulatory mechanism of *MIH* expression during embryogenesis of *C. maenas* and *C. sapidus* may be slightly different. We are currently investigating the presence of ovarian *MIH* to see if its cDNA is identical to that found in eyestalk ganglia in juveniles and adults of *C. sapidus*.

The overall patterns we observed of *CasCHH* and *CasMIH* expression in the eyestalk ganglia mirror the changes of ecdysteroids. Interestingly, the changes in *CasCHH* and *CasMIH* expression during embryogenesis seem comparable to and similar to changes seen in the juveniles where the amounts of

these neuropeptide expressions are closely related to the measured ecdysteroid titers (unpublished data). With this new data and our findings of the current study, we infer that *CasCHH* and *CasMIH* transcription and their hormonal levels may be affected by the presence and amount of ecdysteroids. Our finding suggests that the developing eyestalk during embryogenesis seems to have activity similar to that in the juvenile crabs; therefore, these neuropeptide hormones may function as molt regulator during *C. sapidus* embryogenesis.

Molting results in the increase in the somatic size. Also noteworthy is that there is a difference in the size of decapod crustacean embryos at spawning and just prior to hatching. Similar to the juvenile and adult molt, the hatching process also relies on the uptake of iso-osmotic water that is assisted by the complete discharge of CHH produced by abdominal cells in *C. maenas* [118] and *C. sapidus* (unpublished observation). This increase in the size of embryos may be attributed to the first molt that is initiated by maternal ecdysteroids and occurs within the embryonic envelopes without shedding.

Zygotic ecdysteroids produced by developing Y-organs, as well as CHH and MIH generated in the developing-eyestalk ganglia, may interact and control both the hatching and molting processes. Although the exact timing of the first molt cycle driven by zygotic ecdysteroids is unknown, we suggest that the first molt cycle can be noted by the presence of CHH expression located in the abdominal segments of *C. sapidus* (unpublished data) and *C. maenas* embryos [98], equivalent to gut CHH endocrine cells in adults, occurring only ~ 5-7 days prior to hatching.

Together with the presence of ecdysteroids and related expression of ecdysteroid-responsive factors, we believe that the process of embryos hatching to zoeae accompanying with the cuticular shedding is the first molt of *C. sapidus*. Our data demonstrate the presence of ecdysteroids, their receptors, and both CHH and MIH during the ovarian development and embryogenesis in *C. sapidus*. An inter-relationship between ecdysteroids and the expression of neuropeptides, specifically MIH, still remains to be studied. Since genomic sequences of *Cancer pagurus,* and *Charybdis feriatus* MIH contain the binding sites for BR-C, E74A and RXR in the upstream promoter region, we are currently gathering the sequencing information of the upstream promoter elements of *C. sapidus* [45,47].

3. CHAPTER 3
Ecdysteroids induce the expression of molt-inhibiting hormone of the blue crab, *Callinectes sapidus*

Abstract

Arthropod molt is coordinated through the interplay between ecdysteroids and neuropeptide hormones. In crustaceans, changes in the activity of Y-organs during the molt cycle have been regulated by molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH). Little has been known on the mode of direct effects of ecdysteroids on the levels of MIH and CHH in the eyestalk ganglia during the molt cycle. This study focused on a putative feedback of ecdysteroids on the expression levels of *MIH* using *in vitro* incubation study with ecdysteroids and RNAi in the blue crab, *Callinectes sapidus*. Our results show a tissue-specific expression of *ecdysone receptor* (*EcR)* in which the major isoform in eyestalk ganglia is EcR1. The initial elevation of *MIH* expression at the early premolt stages is replicated by *in vitro* incubations of ecdysteroids that mimic the intrinsic conditions of D_0 stage, in terms of the concentration (75 ng/ml) and the composition (ponasterone A and 20-hydroxyecdysone at a 3:1 ratio). Additionally, multiple injections of *EcR1-dsRNA* reduce *MIH* expression by 67%, compared to the controls. Our data provide evidence on a putative feedback mechanism of hormonal regulation in molting, specifically how the molt cycle may be repeated during the life cycle of crustaceans. The elevated concentrations of ecdysteroids at premolt stage may positively act on the expression levels of *MIH* in the eyestalk ganglia. Subsequently, the increased MIH titers in the

hemolymph at postmolt may inhibit the synthesis and release of ecdysteroids by Y-organs, re-setting, succeeding and subsequently, recapitulating the molt cycle.

3.1 Introduction

Endocrine systems normally have feedback controls to regulate their balance in the organisms. In vertebrates, steroid hormones such as estrogens, glucocorticoids, and androgens regulate their production through negative feedback on neuroendocrine axes [31]. In insect neuropeptides, prothoracicotropic hormone (PTTH) that is produced from the brain and released by corpora cardiaca stimulates the prothoracic gland for ecdysteroidogenesis. PTTH is positively regulated by the ecdysteroids in *Manduca sexta* [24-27]. The ecdysteroids also generate short-loop feedbacks on prothoracic gland: stimulating at lower levels while inhibiting at the higher titers as reported in *M. sexta* and *Pieris brassicae* [10,30-32].

Life stages of arthropods continue through the recapitulated molting process. Molting is complex mechanisms that are hormonally regulated and involves in cell division, synthesis and deposition of new cuticle thereafter shedding of the old shell [36,179,180]. In crustaceans, members of the crustacean hyperglycemic hormone (CHH) family that are originated from the major endocrine tissue, eyestalk ganglia (X-organ-sinus gland) located within the eyestalk, are involved in the regulation of molting: 1) CHH and 2) molt-inhibiting hormone (MIH) [34-36,38,60]. MIH and CHH suppress the synthesis and release of ecdysteroids by Y-organs [178,181]. The hemolymph concentrations of CHH and MIH are closely associated with the levels of ecdysteroids during the molt cycle of the European green crab, *Carcinus maenas* [48].

Ecdysteroids, arthropods' molting hormones, are secreted by crustacean Y-organs that are equivalent to insect prothoracic glands. The levels of hemolymphatic ecdysteroids are positively related to molt stages in the following decapod crustaceans including *Cancer magister, C. maenas*, *Callinectes sapidus, Daphnia magna, Hyas araneus, Homarus americanus*, *Metopograpsus messor*, and *Procambarus clarkii* [3-7,9,59,182]. Y-organs secrete inactive forms of ecdysteroids: ecdysone, and 25-deoxyecdysone (25-dE) [43,49-51] that are subsequently hydroxylated at the peripheral tissues to active forms: 20-hydroxyecdysone (20-HE) and ponasterone A (PoA), respectively [52,57,183,184]. 20-HE is known to be the main active ecdysteroid in insects. However, the hemolymph of a given crustacean species carries more than one active form. In the premolt hemolymph of *C. sapidus* and *C. maenas*, PoA is measured as the major type of ecdysteroid and then 20-HE as the second [9,60,183].

Ecdysteroids have been known to affect the activity of Y-organs as well as that of the eyestalks [14,28]. 20-HE influences the Y-organ activity of *Orconectes limosus* positively or negatively depending on its concentrations [185], while a putative positive feedback of ecdysteroids on eyestalk neuropeptides is suggested in *Cancer antennarius* [39]. Interestingly, at premolt stages, the concentrations of ecdysteroids are changed, as well as the ratio between the two active forms. At the mid-premolt stage (D_2) of *C. sapidus*, the amounts of PoA to 20-HE are observed at a 3:1 ratio [60]. To date, it has not yet been studied in decapod crustaceans how such inherent changes in ecdysteroid

titers and the ratio between PoA and 20-HE may affect the transcription and translation of MIH/CHH in the eyestalk ganglia during the molt cycle.

In addition to the presence of two active ecdysteroids in *C. sapidus*, most of the internal tissues of this species express multiple *CasEcR*s [119]. Specifically, *CasEcR1* distinguishes from *CasEcR2*, as the putative ligand binding pocket (LBP) of the former contains more hydrophilic amino acids (aa) than those of the latter. Such difference in hydrophobicity may play an important role in a ligand specificity and affinity to a particular type of ecdysteroids. However, it has not yet been examined that the elevated levels of total ecdysteroids or a specific type of ecdysteroid may influence in the expression levels of *EcR* in relation to the molt regulation of decapod crustaceans.

In this study, we aimed to better understand the hormonal regulation of molting that recapitulates throughout the life cycle of arthropods using the hatchery-raised blue crab, *C. sapidus* with tractable life and molt stage as a model animal. Specifically, to define a putative feedback mechanism, we focused on the interplay between two endocrine systems involving CasMIH from eyestalk ganglia and ecdysteroids from Y-organs. We first examined to test if ecdysteroids influence the expression of *CasMIH* in eyestalk ganglia using an *in vitro* incubation. We then also determined the effect of multiple injections of *CasEcR-dsRNA* on *CasMIH* expression in the eyestalk ganglia.

3.2. Materials and methods

3.2.1 Animals and sample collections

Male and female young juvenile *C. sapidus* (20-40 mm carapace width, CW) obtained from blue crab hatchery, Aquaculture Research Center (ARC, Institute of Marine and Environmental Technology, Baltimore, MD, USA) were reared in the same conditions as stated [119] until they reached the size of 50-90 mm CW (crab stage 13-16 [114]). Molt stages of these animals were determined: intermolt (C_4), and premolt (D_0-D_{3-4}) stages as stated [114]. After the collection of hemolymph as described [119], the animals were placed on ice for 5-10 min prior to dissection. Eyestalk ganglia and Y-organs were microscopically collected and frozen immediately on dry-ice. The samples were stored at -80^{o}C until further use.

3.2.2 Temporal-spatial distributions of ecdysone receptor (*CasEcR*) isoforms in eyestalk ganglia and Y-organs at different molt stages

Tissues were collected from three animals at the following molt stages: intermolt: C_4, early premolt: D_o, mid-premolt: D_1-D_2, and late premolt: D_{3-4}. Tissues were homogenized with 150 µl ice-cold DEPC treated PBS and 100 µl of homogenized samples were subjected to total RNA extraction, as described [119,153]. After treating with DNase I (Fermentas), the total RNAs (1.5 µg) were subjected to the 1st stranded cDNA synthesis using RevertAid Reverse Transcriptase (Fermentas). The cDNA samples were diluted to the final concentration at 12.5 ng total RNA/µl equivalent. Each sample cDNA (12.5 ng

total RNA equivalent) was examined using an end-point RT-PCR assay with GoTaq® Green Master Mix (Fermentas) and isoform-specific primers at the following PCR condition: initial denaturation at 94℃ for 3 min; 30 cycles at 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 30 s; and a f inal extension at 72℃ for 7 min (Table.3-1). The PCR products were run on a 1.5 % agarose gel and were documented using a Kodak gel-documentation system. The Results shown in Figs. 3-6A and B were the representative pictures of three independent assays.

3.2.3 Effects of *in vitro* incubations of ecdysteroids on *CasMIH* expression in eyestalk ganglia

The animals (80-90 mm CW) at intermolt were ice-chilled; the eyestalks were dissected under a dissection microscope; and, the retinas were removed carefully and rinsed with ice cold DEPC-treated crustacean saline (pH 7.4). The eyestalks were pre-incubated in DEPC-treated crustacean saline, 0.05% M199 media (incubating media) for 30 min at room temperature for pre-conditioning to an experimental condition. After being rinsed with the incubating media again, the eyestalks were then incubated with the incubating medium containing PoA and 20- 20-HE at a 3:1 ratio. These concentrations (75, 150, and 250 ng/ml) of ecdysteroids for the incubation studies were chosen, based on the levels of intrinsic circulating ecdysteroids at D_o, D_1, and D_2 stages, as reported in *C. sapidus* [60,119]. The eyestalk ganglia were incubated on a shaker (30 rpm) for 3 hrs at 23-25^{o}C. The incubated eyestalk ganglia were rinsed twice with DEPC-

treated crustacean saline, blotted on a Kimwipe to remove the saline before being immediately frozen on dry-ice, and kept at -80^{o}C until further analyses.

3.2.4 Total ecdysteroids in tissues using ecdysteroid-radioimmunoassay (Ecd-RIA)

Eyestalk ganglia and Y-organs at various molt stages were homogenized in 100 µl ice-cold DEPC-treated PBS and dissolved in 0.1 M borate buffer (pH 8.0). The homogenized samples were assayed similarly as described [60]. The concentration of ecdysteroids was calculated using AssayZap program (BIOSOFT) and presented in pg/tissue. Protein contents in eyestalk ganglia, Y-organs, and hepatopancreas were estimated using a colorimetric assay, modified Lowry method (Pierce 660).

3.2.5 MIH titers in hemolymph using RIA*

A) *Iodination*

Native CasMIH was isolated and quantified as described (Chung and Webster, 2003). 300 pmol of CasMIH was iodinated by following the procedure as described [118,178]. ^{125}ICasMIH was separated on a PD-10 column (GE Healthcare) and diluted in glycerol (final 50%). Then it was stored at 4^{o}C and used within one or two months after iodination. The specific activity of ^{125}ICasMIH ranged from 300 to 500Ci/mmol.

***** MIH-RIA was performed and analyzed by Dr.Sook Chung

B) *Hemolymph sample preparation*

Hemolymphs were sampled from the animals at various molt stages. Hemolymph was directly withdrawn into a marine anticoagulant [186] at a ratio of 1:1 (v/v) via the arthrodial membrane between a chela and the first walking leg. These samples were extracted in isopropanol (final 60%) and centrifuged as described [150]. The supernatants were recovered in Minisorb tubes (Nunc), dried by vacuum centrifugation (Jouan), and assayed in duplicate. Anti-CasMIH was used at a 1:20,000 final concentration as before [23]. Standards of CasMIH RIAs ranged from1.2 $X10^{-12}$ and 2.5 $X\ 10^{-11}$M with ED_{50} values of $5.7 \pm 0.1 \times 10^{-10}$M (N = 3) and the detection limit of 1.2×10^{-12}M. The RIA data were analyzed as above.

3.2.6 Knockdown effects on *CasMIH* expression in eyestalk and molt interval using double-stranded RNA (*dsRNA*)

CasMIH-, CasEcR1-, and *CasRXR-dsRNAs* were generated using TranscriptAid T7 High Yield Transcription kit (Fermentas) as described [187]. Prior to the experiments, the hemolymph (100 µl) was withdrawn from the all experimental crabs (60-70 mm CW), mixed in a marine anticoagulant at a 1:1 ratio [186] and kept at -20^{o}C until further assayed for ecdysteroid levels. The knock-down effect with dsRNAs was determined in two ways: 1) the effect within the molt cycle by injecting 5-10 times over 20 days and 2) the effect within and after the molt by injecting ~30 times over 60 days. In both cases, animals received 15 µg CasMIH-dsRNA (in 100ul crustacean saline) or CasEcR1-dsRNAs three times a week as described [187]. The controls received the same

volume of crustacean saline. In order to monitor the delivery of dsRNAs into the animals, the crustacean saline was spiked with a 0.01% phenol red as described [187]. At the end of experiments, the animals were at intermolt stage [114] and hemolymph was collected. They were then anesthetized by chilling on ice for 5-10 min and the tissue samples were collected as stated above.

3.2.7 Expression levels of *ecdysone receptor* (*EcR*) and *molt-inhibiting hormone* (*MIH*) using qRT-PCR assays

The eyestalk ganglia and Y-organs at different molt stages, the eyestalk ganglia from incubation and *dsRNA* injection experiments were initially homogenized in 150 µl of ice-cold DEPC-treated PBS. Total RNA extraction and cDNA synthesis were carried out as described above. The cDNA samples were diluted to the final concentration at 12.5 ng total RNA/µl equivalent. The eyestalk cDNA samples (25 ng of total RNA equivalent) were assayed in duplicate to estimate the levels of *CasMIH* and *CasEcR1* using Fast SYBR Green Master Mix (Applied Biosystems) and qRT-PCR primers (listed in Table 3-1) on an Applied Biosystems 7500 instrument. The expression of *arginine kinase* (*AK)* was determined in the same sample cDNAs as a reference gene for endpoint PCR and qRT-PCR assays. The standards of all transcripts analyzed were generated similarly as described [149,150].

Table 3-1. List of primers that were used for qPCR assays and an end-point RT-PCR assay

Primers	Sequences (5'-3')
CasEcR	
qF-short (EcR1/EcR2)	CCAACAAGTCCAATGTCAGCCGGGG
qF-long (EcR1a/EcR2a)	CACTCCTAGCATCGTTCAGACT
qR1 (EcR1/EcR1a)	GGCATCCGAGTCGTCATCACTTATT
qR2 (EcR2/EcR2a)	CATGTCACTTGTATCTTCACCATCG
EcRt-qF	GCATTGTGTTTGGAAATACCTTGCC
EcRt-qR	GCCCTCAATGCATCGAGGTATATTT
CasAK	
qF	TTCCTCCACCCTGTCCAA
qR	GAAGCGGTCACCCTCCTTGA
CasMIH	
qF	TCCCTCGCTCACTCCAAATTTTC
qR	ATTGATAACTCTCGCCGCTGCTT

3.2.8 Statistical analysis

All results represent mean ± 1SE (n), in which n is the number of replicates. SigmaPlot (version 12.3) was used to evaluate the statistical significance of the results. The data were subjected to the normality test using the Shapiro-Wilk test (SigmaPlot) prior to all statistical tests. The data that did not show a normal distribution were analyzed using nonparametric ANOVA (Mann-Whitney Rank Sum test). In all cases, statistical significance was accepted at $P < 0.05$ and noted with letters or asterisks.

3.3. Results

3.3.1 Levels of molt-inhibiting hormone (*CasMIH*) expression in eyestalk ganglia and of CasMIH in hemolymph during the molt cycle

Levels of *CasMIH* expression during the molt cycle slightly increase (Fig. 3-1). At intermolt (C_4), *CasMIH* expression levels are measured at 2.4 ± 0.2 x 10^6 copies/µg total RNA (n=8). These levels are increased to 6.0 ± 1.5 x10^6 copies/µg total RNA (n=8) at D_0 stage (*P*=0.09). The *CasMIH* expression reaches 7.1 ± 1.4 x10^6 copies/µg total RNA (n=22) at D_1/D_2 stage and declines to 6.1 ± 1.2 x10^6 copies/µg total RNA (n=7) at D_{3-4} stages.

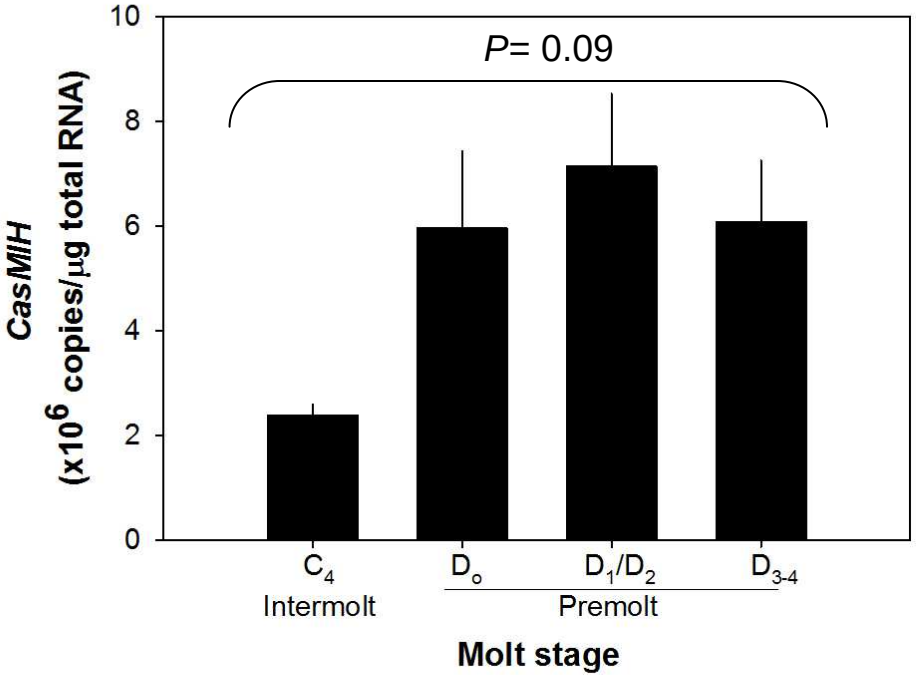


Figure 3-1. Expression levels of *CasMIH* in eyestalk ganglia during juvenile molt by qRT-PCR assay. Each cDNA sample containing 25 ng total RNA equivalent was assayed in duplicate. The expression levels are represented as copies/µg total RNA. The data are presented as mean ± SE (n). Statistical significance was accepted at $P < 0.05$. N.D.= no significant difference

The amounts of CasMIH stored in the sinus gland during the molt cycle are presented in Fig. 3-2. At the intermolt and early premolt (D_0) stages, CasMIH levels are similar and range from 53.9 ± 5.0 to 38.4 ± 8.3 pmol/sinus gland (n=10-12), respectively. At the mid-premolt stage (D_1/D_2), CasMIH levels increase significantly to 121.5 ± 13.6 pmol/sinus gland (n=21) and at the late premolt ($D_{3/4}$) stage, remain high at 105.3 ± 18.5 pmol/sinus gland (n=7), compared to those at preceding molt stages.

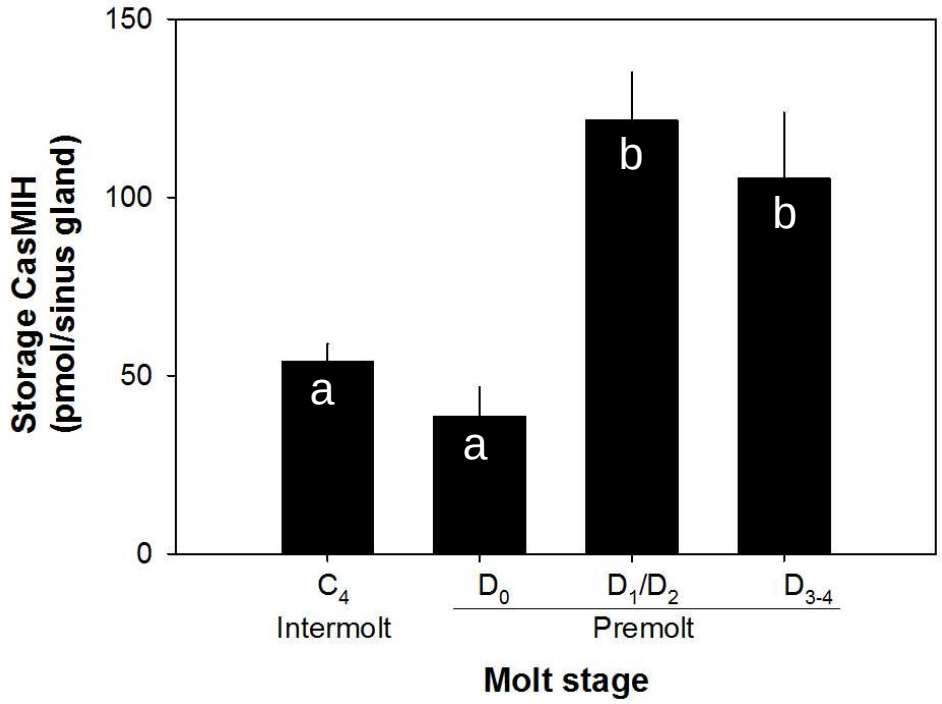


Figure 3-2. Storage levels of CasMIH in sinus gland (SG) by direct ELISA assay developed from Drexler [44,188].

The concentrations of CasMIH in hemolymphs also change during the molt cycle. CasMIH titers are similar ranging from 14.1 to 15.2 pM at intermolt (n=8) and mid-premolt D_2 stages (n=14). At early ecdysis ($E_{0-5\%}$), the CasMIH titers are significantly reduced to 5.8 ± 1.5 pM (n=11), compared to those

measured at intermolt and mid-premolt stages. At post molt (B) stage, the levels of CasMIH are significantly elevated to 36.7 ± 7.0 pM (n= 6) (Fig. 3-3).

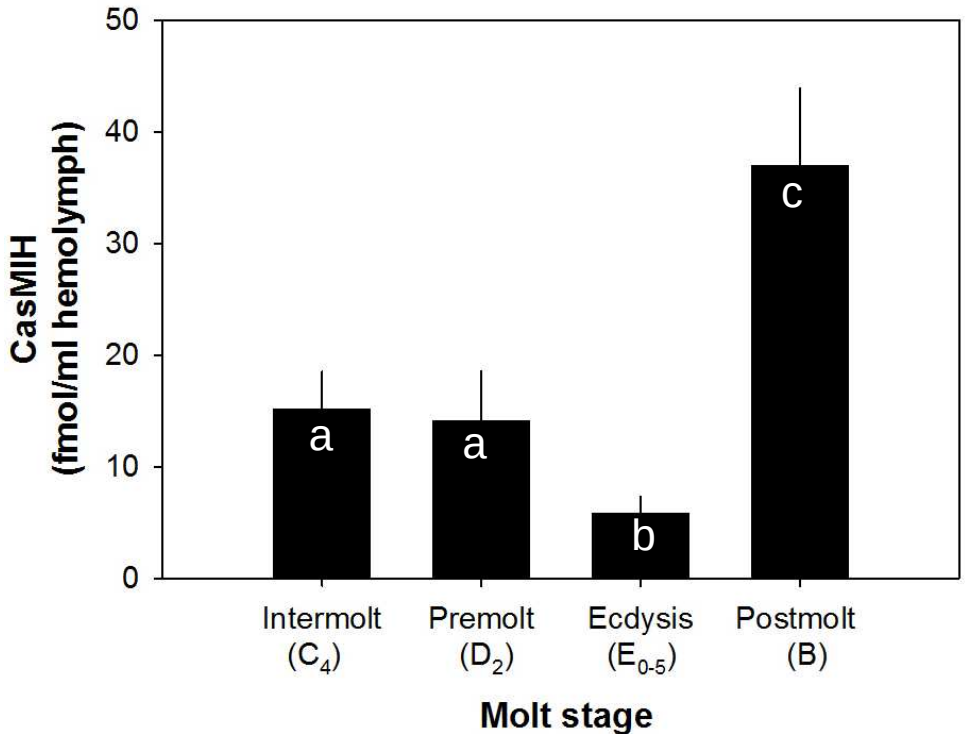


Figure 3-3. Circulating levels in hemolymph of CasMIH during juvenile molt by RIA assays. The data are presented as mean ± SE (n). Statistical significance was accepted at $P < 0.05$ and noted with letters.

3.3.2 Endogenous changes in ecdysteroid concentrations in eyestalk ganglia and Y-organs at different molt stage

The ecdysteroid contents in eyestalk ganglia and Y-organs vary depending on molt stage (Fig. 3-4). Both tissues have similar levels of ecdysteroids at intermolt stage: 2.3 ± 1.0 pg/µg eyestalk ganglia proteins (n=4) and 9.8 ± 3.4 pg/µg Y-organ proteins (n=6). Ecdysteroid levels increase significantly (P =0.05) by four folds to 9.8 ± 3.5 pg/µg eyestalk ganglia proteins (n=6) at early and mid-premolt (D_0/D_1) stages, compared to those estimated at intermolt. However, the ecdysteroid levels in Y-organs are increased only to 17.1 ± 3.0 pg/µg Y-organ proteins at early and mid-premolt (D_0/D_1) stages. These

values do not differ significantly. The hepatopancreas, as a reference tissue has similar levels of ecdysteroids at intermolt: 38.1 ± 5.7 pg/ µg hepatopancreas proteins (n=5) and early and mid-premolt (D_0/D_1) stages: 36.6 ± 4.3 pg/ µg hepatopancreas proteins (n=7).

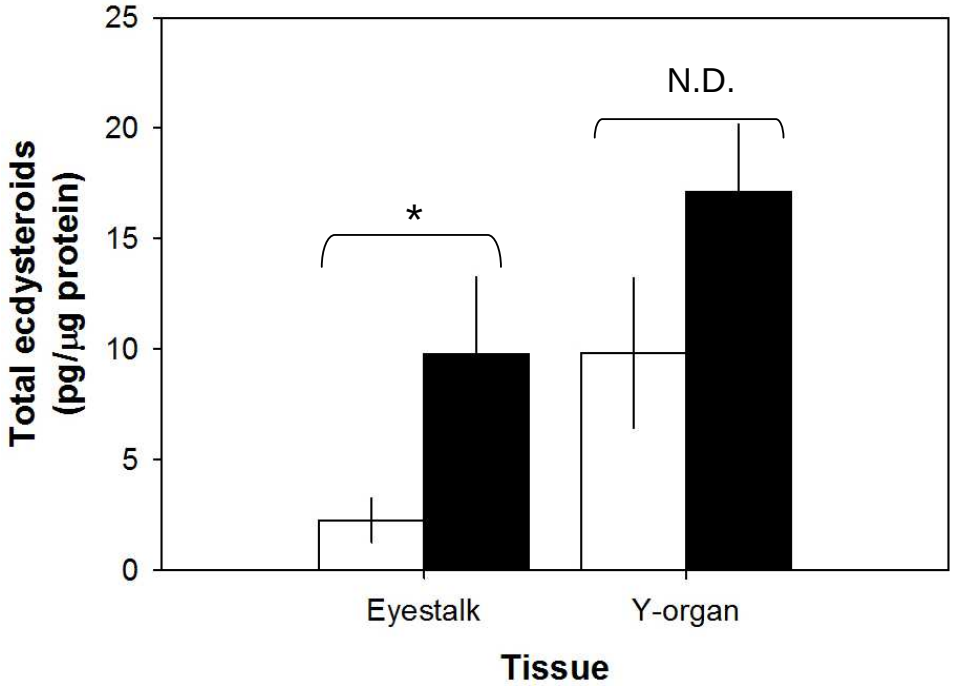


Figure 3-4. Levels of ecdysteroid presence in the tissues assayed by Ecd-RIA. Eyestalk and Y-organ from intermolt (white bar, n=4-6) and premolt (black bar, n=6) stages of juvenile *C. sapidus*. Protein estimation was performed by colorimetric assay modified Lowry method (Pierce 660). Statistical significance was accepted at $P < 0.05$ and noted with asterisk.

3.3.3 Effects of ecdysteroids on *CasMIH* expression

Effects of ecdysteroids on the levels of *CasMIH* expression in the eyestalk ganglia obtained from the animals at intermolt stage differ by the concentrations of PoA:20-HE at a 3:1 ratio that mimic the concentrations at various molt stages (D_0, D_1, and D_2) (Fig. 3-5). At 75 ng/ml concentration (D_0), levels of *CasMIH* expression are significantly up-regulated to 5.0 ± 1.2 x10^6 copies/µg total RNA (n=7), compared to those of the control (1.7 ± 0.6 x10^6 copies/µg total RNA, n= 8). Higher concentrations of ecdysteroids at 150 and 250 ng/ml, levels of

CasMIH expression tend to decrease to 3.9 ± 1.3 x10^6 copies/µg total RNA (n= 16) and 0.3 ± 0.1 x10^6 copies/µg total RNA (n= 5), respectively. However, these reduced *CasMIH* levels do not differ with the controls. Levels of *AK* expression measured in the same sample cDNAs remain constant, ranging from 5.1 ± 0.6 x10^6 copies/µg total RNA (n= 6) with the controls; 4.5 ± 0.5 x10^6 copies/µg total RNA (n= 8) at 75 ng/ml; 3.9 ± 0.6 x10^6 copies/µg total RNA (n= 8) at 150 ng/ml; and 5.7 ± 1.7 x10^6 copies/µg total RNA (n= 8).

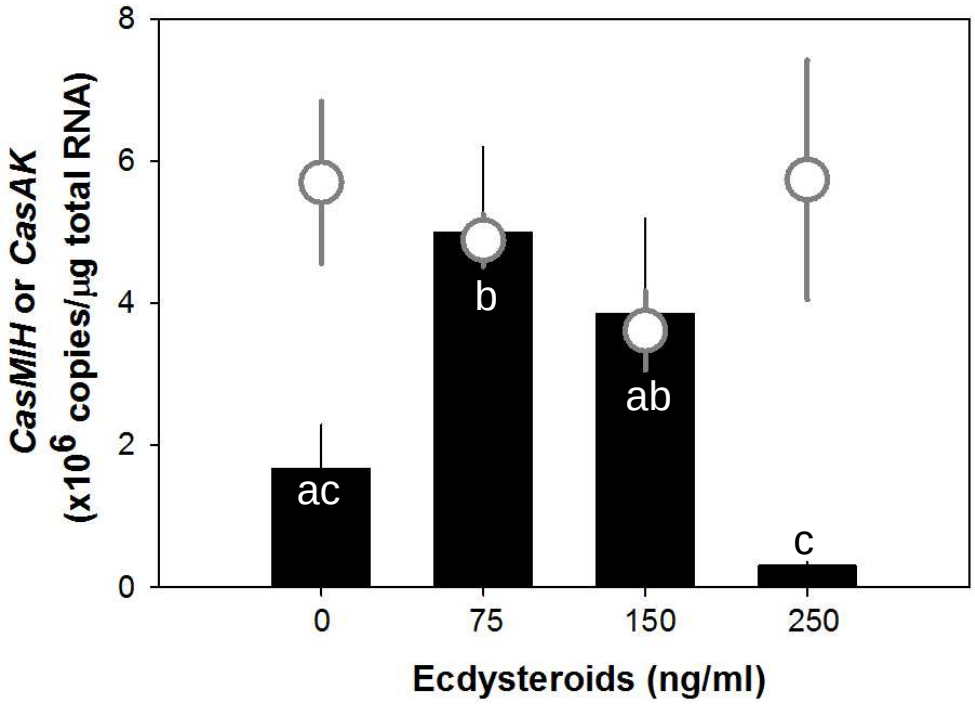


Figure 3-5. Expression levels of *CasMIH* in eyestalk ganglia after incubation with different concentrations of active ecdysteroids by qRT-PCR assay. *CasMIH* expression in different ecdysteroid concentrations that are composed of 3:1 ratio of PoA: 20-HE. The ecdysteroid concentrations of 75, 150, and 250 ng/ml mimic the D_o, D_1, and D_2 stages, respectively. *C. sapidus Arginine kinase* (*CasAK*) expression (Gray dot) was quantified from the same cDNA samples. Each cDNA sample containing 25 ng total RNA equivalent was assayed in duplicate. The expression levels are represented as copies/µg total RNA. Statistical significance was accepted at $P < 0.05$ and noted with letters.

3.3.4 Temporal-spatial distributions of ecdysone receptor (*CasEcR*) isoforms in eyestalk ganglia and Y-organs during the molt cycle

Four forms of *CasEcR*s are differentially expressed in eyestalk ganglia and Y-organs as well as at different molt stages. Overall, the expression of *CasEcRs* is higher in Y-organs than in the eyestalk ganglia, while the *AK* expression in these tissues is constant (Figs. 3-6A and B). In the eyestalk ganglia, *CasEcR1* serves as the major form, while both *CasEcR1* and *CasEcR2* seem to be equally distributed in the Y-organs (Figs. 3-6A and B, respectively).

During the molt cycle, the patterns of *CasEcRs* expression are also different in eyestalk ganglia and Y-organs. In eyestalk ganglia, the *CasEcR1* and *CasEcR2* tend to be gradually increased at premolt stages (D_0-D_{3-4}). However, both *CasEcR1* and *CasEcR2* in the Y-organs seem to be constitutively expressed throughout the molt cycle. *CasEcR1a* in Y-organs shows a molt-stage dependent expression with greater expression at D_1 and D_2 stages than at the other stages.

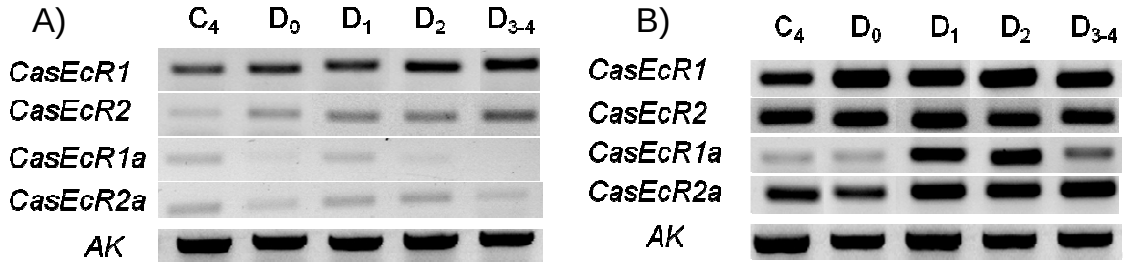


Figure 3-6. Spatial and temporal distributions of four *CasEcR* forms in eyestalk ganglia (A) and Y-organs (B) during the juvenile molt by end-point PCR assay (n=3). *CasAK* serves as the loading control and obtained from the same cDNA samples. Isoform specific primers are listed in Table 3-1.

3.3.5 Levels of ecdysone receptor (*CasEcR*) expression in eyestalk ganglia during the molt cycle

The expression pattern of *CasEcR1*, which is the major form in eyestalk ganglia, is similar with Fig. 3-6. Levels of *CasEcR1* at the intermolt and mid-premolt (D_1) stages remain constant and range from 2.2 - 2.7 x10^4 copies/µg total RNA (n=6-7). These levels are elevated to 7.0 ± 2.4 x10^4 copies/µg total RNA (n=8) at mid-premolt (D_2) stage and continue to significantly increase (P <0.01) to 1.2 ± 0.2 x10^5 copies/µg total RNA (n=7) at the late premolt (D_{3-4}) stage, compared to those measured at intermolt (Fig. 3-7).

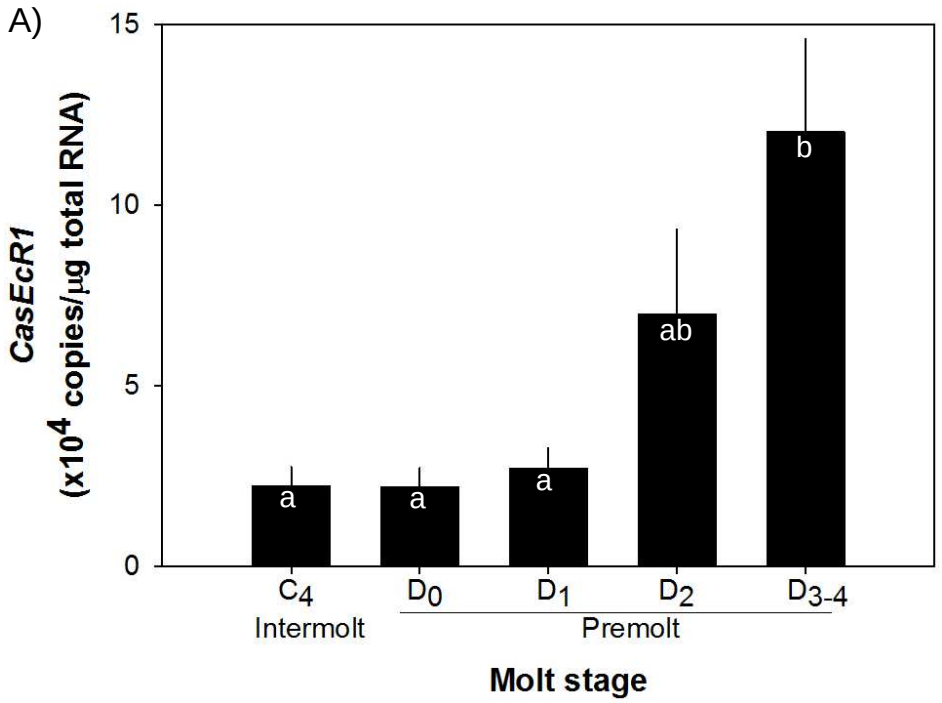


Figure 3-7. Expression levels of *CasEcR1* in eyestalk ganglia during juvenile molt by qRT-PCR assay using isoform specific primers (Table 3-1). Each cDNA sample containing 25 ng total RNA equivalent was assayed in duplicate. The expression levels are represented as copies/µg total RNA. The data are presented as mean ± SE (n). Statistical significance was accepted at $P < 0.05$ and noted with letters.

3.3.6 Effects of *CasMIH- and CasEcR1-dsRNA* on *CasMIH* expression in the eyestalk ganglia and the molt interval

A preliminary time-course study with multiple injections of *CasMIH-dsRNA* significantly reduced levels of *CasMIH* expression (Suppl. Fig. S3-1). *CasMIH-dsRNA* injections reduce *CasMIH* expression by ~50-time: 8.0 ± 3.0 $x10^4$ copies/µg total RNA (n= 9), compared to those that received saline (4.0 ± 1.3 $x10^5$ copies/µg total RNA, n= 6) and *CasRXR*-dsRNA (3.4 ± 1.0 $x10^5$ copies/µg total RNA, n= 5). Interestingly, *CasEcR1-dsRNA* significantly reduces the *CasMIH* levels (1.2 ± 0.4 $x10^5$ copies/µg total RNA (n= 9), compared to the controls. However, *CasRXR-dsRNA* injections have no effect on the levels of *CasMIH* expression (Fig. 3-8).

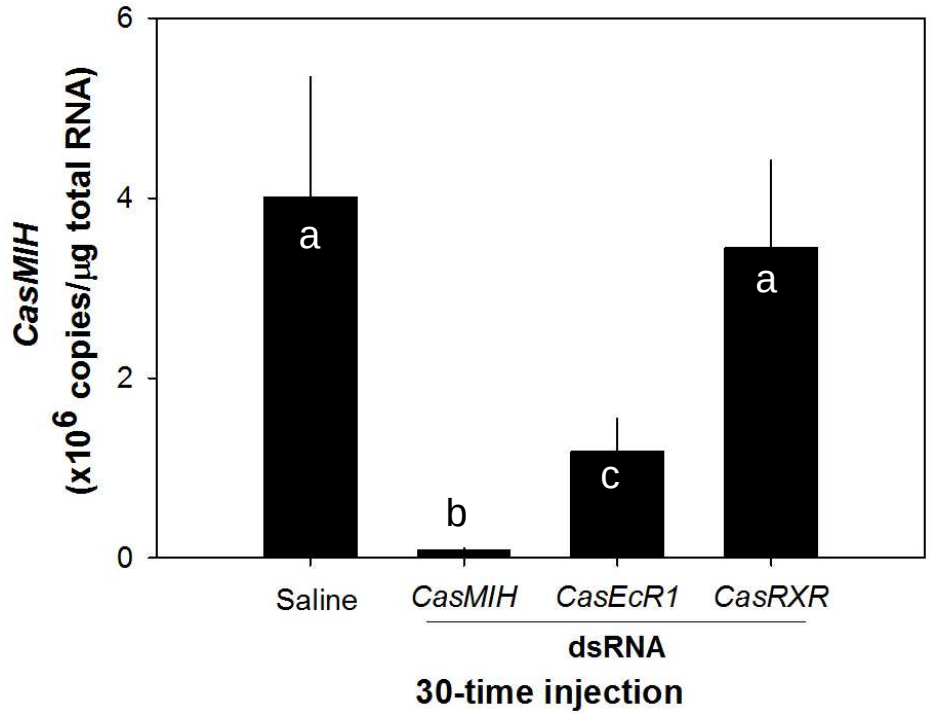


Figure 3-8. Expression levels of *CasMIH* in eyestalk ganglia after being administered with dsRNAs by qRT-PCR assay. Each cDNA sample containing 25 ng total RNA equivalent was assayed in duplicate. The expression levels are represented as copies/μg total RNA. The data are presented as mean ± SE (n). Statistical significance was accepted at $P < 0.05$ and noted with letters.

The molt interval of these experimental groups is shown in Fig. 3-9. The animals injected with *CasMIH-dsRNA* have the molt interval with 14.5 ± 3.0 days (n=4) shorter (P= 0.0737) than the other groups including the control with 20.0 ± 3.2 days (n=4), *CasEcR1-dsRNA* with 20.8 ± 1.2 days (n=5), and *CasRXR-dsRNA* with 23.0 ± 3.0 days (n=5).

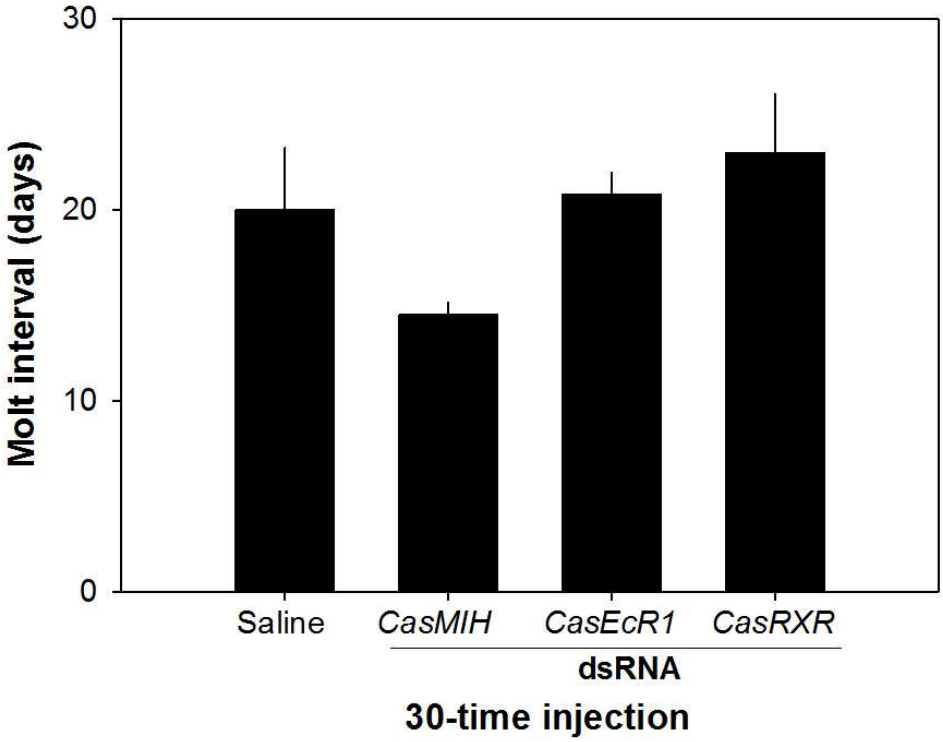


Figure 3-9. Molt interval after administered with dsRNAs. The data are presented as mean ± SE (n). All data were subjected to a normality test using the Shapiro-Wilk test (SigmaPlot). When the data did not show the normal distribution, a nonparametric test (Mann-Whitney Rank Sum test) was employed. Statistical significance was accepted at $P < 0.05$.

3.4 Discussion

Endocrine systems usually involve feedback mechanisms to maintain physiological homeostasis. The present study describes the interplays between the levels of ecdysteroids and *MIH* in the blue crab, *C. sapidus*. Our results demonstrate that eyestalk ganglia and Y-organs, although containing all four *CasEcR* expression, elevate the expression of a tissue-dependent major form during the molt cycle. Effect of ecdysteroids on the levels of *CasMIH* expression in the eyestalk ganglia has been investigated by *in vitro* incubation studies with different concentrations and ratios of ecdysteroids. In addition, the effects of ecdysteroids acting through EcR were examined by *in vivo* RNAi studies.

Molting is one of the most fundamental physiological processes in the animals belonging to the phylum Arthropoda and recapitulates throughout their life cycle. Crustacean molting and the molt cycle are coordinated and controlled by the levels of hormones produced by two endocrine systems: eyestalk ganglia and Y-organs. It has been suggested in a simple way that animals at the intermolt stage carry low levels of ecdysteroids due to high levels of MIH that suppresses the activity of Y-organs. The suppression of MIH on Y-organs is noted in a reduction of protein synthesis and cholesterol uptake in these organs, resulting in the reduction of ecdysteroid synthesis and secretion [39,181]. The MIH binding affinity to the Y-organ membranes prepared from different molt stage as well as the levels of its second messenger, cGMP do not change significantly [178]. On the other hand, the maximal number of binding sites increase significantly at post molt (B) stage [178]. These data indicate that the

inhibitory effects of MIH on the Y-organ may not result from the binding capacity or affinity of receptors. Interestingly, it is reported at the premolt stage that the phosphodiesterase (PDE) in Y-organs increase and affect the levels of cAMP and cGMP in *P. clarkii*, thus alleviating the MIH/CHH actions [20]. However, the exact mechanism by which MIH regulates Y-organ activity through the molt cycle, remain still unknown.

The levels of *CasMIH* expression in the eyestalk ganglia vary and increase at early premolt stage. This result is rather different from those reported in *C. maenas* in which the expression levels remain constant [178]. In addition, the levels of CasMIH in the hemolymph and sinus gland appear to be higher at premolt than those of *C. maenas* [48] and CHH in *C. sapidus* [153]. Our results indicate that the transcription of *CasMIH* may be under the regulation of ecdysteroids as the upstream promoter region of *CasMIH* gene may contain EcR binding site. Indeed, it is noted that the sequence of genomic amplification of *CasMIH* upstream promotor region (GenBank accession no KJ813010, Suppl Fig. S3-2) includes the binding site of RXR, and broad complex , similar as reported in *C. pagurus* [45] and *Charybdis feriatus* [47].

If *CasMIH* expression is under the control of ecdysteroids, the eyestalk ganglia should respond to ecdysteroids in the hemolymph and show the increase in its binding capacity to ecdysteroids at premolt stage, compared to the intermolt. To answer this question, the ecdysteroid contents in the eyestalk ganglia and Y-organs that were obtained from the animals at different molt stages were measured. The results show that there are different levels of

ecdysteroid concentrations between these tissues. The Y-organs contain higher concentrations of ecdysteroids than the eyestalk ganglia at both molt stages. However, the eyestalk ganglia at premolt seem to be able to concentrate or accumulate ecdysteroids by increasing four folds, whereas Y-organs show only ~1.7-fold increase. This indicates that the eyestalk ganglia are more sensitive to the hemolymphatic ecdysteroids than the Y-organs and may elevate their binding capacity to ecdysteroids. The exact type of ecdysteroids in the tissues has not been characterized. However, we infer that PoA and/or 20-HE may be the ones present in these tissues.

Levels of hemolymphatic ecdysteroids change during the molt cycle, along with increase in the total concentrations as well as a particular active ecdysteroid [60]. The intrinsic concentrations of ecdysteroids mimicking the levels of early premolt stage with PoA:20-HE at a 3:1 ratio increase *CasMIH* expression in the eyestalk ganglia that were obtained from the animals at intermolt stage. Our results are pertinent to an earlier finding in *C. antennarius* that ecdysteroids stimulate neuropeptide hormone production [28]. Interestingly, ecdysteroid concentration similar to that of early premolt stage is able to stimulate the *CasMIH* expression, but not with the highest concentrations (250ng/ml). This implicates that there may be difference in a molt-dependent binding capacity of the eyestalk ganglia to ecdysteroids.

At premolt, the increase in the total concentration of ecdysteroids is due to the elevated levels of PoA, yielding the ratio at 3:1 between PoA and 20-HE. Such changes in total levels and PoA may indicate the relative changes in

EcR/RXR expression. The signal transduction of ecdysteroids requires a heterodimer complex of EcR and its binding partner RXR. Interestingly, our results show that the distribution of *CasEcR* isoforms differs by tissues and molt stages. In eyestalk ganglia, *CasEcR1* expression contributes around 70% towards total *CasEcR* expression. Structural property of the putative ligand binding pocket of CasEcR1 seems to be less hydrophobic than that of CasEcR2; therefore, CasEcR1 may prefer to bind to 20-HE rather than PoA. However, levels of *CasMIH* expression in the eyestalk ganglia incubated with of a 1:3 ratio of PoA to 20-HE do not differ from the controls (Suppl. Fig. S3-3). On the other hand, incubation of these two active ecdysteroids at a 3:1 ratio shows that only the ecdysteroids that mimicked intrinsic condition of early premolt (75 ng/ml) significantly and specifically increase the levels of *CasMIH* expression, while it has no effect on the levels of *CasAK* expression. Thus, it appears that the initial elevation of ecdysteroids, particularly PoA, may be important, specifically in regulation of *CasMIH* expression.

As our aim was to examine the initial stimulatory effect of ecdysteroids on *CasMIH* expression, we carried out 3 hr incubation studies. Levels of *CasEcR* in the eyestalk ganglia do not change compared to those measured in the control group. EcR is in general known to be early-ecdysteroid responsive gene. However, there may be a critical and limited window of its expression after the exposure to ecdysteroids. Indeed, up-regulation of *EcR* expression in limb buds of *U. pugilator* occurs only at 1.5 hr incubation with 20-HE but not at 2 or 2.5 hr incubation [189]. Thus, it is implied that up-regulation of *CasEcR* might have

taken placed earlier, which was then subsequently translated for transducing the signal and trans-activating *CasMIH* expression as shown in Fig. 3-5. However, further studies are required particularly for establishing a relationship between the levels of transcription and translation of EcR in crustaceans using a time-course incubation study.

RNAi technique, specifically dsRNA, has been successfully employed in decapod crustaceans for functional study of a gene of interest [187,190]. Prior to carrying out the current experiment, injections of 5 and 10 µg *CasMIH-dsRNA* were tested in order to find an optimal dose that yielded the maximal reduction of the target gene expression. Our preliminary data obtained from three injections at a two-day interval of 10 µg *CasMIH-dsRNA* showed ~ 90% reduction in *CasMIH* expression. Our study with 30-time injections to the animals normally exhibiting molt interval (~20 days) shows that molting is slightly induced after *CasMIH-dsRNA* injections, having the shorter molt interval (14. 5 ± 0.6 days, P = 0.0727), compared to other groups. Our finding is pertinent to an earlier finding in the much smaller sized crayfish, *C*. *quadricarinatus* where multiple injections of *CqMIH-dsRNA* induce molting [190].

CHH neuropeptides specifically produced in crustacean eyestalk ganglia tend to accumulate in the sinus gland, as similar to adipokinetic hormone in corpora cardiaca in insects [191]. The progress of life cycle accentuates their accumulation, suggesting that the amounts of MIH/CHH translation are far greater than what is actually required by the animal. Although *CasMIH* levels are reduced >90% by multiple injections of *CasMIH-dsRNA*, it does not yield

dramatic molt inductions. Perhaps the prolonged reduction of *CasMIH* levels over 60 days may induce the release of CasMIH stored in the sinus gland, although the newly translated materials in the neurosecretory neuronal cells are preferentially released first from the neurosecretory terminals in crustaceans and mammals [192-194].

Interestingly, a long-term administration of *CasMIH-dsRNA* seems to be lethal as half of the animals died during the mid-premolt (D_1 and D_2) stage. This might have occurred as off-target effects of *CasMIH-dsRNA* [195-197]. However, it is suggested that the lack of CasMIH may impair the Y-organ activity in terms of the ecdysteroid levels as well as the specific type of an active ecdysteroid. The eyestalk ablated *C. sapidus* at premolt stage carry 20-HE as the major ecdysteroid in their hemolymph, in contrast to the intact animals that use PoA as the major form [60]. Although it is not clarified in this current study, it will be intriguing to examine in the future, if indeed multiple injections of *CasMIH-dsRNA* may replicate a similar effect of the eyestalk ablation.

We have also observed that *CasCHH-dsRNA* administrations produced a high mortality of the animals, particularly those that were undergoing the ecdysis process. These animals were unable to shed the old exoskeleton. As a result, they were usually trapped in the old skeleton. Crustacean ecdysis relies on the hormonal regulation of a large uptake of iso-osmotic water [118]. The expression and release of gut CHH from the endocrine cells present in the fore- and hindgut are required for the completion of successful ecdysis [118,198]. It has been often observed in *C. maenas* that untimely discharged CHH from these

endocrine cells causes the death of animals during the ecdysis, exhibiting by the typical symptom of swelling of the body trapped in the old exoskeleton. What we have observed is similar to what was reported in *C. maenas* [118]. And, we consider that *CasCHH-dsRNA* may affect the expression of the gut CHH expression, possibly its release, which results in a high mortality of these animals during ecdysis.

The same amounts of multiple injections of *CasEcR1- dsRNA* indeed reduce *CasMIH* expression, strongly suggest that transcription of *CasMIH* may be under the control of ecdysteroids. However, *CasEcR1-dsRNA* injections seem to be less effective in knock-down of *CasEcR1* expression and produce much less reduction, compared to those determined with *CasMIH-dsRNA* injections. The levels of knock-down of a target gene by RNAi vary, depending on the levels of its expression as well as the function or property of that particular gene product [199-201]. We suggest, however, that this result may be due to the low translation rate of *CasEcR* or a slow turnover of CasEcR in the eyestalk ganglia.

Indeed, translation of crustacean *EcR* including *CasEcR* appears to be tightly regulated. The 5´untranslated region (5´UTR) of *CasEcR* cDNA contains upstream open reading frame as well as internal-ribosome entry site [119]. This indicates that translation of the *CasEcR* mRNAs may require a particular physiological condition(s). Reduction of *CasMIH* expression upon *CasEcR1-dsRNA* injection supports the fact that levels of *CasMIH* expression respond to the presence of ecdysteroids. 30-time injections of *CasEcR1-dsRNA*, but not of

CasRXR-dsRNA reduce by 70% of *CasMIH* expression. EcR binds to ecdysteroids, while its partner, RXR, facilitates the liganded EcR to bind to the ecdysteroid-responsive elements. As alluded to earlier, the upstream promoter regions of *MIH* genes of *C. pagurus* [45]*, C. feriatus* [47], and *C. sapidus* (GenBank accession no. KJ813010, Suppl. Fig. S3-2) contain the binding sites for ecdysteroid-responsive factors. Hence, it is suggested that reduced levels (~67%) of *CasEcR* might have generated the downstream effect on decreased levels (three folds) of Cas*MIH* expression.

With the data obtained from this study including the direct effects of ecdysteroids on the *CasMIH* expression, together with the previous findings [14,28,39,45], a regulatory mechanism for molt control in crustaceans is proposed using *C. sapidus* as a model (Fig. 3-10).

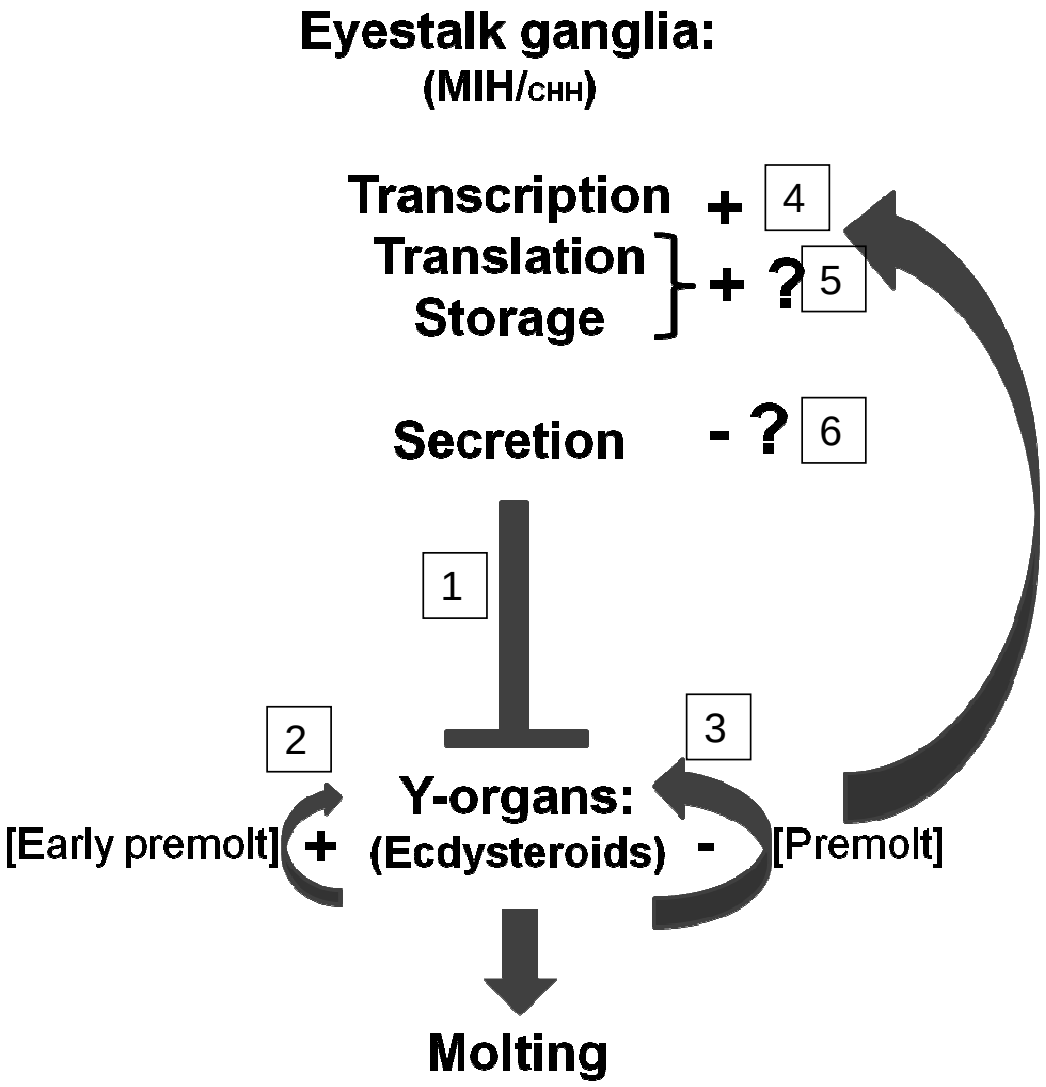


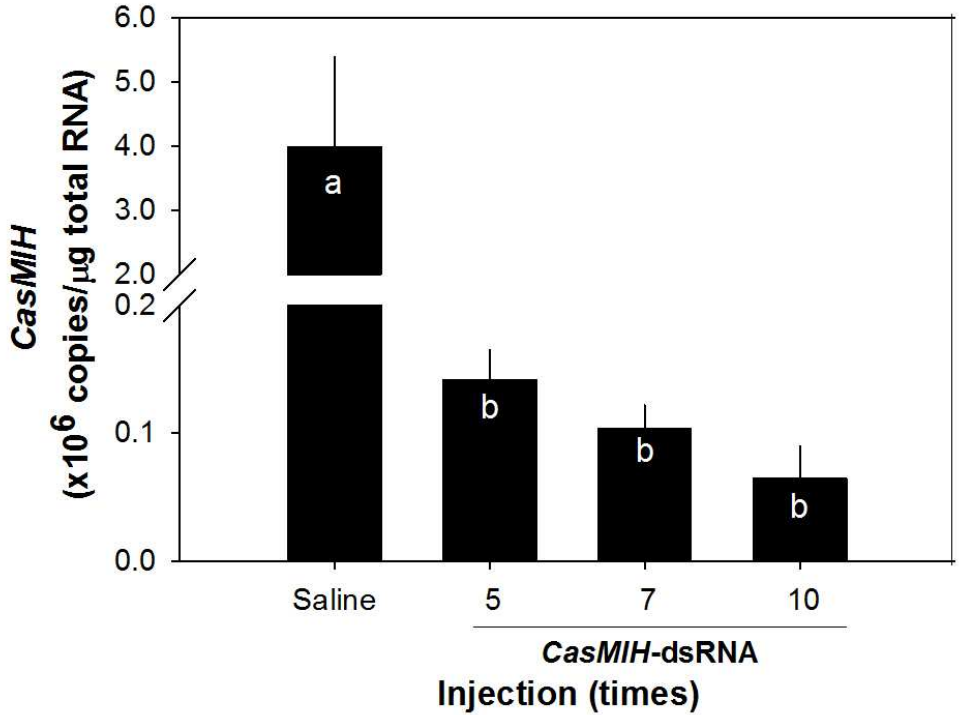
Figure 3-10. Proposed model of molting control in crustaceans. (1) Neuropeptides (MIH and CHH) secreted from the eyestalk ganglia suppress the ecdysteroidogenesis in Y-organs. Autocrine feedbacks of ecdysteroids on the Y-organs may be generated from different ecdysteroid levels: (2) ecdysteroid concentrations at early premolt stage generate positive feedback while the levels at the premolt stages (3) inhibit the ecdysteroidogenesis in the Y-organs. This study provides supporting evidence that premolt ecdysteroids have a stimulatory effect on eyestalk ganglia for neuropeptide production, particularly at transcriptional level (4), generating the long-loop feedback on Y-organs. Based on the endogenous changes of CasMIH expression and storage in this study, it suggests that ecdysteroids might have positive effect on translational and storage processes (5). On the contrary, ecdysteroids might suppress the secretion of neuropeptides as reported in *Cancer antennarius* [28](6). Overall, this define model of molting control suggests that the interactions of ecdysteroids and neuropeptides coordinate the molting into the repetitive cycle. (Modified from Hopkins [14])

At the early premolt, elevated levels of ecdysteroids may stimulate the *MIH* expression in eyestalk ganglia which is confirmed by an *in vitro* incubation of ecdysteroids as well as *CasEcR1-dsRNA* injections. The exact cue for MIH release has not yet been identified in crustaceans. However, the levels of hemolymphatic ecdysteroids may negatively affect the release of MIH. The low levels of CasMIH at D_2 stage are measured at the peak concentrations of hemolymphatic ecdysteroids, as an earlier report in *C. antennarius* [39]. The highest levels of CasMIH in the hemolymph are found at post-molt stage during which animals have the lowest levels of hemolymphatic ecdysteroids (Fig. 3-3). Such high levels of CasMIH preceding intermolt stage inhibit the synthesis and release ecdysteroids by the Y-organs, which may result in re-setting the molt cycle.

This overall proposed model for molt control describes the two feedback mechanisms involving MIH in the eyestalk ganglia and ecdysteroids produced by Y-organs. Firstly, the long-loop feedback includes the neuroendocrine axis between the eyestalk ganglia and Y-organs in that MIH inhibits the activity of the Y-organs at intermolt and post molt stage. At premolt stage, elevated levels of ecdysteroids stimulate the synthesis of the MIH and may suppress its release, while ecdysteroids and their signaling pathway through EcR may have the short-loop feedback for stimulating and inhibiting the ecdysteroidogenesis. However, further studies for examining these following topics are required: 1) the levels of CasMIH synthesis in X-organs at different molt stages in response to the varying concentrations of ecdysteroids; 2) the binding affinity of a specific EcR on each

active ecdysteroid type; and, 3) consequent effects on *MIH* expression in the eyestalk, and the direct effects of PoA and 20-HE on Y-organs.

3.5 Supplement figures



Figures S3-1. Expression levels of *CasMIH* from a time course study of *CasMIH-dsRNA* injections in eyestalk ganglia by qRT-PCR assay. Each cDNA sample containing 25 ng total RNA equivalent was assayed in duplicate. The expression levels are represented as copies/μg total RNA. The data are presented as mean ± SE (n). Statistical significance was accepted at $P < 0.05$ and noted with letters.

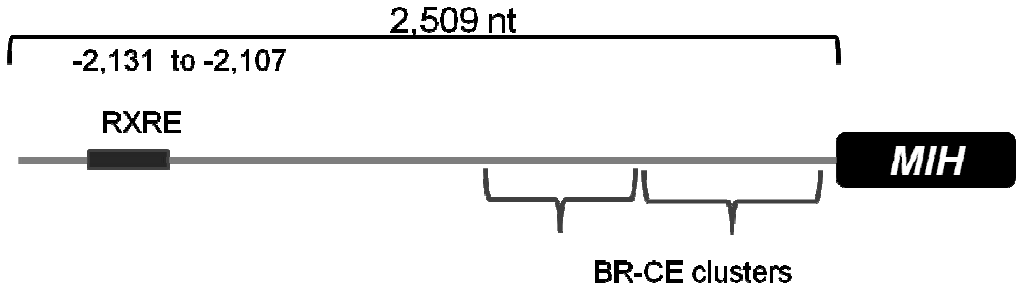


Figure S3-2. Genomic arrangement of *MIH* gene (Accession no. KJ813010) in *C. sapidus* using GenomeWalker (Clontech). The upstream promoter region was extended 2,509 nt from the start codon. The binding site for RXR (RXRE) is predicted on the region between -2,131 to -2,107 nt, while the binding site for broad complex (BR-CE) is found at the proximal region and clustered in tandem repeats using web-based prediction of regulatory elements program (http://consite.genereg.net/).

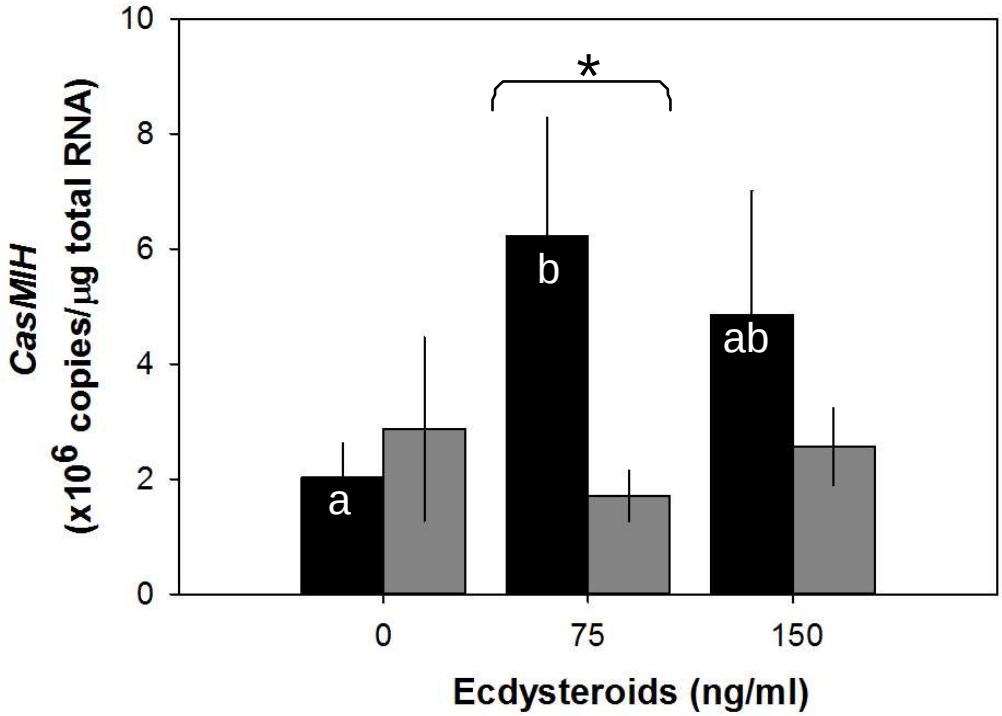


Figure S3-3. Expression levels of *CasMIH* in eyestalk ganglia after incubation with different ratios of active ecdysteroids by qRT-PCR assay. Two different ratios of PoA:20-HE were determined: 3:1 ratio (black bar) and 1:3 ratio (Gray bar). Each cDNA sample containing 25 ng total RNA equivalent was assayed in duplicate. The expression levels are represented as copies/µg total RNA. Statistical significance was accepted at $P < 0.05$ and noted with asterisk

CHAPTER 4

General conclusions and future studies

This study supported the hypothesis that ecdysteroids stimulate *CasMIH* expression as evidenced by the effect of ecdysteroids on neuropeptide production in eyestalk ganglia. Ecdysteroid levels as well as expression levels of *CasMIH* and *CasEcR* were examined in two different molt cycles: embryonic and juvenile molts. In addition, both *in vitro* and *in vivo* experiments were conducted to verify the functional roles of ecdysteroids and the ecdysteroid receptor.

The endogenous changes in ecdysteroid levels as well as expressions of neuropeptides and ecdysteroid-responsive factors during embryogenesis and juvenile molt are similar. These results suggest that embryogenesis includes a molting process that occurs inside the embryonic envelope prior to hatching. During ovarian development, levels of maternal mRNAs encoding for ecdysteroid-responsive factors (*CasEcR*, *CasRXR*, and *CasBTBDP*) are very high, but the levels abruptly decrease when embryonic development is initiated. This suggests that these maternal factors are immediately utilized and involved in the initiation of embryogenesis when the transcription machinery has not yet functioned.

During embryogenesis, the changing of ecdysteroid levels was similar to that found in the juvenile molt. This result indicates that the embryonic ecdysteroids are produced by the newly developed Y-organs. Interestingly, expression levels of *CasEcR* and *CasRXR* peak before ecdysteroids. This

lagged peak of ecdysteroids is also found in the Y-organs of juvenile molt when expression levels of all *CasEcR* forms are peaked at the D_1 stage, while the ecdysteroid peak occurs at the D_2 stage. This pattern is reported as a common response for negative feedback of ecdysteroidogenesis in both insects and crustaceans. Therefore, CasEcR and CasRXR may be involved in the regulation of ecdysteroidogenesis in Y-organs. In addition, temporal changes of ecdysteroid-responsive factors during embryogenesis may be derived mainly from the expressions in embryonic Y-organs.

There appears to be a relationship between *CasMIH* and *CasCHH* expressions and the changes of ecdysteroid levels during embryogenesis. Similarly, during the last juvenile molt cycle, C*asMIH* levels at premolt were higher than those at intermolt. The similar patterns of temporal expressions obtained from two different molt cycles in different life stages indicate that in every molt cycle, the interactions between ecdysteroids and neuropeptides are repeated. Since the embryonic molt is considered the first cycle of molting in the *C. sapidus* lifecycle, interactions between ecdysteroids and neuropeptide hormones are roughly determined from the timing of their expressions. As the ecdysteroid system is initiated before neuropeptides during embryogenesis, it is implied that ecdysteroids may stimulate neuropeptide expressions.

However, much is still unknown about the translation and storage activities of CasMIH in X-organs and sinus glands, respectively. The present findings suggest that high levels of CasMIH expression and storage during the late premolt may be utilized in postmolt and the consecutive intermolt as the secreted

levels at the postmolt stage are higher than other stages. However, all the activities of CasMIH in transcription, translation, and storage are not completed: the expression and storage levels were only examined during intermolt and premolt stages while the secreted levels were covered in one molt cycle. In order to fully understand CasMIH activities during the molt cycle, further studies in the following topics are required: 1) how much *CasMIH* is expressed and stored during the ecdysis and postmolt stages compared to the intermolt and premolt stages; and 2) how much CasMIH is translated in the X-organ in one molt cycle.

An analysis of the effects of ecdysteroids on *CasMIH* expression were assessed via *in vitro* incubation with active ecdysteroids composed of PoA and 20-HE at concentrations and ratios that mimicked the premolt stages of *C. sapidus*. Only the 3:1 ratio of PoA:20-HE at concentrations that simulate early premolt and mid premolt (D_o and D_1) stages induce *CasMIH* expression. This result suggests that PoA has a stimulatory effect on *CasMIH* expression whereas 20-HE may interfere with PoA. It is implicated that PoA may be the key factor in regulation of *CasMIH* expression.

This incubation experiment was performed based on the endogenous ratios of ecdysteroids during the mid premolt (D_2) stage. The endogenous ratio of PoA to 20-HE during intermolt, and early premolt (D_o and D_1) stages are still unknown. Therefore, the intrinsic ratios of PoA:20-HE in these stages need to be evaluated. Thru results in this area, further incubation studies might be attempted to define the effects of PoA and 20-HE on *CasMIH* expression.

In the eyestalk ganglia, CasEcR1 is the major form and has LBP that is more hydrophilic than that of CasEcR2. Considered to be an ecdysteroid property, the hydrophilic LBP of CasEcR1 is predicted to bind with 20-HE in a manner stronger than PoA. The decreased stimulatory effects of ecdysteroids on *CasMIH* expression in higher concentrations of ecdysteroids may result from the increasing amounts of 20-HE in the ecdysteroid mixture that interferes with PoA binding with CasEcR1. This suggestion requires further study to demonstrate the binding affinity of CasEcR1 and/or CasEcR2 to PoA and 20-HE.

Unlike the effects on eyestalk ganglia, it is still unknown which type of active ecdysteroids regulate the feedback on ecdysteroidogenesis in Y-organs. Due to the challenges inherent in distinguishing ecdysteroids in the incubating media and newly secreted ones, experiments that focus on the Y-organ incubation are necessary. By obtaining these results, the regulation process of ecdysteroidogenesis in Y-organs would be better understood.

Based on the knowledge that ecdysteroids transduce signals through CasEcR, a master transcription factor in the ecdysteroid system, transcription of the downstream-responsive genes will be affected by CasEcR depletion. *In vivo* experiments demonstrated that knockdown of *CasEcR1* lead to a reduction in *CasMIH* expression. This indirect effect of reducing CasEcR1 on *CasMIH* expression indicates that CasEcR1 may be involved in *CasMIH* transcription.

In eyestalk, the expression profile of *CasEcR2* is also similar to that of *CasEcR1*, and this putative isoform is predicted to better bind with PoA than CasEcR1. Therefore, it would be interesting to know whether CasEcR2 is

involved in *CasMIH* expression. Since the expression levels of *CasEcR2* are much lower than *CasEcR1*, it is difficult to measure thru a SYBR green qRT-PCR assay. This suggests a need to find other methods that are able to detect very low transcript amounts. Therefore, changes of *CasEcR2* expression in incubation studies and knockdown experiments could be analyzed and considered in *CasMIH* expression.

Moreover, translation of crustacean EcR including CasEcR appears to be tightly regulated as its 5´untranslated region (5´UTR) contains an upstream open reading frame as well as internal-ribosome entry site [119]. This indicates that translation of *CasEcR* mRNAs may require a particular physiological condition. Protein levels of CasEcR during the molting and experiment need to be considered.

When the investigation focused on the genomic arrangement of *MIH* genes in crustaceans, it was discovered that an upstream promoter of *MIH* contained the binding site for RXR and broad complex (Suppl. Fig. S3-2). Thus, there is great evidence to support the hypothesis that ecdysteroids are acting through their responsive factors (EcR, RXR, or BTBDP) to trans-activate the *MIH* expression.

Trans-activation of ecdysteroids on *MIH* expression could be determined through binding experiments in which the ecdysteroid-responsive elements of the *MIH* gene are tested for their ability to bind with different ecdysteroid-responsive factors. By performing these experiments, the following questions can be answered: 1) Which forms of EcR have effects on *MIH* expression at the

transcription level? and, 2) What are the pathways through which ecdysteroids trans-activate *MIH* expression. Is it acting through an EcR-RXR complex or other ecdysteroid-responsive factors such as a broad complex or BTBDP?

All together, this investigation provided lines of evidence that demonstrate the effects of ecdysteroids on eyestalk ganglia. Through this expansion of knowledge, the regulation of molting in crustaceans can be re-defined (Fig. 3-10). Premolt ecdysteroids consisting of PoA:20-HE at a 3:1 ratio stimulates *MIH* expression in eyestalk ganglia. This increase of MIH production during premolt stages leads to the accumulation of MIH in the sinus gland as herein reported. This storage hormone could be further utilized in later stages. It seems possible that premolt ecdysteroids also have a positive effect on MIH at the translational and storage levels. On the contrary, secretion of MIH into the hemolymph may be inhibited by ecdysteroids as reported previously in *C. antennarius* [28]. Considering all possible responses in MIH together, large amounts of MIH would be stored in the sinus gland and then are utilized during postmolt and in the consecutive intermolt as this study has shown. A high secretion of repository MIH into the hemolymph at postmolt will inhibit ecdysteroidogenesis in Y-organs, resulting in re-setting of the molt cycle.

This model proposes that long-loop feedback of ecdysteroids generates negative feedback for ecdysteroidogenesis in the Y-organ through the neuroendocrine axis in which ecdysteroids at premolt stages stimulate the synthesis of neuropeptide hormones. In addition, temporal expression of

CasEcR in the Y-organs suggests that ecdysteroids may have short-loop feedback that stimulates and inhibits ecdysteroidogenesis.

In summary, the major findings of this research study were:

- *C. sapidus* experiences one molt cycle during embryogenesis, and this molt is considered the first cycle in its lifecycle.
- Maternal ecdysteroids as well as *CasEcR*, *CasRXR*, and *CasBTBDP* are deposited in oocytes during ovarian development. The presence of these maternal factors indicates that ecdysteroids may be required for initiating embryogenesis.
- In both embryonic and juvenile molts, the levels of ecdysteroids and the expression levels of neuropeptide hormones (*CasMIH* and *CasCHH*) were correlated. Higher levels of *CasMIH* expression were observed when ecdysteroid titers increase.
- *In vitro* incubation studies revealed that only the combinations of PoA:20-HE ratios that mimic premolt ecdysteroids stimulate *CasMIH* expression in eyestalk ganglia.
- *In vivo stu*dies indicated that the stimulatory effect of ecdysteroids on *CasMIH* expression is acting through *CasEcR*.

This study still requires further research to detail the stimulatory effect of ecdysteroids on the neuropeptide axis. The following lists present remaining questions that need to be answered.

- The endogenous ratios of PoA and 20-HE at other molt stages such as intermolt (C_4) and premolt (D_0 and D_1) stages: different forms of hemolymphatic ecdysteroids from these stages could be isolated and assayed by HPLC-RIA as described [60]. By having this result, the response of eyestalk ganglia on these ecdysteroid conditions could be tested and compared with the recent finding.
- The expression of CasMIH including transcription, translation, and storage at ecdysis and postmolt stages. By obtaining these results, effects of ecdysteroids on CasMIH at different levels could be determined.
- Effects of different ecdysteroid ratios and levels on the sinus gland in eyestalk ganglia for CasMIH secretion. As proposed, the inhibitory effect of ecdysteroids on the CasMIH secretion could be determined. This result would determine whether ecdysteroids have an effect on CasMIH at the secretion levels.
- Expression of CasEcR at protein level in eyestalk ganglia and Y-organ during molting cycle and in experimental conditions. Since the 5'UTR of *CasEcR* mRNA contains uORF and IRES, the translation of *CasEcR* seems to be tightly regulated and depends on specific conditions.
- Localization of CasEcR isoform in eyestalk ganglia and Y-organ. Antibodies that recognize CasEcR1/CasEcR1a and CasEcR2/CasEcR2a are required to detect the localization of CasEcRs in different molt stages and in experimental conditions. This result will provide evidence to

support the presence of specific isoforms that transduce ecdysteroids in different tissues and conditions.

- Binding ability of different CasEcRs with 20-HE and PoA as the putative CasEcR1/CasEcR1a and CasEcR2/CasEcR2a are predicted to bind with 20-HE and PoA, respectively. By obtaining this knowledge, the induction of *CasMIH* expression in eyestalk ganglia will be determined in a CasEcR form where the PoA exerts its signal. In addition, as CasEcR expression in Y-organ is molt-stage dependent, the binding result would also provide information about the possible ecdysteroid that regulates ecdysteroidogenesis in Y-organs.
- Binding of ecdysteroid-responsive factors (CasEcR, CasRXR, and CasBTBDP) on the upstream promoter of *CasMIH*. Binding of these factors on regulatory elements can be detected by Pull-down assay or Gel Shift assay (EMSA). This result will provide strong evidence supporting the hypothesis that ecdysteroids stimulate *CasMIH* expression at a transcriptional level.

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