ABSTRACT

Title of thesis: ECDYSONE AND RETINOID-X RECEPTORS OF THE BLUE CRAB, *CALLINECTES SAPIDUS*: CLONING AND TEMPORAL EXPRESSION IN EYESTALK GANGLIA AND Y-ORGANS DURING THE MOLT CYCLE

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The molt cycle in decapod crustaceans, including the blue crab, *Callinectes sapidus,* is supressed by the crustacean hyperglycemic hormone (CHH) neuropeptide family and stimulated by ecdysteroids (Ecds). The Ecds are thought to act on both eyestalk ganglia (ES) and Y-organs (YO) where the CHH neuropeptides and Ecds are synthesized, respectively. Since the resultant responses of Ecds are mediated through their nuclear receptors: ecdysone receptor (EcR) and retinoid-X receptor (RXR), the temporal expression of *EcR-RXR* in ES and YO during the molt cycle may reveal the regulatory role of Ecd on the activity of these two organs. In this study, the full-legnth cDNA sequences of *C. sapidus EcR1a* and *RXR1*, and the coding region of three other isoforms of EcR and RXR, were isolated. Moreover, temporal distributions of *C. sapidus* specific isoforms: *CasEcR* and *CasRXR,* were examined in ES and YO at different molt stages.

ECDYSONE AND RETINOID-X RECEPTORS OF THE BLUE CRAB, *CALLINECTES SAPIDUS*: CLONING AND TEMPORAL EXPRESSION PATTERNS IN EYESTALK GANGLIA AND Y-ORGANS DURING THE MOLT CYCLE

By

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LIST OF ABBREVIATION

AF1	Activation Function-1
AF2	Activation Function-2
AK	Arginine Kinase
CHH	Crustacean Hyperglycemic Hormone
CPE	Cytoplasmic Polyadenylation Element
Ecd	Ecdysteroid
EcR	Ecdysone Receptor
ES	Eyestalk ganglia
DBD	DNA-Binding Domain
HR	Hinge Region
IRES	Internal Ribosome Entry Site
LBD	Ligand-Binding Domain
MIH	Molt-Inhibiting Hormone
MOIH	Mandibular Organ- Inhibiting Hormone
mORF	Major open reading frame
NR	Nuclear receptor
RACE	Rapid amplification of cDNA ends
RXR	Retinoid-X receptor
uORF	upstream open reading frame
UTR	Untranslated region
YO	Y-organs

1. CHAPTER 1. INTRODUCTION

1.1. The blue crab, *Callinectes sapidus*

Blue crabs, *Callinectes sapidus* are classified into the Phylum Arthropoda, Subphylum Crustacea, Class Malacostraca, Order Decapoda, and Family Portunidae. The life cycle of *C. sapidus* continues through 27 to 29 cycles: molting from hatching to adulthood (**Fig.1**) [1], as each molt increases 30-50% of the body size. Females undergo the terminal-pubertal molt and halt molting during the active reproductive period, while males continue to molt during their adulthood.

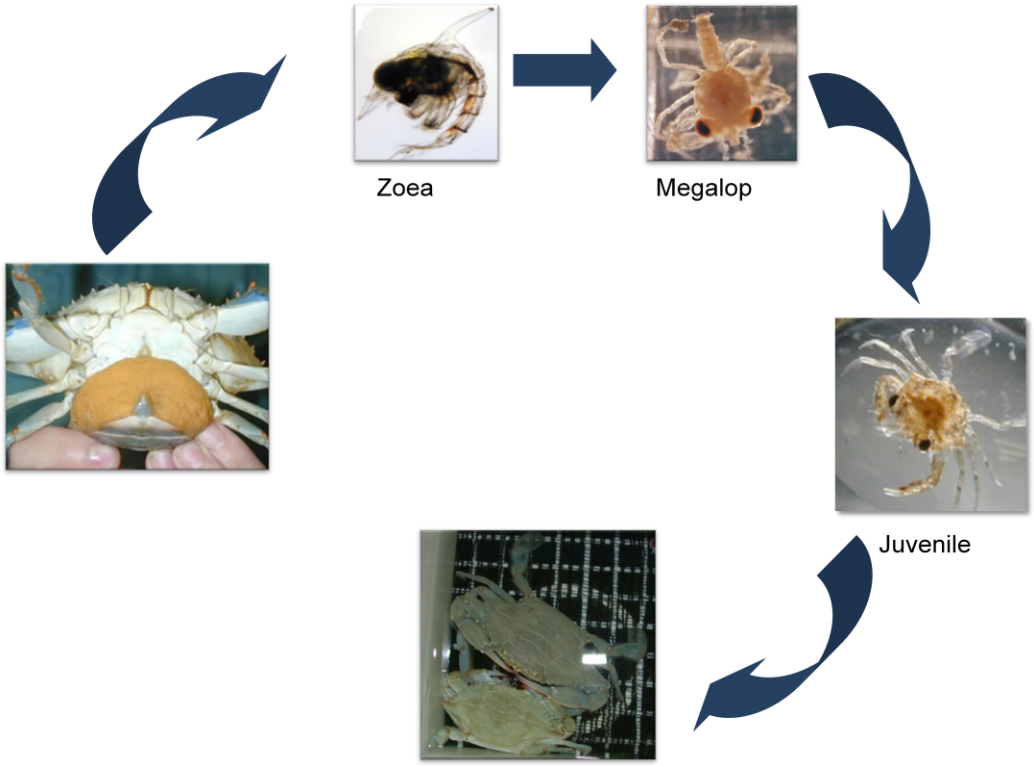


Figure.1. Life cycle of *C. sapidus* starting from zoea, megalopa, juvenile and adult crab. http://www.bluecrab.info/lifecycle.html

1.2. Molt stages and physiological changes

Molting is the shedding process that is required for somatic growth and reproduction in animals belonging to the Phylum Arthropoda. During the molt cycle, physiological and structural changes are closely related to molt stages, and molting can be classified into four different stages: intermolt (C_4), premolt ($D_{0\text{-}4}$), ecdysis (E), and postmolt (A-C_3). Premolt can be further characterized into three stages: early premolt (D_0), mid premolt (D_1-D_2), and late premolt (D_3-D_4) [2,3], as shown in **Fig.2**.

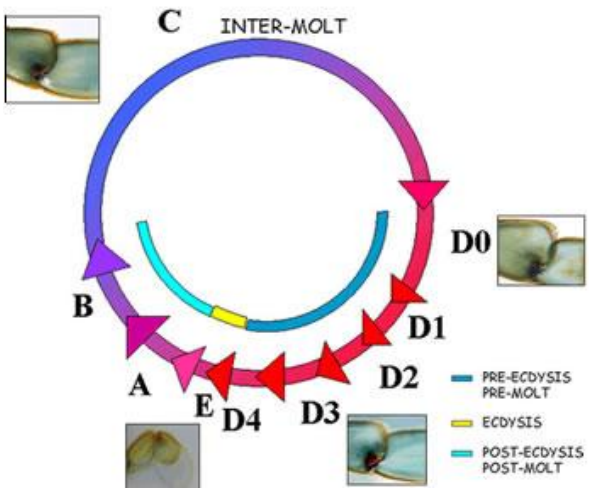


Figure.2. A schematic diagram of the molt cycle of *C. sapidus* with associated pheotypic changes of the swimming leg. Inner ring: blue= premolt, yellow= ecdysis, and light blue= postmolt; outer ring: color represents the progression of molting: blue= intermolt (C_4), magenta= premolt (D_0-D_4), red= 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 completely rigid, and the membranous tissue is attached to the hypodermis and the cuticle [4]. During premolt, the old cuticle is detached from the hypodermis by actions of digestive enzymes (chitinases) secreted by epithelial cells [5], and new cuticles

composed of an organic matrix are laid. While the new cuticle is continuously formed, organic materials from the old one are reabsorbed, resulting in a breakable shell. The 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 crustacean hyperglycemic hormone [6,7]. After tanning and calicification during postmolt (A, B, C_1-C_3 stages), the animals resume normal activities.

1.3. Hormonal regulation of molting in crustaceans

The molt cycle is regulated by an interaction between neuropeptide and steroid hormones, *i.e.* molt-inhibiting hormone (MIH)/crustacean hyperglycemic hormone (CHH) and ecdysteroids (Ecds), respectively.

1.3.1 Neuropeptide hormone family (MIH/CHH)

MIH and CHH are members of the CHH neuropeptide family, consisting of 72 to 83 amino acids (aa) and containing six residues of Cys that form three intra-disulfide bridges [8,9]. MIH/CHH and their respective peptides are synthesized in neurosecretory cells in the medulla terminalis of the eyestalk ganglia (ES). These neuropeptides are transported through the axonal tract and stored in the sinus gland (SG). The stored neuropeptides are secreted into the hemolymph whereupon they suppress activities of the Y-organs (YO). Since YO is the ecdysteroid (Ecd) synthesizing organ, the suppression of YO by MIH/CHH results in reduction of Ecd synthesis and secretion. The inhibitory actions of MIH/CHH on the activity of the YO during the molt cycle have been studied in

several decapod crustaceans including *Carcinus maenas*, *C. sapidus*, *Gecarcinus lateralis, Procambarus clarkii,* and *Uca pugilator* [10-22]

Compared to the effects of MIH/CHH on the YO during the molt cycle, the regulation of these neuropeptide expressions is not fully defined. A previous study of the *Cancer anternarius* showed the effects of Ecds on MIH synthesis and secretion [23]. The extract of ES obtained from Ecd-injected crabs and ES-cultured media had greater effects to suppress the activities of the YO *in vitro*. In addition, Hopkins [11] found that Ecds were concentrated in ES and the levels were higher than those in the hemolymph, indicating that Ecds may have interactions on the ES and stimulate MIH/CHH synthesis [22,24]. In genetic compartmentalization of *MIH* and mandibular organ-inhibiting hormone (*MOIH*) genes in *Cancer pagurus,* the up-stream promoter region of both of these genes contained ordered putative binding sites of the following transcription factors: chronic factor-1 (CF1), a homolog of retinoid-X receptor (RXR)/ultraspiracle (USP), and broad-complex factor (BR-C). Furthermore, a study of MIH/CHH activities during the molt cycle of *C. maenas* showed that both *CHH* and *MIH* transcripts were slightly higher, although not significant, at the premolt than those at the intermolt stage [25]. Altogether, these findings indicate there may be some crosstalks between Ecds and neuropeptide hormones in order to regulate the molt cycle.

1.3.2. Ecdysteroid hormones

Ecdysteroids are a class of steroid hormones that is derived from cholesterol and present in animals, plants, and fungi [26- 31]. Ecds play important roles in several processes, especially in molting. For insects, 20-hydroxyecdysone (20-HE) is the major

form, while other Ecds can be synthesized depending on the precursors present. Ecds in crustaceans are generated in the YO in four major forms depending on the species, including ecdysone (E), 3-dehydroxyecdysone (3DE), 25-deoxyecdysone (25-dE), and 3-dehydro-25-deoxyecdysone (3D25dE) [32-36], as shown in **Table.1**. In the peripheral tissues, these compounds are modified by 3β-reductase, 20-hydroxylase and 25-hydroxylase to an active form [35].

Table 1. Major Ecd secreted by the Y-organs of decapod crustaceans (Mykles, 2011)

Species	E[a]	3DE[a]	25dE[a]	3D25dE[a]
Pachygrapsus crassipes	•			
Cancer antennarius	•	•		
Orconectes limosus	•	•		
Procambarus clarkii	•	•		
Penaeus vannamei	•	•		
Macrobrachium rosenbergii	•	•		
Carcinus maenas	•		•	
Callinectes sapidus	•		•	
Uca pugilator	•		•	
Menippe mercenaria		•		•

[a] *Abbreviations*: E, ecdysone; 3DE, 3-dehydroecdysone; 25dE, 25-deoxyecdysone; 3D25dE, 3-dehydro-25-deoxyecdysone.

1.3.2.1. Ecdysteroids and the molt cycle

It is accepted that molting is induced by Ecds, and molt stages are correlated and defined by Ecd titers [14,37-42]. During the molt cycle, Ecd titers flunctuate widely: low during postmolt and intermolt (C_4), slightly increased during early premolt (D_0), surging suddenly at D_1, reaching a peak at D_2, and dropping suddenly to the basal levels at D_3/D_4, as reported in *Hyas araneu***,** *Metopograpsus messor, C. magister, C. maenas*, and *C.sapidus* as shown in **Fig.3**. In addition, the clearance of Ecds in *Homarus americanus*, measured by percentage of radiolabeled 20-HE in the hemolymph and excretion after injection, is molt-stage dependent: lowest at early premolt (D_0/D_1), followed by mid

premolt (D_2) and highest during post molt and intermolt [43]. Therefore, the levels of Ecds circulating in hemolymphs result from dynamic relationship between synthesis and degradation of Ecds.

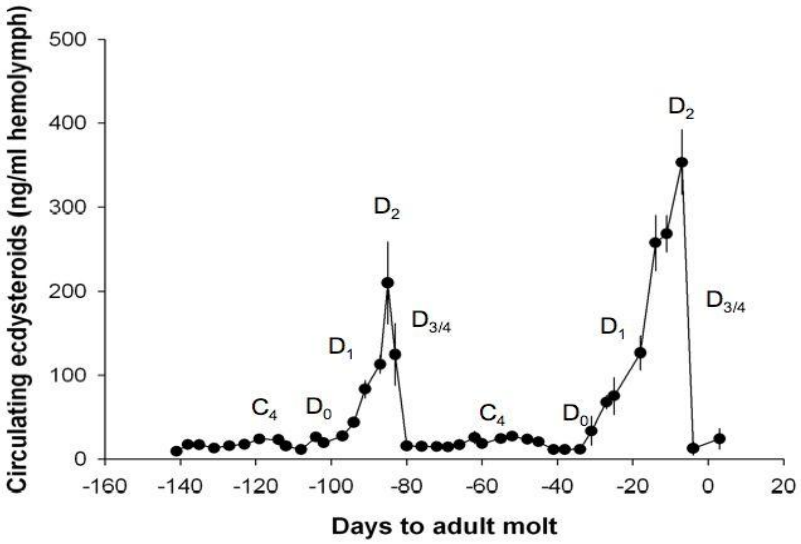


Figure.3. Circulating Ecds from a *C. sapidus* female during the last two molts: pre-pubertal molt (-140 to -80) and adult molt (-80 to 0) assayed by Ecd-RIA. Numbers on the X-axis represents days before and after adult molt. C_4= intermolt, D_0= early molt, D_1-D_2= mid premolt, $D_{3/4}$=late premolt

1.3.2.2. Ecdysteroidogenesis and regulation of Y-organ activity

The YO are the Ecd synthesizing site located in the cephalothorax anterior to the branchial chamber. Y-organ cells typically contain high levels of mitochondria and endoplasmic reticulum [34]. Arthropods synthesize Ecds from cholesterol that is carried by high-density lipoproteins (HDL) in the hemolymph and taken up by the YO. This process, as an important, rate-limiting step, is controlled by CHH neuropeptides that decrease the cholesterol uptake by depressing the number of HDL receptors [34,35,44].

Ecdysteroidogenesis in the YO of crustaceans and the prothoracic glands of insects are similar, but the biosynthetic pathway in the former appears to be more complex and produces many forms of Ecds [35]. Ecdysteroidogenesis can be divided into two stages: 1) conversion of cholesterol to 5β-diketol by a series of enzymes; and 2) conversion of 5β-diketol to the secreted products by four different pathways. Among the key enzymes involved in stage 2, cytochrome P450 monooxygenases (P450) encoded by the *phantom* gene or *Cyp306a1* in decapods [45] are a target for suppression by neuropeptide hormones. Transaldolase, an enzyme involve in production of NADPH (coenzyme of P450), is translationally suppressed by MIH [13]. Therefore, CHH neuropeptides may suppress ecdysteroidogenesis in the YO through reducing HDL uptake and/or activities of Ecd-synthesizing enzymes.

A recent study by Chung [1] reports that, at the premolt stage of *C. sapidus*, ponasterone A (PonA) is the major form followed by 20HE, while 3DE and 25-dE are the minor forms (**Fig. 4**). Moreover, it appears that Ecd ratios in three molt cycles vary in the ratios of 20HE: PonA. This ratios increase with each successive molt: from 0.25 to 0.44, although the functional significance of the changes in the ratios between these two Ecds is not defined.

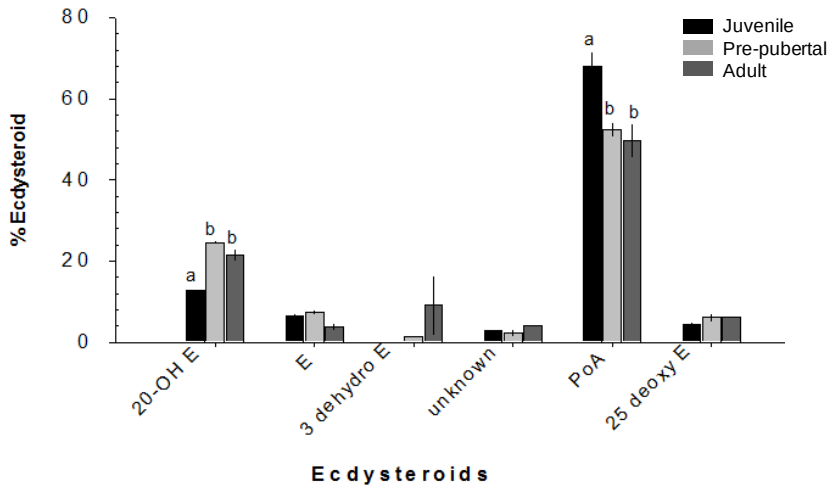


Figure.4. Different forms of Ecds from hemolymph during mid premolt (D_2) from juvenile (black bar), pre-pubertal (light-grey bar), and adult (grey bar) molts, assayed by HPLC-RIA. % Ecd represents percentages of 20-hydroxyecdysone (20-OH E), ecdysone (E), 3-dehydroxyecdysone (3 dehydro E), Ponasterone A (PoA), 25-deoxyecdysone (25 deoxy E), and unknown from total Ecds [1].

1.4. Nuclear hormone receptor superfamily

Ecdysteroids bind to a heterodimer EcR/RXR complex, both of which belong to the superfamily of nuclear receptors (NRs) known for hydrophobic molecules such as steroid hormones, retinoic acids (all *trans* and 9-*cis* isoforms), thyroid hormones, fatty acids, leukotrienes, and prostaglandins [46]. The NRs are phylogenetically related proteins and are commonly found from sponges, echinoderms, tunicates, arthropods, to vertebrates [47]. The NRs are characterized by five independent functional domains (A-F; originally defined by Krust [48]; 1) N-terminal A/B domains or activation function-1 (AF1), 2) C or DNA-binding domain (DBD), 3) D or hinge region (HR) domain, 4) E or ligand-binding domain (LBD), and 5) C-terminal F or AF2 domain [49,50] as shown in **Fig.5**.

Each domain has a specific function: 1) AF1 displays ligand-independent transactivation; 2) DBD binds to regulatory elements (REs) of the target genes; 3) HR provides flexibility within the molecule; 4) LBD serves as docking sites for its cognate ligands and transcriptional regulators (coactivator or corepressor); and 5) AF2 exhibits ligand-dependent activation. Since NRs contain DBD and AF domains in the same molecule, the NR superfamily can directly transactivate their target genes upon the binding of cognate ligands [51]. The NRs exhibit a highly conserved DBD in which two zinc-finger motifs are located and are responsible for binding to the hormone responsive elements (HREs) [46,52,53].

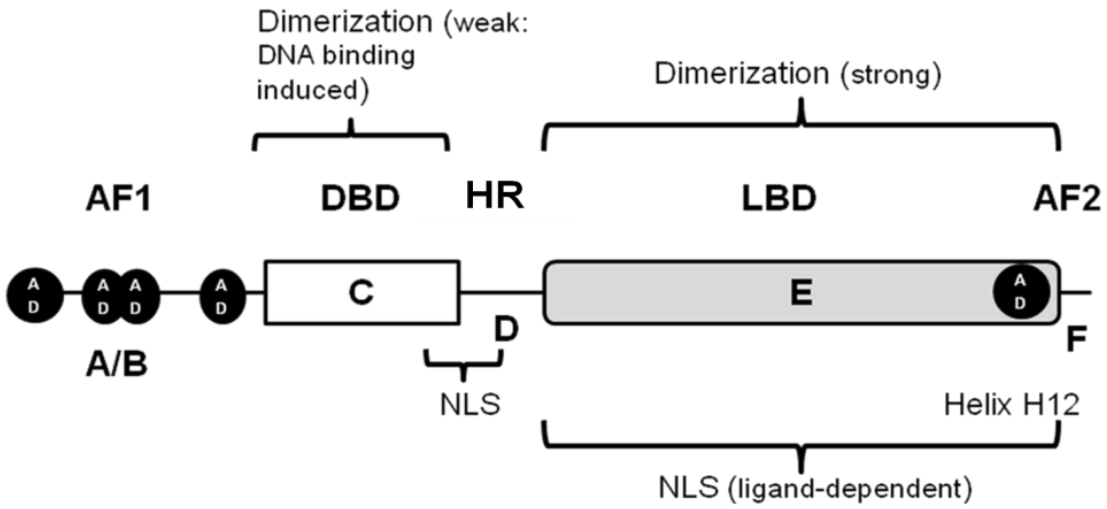


Figure.5. Schematic illustration of the structural and functional organization of nuclear hormone receptor family. The evolutionarily conserved regions C and E are indicated as white and grey boxes, respectively, while the line represents the divergent regions A/B, D, and F. Two transcription activation functions (AFs) have been described: a constitutively active AF1 in the A/B region and a ligand-inducible AF2 in region E or F. AD: autonomous transactivation domain, DBD: DNA binding domain, HR: hinge region, LBD: ligand binding domain, NLS: nuclear localization signal [54].

1.4.1. Transcriptional regulations by nuclear receptors

The molecular mechanisms of NRs upon ligand binding are similar in that conformational changes trigger a cascade of events. The localization of NRs also has an effect on transcriptional activities. In cytosol-localized NRs, binding to a ligand results in the dissociation of a chaperone followed by translocation into the nucleus and formation of a homodimer with their REs. Ligand binding to the nuclear-residing NRs stabilizes the dimer on its REs [28]. The ligand-receptor complexes on HREs will recruit coactivators that have histone acetytransferase (HAT) activities, resulting in local chromatin remodeling. Subsequently, TATA-binding protein (TBP)-associated factors (TAFs) will target the TBP and TFIIB on the TATA-box, stabilize the formation of pre-

initiation complex, and position general transcription factors and RNA polymerase II on the core-promoter (**Fig.6.)** [28,52].

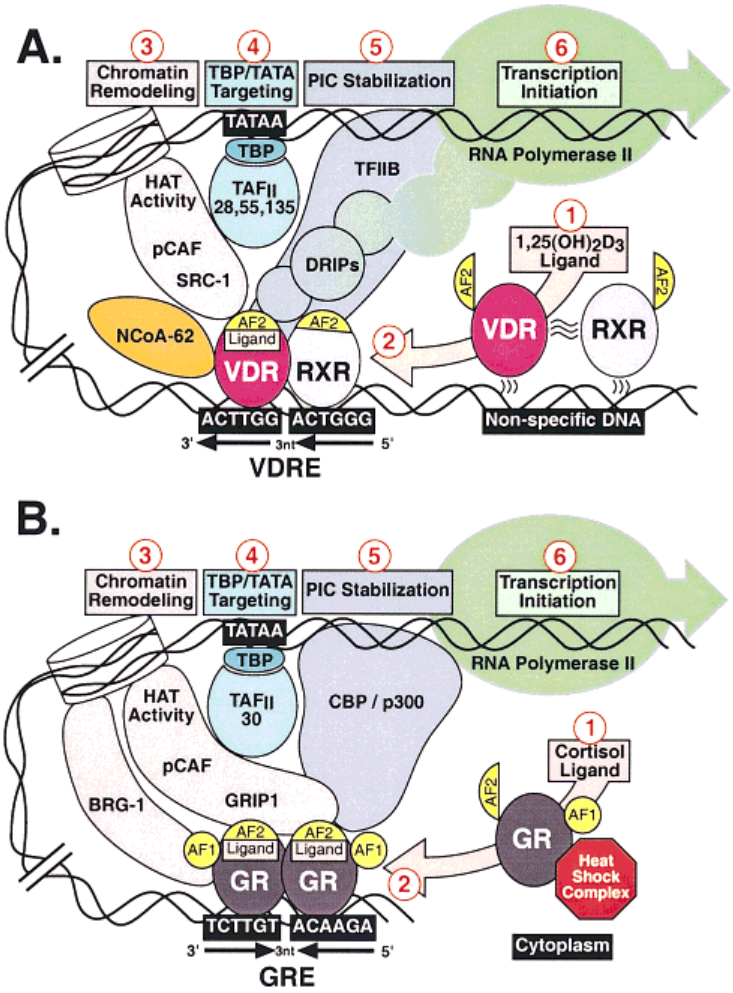


Figure.6. Simplified models of transactivation by heterodimerizing (**A**) or homodimerizing receptors (**B**) **A**: 1) unliganded vitamin-D receptor (VDR) weakly associates with retionoid-X receptor (RXR) and binds non-specifically to DNA [55]. Upon binding to its ligand, a strong heterodimer is formed and binds to its direct repeat VDRE. 2) Conformational changes in LBD allow the AF2 domain to recruit coactivators: SRC-1, DRIPs 3) With HAT activity in some coactivators, chromatin remodeling is catalyzed. 4) TAFs are attracted to TBP/TATA. 5) Binding of TFIIB and DRIPs stabilizes preinitiation complex (PIC). 6) Transcription initiation by RNA polymerase II is initiated at the core promoter. **B**: 1) Unliganded monomer (GR) associates with chaperones in cytosol. 2) Upon binding to the cognate ligand, GR is translocated into the nucleus and forms homodimer on its palindromic GREs. Conformational changes in AF1 and AF2 domains promote series of events (3-6) as described in (**A**) [28]**.**

1.4.2. Subcellular localization of Ecdysone receptor (EcR) and retinoid-X receptor (RXR)

As members of the NR superfamily, both EcR and RXR/USP contain a nuclear localization signal (NLS) and/or a nuclear export signal (NES) that determine whether they are localized in the cytosol or the nucleus [56]. NLS is located in the D-domain adjacent to the second zinc-finger motif [57]. Each NR is regulated differently for nuclear transport and can be categorized as: 1) exclusively cytoplasmic, 2) both cytoplasmic and nuclear, and 3) exclusively nuclear [57,58]. EcR and RXR/USP have differential distributions; RXR/USP is found exclusively in the nucleus, while EcR will be translocated into the nucleus which subsequently heterodimerizes to its partner, USP/RXR, in the presence of its ligand [56,57].

1.5. Post-transcriptional controls in eukaryotic messenger RNA

The central dogma of genetic codes states that DNA gives rise to RNA followed by protein synthesis [59]. However, recent data shows that the flow of genetic information is not as simple as proposed initially. Genetic regulations after post-transcription also play important roles in the control of gene expressions as shown in **Fig.7** [60-67]. In general, post-transcriptional modifications are as follows: 1) a 5´cap is added to the nascent pre-mRNAs after transcription initiation; 2) spliceosomes assemble and introns are spliced out from pre-mRNAs; and 3) a specialized 3´end is generated by cleavage and polyadenylation. After the pre-mRNA has been fully processed to mature mRNA, the ribonucleoproteins will be complexed with export proteins for transporting

into the cytosol [64,68]. Furthermore, a steady state level of mRNAs will be determined by mRNA localization, turnover, and surveillance [63,65].

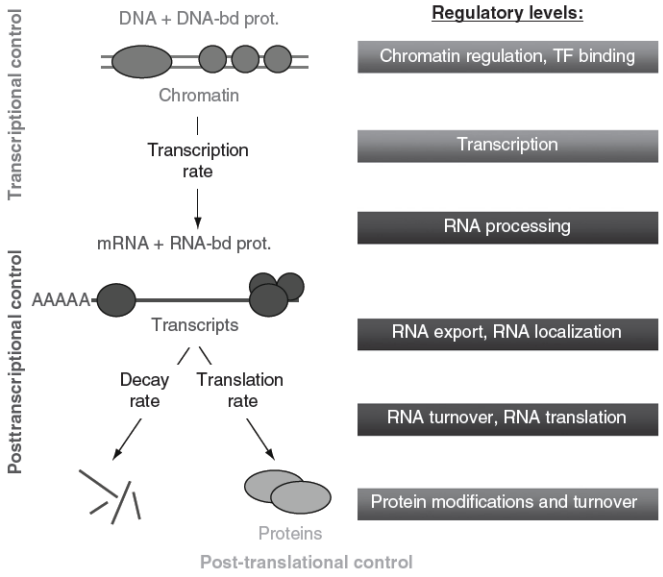


Figure.7. A Schematic diagram of genetic regulations: regulatory processes are listed corresponding to transcriptional, post-transcriptional, and post-translational controls [64].

Unlike prokaryotes, eukaryotic mRNAs also contain 5´ and 3´ untranslated regions (UTR) that serve as *cis*-regulatory elements (*cis*-REs). Decoding nucleotides to amino acids (aa) during translation requires energy. Therefore, cells will tune translation of the transcripts according to their physiological needs [64]. In general, translation initiation of mRNAs can be modulated globally or specifically [64,69]. In the former, translational control is tuned by reversible modifications of general translation factors by phosphorylation [63,64]. For the latter, translational regulation is managed through *cis*-REs. For most well-characterized regions, *cis*-REs are normally present in either the 5´ or 3´ UTR (**Fig.8.**).

1.5.1. 5´untranslated region (5´UTR)

cis-REs located in the 5´UTR influence translation rates (**Fig.8.**). The majority of eukaryotic mRNAs have 20-100 nucleotides in their 5´UTRs. However, unusually long 5´UTRs containing upstream AUGs, uORFs and/or secondary structure will affect translation efficiency during translation initiation [69,70]. Interestingly, these *cis*-REs are often found in mRNAs encoding proto-oncogenes, transcription factors, growth factors, and their receptors [70,71].

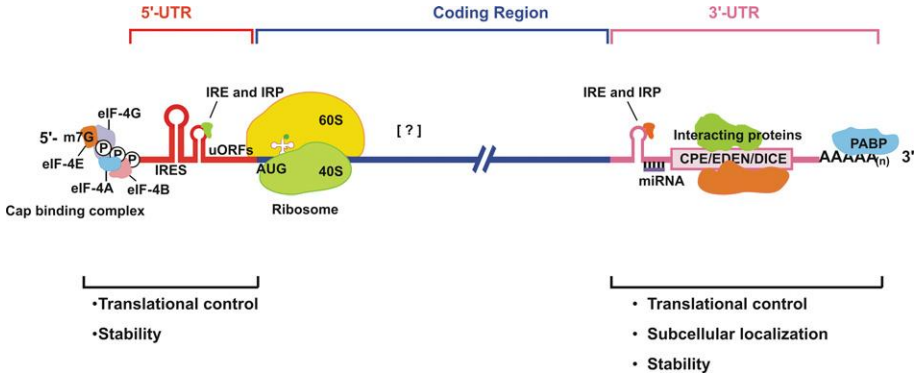


Figure.8. A structural organization of eukaryotic mRNA and the possible elements of translational regulation through various *trans*-acting factors: 5´-m7G, cap structure; eIF, eukaryotic initiation factor; CPE, cytoplasm polyadenylation element; EDEN, embryonic deadenylation signal; DICE, differential control element; PABP, poly(A)-binding protein. [?], possible sites of interaction of transacting factors (yet unknown) in the coding sequence. Regions of the mRNA involved in subcellular localization and stability are also indicated [72].

1.5.1.1. Upstream open reading frame (uORF)

Messenger RNAs containing uORFs are often up-regulated in their translations under stress or starvation condition, while the translations of other mRNAs are down-regulated by the phosphorylated Eukaryotic Initiation Factor 2 (eIF2) [64]. For example, the *GCN4* gene in yeast, encoding a master transcriptional activator for aa-biosynthesis

genes, is translated when eIF2 activity decreases. Reducing the levels of ternary complexes (Met-tRNA$_i^{Met}$ and eIF2-GTP) will slow the reinitiation on four upstream uORFs and allow the 40S preinitiation complex to scan through the encoding ORF of the *GCN4* gene. As a result, the *GCN4* gene is translated during the starvation (**Fig.9.**).

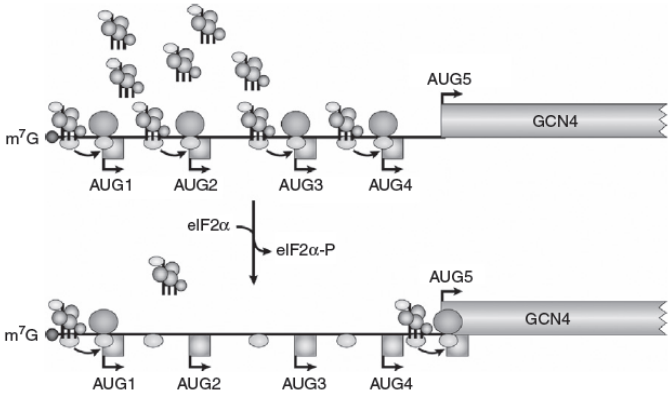


Figure.9. Translational regulation of *GCN4* by upstream open reading frames (uORFs). With low levels of eIF2α-phosparylation and abundant active ternary complex, ribosomes initiate translation at uORF1, resume scanning and reinitiate at uORF2, uORF3, or uORF4, but not at the start codon of GCN4. When cells are starved for aa, eIF2α becomes phosphorylated and the number of active ternary complexes decreases. As a result, reinitiation at uORF2-uORF4 occurs less frequently and scanning can resume at the authentic initiation codon [64].

1.5.1.2. Internal ribosome entry site (IRES)

During translation initiation, the 40S ribosomal subunit is recruited to the mRNA and positioned at the correct initiation codon by a series of intermolecular events involving a group of protein factors that assemble on the capped 5´ end of the mRNA [73]. Then, the recruited 40S ribosome will scan for the translation start site [74]. The translation initiation through this canonical pathway will be down-regulated under stress conditions. Alternatively, some mRNAs contain a secondary or a tertiary structure at the 5´UTR, called the internal ribosome entry site (IRES) that provides an alternative pathway for translation initiation. Specific mRNAs containing IRES can be continuously translated when the cap-dependent mechanism is impaired as shown in **Fig.10.** [69,75,76].

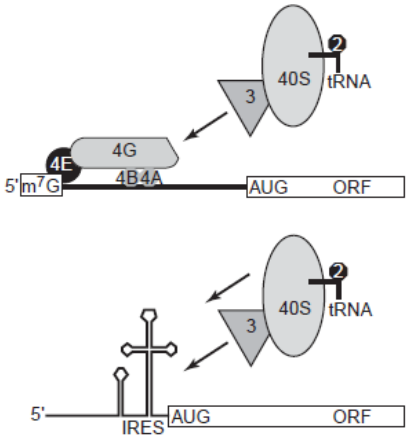


Figure.10. A schematic representation of cap-dependent (top) and HCV IRES-driven internal initiation of translation (bottom) [74].

1.5.2. 3´untranslated region (3´UTR)

The 3´UTRs contain several highly conserved REs that are responsible for stability, translation efficiency, and spatial and temporal expression of mRNAs [61,63,65,77]. In addition, the 3´UTR also plays an important role in the control of variations in gene expression during early development when there is little or no transcriptional regulation [61]. In eukaryotes, translational initiation requires the formation of circular mRNAs that are generated by binding of the polyA-binding protein (PABP) and eIF4G at the 3´UTR and 5´UTR, respectively. The 3´UTR also contains a signal for polyadenylation and serves as the binding sites for other translational regulators. In general, nuclear-exported mRNAs will have 250 nts of poly (A) tail that will be slowly removed upon entering the cytoplasm [69]. Poly (A) tails less than 50 nts will decrease translation rate, while the longer tails, more than 200 nts, will stimulate translation [78]. This translational control is normally found in early development and oocyte maturation when transcription is absent. Differential translation of maternal mRNAs is regulated in part through polyadenylation by 3´UTR *cis*-acting elements: cytoplasmic polyadenylation elements (CPE) and polyadenylation hexanucleotides (AAUAAA) [78,79]. These REs are bound by the CPE-binding protein (CPEB) and cleavage and polyadenylation specificity factor (CPSF), respectively [80,81]. Polyadenylation is initiated by phosphorylation of CPEB to enhance the association between CPEB and CPSF. As a result, poly(A) polymerase is recruited to the end of the mRNA and initiates poly(A) addition as shown in **Fig.11** [78,82].

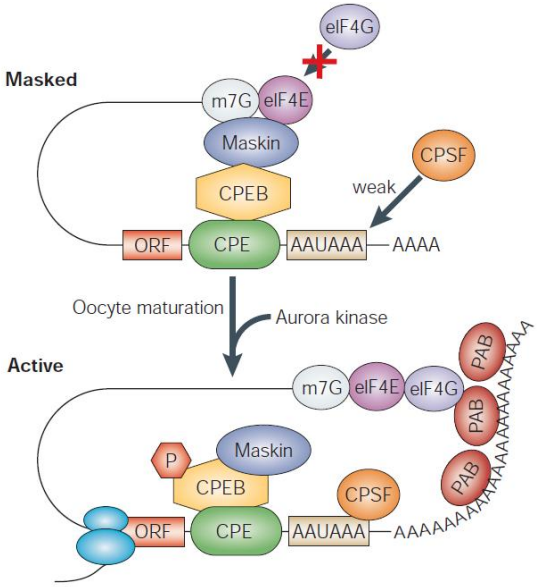


Figure.11. A schematic diagram of translational control by *cis*-regulatory elements in the 3´UTR through trans-factors: cytoplasmic polyadenylation element binding protein (CPEB) and cleavage and polyadenylation specificity factor (CPSF) [61].

2. CHAPTER 2. Molecular cloning ecdysone receptor (EcR) and retinoid-X receptor from the blue crab, *Callinectes sapidus*, and expression patterns in eyestalk ganglia and Y-organs

Abstract

Crustacean molting is regulated by interactions between the levels of ecdysteroids (Ecds) and the crustacean hyperglycemic hormone (CHH) neuropeptide family including molt-inhibiting hormone (MIH) and CHH. Ecds exert their signals through a heterodimer, nuclear receptor complex consisting of ecdysone receptor (EcR) and its partner, Ultraspiracle (USP)/retinoid-X receptor (RXR). As an Ecd peak is recapitulated in each molt cycle during the life cycle of *Callinectes sapidus*, we undertook to gain a better understanding in the regulatory role of Ecds on molt cycle by isolating cDNAs of *C. sapidus EcR* and *RXR* (*CasEcR* and *CasRXR*). Consequently, four structural isoforms of *CasEcR* and *CasRXR* cDNAs were isolated from eyestalk ganglia (ES) and Y-organs (YO). The deduced amino acid (aa) sequences of four CasEcR and CasRXR isoforms consist of five structural domains that are typically found in nuclear receptors: 1) activation function-1 (AF1), 2) DNA binding domain (DBD), 3) hinge region (HR), 4) ligand-binding domain (LBD), and 5) AF2. Four CasEcR isoforms are encoding 503-530 aa residues and show variations in the HR with a 27-aa insert and the LBD with a 49-aa substitution. On the other hand, the isoforms of CasRXRs with 399-449 aa residues are varied by a 5-aa and/or a 45-aa insert in the DBD and LBD, respectively. It appears that expression levels of *CasEcR* and *CasRXR* isoforms are tissue-specific and molt-stage dependent in the ES and YO. This indicates that different isoforms of CasEcR and CasRXR may be involved in transducing the Ecd signal during the molt cycle.

2.1. Introduction

Molting is the shedding process that is required for somatic growth and reproduction in animals belonging to the Phylum Arthropoda. Molting control was first characterized in insects where it is stimulated by the molting hormone named ecdysteroids (Ecds) [26,27,83,84]. In crustaceans, molting is regulated by interactions between the hormones that act and counter-act: stimulation by Ecd and suppression by the neuropeptide hormone family named molt-inhibiting hormone (MIH) and the crustacean hyperglycemic hormone (CHH) [85-88]. Ecds are synthesized and released from Y-organs (YO), while MIH/CHH is produced in eyestalk ganglia (ES) [89-91]. Although the inhibitory effects of MIH/CHH on ecdysteroidogenesis in the YO are established, regulation of MIH/CHH synthesis and release during the molt cycle still remains unknown [45,92,93].

Ecd transduces its signal through a heterodimer complex consisting of the ecdysone receptor (EcR) and the retinoid-X receptor (RXR) [94-98]. EcR and RXR are members of the nuclear receptor (NR) superfamily [28,47,99-101]. The NR superfamily can directly transactivate the target genes upon the binding to cognate ligands [51]. Members of the nuclear hormone receptor family are characterized by their five independent functional domains (A-F; [48]: 1) N-terminal A/B domains or activation function-1 (AF1), 2) C or DNA-binding domain (DBD), 3) D or hinge region (HR), 4) E or ligand-binding domain (LBD), and 5) C-terminal F or AF2 domain [49,50,102]. The specific function of each domain is as follows: 1) AF1 possesses ligand-independent transactivation, 2) DBD binds to regulatory elements (REs) of the target genes, 3) HR provides flexibility within the molecule, 4) LBD serves as docking sites for its cognate

ligands and transcriptional regulators (coactivator or corepressor), and 5) AF2 has ligand-dependent activation. The NRs are highly conserved in the DBD containing two zinc-finger motifs that are responsible for binding to the hormone responsive elements (HREs) [46,53,54,103]. In general, binding of the ligands to LBD renders conformational changes in the LBD and AF2, resulting in stabilization of dimerization or release of a co-repressor.

It has been proposed that Ecds may give a signal to the ES for MIH/CHH production [11]. The genetic compartmentalization of *MIH* in *Cancer pagurus* lists that he up-stream promoter region of *MIH* contains putative binding sites for transcription factors: chronic factor-1 (CF1), a homolog of RXR/ultraspiracle (USP), and broad-complex factor (BR-C) [22,24]. Furthermore, MIH/CHH activities in the molt cycle of *C. maenas* showed that both *CHH* and *MIH* transcripts were slightly higher during the premolt stage [25].

Overall, these data suggest that there may be some crosstalks between Ecds and neuropeptide hormones to control the molt cycle. Specifically, Ecds may intimately regulate the levels of MIH/CHH in the ES through the EcR-RXR complex. We aimed to examine this proposed model in *Callinectes sapidus*. *EcR* and *RXR* cDNAs were isolated. And, the levels of *EcR* and *RXR* transcripts were determined in ES and YO during the molt cycle.

2.2. Materials and methods

2.2.1. Animals and sample collection

Juvenile crabs of *C. sapidus*, (20-40 mm carapace width, CW) obtained from the blue crab hatchery at the Aquaculture Research Center [Institute of Marine and Environmental Technology (IMET), Baltimore, Maryland] were reared in individual compartments (15x15 cm) in a re-circulating closed artificial seawater system at 25 ppt salinity and 23-25 ^{o}C. The animals were fed daily with chopped squid and monitored for their growth. Animals with 70-90 mm CW were molt-staged initially [3]. The hemolymph (100ul) was withdrawn, while eyestalks (ES), and Y-organs (YO) were dissected and frozen immediately on dry-ice and kept at -80 ^{o}C until further use.

2.2.2. Total RNA extraction

Total RNA from collected tissues was extracted with QIAzol Lysis Reagent according to the manufacturer's procedure (Qiagen, Maryland, USA). In brief, tissues were lysed in 1 ml of QIAzol reagent, and 0.2 ml of chloroform was added for organic extraction. After centrifugation at 14,000 g for 15 min at 4 ^{o}C, , the upper phase containing RNA was taken and mixed with isopropanol at a 1:1 ratio. RNA was recovered by centrifugation at 14,000 g for 15 min at 4^{o} C. The supernatant was discarded, and the pellet was washed with 70% diethyl pyrocarbonate (DEPC)-treated ethanol and air-dried. The extracted RNAs were re-suspended in DEPC-treated water and quantified with a NanoDrop ND-1200 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA).

2.2.3. Messenger RNA extraction from total RNAs

Total RNAs (10-100 µg) were used for mRNA extraction using the NucleoTrap® mRNA kit (BD Biosciences Clonetech, Mountain View, CA USA) in accordance with the manufacturer's protocol. In detail, the total RNA was adjusted to 1 µg/µl with DEPC-treated water. Oligo-dT beads were added and volume adjusted to reach 100 µg of total RNAs/15 µl, mixed, and denatured at 68 ^{o}C for 5 min. The mixture was incubated at room temperature for 10 min, and briefly centrifuged at 2,000 x g for 15 sec at 4 ^{o}C and at 11,000 x g for 2 min at 4 ^{o}C. After discarding the supernatant, the pellet was washed with 3 volumes of buffer RM2 and gently mixed until it became milky. The suspension was transferred into a Nucleotrap Column and centrifuged under the same conditions as above. After discarding the flow through, the column was washed with 5 volumes of buffer RM3, and the pellet was re-suspended by pipetting, and centrifuged under the same conditions as above. The pellet was washed another time with buffer RM3 as described, and dry-centrifuged at 11,000 x g for 1 min at 4 ^{o}C. The mRNA pellet was eluted with DEPC-treated water pre-warmed to 68 ^{o}C (20 µl/10 µl Oligo-dT beads), resuspended by pipetting until it became milky, incubated at 68 ^{o}C for 7 min, and centrifuged at 11,000 g for 1 min at 4 ^{o}C. The concentration of resultant mRNAs was measured using a NanoDrop Spectrometer.

2.2.4. 5´ and 3´ Rapid Amplification of cDNA ends (RACE)

The 5´ and 3´RACE cDNAs were synthesized using a BD SMART^{TM} RACE cDNA Amplification Kit (BD Biosciences Clontech) and RevertAid^{TM} Reverse Transcriptase (Fermentas). For 5´RACE, 1 µg mRNA, 1 µl random primer (12 µM), and 1 µl BD Smart II A Oligo (12 µM: 5´–AAGCAGTGGTATCAACGCAGAGTACGCGGG–3´) were mixed and the volume was finalized to to 5 µl with DEPC-treated water. For 3´RACE, 1 µg mRNA and 1 µl of 3´CDS primer (12 µM: 5´–AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN–3´) were mixed and the volume was finalized to 5 µl with DEPC-treated water. The RNA mixtures were incubated at 70 ^{o}C for 2 min and cooled on ice for 2 min. The mixture was added 4 µl of 5X Reaction Buffer, 0.5 µl of RiboLock^{TM} RNase Inhibitor (20 U), 2 µl of dNTP mix (10 mM), and 1 µl of RevertAid^{TM} Reverse Transcriptase (200 U) and the volume was finalized to 20 µl with DEPC-treated water. After gentle mixing, the reaction was incubated at 42 ^{o}C for 1 h and terminated by incubation at 70 ^{o}C for 10 min.

2.2.5. Design of degenerate primers for cloning the full-length cDNAs of CasRXR *and* CasEcR

Degenerate primers of *RXR* (listed in **Table 2**) were designed based on the conserved regions identified from the multiple alignment (ClustalW: www.genome.jp) of crustacean *RXR* sequences including *Carcinus maenas* (ACG63787.1, ACG63788.1), *Gecarcinus lateralis* (AAZ20371.1, AAZ20368.1), *Celuca pugilator* (AAC32789.3), and *Marsupenaeus japonicus* (BAF75376.1). The first *EcR* sequence of *C. sapidus* was initially obtained by Zmora and Chung (unpublished data).

2.2.6. PCR with degenerate primers and 5´ and 3´RACE cDNA

The initial *RXR* sequence was obtained from a two-step PCR assay with degenerate primers [104]. In brief, a touch-down (TD) PCR was carried out with 3´RACE cDNA from the YO and RXR-dF1 primers and universal primers (UPM) with an Advantage Taq Polymerase (BD Biosciences). Annealing temperatures at 47 °C, 45 °C, and 43 °C were used for 3 cycles with extension at 68 °C, for 1 min, while the final annealing was achieved at 48 °C for 27 cycles. The TD products were diluted 20-fold in sterilized deionized water and used for the nested PCR with RXR-dF2 and RXR-dR1 primers with annealing at 50 °C and extension for 1 min at 68 °C. After electrophoresis, the PCR product with the expected size was excised using the QIAquick Gel extraction kit (QIAgen). The remaining procedures for cloning and sequencing were performed as described previously [104]. Based on these results from the initial sequence of *CasRXR1*, gene specific primers (RXR-5R1, RXR-5R2, RXR-3F1, and RXR-3F2) were produced for 5´ and 3´ RACE of *CasRXR*, respectively (**Table. 2**).

The full-length cDNA sequence of *CasRXR1* was obtained from 5´and 3´ RACE. For the remaining isoforms, primers specific to the start and the end of open reading frame (ORF): *CasEcR* with EcR-3Fi and EcR-Rend primers; and *CasRXR* with RXR-3Fi and RXR-Rend primers, were amplified using a high-fidelity Taq polymerase (Clontech). The rest of the procedures for cloning and sequencing were the same as stated above.

Table. 2. Lists of primer sequences used for cloning of *RXR* and *EcR*.

Primers	Primer Sequence (5´-3´)	Tm (ºC)
RXR primers		
RXR dF1	GGNAARCAYTAYGGNGTNTA	**54.3**
RXR dF2	TGYGARGGNTGYAARGGNTT	**62.5**
RXR dR1	TCNARRCAYTTNARNCCDAT	**55.3**
RXR dR2	AARTGNGGDATRTGYTTNGCCCA	**67.4**
RXR 3Fi	CTGGTGGCAGTGTTTCCCGAAGAGA	**62.7**
RXR 3F1	CACCATCGACAAGAGACAGAGGAA	**65.0**
RXR 3F2	AGGAACGTCAGAGGACAAAAGGTG	**64.9**
RXR 5R1	TATTGCTTACCACATCCCCTTGGT	**64.7**
RXR 5R2	AGATAGCGCCACAGGAGGACTCT	**64.9**
RXR 5Rend	CTAGCTGGTGGGGGAGGTGTTGCT	**65.1**
EcR primers		
EcR start	AGAGCCGCCGCGTCTCAGTGT	**66.7**
EcR Fi	ATGTTTGTGTTGGGCTCTGGTGTGG	**61.8**
EcR 3F1	ATGAGTCCTCCGACCTCCCTGGCC	**73.3**
EcR 5R1	GCAACCAGCAACATCCCAGATCTC	**68.2**
EcR Rend	ATATGGCACCCAGGAGGTGGC	**61.9**

"d" represents degenerate primers. Forward primers are designated with "F" and used for 3´ RACE, while reverse primers are coded with "R" and used for 5´RACE.

2.2.7 Spatial distributions of *C. sapidus EcR* and *RXR*

ES and YO collected from the animals at molt stages C_4, D_0, D_1, D_2, and $D_{3/4}$ were extracted for total RNAs, subsequently treated with DNase I, and used for cDNA synthesis [104]. Sample cDNAs, each containing 12.5 ng of total RNA, were amplified with a combination of primers (**Table. 3**) for each isoform of *CasEcR* (CasEcR1: F-R, CasEcR2: F-R, CasEcR1a: F-R, CasEcR2a: F-R, and CasEcRt: F-R) and *CasRXR* (CasRXR1: F-R, CasRXR2: F-R, CasRXR1a: F-R, CasRXR2a: F-R, and CasRXRt: F-R) by using GoTaq® Green Master Mix (Promega) with an annealing temperature at 58 °C and extension at 72 °C, 30 sec for 33 cycles. The expression of arginine kinase (*AK*) was determined in the same sample cDNA as a reference gene [104]. PCR products were

visualized by staining with ethidium bromide after electrophoresis on a 1.5% agarose gel and documented using a Kodak Gel Logic 200 Imaging system.

Table.3. Lists of primer sequences used for screening *CasEcR* and Cas*RXR* isoforms.

Primers	Primer sequences (5´-3´)	Tm (°C)
EcR1		
EcR1 F	CGACTCGGATGCCAAGTTTAAACAC	**58.3**
EcR1 R	GAACCGGGCTGCTCGGAGCATCATG	**65.7**
EcR2		
EcR2 F	CAGGCACATAACCGAGATGACGATC	**59.2**
EcR2 R	GAACCGGGCTGCTCGGAGCATCATG	**65.7**
EcR1a		
EcR1a F	CACTCCTAGCATCGTTCAGACTCCT	**59.6**
EcR1a R	GGCATCCGAGTCGTCATCACTTATT	**58.7**
EcR2a		
EcR2a F	CACTCCTAGCATCGTTCAGACTCCT	**59.6**
EcR2a R	CATGTCACTTGTATCTTCACCATCG	**55.7**
Total EcR		
EcRt F	GCATTGTGTTTGGAAATACCTTGCC	**57.4**
EcRt R	GCCCTCAATGCATCGAGGTATATTT	**57.2**
RXR1		
RXR1 F	GAAGCGGTCCAGGAGGAACGTCAGA	**64.0**
RXR2 R	TATTGCTTACCACATCCCCTTGGT	**64.7**
RXR2		
RXR2 F	CGGTCCAGGTAGGGGCAGTAGAGGA	**65.0**
RXR2 R	TTCCTCCTCTACTGCCCCTACCTGGAC	**64.2**
RXR3		
RXR3 F	GAAGCGGTCCAGGAGGAACGTCAGA	**64.0**
RXR3 R	GAACTTGACAATGGTTGACAAGCCTC	**58.4**
RXR4		
RXR4 F	CGGTCCAGGTAGGGGCAGTAGAGGA	**65.0**
RXR4 R	GAACTTGACAATGGTTGACAAGCCTC	**58.4**
total RXR		
RXRt F	TTCCCATAGAAGACCAAGTTGTATTA	**59.41**
RXRt R	CTGGGTTGAACAGGACAATGGCACG	**62.2**

2.2.8. Sequence analyses of *C. sapidus EcR* and *RXR*

The open reading frame (ORF) of *CasEcR* and *CasRXR* nucleotide sequences were identified by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Both the nucleotide and deduced aa sequences of *CasEcR* and *CasRXR* isoforms were aligned using ClustalW (http://align.genome.jp/). The nucleotide sequences of *CasEcR* and

CasRXR isoforms were further analyzed for putative RNA regulatory elements in the 5´ and 3´ UTR using RegRNA (http://regrna.mbc.nctu.edu.tw/html/prediction.html) [105]. Post-translational modifications including glycosylation and phosphorylation were predicted by YinOYang and NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/), respectively.

For secondary-structure prediction, the aa sequences of *CasEcR* and *CasRXR* isoforms were analyzed by PREDATOR 2.1.2 [106]. The deduced aa sequences of *CasEcR* and *CasRXR* isoforms were formatted into PDB sequences using the Phyre program (http://www.sbg.bio.ic.ac.uk/Phyre^2/, using hemipteran and *T. castaneum* as references [107]) before viewing by PyMol program (Version 1.2r3pre) for tertiary structure simulation. A phylogenetic tree of the putative EcR and RXR/USP obtained from arthropods including the CasEcR and CasRXR isoforms were generated and presented as phylograms [108] (http://www.phylogeny.fr).

2.3. Results

2.3.1. *EcR* isolation from *C. sapidus*

2.3.1.1. Full-length sequences of *EcR* gene

The full-length cDNAs of the four *CasEcR* isoforms obtained posess same 5´UTR (917 nt) and 3´UTR (759 nt), but have variations in the ORF (1,509 nt in *CasEcR1/CasEcR2* and 1,590 nt in *CasEcR1a/CasEcR2a*) (**Figs. 12 A-C**). The ORFs are predicted to encode the NR superfamily that displays the five putative conserved domains: AF1, DBD, HR, LBD, and AF2. All putative CasEcR proteins share identical AF1 (141 aa residues), DBD (90 aa residues), and AF2 (3 aa residues). However, the HR

and LBD are varied in each isoform where a 27-aa insert and a 49-residue substitution are located, respectively (**Figs. 12** and **13**). Isoforms containing the 27-aa insert in HR (named as HR^{i}) are designated with "a," while those which have a variation in the LBD are referred as "1" or "2."

The 5´UTR of *CasEcR* analyzed by RegRNA appears to contain translational regulation sites with two internal ribosome entry sites (IRES) and two upstream open reading frames (uORFs). Two IRES are located at C_{286}-G_{366} and C_{505}-C_{692} (underlined in **Fig. 12 A**), while two uORFs are located in different frames: uORF1 (A_{706}-A_{777}) and uORF2 (A_{913}-$A_{1,068}$) (highlighted in grey in **Fig. 12 A**). Both uORF1 and uORF2 are located downstream of the second IRES, while uORF2 overlaps the major ORF (mORF). The uORFs are predicted to encode two short peptides: uORF1 with 6 aa residues (MALALT) and uORF2 with 51 aa residues (MECLCWALVWPHSTSPPWGTRVAPRCPAHHRSPALVPSPRQHSSASASTLA).

(A)
CasEcR1 (HQ630857)

```
   1  AGAGTTGCCCGCCAGCCGTCGCCGGGTGAGCACCTCATCGCTCGTGTCCGCCTCTCTCCACTACGTTTCG
  71  TGTCCTTCCTCGCGCGTGTTTCGTGCTTTACATTGACTCTTGAGTGCTGCAGTAAAAGAACACCTTGTCG
 141  CGTGTGACAACAATACGTATATTGTTGGCGACAACTTTCTTTTACTGTCAGAGAAACTTTGTCATTCCGT
 211  CGCGTTTGTTTTGTTGGTGCAAGAGAGTGCCGGACGCTATTGTAAGCCAAGTTTTTACTTACCTTTGATT
 281  CTTGACATTGGCTCAGTAACAGACAACCGCAGTCTCACGACCCCGCATCAGTTTAACCGATAACAAGACG
 351  TGTAAGATAAGATAAGTCACCAATAGTCCGTCGTTGTGAACGAGAGCCGCCGCGTCTCAGTGTTGGAGTG
 421  ACTGGCCTCGCGAGAGTTCCAGCGGCACACAAGGTCAAGTTCATTCGCCGCCTCCACCCCCGCACCGTTG
 491  TCCCTTCACTCCTTCATAAACTGCTATTGAACCTGTTGCTCGAGGAAAGCCAAGTGAAAGGAAAAGTTGC
 561  TTTCGACCTGACCTGGTAACCGACGCCATTTGACCCAAATACTACGCCACCCATTCACCAACACTGACAG
 631  GGCACACTTGACCTACACGAGCCACTCCACCACCACTACAGTGAGAGTGGGAGGAGCAGCACACACCCGC
 701  TGAAGATGGCTCTGGCCCTTACATAAGTGATCCAGAGTCATGACCTAAGATGAGCCCTGCTGAATACTAC
 771  CAATTGATCCTCGTGAACTCACCACCCACTACCAAGTGATCTATTGGACGGGCCATTGTTTCTCACGTGT
 841  GACAGTCAGTGGACGACGCGTGACGGCCGCCGCGCCAACGTTTGGCCAAAGTGCCGGCGACAGCGCGTGT
 911  CGATGGA
```

```
 918 atgtttgtgttgggctctggtgtggccacactcaacctctccgccatgggggacgagagt
     M  F  V  L  G  S  G  V  A  T  L  N  L  S  A  M  G  D  E  S  20
 978 tgctccgaggtgtccagctcatcaccgctcaccagccctggtgccctctccccgccagca
     C  S  E  V  S  S  S  S  P  L  T  S  P  G  A  L  S  P  P  A  40
1038 ctcgtcagcgtcggcgtcaacgttggcatgagtcctccgacctccctggcctcctcagac
     L  V  S  V  G  V  N  V  G  M  S  P  P  T  S  L  A  S  S  D  60
1098 atcggcgaggtggacctggacttctgggatcttgatctcaactccccaagccccccacac
     I  G  E  V  D  L  D  F  W  D  L  D  L  N  S  P  S  P  P  H  80
1158 ggcatggcatccatcgcttccaccaacgccctcttgctcaacccccgtgctatggcctca
     G  M  A  S  I  A  S  T  N  A  L  L  L  N  P  R  A  M  A  S  100
1218 ccctccgacacctcctccctatcagggcgagatgacatgtcgccgccctcttcagtgggc
     P  S  D  T  S  S  L  S  G  R  D  D  M  S  P  P  S  S  V  G  120
1278 aactacagcgccgattccttcggcgatctgaagaagaaaaaaggccccatccctcgccag
     N  Y  S  A  D  S  F  G  D  L  K  K  K  K  G  P  I  P  R  Q  140
1338 caggaggaactgtgtttagtgtgcggggaccgggcgtcaggctaccaccacaatgcactc
DBD  Q  E  E  L  C  L  V  C  G  D  R  A  S  G  Y  H  H  N  A  L  160
1398 acctgcgagggatgcaaaggcttcttccgaagatccatcacaaagaatgctgtgtaccag
     T  C  E  G  C  K  G  F  F  R  R  S  I  T  K  N  A  V  Y  Q  180
1458 tgtaaatatggtggcaactgtgaaatggacatgtacatgcgacgcaagtgtcaagagtgt
     C  K  Y  G  G  N  C  E  M  D  M  Y  M  R  R  K  C  Q  E  C  200
1518 cgcctcaaaaagtgtcttggtgtgggcatgcgaccagaatgtgtcgtgccagagtctcag
     R  L  K  K  C  L  G  V  G  M  R  P  E  C  V  V  P  E  S  Q  220
1578 tgcgtggtgaagagagagcagaagaagctacgagacaaggataagaaggactatccaagt
     C  V  V  K  R  E  Q  K  K  L  R  D  K  D  K  K  D  Y  P  S  240
1638 caaggctccccagtagctgaggaaaaggccgttccaacaagtccaatgtcagccgggggcc
     Q  G  S  P  V  A  E  E  K  A  V  P  T  S  P  M  S  A  G  A  260
                                                 +81 nt
1698 agatcaaatgtcaaaccactcactcgagagcaggaggagctgatcaacacactagtctac
     R  S  N  V  K  P  L  T  R  E  Q  E  E  L  I  N  T  L  V  Y  280
                                LBD
1758 taccaagaacagtttgaacagcctactgaagcagacatcaaaaagataagagattacgaa
     Y  Q  E  Q  F  E  Q  P  T  E  A  D  I  K  K  I  R  D  Y  E  300
1818 ataagtgatgacgactcggatgccaagtttaaacacatcacagacatgaccatcctcaca
     I  S  D  D  D  S  D  A  K  F  K  H  I  T  D  M  T  I  L  T  320
1878 gtgcagctgatcgtagagttctccaagcgcctgcccggctttgacacactactcagagag
     V  Q  L  I  V  E  F  S  K  R  L  P  G  F  D  T  L  L  R  E  340
1938 gatcagattacactgctaaaggcttgctcatcagaagtcatgatgctccgagcagcccgg
     D  Q  I  T  L  L  K  A  C  S  S  E  V  M  M  L  R  A  A  R  360
1998 ttctatgatgccaaaacagactgcattgtgtttggaaataccttgccatacacacaaaca
```

```
     F  Y  D  A  K  T  D  C  I  V  F  G  N  T  L  P  Y  T  Q  T 380
2058 tcatatgagtttgctggcttgggagaatcatcacaaatactcttccgtttttgtcgcaac
     S  Y  E  F  A  G  L  G  E  S  S  Q  I  L  F  R  F  C  R  N 400
2118 ctatgtaaaatgaaagttgataatgctgagtatgcacttctgtctgccataatcatattt
     L  C  K  M  K  V  D  N  A  E  Y  A  L  L  S  A  I  I  I  F 420
2178 tcagagaggccaaacctaaaggaactccagaaggtggaaaagcttcaagaaatatacctc
     S  E  R  P  N  L  K  E  L  Q  K  V  E  K  L  Q  E  I  Y  L 440
2238 gatgcattgagggcatacgtatgtaatcaaaggtttccccgacctggcatggtgtttgca
     D  A  L  R  A  Y  V  C  N  Q  R  F  P  R  P  G  M  V  F  A 460
2298 aagttgcttaatatcctcactgagttacgaacccttggaaacttgaactcagaggtatgc
     K  L  L  N  I  L  T  E  L  R  T  L  G  N  L  N  S  E  V  C 480
2358 ttctccctcaaactcaagaacaaaagactcccaccatttttggctgagatctgggatgtt
     F  S  L  K  L  K  N  K  R  L  P  P  F  L  A  E  I  W  D  V 500
2418 gctggttgctga
     A  G  C  *  503

2430 GCTTCAGCCACCTCCTGGGTGCCATATTACCGAGGTAAAATATTGGCGCAAGAAGGTTGTGAGCCCTTTG
2500 GGGGTGGTGGATGGACTCTACACTATGTACCTGTCGGCTGTAAGTGAGAGACTGTTGATGTTGTGGCAGC
2570 AGCTTCACACTGCCACCCCAGGGCCCACTTTCAGCCACCTGACGTGTTTATGGTTCAGGCCACCACTGTG
2640 ACAGTGCTGTGTAGCACTCTATTAGTGCAATGATAAGTGTATAATGATGCACTTTAGATGTGAAGGGCAG
2710 CAGTATGGGCATTGTAGCCATTTTTATTAACACATTTTATAAAACCTTCAGATATAATGAAAGTGGTGTG
2780 CATATGACTGTTTCATATACGTATACCTACATGTATGTGTAAAATGTTTTTTGCTATGTAATTTTTTTAT
2850 ATAATATTTAAAGTAAAAAGGGCTACATTGCACTGTTGTTCAGACACTGCGCAAGCCACATTTTGGCAGG
2920 CAACGAACTCCCCGTGCCTTGCTTACGGTCAAGCCTCTGTTTCCTACACTATTCTCAAGGTGCTCCTGCG
2990 GGACTGAGGAGAGGGGAATCTAAACACAAGGAACAGTGCGATGTCTTTTGATCCATATTTTTATATGAAT
3060 TCTTGTTAATTTAATGAGCTGGTATGCCGTCCTTACAAGTAGTAAACTATCGAAAATATATGTTAATATT
3130 ACTGTAATATTATTAATACTGCTATGAAATGTAAAAAAAAAAAAAAAAAAAAAAAAA 3188
```

(B)

+81-nt insert within HR[i] of *CasEcR1a* (HQ630859) and *CasEcR2a* (JQ771939) isoforms

```
tgtaaatccaaaggtccatcacctgcatgtgatatgcagttcaaaaatcttgttgacact
C  K  S  K  G  P  S  P  A  C  D  M  Q  F  K  N  L  V  D  T
cctagcatcgttcagactcct
P  S  I  V  Q  T  P
```

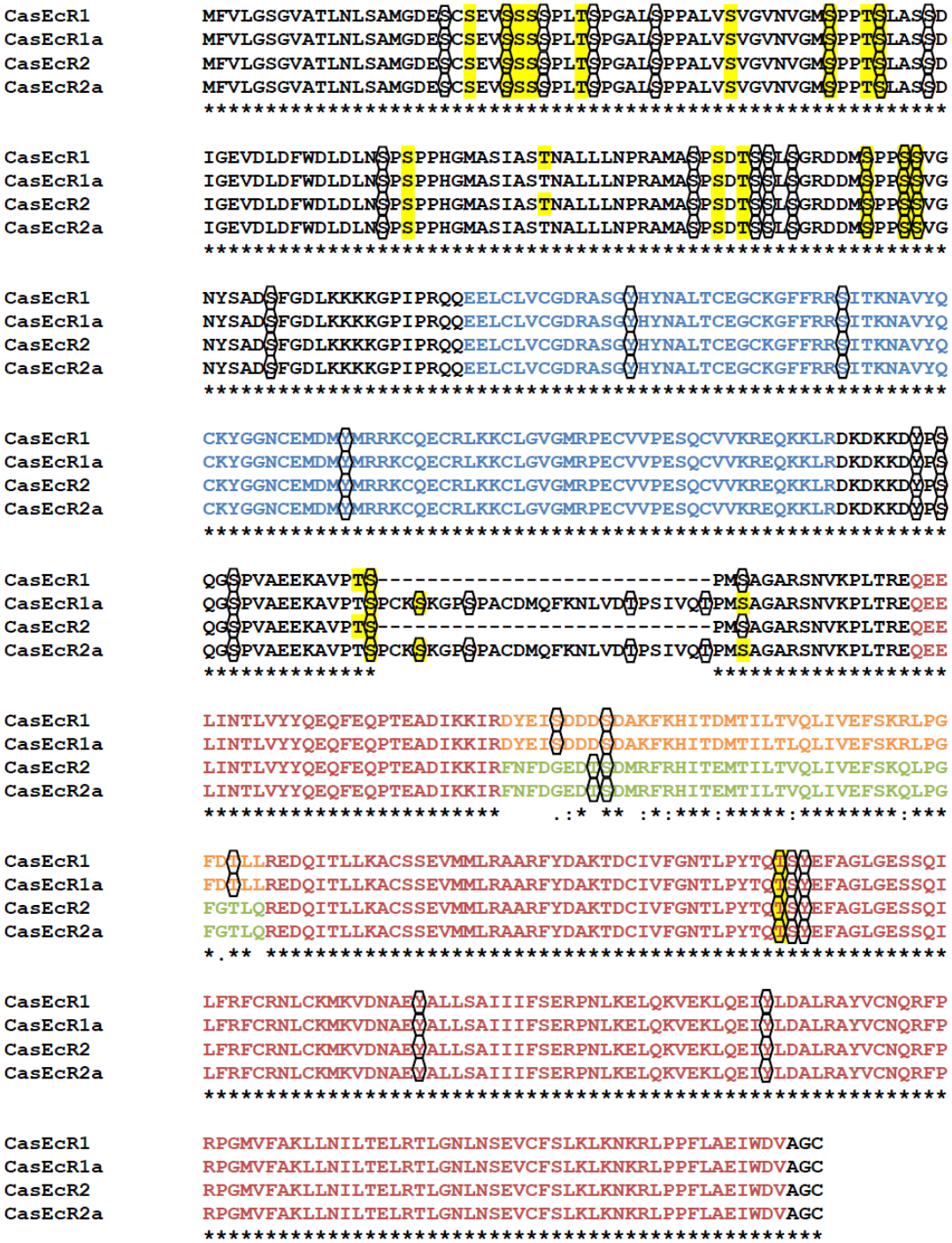
(C)

147-nt substitution in *CasEcR2* (HQ630858) and *CasEcR2a*

```
tttaacttcgatggtgaagatacaagtgacatgagattcaggcacataaccgagatgacg
F  N  F  D  G  E  D  T  S  D  M  R  F  R  H  I  T  E  M  T
atcctcacagttcagctcatagtggaattctccaagcaactaccaggtttcggcacactt
I  L  T  V  Q  L  I  V  E  F  S  K  Q  L  P  G  F  G  T  L
cagcgagaagaccagattaccctgctc
Q  R  E  D  Q  I  T  L  L
```

Figure.12. (A) Full-length sequence of *CasEcR1* and its putative regulatory elements analyzed by RegRNA; grey hightlight = uORF; underline = IRES; bold underline = DBD and LBD; triangle = insertion in *CasEcR1a*/*CasEcR2a* isoforms (B), and arrows = nucleotide substitution in *CasEcR2/CasEcR2a* isoforms (C). The numbers on the left designate the nucleotides whereas those on the right refer to the amino acid residues encoded in the above nucleotides.

(A)



(B)

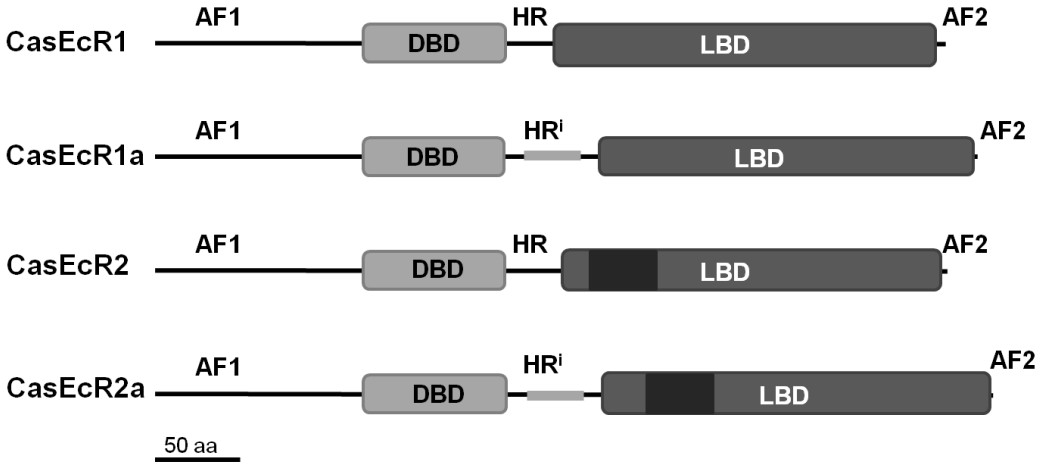


Figure. 13. (A) Multiple alignment of deduced aa sequences of four *CasEcR* isoforms (ClustalW). Blue and magenta alphabets are DBD and LBD, respectively. A 49-aa substitution in the LBD of CasEcR1/CasEcR1a and CasEcR2/CasEcR2a is presented in orange and green, respectively. Glycosylation sites are highlighted in yellow (YinOYang), and phosphorylation sites are marked with hexagons (NetPhos 2.0 Server). (B) Schematic diagram shows variations in four isoforms regarding to structure compartmentalization.

2.3.1.2. Post-translational modification predicted in *CasEcR*

The glycosylation sites predicted are marked in yellow in **Fig. 13 A**. The AF1 domain has the highest number of glycosylation sites: 16 residues in CasEcR1/CasEcR2 and 15 residues in CasEcR1a/CasEcR2a, while the HR^i within CasEcR1a/CasEcR2a has one more glycosylation site than the HR of CasEcR1/CasEcR2. The LBDs of all four isoforms show only one glycosylation site, whereas none is found in the DBD and AF2.

The majority of predicted phosphorylation sites are found in the AF1 domain (17 aa residues, marked with hexagon in **Fig. 13 A**). The insert fragment in HR^i of CasEcR1a/CasEcR2a provides three more sites, and the LBD of CasEcR1/CasEcR1a has one additional site compared to that of CasEcR2/CasEcR2a. For the DBD, all four

CasEcR isoforms contain three phosphorylation sites at Y_{155}, S_{172}, and Y_{192}, while none are found in the AF2 domain. The number of the predicted sites in each domain of CasEcR is listed in **Table.4.**

Table. 4 Post-translational modifications in CasEcR: O-linked glycosylation predicted by YinOYang 1.2 and phosphorylation predicted by NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/)

Isoforms	O-linked glycosylation (residues)					Phosphorylation (residues)				
	AF1	DBD	HR	LBD	AF2	AF1	DBD	HR	LBD	AF2
CasEcR1	16	0	2	1	0	17	3	5	8	0
CasEcR2	16	0	2	1	0	17	3	5	7	0
CasEcR1a	15	0	3	1	0	17	3	8	8	0
CasEcR2a	15	0	3	1	0	17	3	8	7	0

2.3.1.3. Structural prediction and computational analysis of CasEcR isoforms

When isoforms of CasEcR are analyzed for secondary structure within the encoding sequences by PREDATOR 2.1.2 [106], helices (H) and β-sheets (E) are formed in every domain except AF2 (see **Table.5.**). The structural conformation of the AF1 domain is similar in all four isoforms, and the domain is predicted to form three sheets and one helix. On the other hand, the DBD has number of β-sheet that relate to the 27-aa insertion at the HR in which the *CasEcR1/CasEcR2* has four sheets while *CasEcR1a/CasEcR2a* has five, but all isoforms have the same three helices. On the HR itself, the inserted fragment has likely adopted one more sheet and helix as shown in CasEcR1a/CasEcR2a. For the LBD, all four isoforms have four sheets but contain different helices. However, none of the secondary structures are predicted to form in the AF2 domain.

Table. 5 Secondary structure of deduced aa sequences of CasEcR predicted by PREDATOR 2.1.2. H: helix, E: β-sheet, AF1: activation function-1, DBD; DNA-binding domain, HR: hinge region, LBD: ligand-binding domain, and AF2: activation function-2

Isoforms	**AF1**		**DBD**		**HR**		**LBD**		**AF2**
	H	E	H	E	H	E	H	E	
CasEcR1	1	3	3	4	1	-	7	4	-
CasEcR2	1	3	3	4	1	-	8	4	-
CasEcR1a	1	3	3	5	2	1	9	4	-
CasEcR2a	1	3	3	5	2	1	9	4	-

The computational analysis of the putative LBD of CasEcR1/CasEcR1a (D_{298}-L_{338}) and CasEcR2/CasEcR2a (F_{298}-Q_{338}) reveals that the variable residues are located in the ligand-binding pocket (LBP) and appear to have an effect on the hydrophobic field of the LBP (**Figs. 14 A** and **B**). The simulated LBP of CasEcR2/CasEcR2a isoforms is more hydrophobic than that of CasEcR1/CasEcR1a.

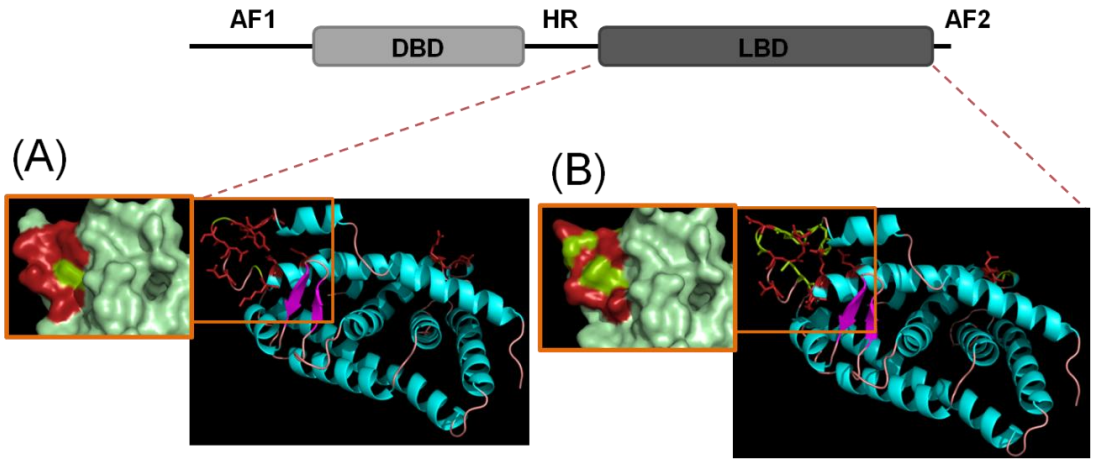


Figure.14. Putative protein structure of CasEcR1 and CasEcR2 by PyMol program base on X-ray crystallography of EcR-USP complex bound to ponasterone A of *Heliothis virescens* [107]. The Number in the structural compartment represents aa residues in each domain. Variations in *CasEcR1* and *CasEcR2* are labeled in the ball-and-stick model (green and red representing hydrophobic and hydrophilic residues, respectively). The legends in (**A**) and (**B**) represent surface model in variations of CasEcR1 and CasEcR2, respectively.

2.3.1.4. Relationship of arthropod EcRs

The relationship of four putative CasEcR sequences were compared with other arthropod EcR sequences using human farnesoid X receptor (HosFXR) as an outgroup, which resulted in two clades: one for crustaceans and the other for insects (**Fig. 15**). CasEcRs are most closely related to *C. maenas* EcR and with *Apis mellifica* EcR among the crustaceans and insects, respectively.

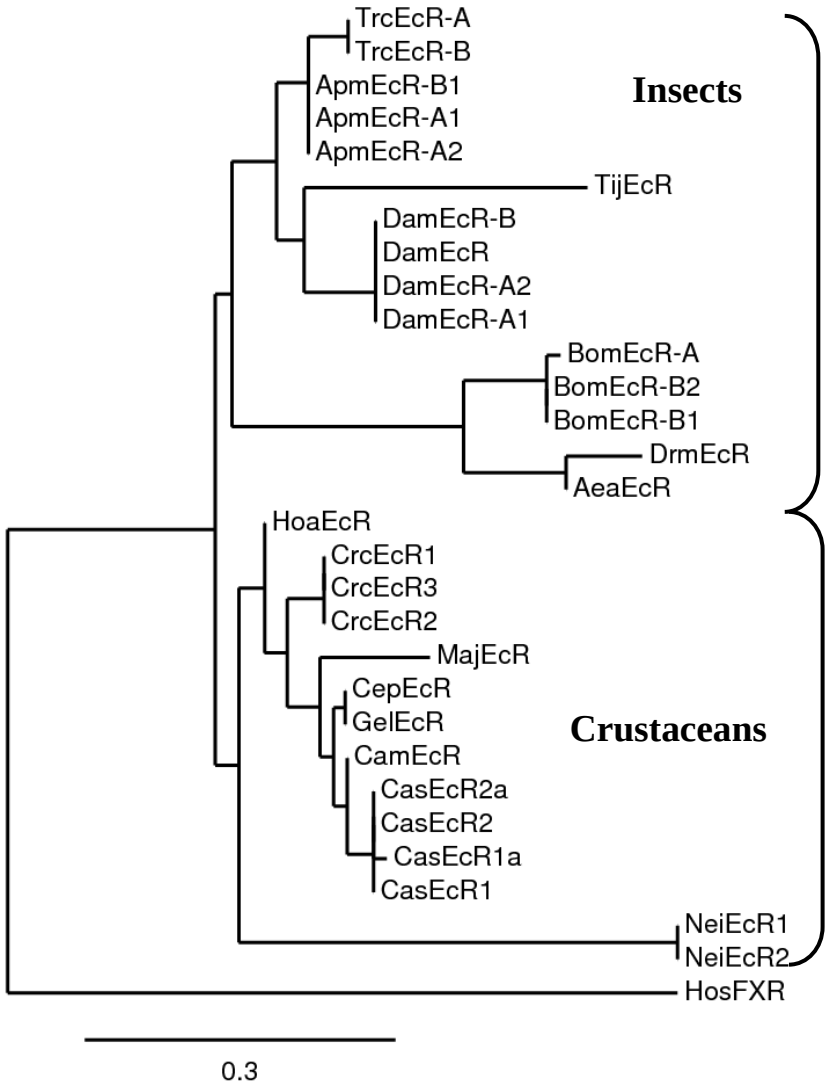


Figure.15. A phylogram of ecdysteroid receptor (EcR) in arthropods generated by *Phylogeny.fr*: EcR homolog in human (HosFXR accession: AAH71778.1) is served as the outgroup. *A. aegyptii* (AeaEcR: AAA87394.1), *A. mellifica* (ApmEcR: NP_001152827.1, NP_001091685.2, BAF46356.1), *B. mori* (BomEcR: NP_001037331.2, NP_001166846.1, NP_001166848.1), *C. sapidus* (CasEcR: HQ630857, HQ630858, HQ630859, JQ771939), *C. maenas* (CamEcR: AAR89628.1), *C. pugilator* (CepEcR: AAC33432.2), *C. crangon* (CrcEcR: ACO44665.1, ACO44666.1, ACO44667.1), *D. magna* (DamEcR: BAF49032.1, BAF49033.1, BAF49030.1, ABP48741.1), *D. melanogaster* (DrmEcR: AAA28498.1), *G. lateralis* (GelEcR: AAT77808.1), *H. americanas* (HoaEcR: AEA29831.1), *N. integer* (NeiEcR: ACO92359.1, ACJ68423.1), *T. japonicus* (TijEcR: ADD82902.1), *T. castaneum* (TrcEcR: CAL25730.1). The scale bar represents sequence variation among the analyzed sequences.

2.3.2. Isolation of *C. sapidus RXR* cDNA

2.3.2.1. Full-length cDNA sequences of *C. sapidus RXR*

The full-length cDNA sequences of the four *CasRXR* isoforms obtained posess the same nucleotide sequence in the 5´UTR (181 nt) and the 3´UTR (209 nt) but have a different sized mORF ranging from 1,200 to 1,350 nt (**Figs. 16 A-C**). Similar to CasEcR, the four putative CasRXR proteins contain five functional domains in the following order: AF1, DBD, HR, LBD, and AF2. These four deduced CasRXR proteins have identical AF1 (71 aa residues), HR (20 aa residues), and AF2 (28 aa residues). However, variations can be seen in the DBD and/or LBD with a 5- and/or a 45-aa insert, respectively (**Figs. 16** and **17**). The 5-aa insert is positioned at the T-box of the DBD, where the dimerized interface with the DNA target sequence is located, (called DBD^T), while the 45-aa insert generates a longer LBD (LBD^L). Therefore, four CasRXR isoforms can be classified as the following: CasRXR1, CasRXR1a (+5: DBD^T), CasRXR2 (+45: LBD^L), and CasRXR2a (+5, +45: DBD^T, LBD^L).

Translational regulation sites were found in the 5´UTR of *CasRXR*, when it was analyzed by RegRNA. Both an IRES and an uORF are predicted and located at A_{17}-A_{88} and T_{99}-G_{184}, respectively (**Fig. 16**). The last 3 nt (ATG) of the IRES are overlapped with the first codon of the mORF. The uORF is predicted to encode a putative19-aa peptide (MNAGGGRPCLYSLFVNNLF).

(A)
CasRXR1 (HQ630860)

```
  1   ACAGCGGCAGGCAGACATGAACGCTGGTGGTGGTCGGCCATGTTTATATTCTCTGTTTGTCAATAATCTC
 71   TTCTAACTACCGATTTGACTAACATTTTTTTTTTACTCCGAACGTTACTTCCGCGGCGAGTGACAGCTGG
141   TGGCAGTGTTTCCCGAAGAGAGTCCAGGTGTGGATATCGGC

182 atgtccggctccctggatcgccagtccccccctcagcgtggcgccagacaccgtgtccctc
    M  S  G  S  L  D  R  Q  S  P  L  S  V  A  P  D  T  V  S  L  20
242 ctctcccccgcgcccagcttctccaatgccaatggtggaccggcatcgcccagcatatcg
    L  S  P  A  P  S  F  S  N  A  N  G  G  P  A  S  P  S  I  S  40
302 acatctcccttcaccattggctcaagcaacaccaccagcttgagtacctcccccacccag
    T  S  P  F  T  I  G  S  S  N  T  T  S  L  S  T  S  P  T  Q  60
362 tacccccccagccacccactgtccggctccaagcacctctgctccatatgtggtgaccgg
    Y  P  P  S  H  P  L  S  G  S  K  H  L  C  S  I  C  G  D  R  80

                          DBD
422 gcctcaggcaaacactatggcgtgtacagttgtgaagggtgcaaggggttcttcaagcgg
    A  S  G  K  H  Y  G  V  Y  S  C  E  G  C  K  G  F  F  K  R  100
482 acagtgcgcaaggacctgacatatgcctgtcgagaagagaggtcatgcaccatcgacaag
    T  V  R  K  D  L  T  Y  A  C  R  E  E  R  S  C  T  I  D  K  120
542 agacagaggaaccgttgccagtattgccggtaccagaagtgcttgtccatggggatgaag
    R  Q  R  N  R  C  Q  Y  C  R  Y  Q  K  C  L  S  M  G  M  K  140
602 agagaagcggtccaggaggaacgtcagaggacaaaaggtgacaaaggagacggagacaca
    R  E  A  V  Q  E  E  R  Q  R  T  K  G  D  K  G  D  G  D  T  160
                    +15
                          LBD
662 gagtcctcctgtggcgctatctccgacatgcccatcgccagcattcgtgaggccgagctc
    E  S  S  C  G  A  I  S  D  M  P  I  A  S  I  R  E  A  E  L  180
722 agtgtggatcccatcgatgagcagccactggaccaaggggatgtggtaagcaatatctgc
    S  V  D  P  I  D  E  Q  P  L  D  Q  G  D  V  V  S  N  I  C  200
                                +135
782 caggcagctgatcgacatctggtgcagctggtggagtgggccaaacatatcccacacttc
    Q  A  A  D  R  H  L  V  Q  L  V  E  W  A  K  H  I  P  H  F  220
842 acagaccttcccatagaagaccaagttgtattactcaaggctggctggaatgagctgctc
    T  D  L  P  I  E  D  Q  V  V  L  L  K  A  G  W  N  E  L  L  240
902 attgcttcattctcacaccgcagcataggagtggaggatggcattgtgctggccacaggc
    I  A  S  F  S  H  R  S  I  G  V  E  D  G  I  V  L  A  T  G  260
962 cttgtggtgcacagaagtagtgctcaccaggctggggtgggagccatatttgaccgtgtc
    L  V  V  H  R  S  S  A  H  Q  A  G  V  G  A  I  F  D  R  V  280
1022 ttatccgagctggtgtccaagatgaaagaaatgaagatggataagacagaactgggttgt
    L  S  E  L  V  S  K  M  K  E  M  K  M  D  K  T  E  L  G  C  300
1082 ctccgtgccattgtcctgttcaacccagatgccaaaggagtgaactgctgcagcgatgtg
    L  R  A  I  V  L  F  N  P  D  A  K  G  V  N  C  C  S  D  V  320
1142 gagatcctgcgtgaaaaagtgtatgcagcccttgaggagtacacccgcaccacctaccca
    E  I  L  R  E  K  V  Y  A  A  L  E  E  Y  T  R  T  T  Y  P  340
1202 gacgagcccggccgcttccccaagctgctgctgcgactcccatcactcaggtccatcggg
    D  E  P  G  R  F  P  K  L  L  L  R  L  P  S  L  R  S  I  G  360
1262 ctcaagtgtcttgaatacctcttcttcttcaagctcattggagacacacccctggatagc
    L  K  C  L  E  Y  L  F  F  F  K  L  I  G  D  T  P  L  D  S  380
1322 tacttgatgaaaatgctggtggacaacccaagtcccagcaacacctcccccaccagctag
    Y  L  M  K  M  L  V  D  N  P  S  P  S  N  T  S  P  T  S  *  399

1382 GCCCCCACCAGCCACCAAGTGGTGTTCGCAATCGGCGTTCCCCAAATACTCGTGAAGTCTGTTTTTATTG
1452 CAAGGGGAGGTCCAGTGGAATGCTTTCAGTCCTCTGTGCTCACTTGTGGTCTGAATGTTGCCCATCTTGA
1522 TGTGGAGTTCTGCCAGTCAAGTGTGTCACCCCTGCATGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

(B)

+15-nt insert within DBD^T of *CasRXR1a* (JQ771940) and *CasRXR2a* (JQ771942)

```
gtaggggcagtagag
V  G  A  V  E
```

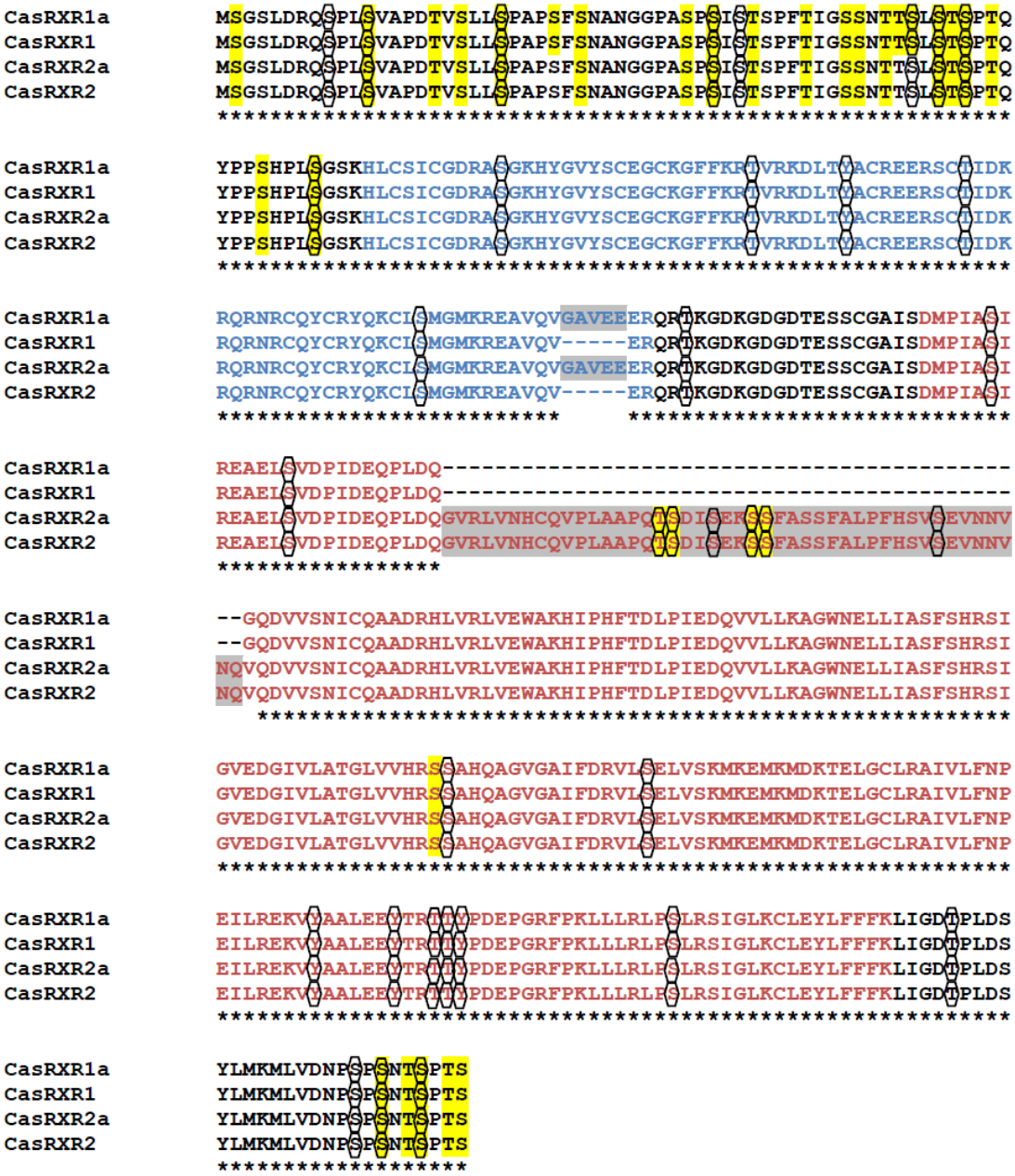
(C)

+135-nt insert within LBD^L of *CasRXR2* (JQ771941) and *CasRXR2a*

```
gtgaggcttgtcaaccattgtcaagttccactggcagcaccacagaccagt
V  R  L  V  N  H  C  Q  V  P  L  A  A  P  Q  T  S
gatataagtgaaaagagtagctttgccagtagctttgcattacctttcat
D  I  S  E  K  S  S  F  A  S  S  F  A  L  P  F  H
tctgtcagtgaagttaacaacgttaatcaagtggatgtg
S  V  S  E  V  N  N  V  N  Q  V  D  V
```

Figure.16 A) Full-length sequence of *CasRXR1* and its putative regulatory elements analyzed by Regulatory RNA analysis program [105]; grey highlight = uORF; line = IRES; bold line = DBD and LBD; and triangles = insertion sites. B) 15-nt insert in DBD^T of CasRXR1a and CasRXR2a, and C) 135-nt insert in LBD^L of CasRXR2 and CasRXR2a. The numbers on the left designate the nucleotides whereas those on the right refer to the amino acid residues encoded in the above nucleotides.

(A)



(B)

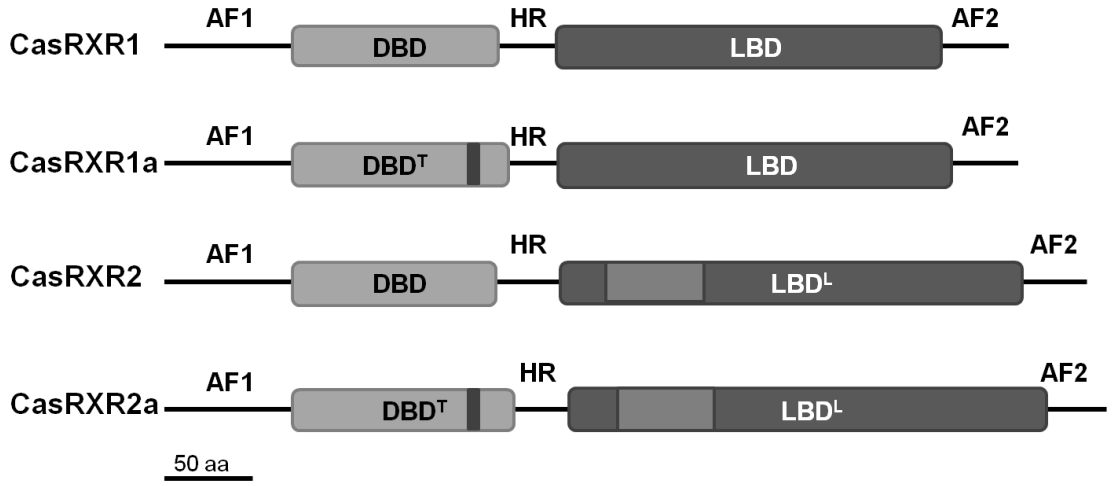


Figure.17. (A) A multiple sequence alignment of putative aa sequences of CasRXR isoforms. Alphabets in blue and magenta represent DBD and LBD, respectively. Grey highlight indicates the insertion fragment. Glycosylation sites are highlighted in yellow (YinOYang), and phosphorylation sites are marked with hexagons (NetPhos 2.0 Server). (B) Schematic diagrams show structural variations in the compartmentalization of four isoforms.

2.3.2.2. Post-translational modification predicted in CasRXR

The four encoded CasRXR proteins were examined for putative O-linked glycosylation and phosphorylation (**Fig. 17 A)**. The highest number of glycosylation sites is predicted at the AF1 domain (19 to 22 aa residues), followed by the AF2 domain (5 aa residues) and LBD (2 to 6 aa residues). No glycosylation site is predicted in the DBD or the HR. In contrast, phosphorylation sites are found in the LBD (10 to 16 aa residues), the AF1 domain (9 aa residues), the DBD (5 aa residues), the AF2 domain (4 aa residues), and the HR (1 aa residue). The number of the predicted sites in each domain of CasRXR is listed in **Table. 6**.

Table.6. Post-translational modifications in CasRXR: O-linked glycosylation predicted by YinOYang 1.2 and phosphorylation predicted by NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/)

Isoforms	**O-linked glycosylation (residues)**					**Phosphorylation (residues)**				
	AF1	DBD	HR	LBD	AF2	AF1	DBD	HR	LBD	AF2
CasRXR1	22	0	0	2	5	9	5	1	10	4
CasRXR1a	22	0	0	2	5	9	5	1	10	4
CasRXR2	19	0	0	6	5	9	5	1	16	4
CasRXR2a	19	0	0	6	5	9	5	1	16	4

2.3.2.3. Relationship of arthropod USP/RXR

The relationship of the four putative CasRXR shows a similar pattern to that of CasEcRs in which two clades are generated: one for crustaceans and the other for insects, having human RXR-β as an outgroup (**Fig. 18**). CasRXRs are most closely related to *C. maenas* RXR and with *A. mellifica* USP among the crustaceans and insects, respectively.

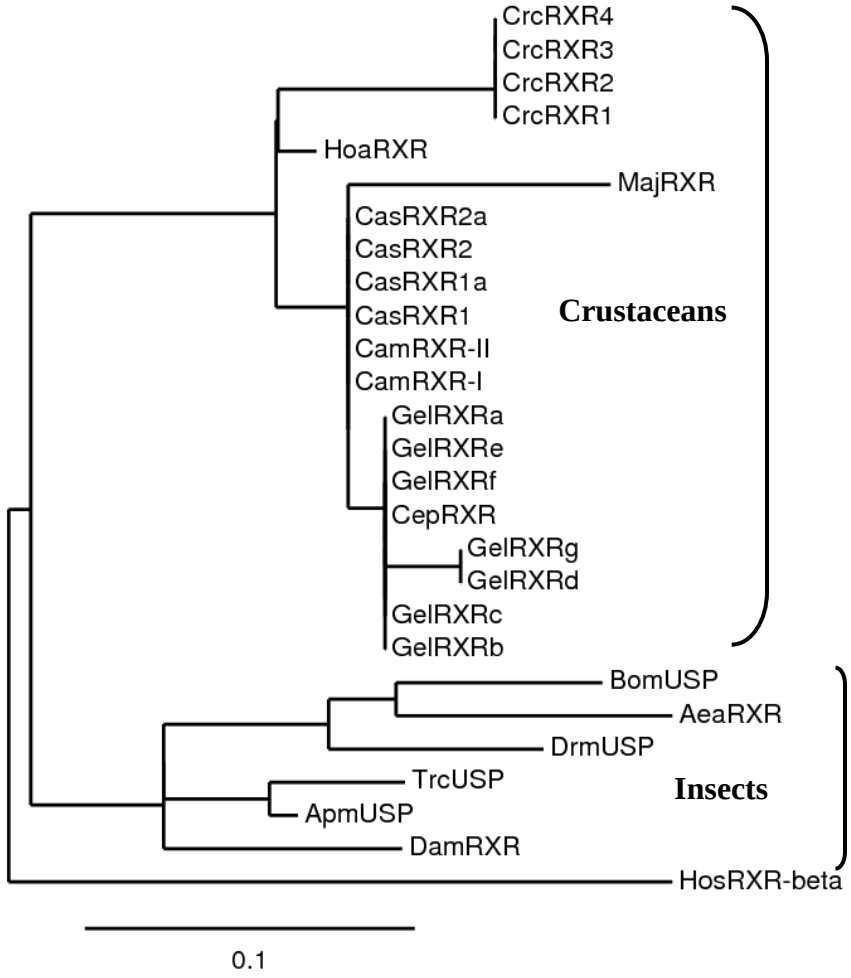


Figure.18. A phylogram of RXR/USP sequences in arthropods analyzed by *Phylogeny.fr* with human RXR-β (AAB24275.1) as the out-group. *A. aegyptii* (AeaRXR: AAG24886.1), *A. mellifica* (ApmUSP: NP_001011634.1), *B. mori* (BomUSP: NP_001037470.1), *C. sapidus* (CasRXR: HQ630860, JQ771940, JQ771941, JQ771942), *C. maenas* (CamRXR: ACG63787.1, ACG63788.1), *C. pugilator* (CepRXR: AAC32789.3), *C. crangon* (CrcRXR: ACO44671.1, ACO44668.1, ACO44669.1, ACO44670.1) , *D. magna* (DamRXR: ABF74729.1) , *D. melanogaster* (DrmUSP: AAF45707.1), *G. lateralis* (GelRXR: AAZ20368.1, AAZ20369.1, AAZ20370.1, AAZ20371.1, AAZ20372.1, AAZ20373.1, AAZ20374.1), *H. americanas* (HoaRXR: AEA29832.1), *M. japonicus* (MajRXR: BAF75376.1), *T. castaneum* (TrcUSP: NP_001107650.1). The scale bar represents sequence variations among the analyzed sequences.

2.3.2.4. Computational prediction and structural modeling of CasRXR

The secondary structure of the four CasRXR isoforms obtained was analyzed using PREDATOR 2.1.2. All four CasRXR isoforms (**Table.7**) contain three and six helices (H) in the DBD and the LBD, respectively, and two β sheets (E) in the DBD. However, the 45-aa insert found in the LBD^{L} of CasRXR2/CasRXR2a forms two more β sheets. The other domains (AF1, HR, and AF2) were not predicted to contain either H or E structures.

In order to predict the 3-dimentional (3D) structure of the four CasRXR isoforms, crystal structures of other USPs/RXRs are required for converting the CasRXR into the PDB format. Base on the PDB format of all four CasRXR isoforms using *Bemisia tabaci* and *T. castaneum* as references, their PDF are started after the insert site in the LBD (**Fig. 17 B**). Therefore, there is no difference among the four CasRXR isoforms when they are analyzed for structural simulation.

Table. 7. Secondary structure of deduced aa sequences CasRXR predicted by PREDATOR 2.1.2. H: helix, E: β-sheet, AF1: activation function-1, DBD; DNA-binding domain, HR: hinge region, LBD: ligand-binding domain, and AF2: activation function-2

Isoforms		**DBD**			**LBD**		
	AF1	H	E	**HR**	H	E	**AF2**
CasRXR1	-	3	2	-	6	7	-
CasRXR1a	-	3	2	-	6	7	-
CasRXR2	-	3	2	-	6	9	-
CasRXR2a	-	3	2	-	6	9	-

2.3.2.5. Genomic arrangement of *CasRXR* gene

The genomic arrangement of *CasRXR* was mapped from the alignment of overlapping sequences obtained from endpoint PCR assays of genomic DNA amplication with different pairs of gene specific primers. Exon-intron boundaries are located by aligning all sequences obtained from genomic DNA amplications against the full-length cDNA of *CasRXR* (**Fig. 19**). The analyses of some intronic sequences have not been completed yet. Thus far it was revealed that the full length cDNA of *CasRXR* is derived from eight exons and seven introns with a total size of approximately 6 kb. Exon 1 and exon 2 form the AF1 domain, exon 3 and a partial exon 4 generate the DBD, exon 4 spans from the DBD to the HR, while exons 5-8 encode the LBD and the AF2 domain. Variations by insertion (+5 aa and +45 aa residues) are found in exons 4 and 5 that are encoding for the DBD and the LBD, respectively.

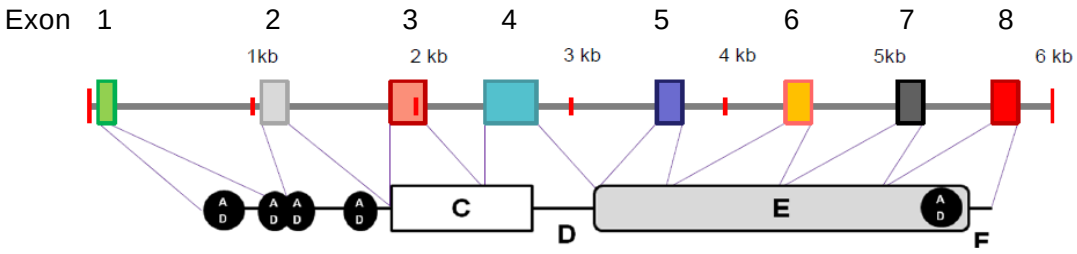


Figure.19. Genomic map and arrangement of *CasRXR* constructed by sequence analysis and size comparison from PCR amplification using cDNA and genomic DNA. The red vertical lines correspond to total lengh in kb.

2.3.3. Expression patterns of CasEcR and CasRXR during the molt cycle

2.3.3.1. Eyestalk (ES)

Transcript levels of overall *CasEcR* and *CasRXR* isoforms are different during the molt cycle in that the former levels are molt-stage dependent, with lower expression at intermolt and higher expression at premolt, whereas the latter remains constant. The expression levels of *CasRXR* isoforms appear to be greater than those of the *CasEcRs* (**Figs. 20 C-D** and **A-B**, respectively). In detail, the expression of *CasEcR1* is the highest among four isoforms, followed by expression of *CasEcR2*, *CasEcR2a* and *CasEcR1a* (**Fig. 20 A**). The expression of *CasEcR1* is constant and high throughout the molt cycle, while *CasEcR2* gradually increases as the molt cycle proceeds. *CasEcR1a* and *CasEcR2a* are near the detection limit during the molt cycle. *CasRXR1* is found to be the major isoform, followed by *CasRXR1a* and *CasRXR2*, while the longest form (*CasRXR2a*) is near the detection limit.

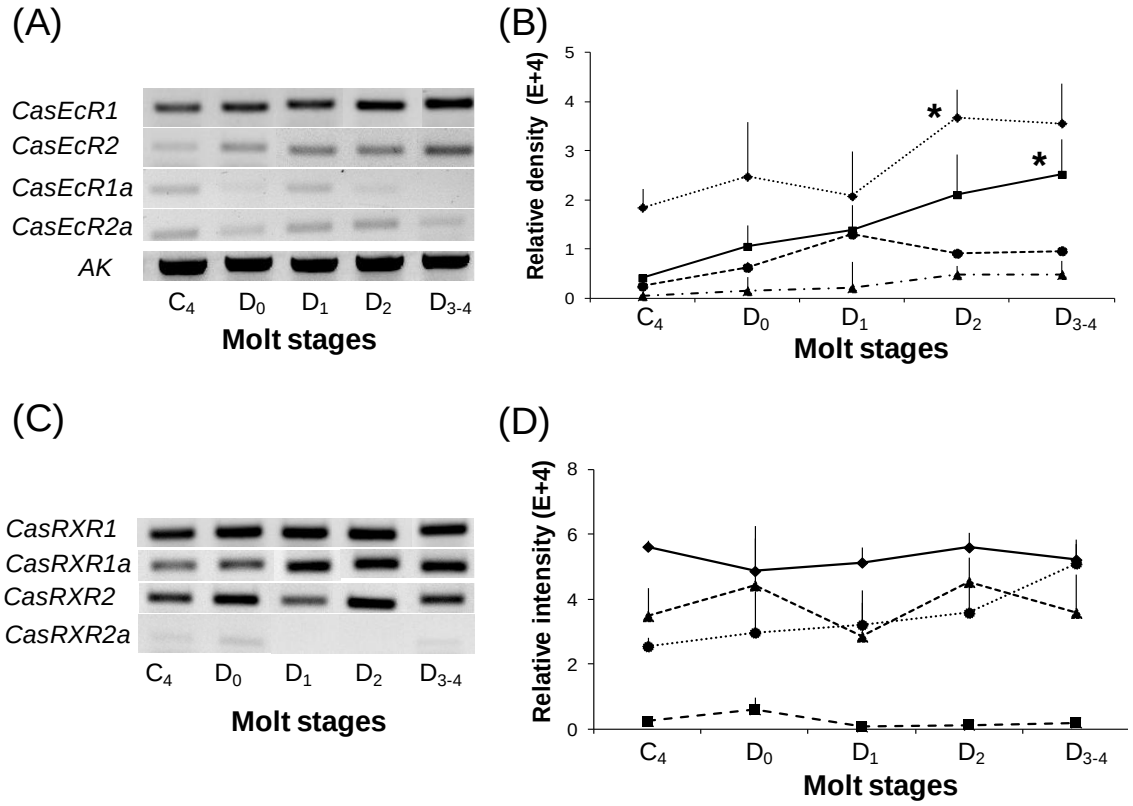


Figure. 20. Temporal expression of the four isoforms of *CasEcR* (A) and *CasRXR* (C) in eyestalk ganglia (ES) during the molt cycle using an end-point PCR assay. For each molt stage, three cDNA samples obtained from different animals were amplified along with the reference gene (*AK*= arginine kinase). The relative intensity in (B) and (D) was normalized using the ImageJ software program [109]. (B) *CasEcR* transcripts: diamond = *CasEcR1*, rectangular = *CasEcR2*, triangle = *CasEcR1a*, circle = *CasEcR2a* (D) *CasRXR* transcripts: diamond = *CasRXR1*, circle = *CasRXR2*, triangle = *CasRXR1a*, regtangular = *CasRXR2a*; C_4 = intermolt, D_0 = early premolt, D_1 = premolt, D_2 = mid premolt, and D_{3-4} = late premolt. The *p* value analyzed by One-way ANOVA with post test (The *p* value *, $p<0.05$)

2.3.3.2. Y-organs (YO)

The transcript levels of *CasEcR* and *CasRXR* isoforms in the YO, shown in their band intensities are much higher than those found in the ES. YO at C_4 to D_4 stages have constitutively strong expression of almost all of *CasEcR-CasRXR* isoforms, except for *CasEcR1a* and *CasRXR2a* (**Figs. 21 A** and **B**). The expression levels of *CasEcR1a* at D_1

and D_2 stages are higher than the rest of the molt stages. *CasRXR2a* expression is highest at the early premolt (D_0-D_1) compared to all the other stages (**Fig. 21**).

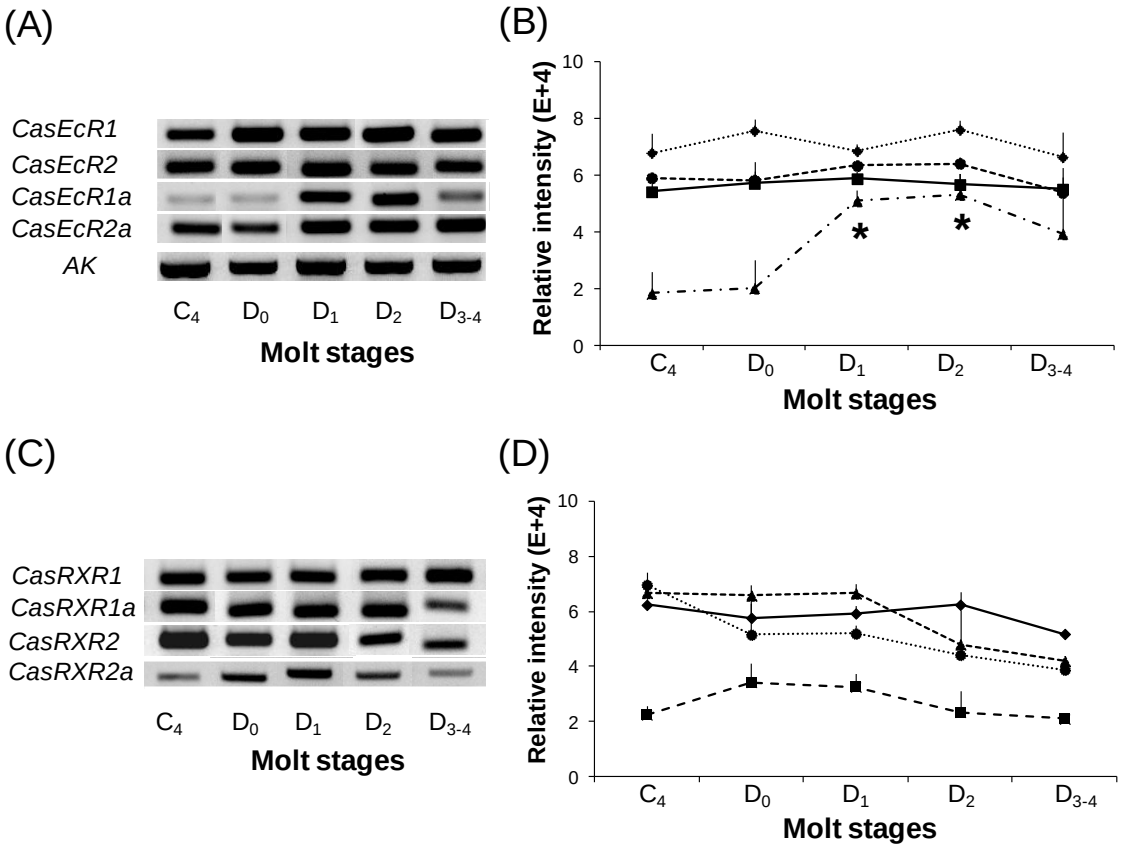


Figure. 21. Temporal expressions of four isoforms of *CasEcR* (A) and *CasRXR* (C) in Y-organs (YO) during a molt cycle using an end-point PCR assay. In each molt stage, three cDNA samples obtained from different animals were utilised along with the reference gene (AK = arginine kinase). The relative intensity in (B) and (D) is converted by ImageJ program [109]. (B) *CasEcR* transcripts: diamond= *CasEcR1*, rectangular = *CasEcR2*, triangle = *CasEcR1a*, circle = *CasEcR2a* (D) *CasRXR* transcripts: diamond = *CasRXR1*, circle = *CasRXR2*, triangle = *CasRXR1a*, regtangular = *CasRXR2a*. C_4= intermolt, D_0 = early premolt, D_1 = premolt, D_2 = mid premolt, and $D_{3\text{-}4}$ = late premolt.
The *p* value analyzed by unpaired t-test (*, $p<0.05$) was considered as significant.

2.3.4. Regulatory elements in untranslated regions (UTR) of arthropod EcR and RXR/USP

Since the 5´UTRs of *CasEcR* and *CasRXR* contain translational regulation sites, we further examined whether this is a common feature in the sequences of other arthropod *EcR* and *RXR/USP*. *EcR* sequences contain uORF as a common element in the 5´UTR, where crustacean *EcR*s have one to two uORF, while those of insects display one to six uORFs (**Table 8**). In both insects and crustaceans, when the 5´UTR has more than one uORF, the last uORF overlaps with the mORF.

Both crustacean and insect uORFs encode a short peptide sequence ranging from 6 to 51 residues. In *C. pugilator*, the uORF encodes a either hexapeptide (MALAFT) or a tetradecapeptide (MIPPLAASPPTNG), while those of *T. japonicus* and *M. japonicus* are predicted for a nonapeptide (MLAIKHLLT) and a decapeptide (MGERGRGDQD), respectively. These peptides encoded in the uORF(s) are predicted for putative roles, but none has functions relating to the Ecds. The 3´UTR of crustacean *EcR*s do not contain a cytoplasmic polyadenylation element (CPE), while those of insects have CPEs that are arranged in tandem repeats (**Table 8**).

Table 9 lists the IRES and uORF predicted in the 5´UTR of *RXR/USP*. Crustacean *RXR*s contain a single IRES site and/or one uORF, while the insect/arachnid *RXR/USP* encodes more than one IRES and uORF. Similarly, when these encoded peptides were predicted for putative functions, there was no correlation to Ecds.

Table. 8. Putative regulatory elements in the 5´ and 3´UTR of *EcR* in arthropods, analyzed by RegRNA

species	*EcR*		
	5´UTR		3´UTR
	IRES (n)	uORF (n)	CPE (n)
Crustaceans			
C. sapidus (HQ630857)	2	2	
C. pugilator (AF034086.2)	1	1	
D. magna:- AB274824.1	1		
EF363705.1	1		
T. japonicus (GQ351503.1)	1	1	
M. japonicus (AB295492.1)		1	
Lepidoptera/Diptera			
A. aegypti:- AY345989.1	2	4	
AAU02021	1	4	tandem
B. mori:- NM_001043866.2	1		
NM_001173377.1	2	1	1
D. melanogaster:-NM_001169590.1)	1	5	tandem
NM_165464.2	1	3	
NM_165465.2	1	5	
M74078.1	1	5	tandem
AB274824.1	1		
L. cuprina (LCU75355)		3	tandem
P. interpunctella (AY489269.2)	7	5	tandem
M. sexta (MSU19812)	1	1	
A. albopictus (AF210733.1)	3	5	tandem
C. fumiferana (AF092030.2)	2	1	tandem
A. silvatica (GQ281317.1)	2	1	
B. coprophila: GQ427083.1	1	3	tandem
GQ427082.1	2	4	tandem
O. fuscidentalis (EF667891.1)	1	1	
P. xylostella (EF417852.1)	1	1	
C. capitata (AJ224341.1)	1	1	tandem
C. suppressalis (AB067812.1)	1	1	
H. virescens (Y09009.1)	1	1	
Arachnida/Chilopoda			
A. americanum: AF020188.1	1	1	
AF020187.1	4	10	
L. australasiae (AB297929.1)		1	
O. moubata (AB191193.1)	1	2	
Coleoptera/Hymenotera/Hemiptera/Orthoptera			
T. emma (HQ131915.1)	1	4	
N. vitripennis (NM_001159357.1)	1		
A. mellifera (NM_001159355.1)	1		
L. migratoria (AF049136.1)	1		
C. japonicus: AB296081.1		1	tandem
AB296080.1	2	6	tandem
B. germanica (AM039690.1)	1	1	tandem
L. decemlineata: AB211192.1	1	2	
AB211191.1		3	
T. molitor (Y11533.1)	3	3	

Table.9. Putative regulatory elements in the 5´ and 3´UTR in *RXR/USP* obtained from arthropods

species	*USP/RXR*		
	5´UTR		3´UTR
	IRES (n)	uORF (n)	CPE (n)
Crustacean			
C. sapidus (HQ630860)	1	1	
C. maenas (EU683888.1, EU683889.1)		1	
D. magna (DQ530508.1)		1	
M. japonicus (AB295493.1)		1	tandem
Lepidoptera/Diptera			
S. littoralis (HM445735.1)	1	1	
H. armigera (EU526832.1)	1	1	
B. mori (NM_001044005.1)	1	1	tandem
M. sexta (MSU44837)	2	2	
A. aegypti: AF305213.1	3	2	tandem
AF305214.1	1	4	tandem
D. melanogaster (NM_057433.3)	2	2	tandem
C. suppressalis (AB081840.1)	3		tandem
P. interpunctella (AY619987.1)	1		
B. dorsalis (HM195185.1)	1		tandem
A. silvatica (GQ281318.1)	1		
A. albopictus (AF210734.1)	1		
S. exigua (EU642475.1)	1	2	
Arachnida			
L. australasiae (AB297930.1)	1	2	
O. moubata (AB353290.1)	1		1
A. americanum (AF035577.1, AF035578.1)	1		tandem
Coleoptera/Hymenotera/Hemiptera/Orthoptera			
C. fumiferana (AF016368.1)	1	2	tandem
N. vitripennis: XM_003426091.1	5	5	tandem
XM_001605769.2	5	4	tandem
L. migratoria (AY348873.1)	1	1	tandem
T. molitor (AJ251542.1)	2	2	tandem
T. castaneum (NM_001114294.2)	2	3	tandem
B. impatiens XM_003484414.1	1	2	
XM_003484415.1	1	1	
A. pisum (NM_001161668.1)			1
B. germanica (AJ854490.1)			
A. mellifera (NM_001011634.2)			tandem
G. firmus (GU201493.1)	1	1	tandem
B. terrestris (XM_003397944.1)	1	1	
X. pecki (AY827155.1)	1	2	

2.4. Discussion

2.4.1. Isolation of cDNAs of *C. sapidus EcR* and sequence analyses

Since EcR and RXR mediate the signaling of Ecds by acting as transcription factors of Ecd-responsive genes, expression patterns of these two genes in ES and YO, where MIH/CHH and Ecd are produced, respectively, may elucidate a regulatory relationship between these molecules at various molt stages.

The four isoforms of *CasEcR* are similar in their deduced aa sequences except for HR and the LBD. The HR regions are known to affect their molecular flexibility and ligand-binding affinity. The 27-aa insert (CKSKGPSPACDMQFKNLVDTPSIVQTP) in the HR^i of CasEcR1a/CasEcR2a contains four residues of Pro. Interestingly, Pro is reported to prevent conformation changes within the molecule [110,111]. Thus, it is likely that the rotation between the DBD and the LBD of the CasEcR1a/CasEcR2a isoforms may be more rigid than that of CasEcR1/CasEcR2. Generally, conformational changes in whole molecule are induced by ligand binding at the LBD [49,56,112-114] but these mechanisms may be inhibited by the insert fragment in HR^i of CasEcR1a/CasEcR2a. In addition, post-translational prediction of the putative CasEcR revealed that the HR^i contains a greater number of potential phosphorylation and glycosylation sites, indicating a greater steric hindrance. As a result, CasEcR1a/CasEcR2a might be more recalcitrant to the transduction of Ecd signals than CasEcR1/CasEcR2.

In *silico* structural analyses revealed that there are differences between the LBD of CasEcR1/CasEcR1a and CasEcR2/CasEcR2a. The LBD of CasEcR2/CasEcR2a, which is composed of more hydrophobic aa residues, may generate a favorable site for

Ecd binding. This structural model is consistent with the post-translational prediction which shows that the LBD of CasEcR1/CasEcR1a seems to be more negatively charged resulting from phosphorylation. It is reasonable to suggest that CasEcR2/CasEcR2a may show a greater affinity to Ecd than CasEcR1/CasEcR1a. All together, each CasEcR isoform presents somewhat different molecular properties which may affect the level of signal transduction. Considering steric hindrances on the HR and overall Ecd-binding affinity, the ability to transduce the Ecd into a transcriptional response may be predicted in the following order: CasEcR1~CasEcR2 > CasEcR1a~ CasEcR2a.

The variation in the HR and LBD is also found in the isoforms of EcR of other decapod crustaceans. Putative alternative splicing sites are located in the HR and/or the LBD as in *C. crangon*, *H. americanus*, *M. japonicus*, *N. integer*, and *U. pugilator* [97,115-118]. Pro residues are generally included in the insert, and isoforms derived from these aa substitution can be more hydrophobic than the others, as found in the LBD of NeiEcR and HoaEcR (NeiEcR2>NeiEcR1 and HoaEcRa>HoaEcRb: reanalyzed by PyMol). Over all, it appears that the crustacean EcR isoforms share common features of kink formations either in the HR (*C. sapidus*, *H. americanas*, *M. japonicus*, and *U. pugilator*) or the LBD (*C. crangon* and *N. integer*), and/or hydrophobic fields in the LBD (*C. sapidus*, *N. interger*, and *H. americanas*). On the other hand, insect and arachnid EcR isoforms contain variable regions in the AF1 domain where other transcription factors, co-activators, and/or co-repressors can bind [49]. In the AF1 domain, insertions and/or aa substitutions are common isoform progenitors in *D. melanogaster*, *A. mellifica*, *A. pisum*, *N. vitripennis*, *T. castaneum*, *N. lugens*, and *A. aegypti* [98,99,119-126,127]. It

has been found that these variations result from an alternative splicing in the exons encoding the A/B domain [125].

In regards to the distinct differences in EcR-isoform formation between crustaceans and other arthropods, it can be deduced that hormonal systems in crustaceans and insects/arachnids are different. This phenomenon might result from different types of Ecds circulating in the hemolymph as has been reported [1,11,34,43,128,129]. Moreover, the hormonal interactions in crustaceans seem to be more complicated than those of insects/arachnids *e.g.* the number of possible interactions by MIH/CHH. Therefore, a cascade of hormonal responses to Ecd signals in insect systems cannot be used to explain crustacean systems.

2.4.2. Isolation of *C. sapidus RXR* and sequence analyses

RXR is an obligatory partner of EcR during Ecd signal transduction. Therefore, expression patterns and characterization of CasRXR are required for understanding Ecd responses in *C. sapidus*. The different sizes and locations of the inserts create four different CasRXR isoforms. The 45-aa insert in the LBD^{L} of CasRXR2 and CasRXR2a contains three Pro residues that may introduce a kink in this domain, likely hindering accessibility to ligand(s) as predicted above in CasEcR. The actions of CasRXR to transduce Ecd signal cannot be applied because 1) RXR itself does not bind to Ecd in the heterodimer of EcR-RXR, and 2) a cognate ligand for crustacen RXR/USP has yet to be identified [103,130-132]. However, it has been reported that the different isoforms of RXR display their surface residues differentially which can affect the ability or EcR to transduce the Ecd signal [131]. Crustacean RXRs including CasRXR share a distinctive

feature with the location of inserts that contain many Pro residues. As stated above, structural variation in RXR isoforms provided by these inserts may bear some fucntinal significance in flexibility of the LBD. Interestingly, the variations of insect isoforms are different and more diverse than those of crustaceans. The majority of insect isoforms vary in the AF1 domain, as those found in *N. vitripennis (*accession: XM_003426091.1, XM_001605769.2*), A. aegypti*, and *T. castaneum* [125]. Isoforms of *L. peregrinus* show two different inserts, similar to crustaceans [133]. This finding supports the hypothesis that crustaceans have hormonal systems different from insects [134].

2.4.3. Spatio-temporal expressions of *C. sapidus EcR-RXR*

Every crustacean tissue including ES and YO can be a target for Ecds [123,135,136]. Because ES and YO produce counter-acting hormones that regulate molting, expression patterns of *CasEcR* and *CasRXR* might differ in these two tissues.

2.4.3.1. Eyestalk ganglia (ES)

The ES, containing the ganglia responsible for MIH/CHH synthesis and secretion, is proposed to have responses to the Ecd changes during a molt cycle [11,23]. The CasEcR-CasRXR complex in ES may be sensitive to Ecd in the hemolymph. *CasEcR1* is the major isoform that is constitutively expressed throughout the molt stages, while the *CasEcR2* gradually increases as the molting progresses. This finding is consistent with the predicted activity of CasEcR isoforms that the CasEcR2 seems to be the most proficient at both binding and transducing Ecd signals. Therefore, up-regulation of *CasEcR2* during the premolt might result in the increase of *trans*-activation of the Ecd-

responsive genes during this stage. In the ES, only CasRXR2a, the form predicted to have an altered heterodimer interface and flexibility, is reduced during the premolt as compared to the intermolt. This raises the possibility for the other CasRXR isoforms to form a heterodimer with CasEcR.

In addition, it has been reported that the unliganded EcR-USP/RXR heterodimer can act as a silencer or repressor for many genes [28,46,49,107,137-140]; therefore, Ecd-responsive genes including *MIH* might not be activated during the intermolt. However, this hypothesis contradicts the actions of MIH/CHH at the intermolt in which the inhibitory actions of MIH/CHH on molting are highest, so MIH/CHH should be highly secreted into the hemolymph [141]. There are possible hypotheses to explain this contradiction: 1) CasRXR might form homodimer or heterodimer with other NRs to transactivate *MIH*, and 2) high levels of MIH/CHH are stored from the previous premolt and constantly secreted during the next intermolt [25,88,142]. In addition, the knowledge that YO during the intermolt have the highest responsiveness to the MIH/CHH signal needs to be considered [14,19,25,92,141,143]. Although it is possible that the homodimer of CasRXR might stimulate *MIH* transcription during intermolt, cognate ligands are required for releasing CasRXR from its inactive stage [56]. Therefore, this hypothesis remains questionable. During the premolt when ecdysteroid titers are increased, dimerization of CasRXR might be switched to a CasEcR-CasRXR heterodimer resulting from the binding of ligand [144,145]. These activated CasEcR-CasRXR complexes could stimulate the cascade of downstream genes including *EcR* itself [112] and *MIH* as has been proposed [11,22], resulting in an increase in MIH

production during the premolt. However, further experiments need to be performed to validate this assumption.

2.4.3.2. Y-organs (YO)

YO, in which Ecds are produced and secreted, have irregular activities during the molt cycle. Ecds are able to generate both positive and negative feedbacks on the YO [146]. Therefore, it is suggested that different CasEcR-CasRXR complexs may transmit the Ecd signals during both early and mid premolt. In this study, it was found that *CasEcR1a* transcripts, the isoform predicted to be the least effective in transducing Ecd signals, are increased during the mid premolt (D_1-D_2). This increase in *CasEcR1a* transcripts may increase the ratio of poorly binding forms within a pool of CasEcR-CasRXR complexes. As a result, feed-forward interactions of Ecd on YO through CasEcR-CasRXR may be switched, and ecdysteroidogenesis would be reduced. However, to prove this hypothesis, further experiments need to be performed.

Expression levels of *CasEcR* and *CasRXR* in the YO are comparable throughout the molt cycle; therefore, it is suggested that CasEcR and CasRXR are constitutively bound as a heterodimer complex. However, the expression of *CasEcR* and *CasRXR* in this study was assayed by end-point PCR. Therefore, the exact transcript levels of each isoform are required.

2.4.4. Post-transcriptional regulations

Genes encoding for growth factors, transcription factors and their receptors are normally transcriptionally regulated by *cis*-regulatory elements in the 5´UTR

[60,147,148]. The existence of IRES and uORF in the 5´UTR of *CasEcR* and *CasRXR* suggests that the transcripts of these two genes may not directly represent the expression at the protein level. The IRES presenting in both *CasEcR* and *CasRXR* may facilitate translational initiation during cellular stresses [75]. However, the relative positions of IRES and uORF need to be considered because the uORF functions to suppress translation as has been previously reported [149-156]. In the *CasEcR*, two IRES are located upstream of uORF whereas they occur downstream in *CasRXR*. This arrangement may affect the overall effects of IRES and uORF, in which the relative downstream uORF may suppress translation better than the upstream one because the IRES acting as the ribosome binding site can recruit the initiation machinery to the mRNA in a cap-independent manner, and the scanning ribosome may not complete initiation if it encounters the downstream uORF. This could be the case for the mRNA of *CasEcR*. Moreover, the second frame of the uORF in the *CasEcR* overlaps with the mORF, resulting in a more effective translational suppression of the mORF. Therefore, it is suggested that the *CasEcR* may have more pronounced post-translational control than the *CasRXR*.

This is the first time in arthropods that the cDNA sequences of *EcR* and *RXR/USP* have been analyzed for post-transcriptional control and it shows that the occurrance of IRES and uORF is conserved among arthropod *EcR* and *RXR/USP* genes. This supports the hypothesis that *CasEcR* and *CasRXR* may be subjected to post-transcriptional control through elements in the 5´UTR. In order to determine both stimulating and suppressing regulation by IRES and uORF, respectively, further studies are needed to test these hypotheses.

3. CHAPTER 3. General conclusions and future studies

The molt cycle in the *C. sapidus* is resulted of interactions between crustacean hyperglycemic hormone (CHH) neuropeptide family: CHH/MIH and Ecd hormones. It is clearly stated that CHH and MIH have inhibitory effects on the YO, and the YO show differential responses to the CHH neuropeptides during the molt cycle. The YO at the intermolt stage exhibits the greatest inhibitory response to the MIH and CHH, but at premolt stage it exhibits a reduced response.

Previous studies suggest that a crosstalk(s) between MIH/CHH and Ecds may determine a molt stage and regulate molting as a cycle. Suppression of ecdysteroidogenesis by MIH/CHH, particularly *in vitro*, is well established, but the feedback regulation of Ecds on the MIH/CHH production in the ES remains to be further studied. Ecds transduce signals by binding to the receptors of transcription factors. Thus, expression levels of the receptors in these tissues may correlate to the overall response to the Ecd signals. In this study, cDNA sequences encoding for the ecdysone receptor (EcR) and the retinoid-X receptor (RXR) of *C. sapidus* (*CasEcR-CasRXR*) were isolated and characterized for their significant properties *in silico*. It was found that *C. sapidus* expresses four putative isoforms of CasEcR and CasRXR. EcR is especially important since it is known to bind directly to the Ecds. Two putative CasEcR isoforms (CasEcR1a/CasEcR2a) have an insert in the hinge region (HR) that lies between the LBD and the DBD. This insert fragment contains four residues of Pro that may generate steric hindrance which can interfere with conformational changes and can result in inefficient signal transduction of Ecd. In addition, the different hydrophobic fields of CasEcR1/CasEcR1a and CasEcR2/CasEcR2a may differentially affect binding to the

several forms of circulating Ecds which occur during the molt cycle. It was found that during late premolt, PonA is the major Ecd circulated in the hemolymph following by 20HE, and the former is also more hydrophobic than the latter. Therefore, it is predicted that the CasEcR2/CasEcR2a containing more hydrophobic residues in the LBD, may be responsible for binding with PonA, while CasEcR1/CasEcR1a may be more suitable for 20HE. The CasRXR, serving as a partner for CasEcR, appears to have variations consisting of an inserted fragment at the T-box within the DBD and at the anterior of the LBD. Both inserts are predicted to have effects on the dimerization of CasRXR to the CasEcR partner.

CasEcR expressions in both ES and YO are molt-stage dependent. In the ES, the transcript levels of *CasEcR1* and *CasEcR2* were increased during premolt in which the levels of circulating Ecds are high. This finding is consistent with the predicted properties of CasEcR1 and CasEcR2 that are able to transmit the Ecd signals better than CasEcR1a and CasEcR2a isoforms. It suggests that Ecds may able to induce the expression of their receptor for mediating the signals and other Ecd-responsive genes including the CHH neuropeptides in the ES. For the YO, the expression patterns were opposite, in which the *CasEcR1a* transcripts were suddenly increased during the mid premolt. Predicted to be the least effective form to transmit the Ecd signals, the increasing of CasEcR1a may lead to a slowing down of the self-stimulation of Ecds in the YO, resulting in reduction of ecdysteroidogenesis. However, isoform screening in *CasEcR* and *CasRXR* was measured at the transcript levels, and these patterns may not represent the expression in the protein levels because *cis*-regulatory elements for translational control are predicted in their 5´UTRs. Both *CasEcR* and *CasRXR* contain

uORF and IRES, and these regulatory elements seem to be phylogenetically conserved throughout the Phylum Arthropoda. The presence of IRES and uORF in the 5´UTR of *CasEcR* and *CasRXR* appears to facilitate translational initiation during the transient stress when the general translational machinery is impaired, such as during the premolt.

In conclusion, the isolation of cDNA sequences of *CasEcR* and *CasRXR* isoforms provide fundamental knowledge for further investigation into the roles of Ecds in molt cycle regulation. However, the hypotheses that Ecds signal to ES for the production and secretion of the CHH neuropeptide hormones, but slow-down ecdysteroidogenesis in the YO requires further experiments. Transcript levels of *CasEcR-CasRXR* isoforms during the molt cycle need to be determined using quantitative PCR assays, and the direct effects of Ecds on the expressions of *CasEcR1/CasEcR2* and *CasEcR1a* in ES and YO, respectively, need to be studied. Moreover, transactivation of EcR/RXR complex upon binding to Ecds on the levels of MIH/CHH needs to be examined on its transcription, translation, and secretion.

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