DISSERTATION

REGULATION OF HALLOWEEN GENES AND ECDYSTEROID RESPONSIVE GENES IN MOLTING GLAND OF BLACKBACK LAND CRAB *GECARCINUS LATERALIS* BY MOLT-INHIBITING HORMONE, MTOR AND TGFβ SIGNALING PATHWAYS

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ABSTRACT

REGULATION OF HALLOWEEN GENES AND ECDYSTEROID RESPONSIVE GENES IN MOLTING GLAND OF BLACKBACK LAND CRAB *GECARCINUS LATERALIS* BY MOLT-INHIBITING HORMONE, MTOR AND TGFβ SIGNALING PATHWAYS

PATHWAYS

Molting is necessary for growth and development in all arthropods. Halloween genes are expressed in the Y-organs (YO) and encodes cytochrome P450 enzymes. These enzymes catalyze the synthesis of ecdysteroid hormones that regulate the molt cycle. This hormone binds to ecdysteroid receptor to activate a cascade of ecdysteroid-responsive genes that effect tissue responses to hormone. We used *P. leptodactylus* Halloween gene and insect ecdysteroid-responsive gene sequences to extract and characterize the land crab orthologs in the YO transcriptome. This resulted in identification and characterization of eight ecdysteroidogenic genes that encode *phantom*, *disembodied*, *spook*, *shadow*, *Cyp18a1*, *neverland, NADK* and *ALAS* and nine ecdysteroid-responsive genes that encoded *EcR, RXR, broad complex*, *E75*, *E74*, *Hormone receptor 4*, *Hormone receptor 3*, *forkhead box transcription factor (FoxO)* and *Fushi tarazu factor-1*. Sequences were validated by end-point PCR and Sanger sequencing. We used phylogenetic analysis to infer evolutionary relationships among contig sequences and ortholog of Halloween genes and ecdysteroid-responsive genes in other species. The results showed the contig sequences clustered with their corresponding orthologous genes. Tissue distributions for *spook* and *phantom* showed significantly higher mRNA levels in the YO compared to other tissues. By contrast, the mRNA levels of *NADK*, *ALAS*, and all ecdysteroid-responsive genes

were not higher in the YO than those in other tissues. These data show that the YO is the primary source of ecdysteroid production and that the YO can respond to ecdysteroid, suggesting a feedback regulation on ecdysteroid synthesis and secretion. qPCR was used to quantify gene expression of Halloween and ecdysteroid-responsive gene expression in the YO of *Gecarcinus lateralis* induced to molt by multiple limb autotomy (MLA) or eyestalk ablation (ESA). ESA decreased mRNA levels of *Gl-Phm, Gl-E75* and *Gl-RXR* at 3 days post-ESA. *Gl-Dib* and *shadow* increased at 3 days post ESA and decreased at 7 and 14 days post-ESA. *Gl-Cyp18a1*, *Gl-BR-C*, *Gl-NADK* and *Gl-ALAS* mRNA were higher at Day 0 and 1 post-ESA and lower at Day14 post ESA. *Gl-HR3*, *Gl-HR4*, and *Gl-E74* were expressed at low levels. MLA lowered mRNA levels of Halloween genes, *Gl-Nev,* and *Gl-E75,* except *Gl-dib*, at premolt and postmolt stages. *Gl-Dib*, *Gl-NADK*, *Gl-ALAS,* and *Gl-BR-C* mRNA levels were not affected by molt stage. *Gl-EcR, Gl-HR4* and *Gl-HR3* mRNA levels were highest in premolt and lowest in postmolt. *Gl- FOXO* mRNA levels were highest in premolt and lowest in intermolt. These data suggest that molting has an indirect effect on the regulation of Halloween genes and that molting directly regulates *HR3, HR4, RXR* and *FOXO* to increase ecdysteroid synthetic capacity of the YO in premolt animals. The presence of *EcR*/*RXR* and ecdysteroid-responsive genes suggest that elevated ecdysteroid represses the YO at the end of premolt. TGFβ/activin signaling mediates the transition of the YO from the activated to the committed state, as SB431542 blocks this transition. *G. lateralis* were eyestalk-ablated to induce molting and injected with vehicle (DMSO) or SB431542 at Day 0. In controls, ESA increased hemolymph ecdysteroid titers at 3, 7 and 14 days post-ESA. There were significant increases in the mRNA levels of *Gl-Nvd* at 7 and 14 days post-ESA and other Halloween genes (*Gl-Spo, Gl-Phm, Gl-Dib,* and *Gl-Sad*), as well as *Gl-CYP18a1, Gl-ALAS, Gl-NADK*, *Gl-BR-C,Gl- FOXO, Gl-EcR*, and *Gl-RXR*, at 14 days post-

ESA. SB431542 reduced hemolymph ecdysteroid titers at 7 and 14 days post-ESA compared to control animals, but titers were no different from controls at 1, 3, and 5 days post-ESA, indicating that SB431542 had no effect on YO activation. SB431542 blocked the increases in mRNA levels of *Gl-Nvd*, *Gl-Spo, Gl-Phm, Gl-Dib, Gl-Sad*, *Gl-CYP18a1*, *Gl-ALAS, Gl-NADK*, *Gl-BR-C, Gl-EcR*, and *Gl-RXR* by ESA. SB431542 had no effect on mRNA levels of the ecdysteroid-responsive genes *Gl-HR3 Gl-HR4, Gl-E74, Gl-E75* and *Gl-FTZ-F1*. These data suggest that an Activin-like TGFβ factor stimulates YO ecdysteroidogenesis in the committed YO by up-regulating Halloween, *Gl-BR-C,* and *Gl-FOXO* genes.

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Chapter 1

Introduction

Molting and its stages

Arthropods have a hard exoskeleton that must be shed periodically for the animals to grow; this process is called molting or ecdysis. Molting is controlled by a complex interplay of hormones (Covi et al., 2009). The Y-organ (YO) is the crustacean molting gland that secretes ecdysteroids (molting hormones), particularly ecdysone. Molt-inhibiting hormone (MIH) is an inhibitory peptide that suppresses ecdysteroidogenesis in the YO during intermolt (Covi et al., 2009; Mykles, 2011; Webster, 1991; Webster and Keller, 1986). The molt cycle consists of four major stages. During premolt or proecdysis ($D_{1–4}$), the epidermis separates from the old exoskeleton and the outer two layers (epicuticle and exocuticle) of the new exoskeleton are deposited and limb regenerates grow. The second stage is ecdysis (E) or molting, which involves the active shedding of exoskeleton. During postmolt or post-ecdysis (A, B, and C_{1-3}) the endocuticle is secreted and the exoskeleton becomes calcified. The crab consumes the old exoskeleton to recycle minerals, proteins, and chitin for cuticle synthesis and calcification. Intermolt or anecdysis (C_4) begins after the final layer of the exoskeleton, the membranous layer, is deposited. It is characterized by a hard exoskeleton. During this stage the animal feeds and reproduces. It is the longest period; it can last months or years in adults (Chang and Mykles, 2011; Skinner, 1962) (Fig.1. 1,2).

During the molt cycle, the YO transitions through different physiological states. The YO at intermolt stages (C_4) is in the basal state with low levels of ecdysteroid hormone. When the animal enters the molt cycle, the YO transitions from the basal state to the activated state at the early premolt stage (D_0). This transition is triggered by a decrease in the release of MIH. At this stage, the YO requires mTOR activity to start the synthesis of ecdysteroid hormone. At the

midpremolt stage (D_1) the YO transition to the committed state and the ecdysteroid biosynthesis levels increase and reach a peak (>300 pg/µl). At this stage mTOR activates TGFβ signaling that is required to stimulate of mTOR and inhibit MIH signaling to facilitate an increased synthesis of ecdysteroid hormone level. The YO transitions from the committed state to the repressed state in late premolt ($D_{3–4}$) and continues to produce high levels of ecdysteroid hormone. Finally, the YO will transition once again to the basal state in post molt when ecdysteroid levels are low. Thus, high level of ecdysteroids are associated with the transition of the YO from the committed state to the repressed state at end of premolt, whereas low levels of ecdysteroid hormone transition the YO from postmolt to the basal state (Chang and Mykles, 2011; Liu and Feng, 2010; Nakatsuji and Sonobe, 2004; Skinner, 1962; Tsuchida et al., 2009) (Fig. 1. 2). We hypothesize that ecdysteroid hormones regulates its own synthesis through an autoregulatory feedback mechanism.

Regulation of molting in Arthropoda

In crustaceans, molting is regulated by two endocrine organs, the X-organ/sinus gland (XO/SG) complex, which is located within the eyestalks and a pair Y-organs (YOs), which are located in the cephalothorax. The XO secretes the neuropeptide molt-inhibiting hormone (MIH) that inhibits ecdysteroid production by YO (Chang and Mykles, 2011; Nakatsuji and Sonobe, 2004; Skinner, 1985). The molting process is initiated by a reduction in MIH in the hemolymph, which stimulates the YO to synthesize and secrete ecdysone. Ecdysone hormone is converted to the active molting hormone, 20-hydroxecdoysone (20E), by peripheral tissues (Chang and Mykles, 2011). Precocious molts can be induced in two ways. The first method is eyestalk ablation (ESA), which reduces circulating MIH and results in an immediate rise in hemolymph ecdysteroids, which allows the YO to become activated and the animal enter to premolt (Chang

and Mykles, 2011; Covi et al., 2009; McCarthy and Skinner, 1977). The second method is multiple leg autonomy (MLA) where five or more walking legs are autotomized, which triggers a reduction in MIH (Fig.1. 3). This method represents a more natural mode of molt induction by mimicking the effects of a predator or injury (Skinner and Graham, 1972). However, molting is a complicated process in which the two glands contribute to complete this important process (Chang and Mykles, 2011; Covi et al., 2012).

In insects, molts and metamorphosis is control by a rise in the titer of the steroid hormone. Synthesis of ecdysteroid hormone from the molting gland in insect is regulate by a small peptide which is called prothoracicotropic hormone (PTTH). PTTH is synthesized in the brain and releases from the *corpora allata* in *Manduca sexta*. PTTH stimulates ecdysone synthesis in prothoracic glands, the molting gland in insects (Agui et al., 1980; Kataoka et al., 1991; Warren et al., 2006)

Halloween genes and ecdysteroid biosynthesis pathway in arthropod

Ecdysteroid biosynthesis in the YO is mediated by cytochrome p450 (CYP) enzymes encoded by the Halloween genes (Table 1. 1). Halloween genes *phantom*, *cyp306a1* (*phm*), *disembodied*, *cyp302a1*(*dib*) *shadow*, *cyp315a1*(*sad*), *shade*, *cyp314a1*(*shd*) *spook*, c*yp307a1*(*Spo*) and *neverland* (*nvd*) were first identified in *Drosophila melanogaster* through mutagenesis screening. These genes are necessary for normal development, molting, reproduction, metabolism, and homeostasis in insects (Gilbert et al., 2002; Rewitz and Gilbert, 2008). Loss-of-function mutations of Halloween genes in *D. melanogaster* embryos cause a reduction in hemolymph ecdysteroid tiers, morphological abnormalities, failure to express ecdysteroid-responsive genes, and lead to embryo lethality (Chavez et al., 2000).

Halloween genes have characteristic signature domains that place these genes within the CYP450 family. Halloween genes contain main five motifs; the WxxxR motif is located in the C helix, the GxE/ DTT/S motif is located in I helix, the ExLR motifs located in helix K the PERF motif is PxxFxPE/DRF, and the heme-binding domain PFxxGxRxCxG/A (Feyereisen, 2012; Gilbert and Warren, 2005; Rewitz et al., 2006b). P450s are located in mitochondria or endoplasmic reticulum depending on their N- terminal sequence. The N- terminal sequence typically consists of many hydrophobic residues followed by a proline–glycine rich region. Located in the endoplasmic reticulum *phantom* and *spook* are good representatives of genes in this group. However, the mitochondrial P450 enzymes include a mitochondrial targeting sequence at the N-terminus and has charged residues such as those observed in *disembodied* (dib) (CYP302A1) and *shadow* (sad) (CYP315A1) enzymes (Guzov et al., 1998; Iga and Smagghe, 2010) (Fig. 1. 8).

The ecdysteroid biosynthetic pathway is diagrammed in (Fig. 1. 4). Arthropods cannot synthesize cholesterol; they rely on the cholesterol in their diet and/or convert phytosterols, such as β-sitosterol, to cholesterol via dealkylation (Gilbert et al., 2002). The ecdysteroid biosynthetic pathway begins with the dehydrogenation of cholesterol to 7-dehydrocholesterol (7DC) by Neverland (Fig.1. 4). The 7DC enters what is called the "Black Box", because little is known about the details of the chemical reactions that convert 7DC to Δ^4-diketol. Black box reactions are mediated by Non-molting glossy (Nm-g)/Shroud (Sro), spook (spo)/spookier (spok), and spookiest (spot) (Pondeville et al., 2013; Warren et al., 2009). Δ^4-Diketol is converted to 5β-diketol by 5β-(H)-reductase. 5β diketol can be converted to three products by *disembodied* (*dib*), *phantom* (*phm*), and 3-dehydroecdysteroid-3-βreductase, resulting in the potential secretion of four products, three of which are converted to the active hormones 20E and ponasterone A by the

Shade protein expressed in peripheral tissues (Fig.1. 4.). However, the YO usually secretes one or two products, depending on species (Mykles, 2011). The main way to eliminate ecdysteroid (95%) is via excretion in the urine, whereas high polarity metabolites such as 20-hydroxyecdysonoic acid and 20, 26-dihydroxyecdysone and remaining ecdysteroid are excreted as apolar metabolites in the feces (5%) (Snyder and Chang, 1991). Taken together, this physiology suggests mechanism of feedback that involves first synthesis and followed by clearance of ecdysteroid from the hemolymph before ecdysis (Chung, 2010) (Fig.1. 4)

General structural organization of nuclear receptors

Nuclear receptors are a class of ligand activated proteins that are activated by steroid hormones. Nuclear receptors are proteins that belong to a large superfamily, which is divided into three classes: 1-the steroid receptor family; 2-the thyroid/ retinoid family and; 3- the orphan receptor family. The nuclear receptor superfamily are single polypeptide chains containing six domains, designated A-F (Hu et al., 2003; Moras and Gronemeyer, 1998) (Fig.1. 5). The A and B domains, or N terminal domain varies between nuclear receptors. The C domain, which is a highly conserved DNA-binding domain (DBD) contains two conserved cysteine-cysteine zinc fingers. The D domain, which is called the hinge region or the C-terminal extension of the DBD (CTE) gives flexibility to the protein as this domain contains amino acid residues that bind the DNA minor groove. The ligand-binding domain (LBD), or E-domain, is a less conserved domain. The AF2 activation function-2 domain is located within the ligand-binding domain (LBD) at the C-terminus. The activity of the AF2 is ligand-dependent (Bourguet et al., 2000; Wurtz et al., 1996).

NRs bind to specific DNA sequences, and their selectivity for specific targets is mainly encoded by the DBDs (Kumar et al., 1986). DNA sequence recognition and binding to NR,

ligand binding domains (LBDs) is the first step of the transactivation process. NR ligands bind in a hydrophobic cavity within a highly α-helical LBD. In the second step, ligand binding to the LBD causes conformational changes that appear on the receptor surface at the sites for receptor-coregulator interactions (Pawlak et al., 2012) (Fig.1. 5).

Ecdysteroid-responsive genes

Ecdysteroids bind to *EcR* and *RXR* to induce molting processes by activating the expression of downstream transcription factors including *EcR-RXR* itself (Gouveia et al., 2018). In insects *EcR*/*USP* induce different groups of ecdysteroid responsive genes depending on the titer of 20E. Low levels of 20E bind to *EcR*/*USP* and induce early ecdysteroid responsive genes such as *Broad complex*, *E74*, and *E75*. These early genes have two opposing regulatory functions: they repress their own expression, depending on hormone level, and they induce an early-late and late gene such as (*Hormone Receptor 4*, *Hormone Receptor 3*, and *Fushi tarazu factor-1*) which are required during larval development and metamorphosis (Andres and Thummel, 1992; Fletcher et al., 1995; Jindra, 1994). In insects *HR3* gene is an inducer of *βFTZ-F1* expression in mid-prepupae when 20E level is low (Lam et al., 1999). *E75* inhibits the activity of *HR3* gene at late third instar larva when ecdysone hormone at high level (Hannas et al., 2010; Lam et al., 1999). *BR-C E74* and *E75* genes are activated by molting hormone in late third instar larvae then they themselves are quickly inhibited but reactivate again in in late prepupa. These transcriptional fluxuations are required to mediate the developmental dependency on molting hormone levels during metamorphosis (Kiss et al., 1988; Lam et al., 1999) (Fig.1 6-7).

Molt-inhibiting hormone (MIH)

MIH is an inhibitory neuropeptide found in crustaceans (Chang and Mykles, 2011; Mykles, 2011). MIH is neuropeptide hormone belonging to the crustacean hyperglycemic hormone (CHH) family that includes CHH, MIH, and gonad-stimulating and -inhibiting hormone (GS/IH) (Chan et al., 2003; Khayat et al., 1998). Molting is stimulated by removal of the eyestalks, which led to discovery of an eyestalk factor that inhibits molting. The X-organ, located within the eyestalk ganglia, synthesizes these hormones which are stored in the sinus gland and secreted into the hemolymph to inactivate the ecdysteroid activity in the YOs (Mattson and Spaziani, 1985). Inhibition of the YO by MIH and CHH has been established in many of crustaceans, such as *Carcinus maenas, Gecarcinus lateralis*, and *Uca pugilator* (Mykles et al., 2010). To date we still do not know how ecdysteroid hormone affects and regulate MIH in the eyestalk ganglia during the molt cycle.

In insects molting is regulated by PTTH, neuropeptide that is synthesized in the brain and secreted by the corpora allata to stimulate the prothoracic gland (molting gland) (Rewitz et al., 2013). In larvae, the amount of PTTH released depends on the size of larva and the photoperiod. The hormone binds to a receptor tyrosine kinase (RTK) on PG membrane cells. PTTH with its receptor activates a signaling cascade of the protein kinase pathway to induce ecdysteroid biosynthesis (Rewitz et al., 2009). In lepidopterans PTTH regulates ecdysteroidogenesis through a signaling cascade involving Ca^{2+}, cAMP and several protein kinases (Marchal et al., 2010; McBrayer et al., 2007).

***Gecarcinus lateralis*, (Blackback land crab) as a model for the study of molting.**

G. lateralis, is found in tropical terrestrial habitats along the Atlantic coast and the islands of the Caribbean (Bliss, 1979). These crabs spend some of their life in the ocean the when female

crabs release their egg which then remain immersed for several larval stages before emerging on land. Adult crabs make burrows in the sand dune areas above the beach to protect themselves from dehydration and predators (Bliss, 1979). Adult *G. lateralis* molt once per year (Skinner and Graham, 1972)

G. lateralis is good model for the study of molt regulation. We can keep and care for these animals in the lab and molting is easily manipulated in two ways. The first method is eyestalk ablation (ESA) and we can track molt cycle progression by collecting hemolymph and measuring titers of ecdysteroid at specific days. The second method is multiple leg autonomy (MLA) which removes five to eight walking legs and mimics a natural way to induce molting. The regeneration (R) index is used to determine molt stage by measuring the limb bud length as function of carapace width. It is calculated by the equation: regenerate length x 100/carapace width (Chang and Mykles, 2011). Using these methods, we can study the regulation of molting and are able to further understand molt stage procession at the molecular level.

The mechanistic Target of Rapamycin (mTOR) signaling and its role in molting in arthropods:

Mechanistic target of rapamycin (mTOR) is a highly conserved protein kinase that controls global translation of mRNA into protein in animal and plant cells (Proud, 2009). mTOR is a serine/threonine kinase, that regulates translation and transcription in response cell growth and proliferation (Soliman, 2005). mTOR is controlled by multiple signaling pathways, including insulin signaling, growth factors, energy, stress, mitogens, and amino acids (Showkat et al., 2014). mTOR consists of two distinct complexes: mTORC1 contains the accessory protein raptor, whereas mTORC2 is associated with rictor. mTORC1 regulates translation by phosphorylation of 4EBP1 and p70S6K; mTORC2 activates AKT through phosphorylation at

Ser473 (Zoncu et al., 2011). mTORC1 is activated by the Rheb (Ras family GTPase) and inactivated by Rheb-GTPase activating protein (Rheb-GAP or TSC1/2) (Garami et al., 2003). mTORC1 is inhibited by rapamycin, which binds to FKBP12 and through this complex inhibits mTORC1 (Schreiber et al., 2015). In *Drosophila, Rheb* is an essential activator of TORC1 in the insulin/IGF-I (insulin-like growth factor 1) receptor pathway, while TSCs acts as repressor of mTORC1 by functioning as a GTPase-activating protein (Inoki et al., 2003; Saucedo, 2003).

In *G. lateralis,* mTOR signaling pathway components are up-regulated in the YO following ESA. Large increases in the mRNA levels for *mTOR1/2*, *EF2*, *EIF4E*, *RhoA*, *TSC1/2*, *S6K*, *S6*, *Mo25*, *Akt*, and *Rheb* are reported (Abuhagr et al., 2014a; Abuhagr et al., 2014b; Shyamal et al., 2018). In shrimp (*Penaeus japonicus*) an insulin receptor tyrosine kinase and a phosphotyrosyl protein phosphatase is characterized (Chuang and Wang, 1994). Rapamycin inhibits ecdysteroid secretion by the YO in-vitro and in-vivo, indicating that mTOR-dependent protein synthesis is required for ecdysteroid secretion (Abuhagr et al., 2014b; Shyamal et al., 2018). In insects mTOR increases the ecdysteroid biosynthetic capacity of the prothoracic gland (PG) via prothoracicotropic hormone (PTTH and insulin-like peptides (Covi et al., 2012; Hatem et al., 2015; Yamanaka et al., 2013).

TGF-β signaling pathway and its role in molting process in arthropod

The transforming growth factor-β (TGFβ) superfamily of cytokines controls differentiation, morphogenesis, tissue homeostasis and cell proliferation (Massagué and Gomis, 2006). TGFβ-family genes are highly conserved between species. TGFβ signaling components are found in all animal genomes studied to date (Derynck et al., 1985; Derynck and Zhang, 2003). There are two major types of the TGFβ superfamily ligands: the bone morphogenetic protein (BMP) and activin/TGFβ signaling pathways (Hudnall et al., 2016). (TGFβ) receptors are

serine/threonine kinase receptors, which are classified into types I and II receptor kinases. An activin dimer binds to the type II receptor, then recruits and phosphorylates the type I receptor into a heteromeric complex that prompts a signal transduction cascade that includes *Smads* (Covi et al., 2008; Wieser et al., 1995; Wrana et al., 1992). *Smad* proteins act as effector proteins which activate the type 1 receptor then phosphorylates *R-Smads*, including *Smad1/5* and *Smad2/3*. *R-Smads* are activated by the Activin type I receptor kinase, then *RSmad* interacts with the common-mediator *Smad* (*Co-Smad*), and then they translocated into the nucleus. Inhibitory *Smads* (*I-Smads*) bind to the type I receptor to prevent the activation of *R-Smads*. *I-Smads* can also compete with *R-Smad* in binding with *Co-Smads* (Pang et al., 2011; Xu, 2006).

The TGFβ signaling is not necessary for YO activation, although YO commitment requires a TGFβ factor acting through Activin receptor/Smad signaling, resulting in mTOR activation, up-regulation of ecdysteroid biosynthetic enzymes, and down-regulation of MIH signaling (Abuhagr et al., 2016). TGFβ appears to be involved in the transition of the YO from the activated state to the committed state, as SB431542, an Activin receptor antagonist, lowered hemolymph ecdysteroid titers in mid premolt animals (Fig.1. 9)

The purpose of this study was to identify and characterize cDNAs encoding Halloween genes and ecdysteroid-responsive genes from MLA and ESA YO transcriptome from the blackback land crab, *Gecarcinus lateralis*. In addition, we determined the effects of molting on Halloween and ecdysteroid-responsive genes expression in the YO. In the last aim we determined effects of inhibitor TGFβ (SB431542) signaling on Halloween and ecdysteroid-responsive genes in YO of blackback land crab, *Gecarcinus lateralis.*

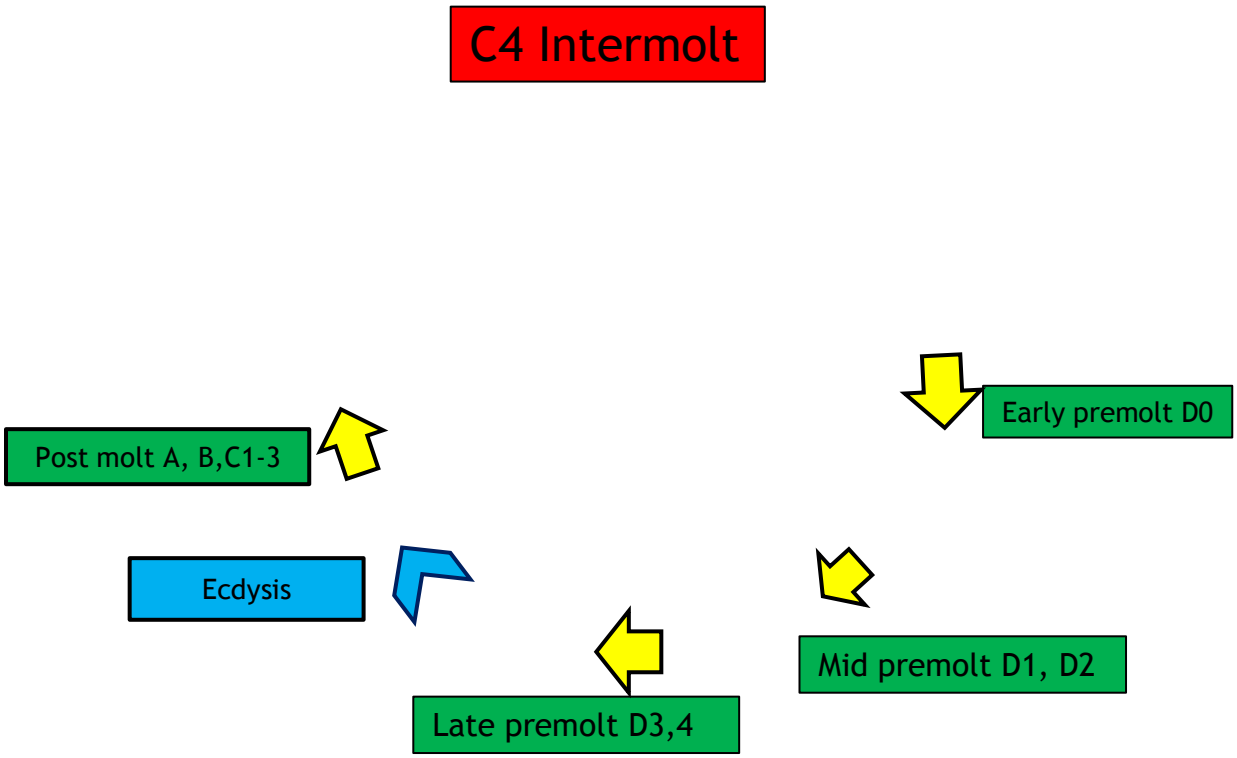

Figure.1.1 A schematic diagram of the molt cycle of *G. lateralis* is presented, showing stages of the crustacean molt cycle. The animal main in intermolt for extended periods = red circle. The green circle shows premolt and ecdysis and post molt. (D_0-D_4), Blue arrow =ecdysis (E).

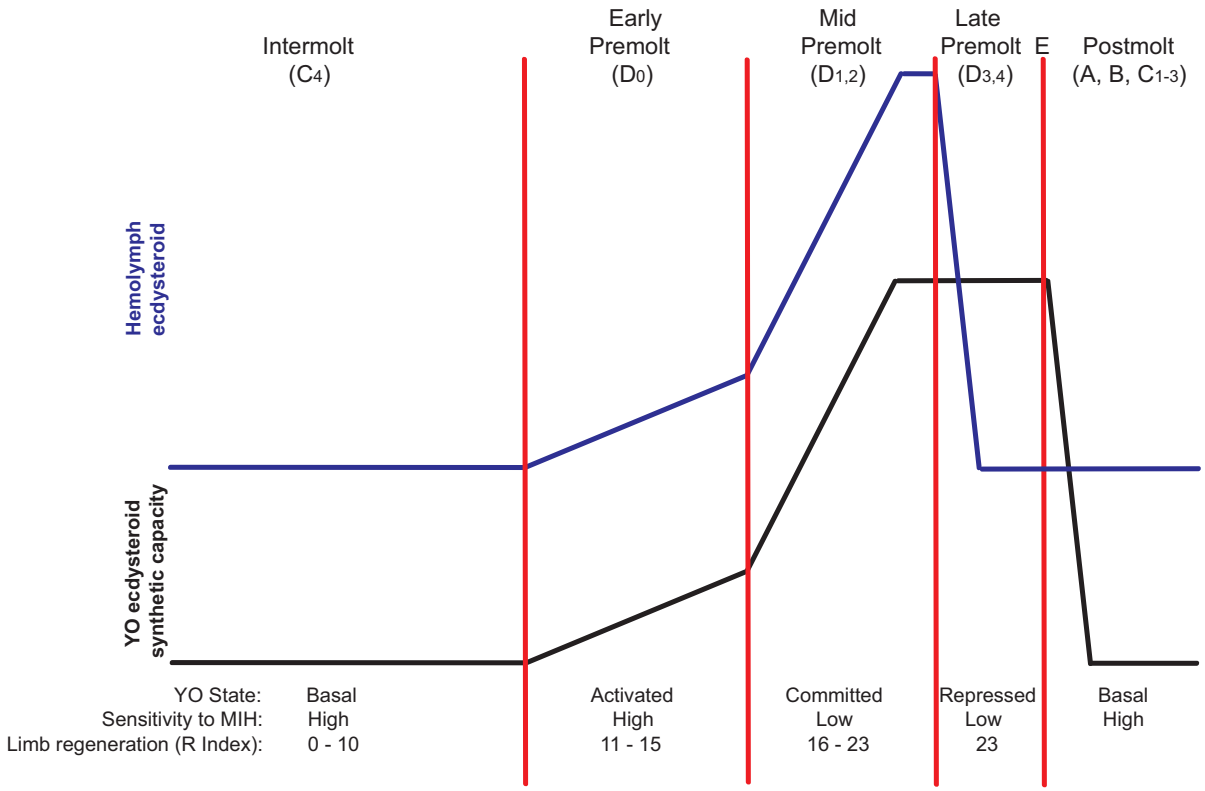

Figure.1. 2. Hormonal regulation of molting. MIH level reduced during molt cycle. Ecdysteroid level increased during premolt cycle. The YO transitions through four physiological states (basal, activated, committed, and repressed) during the molt cycle. YO activation and committed YO are triggered by a reduction in MIH, mTOR, and TGFβ signals (Chang and Mykles, 2011).

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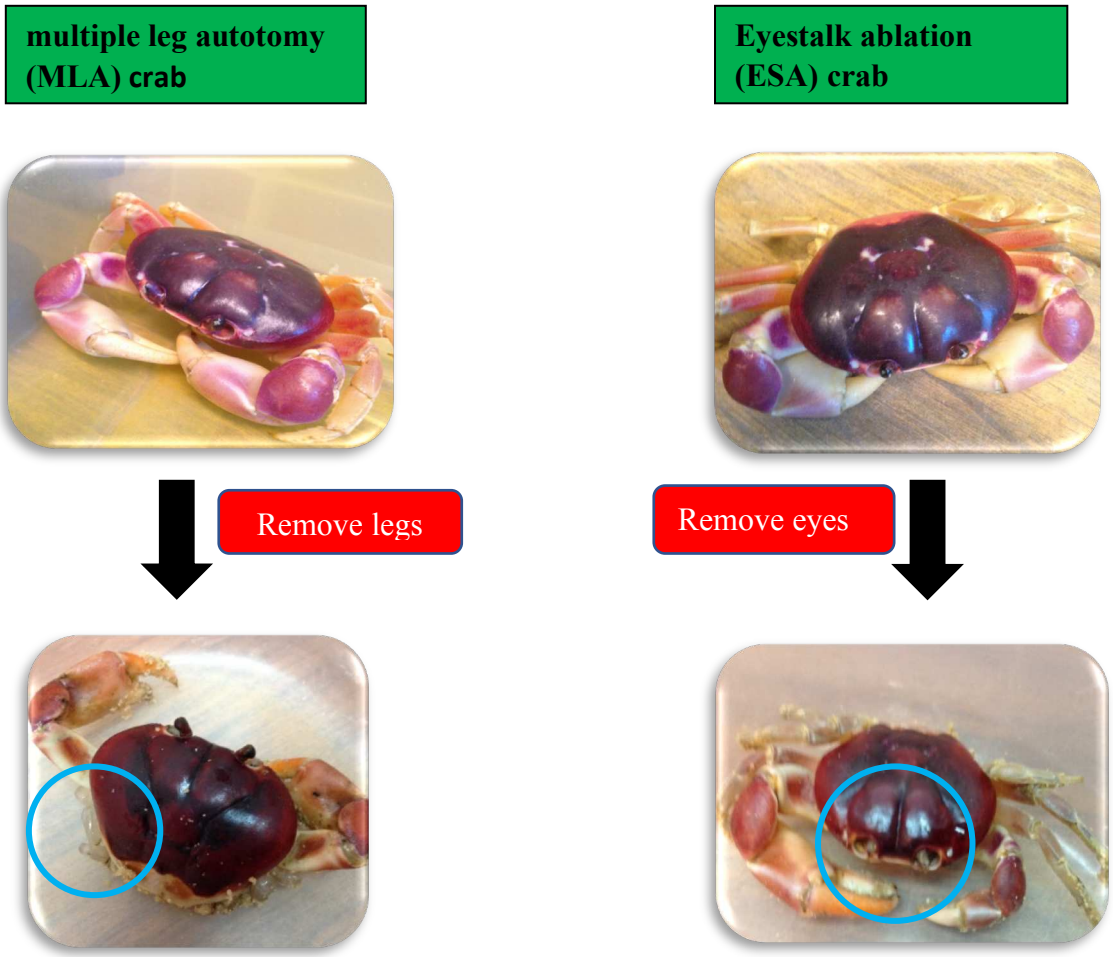
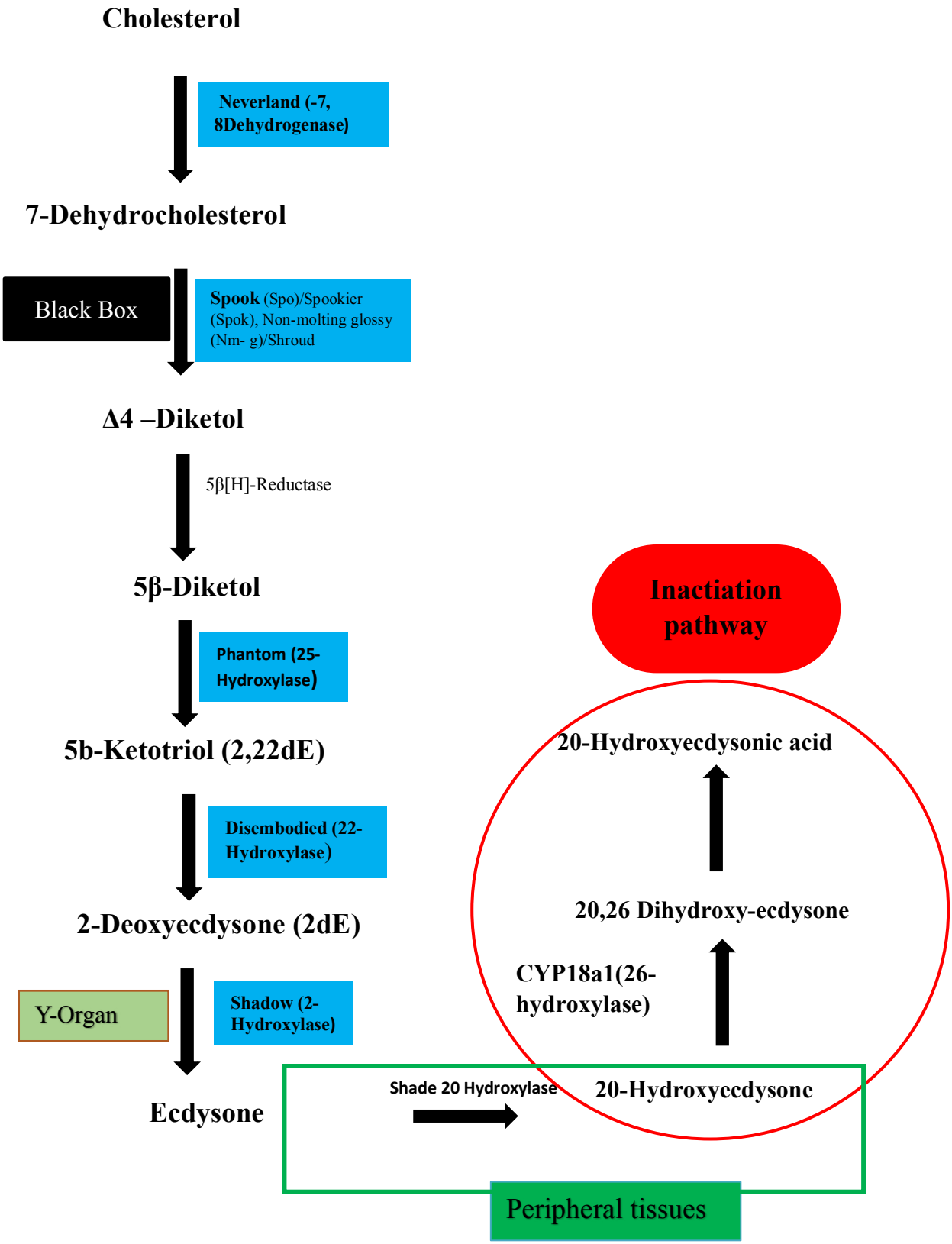
Table 1. 1. Ecdysteroid biosynthesis enzymes and their catalytic activity.

Gene	CYP number	Enzyme activity	Reaction catalyzed
Phantom	cyp306a1	25-hydroxylase	Converts 2,22,25 dE-ketodiol to 2,22dE-ketotriol
Disembodied	cyp302a1	22-hydroxylase	Coverts 2,22 dE-ketotriol to 2-deoxyecdysone
Shadow	cyp315a1	2-hydroxylase	Converts 2-deoxyecdysne to ecdysone
Spook/ Spookier	cyp307a1	Unknown	Converts 7-dehydrocholesterol to □4-diketol
Shade	cyp314a1	20-hydroxylase	Converts ecdysone to 20-hydroxyecdysone
N*everland*	None	7,8-dehydrogenase	Coverts cholesterol to 7-dehydrocholesterol

Ways to induce molting



Figuer.1. 3. Ways to induce molting. Eyestalk ablation (ESA) removes the source of MIH and the animal can enter premolt. Alternatively, multiple leg autonomy (MLA) can be performed, in which five or more walking legs are autotomized.


Cholesterol
Neverland (-7, 8Dehydrogenase)
7-Dehydrocholesterol
Black Box
Spook (Spo)/Spookier (Spok), Non-molting glossy (Nm- g)/Shroud
Δ4 –Diketol
5β[H]-Reductase
5β-Diketol
Phantom (25-Hydroxylase)
5b-Ketotriol (2,22dE)
Disembodied (22-Hydroxylase)
2-Deoxyecdysone (2dE)
Y-Organ
Shadow (2-Hydroxylase)
Ecdysone
Inactiation pathway
20-Hydroxyecdysonic acid
20,26 Dihydroxy-ecdysone
CYP18a1(26-hydroxylase)
Shade 20 Hydroxylase
20-Hydroxyecdysone
Peripheral tissues

Figuer.1. 4. The ecdysteroid biosynthetic pathway in the crustacean molting gland (Y-organ). In the first stage, cholesterol is converted to 20E by enzymes encoded by different genes. The reactions are catalyzed by cytochrome p450 enzymes encoded by the Halloween genes phantom, disembodied, and shadow. Shade activity in peripheral tissues produces the active ecdysteroids 20E a from ecdysone.

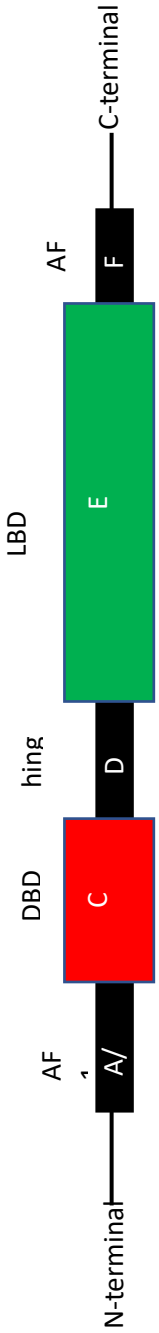


Figure.1 .5. Schematic of the structural organization of a nuclear hormone receptor family. A/B represents the transcriptional activation domain, C the DNA binding domain, D the hinge region, E the ligand binding domain and F the variable carboxyl-terminal domain.

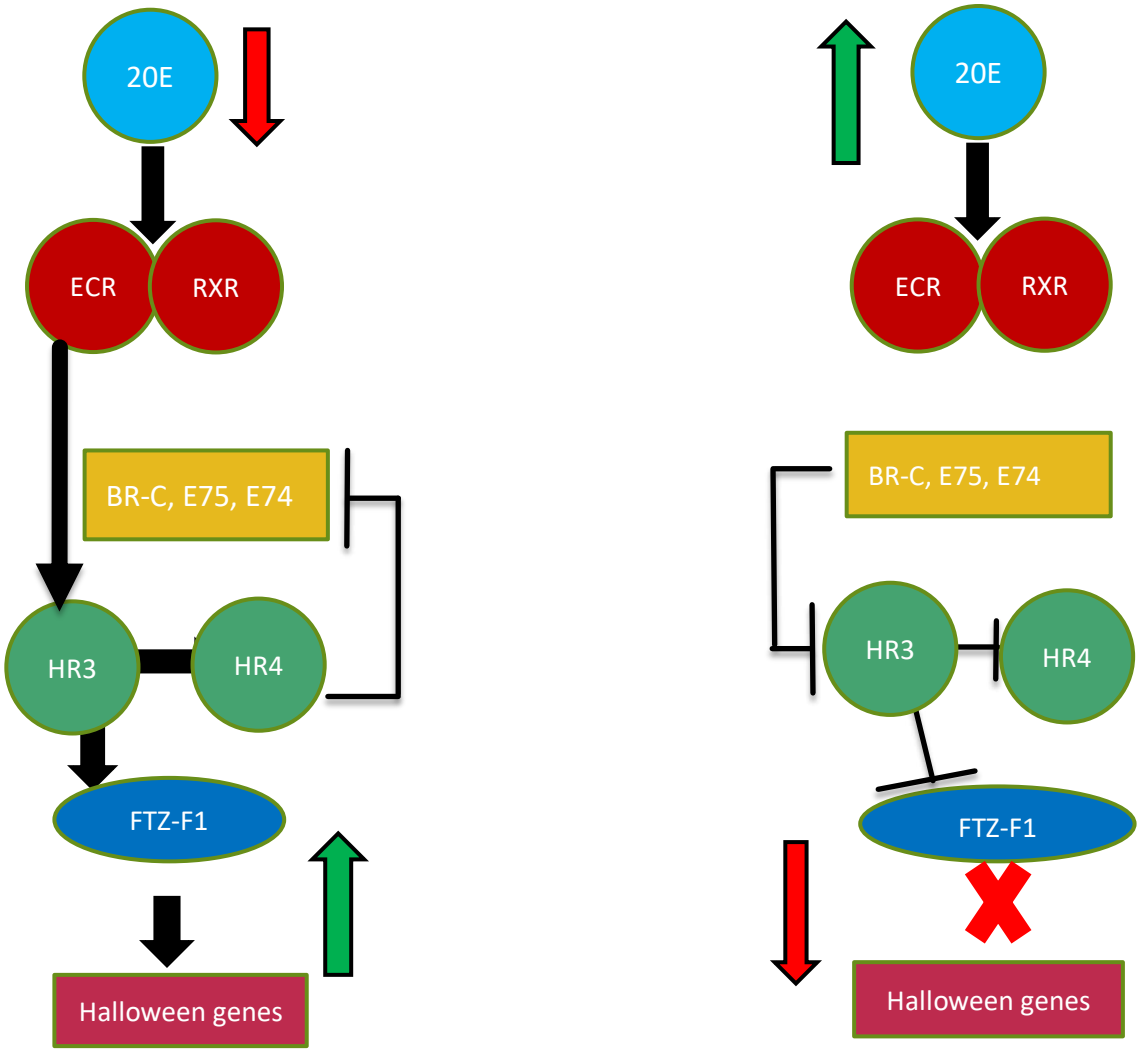


Figure.1. 6 Putative model illustrating the 20E regulation of molting in arthropods. A schematic representation of the 20E is shown at the top. Induction and inhibition of different genes depends on the level of 20E. Ecdysteroid receptor (EcR/RXR) binds active molting hormone, which induces serial activation of ecdysone-responsive genes. 20E: 20-Hydroxyecdysone; EcR: ecdysone receptor; Br-c: broad-complex; E75: nuclear receptor E74: nuclear receptor E74; Hr4: hormone receptor 4; Hr3: hormone receptor 3; Ftz-f1: Fushi tarazu factor-1

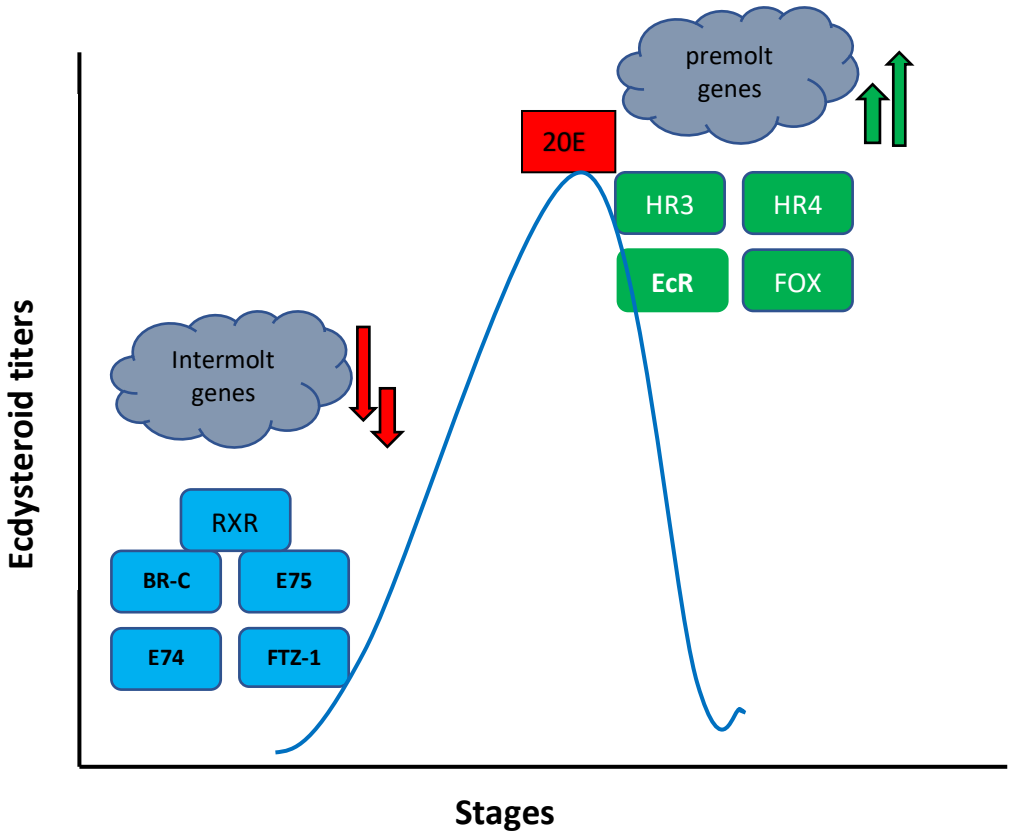


Figure.1. 7. Putative ecdysone regulatory cascade in crustaceans. Ecdysone is secreted by the Y-organ, leading to its active form 20-hydroxyecdysone (20E). The heterodimer *EcR*/*RXR* binds 20E and initiates the transcription of the "early" response genes, which in turn regulates the transcription of "late" genes responsible for initiating molting the ecdysteroid titer profile during molt stages is showed ecdysone responsive genes expression.

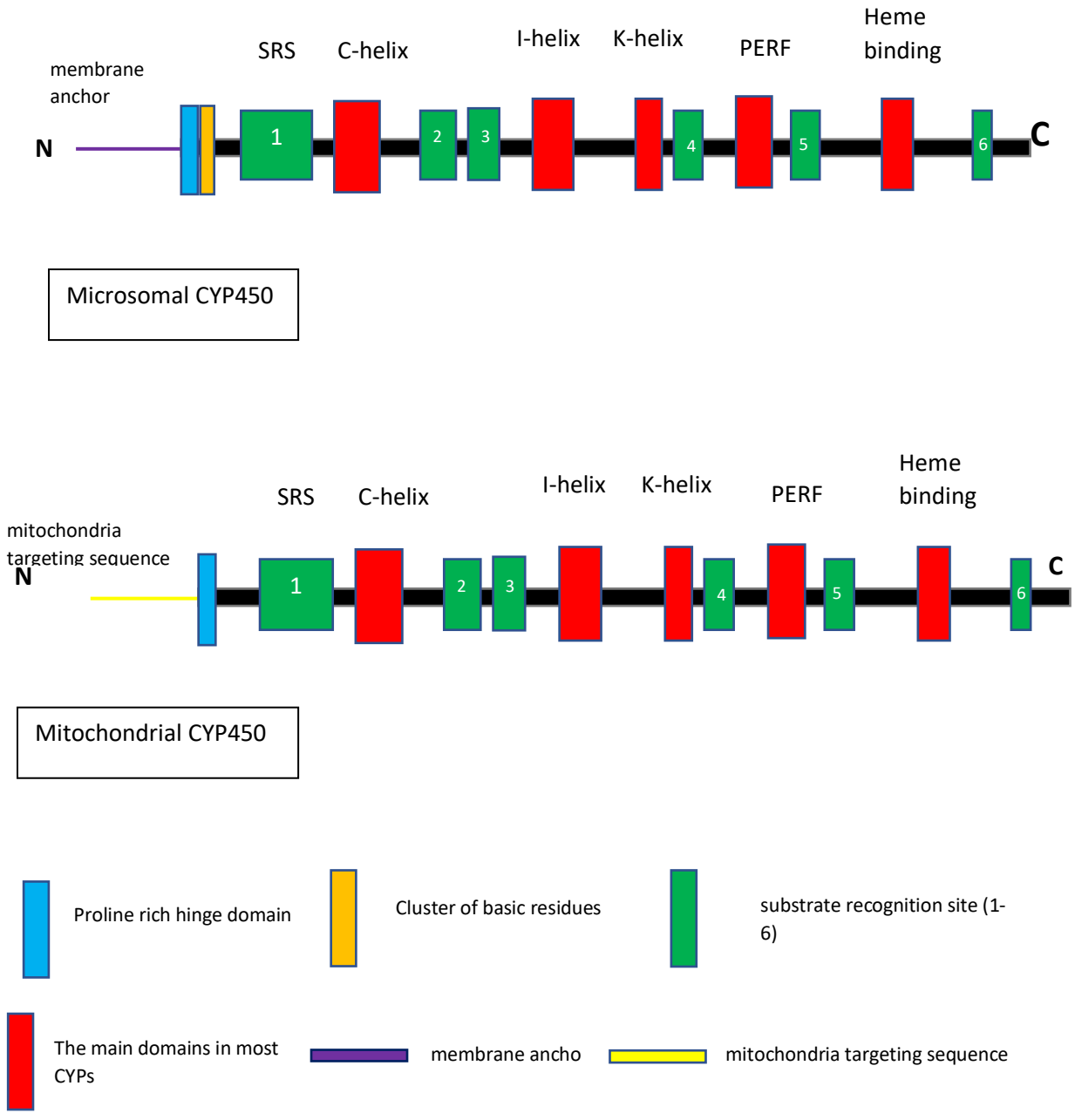


Figure.1. 8. Conserved CYP domains for microsomal and mitochondria CYP450.The main domains observed in most CYPs are shown in red (C-helix, I- helix, helix, PERF, heme binding domain). Green boxes indicate the more variable substrate recognition sites. Purple and light blue boxes indicate the membrane anchor and a key proline residue. Yellow indicate a mitochondrial targetin sequence. Gold indicates to the cluster of basic residues. Modified from (Feyereisen, 2012)

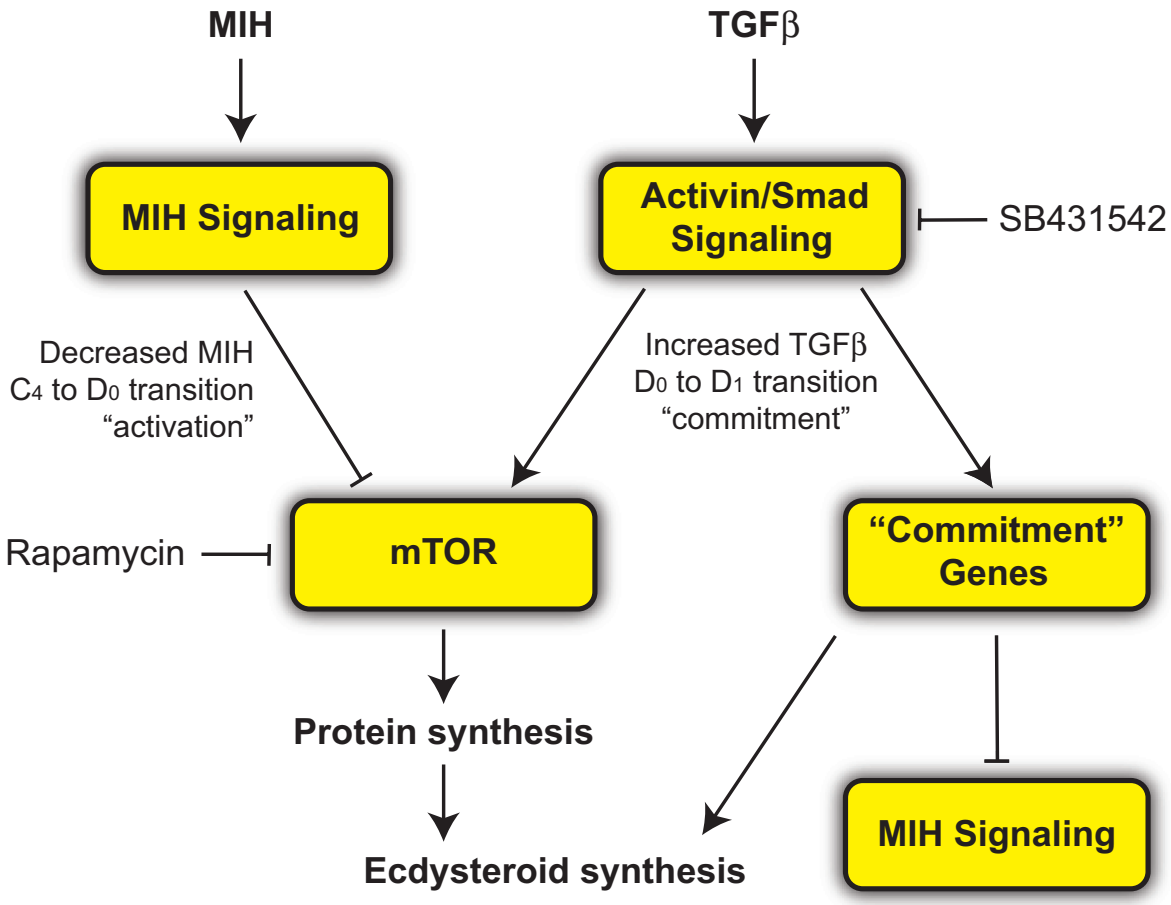


Figure1. 9. Putative signaling pathway of MIH, TGF-β factor and mTOR, in the YO. MIH inhibits activation of the mTOR pathway, which is responsible stimulation ecdysteroid synthesis. In contrast, activin and Smad signaling activate the mTOR pathway to stimulate molting, which transition the YO to the committed state and triggers the animal to enter late premolt

Chapter 2

Identification and characterization of contigs encoding Halloween and ecdysteroid-responsive genes in the *G. lateralis* YO transcriptome

Summary

Molting, a vital physiological process, is necessary for growth and development in all arthropods. Halloween genes are expressed in Y-organs (YO, an endocrine gland) and encodes for P450 enzymes. These enzymes catalyze the synthesis of ecdysteroid hormones that regulate the molt cycle. This hormone binds to ecdysteroid receptor to ecdysteroid-responsive genes that effect tissue responses to hormone. We used *P. leptodactylus* Halloween genes and insect ecdysone responsive genes to extract and characterize the land crab orthologs in the YO transcriptome. This resulted in identification and characterization of eight ecdysteroidogenic genes which are *phantom*, *disembodied*, *spook*, *shadow*, *Cyp18a1*, *neverland, NADK* and *ALAS* and nine of ecdysteroid-responsive genes, which are *EcR, RXR, broad complex*, *E75*, *E74*, *Hormone receptor 4*, *Hormone receptor 3*, *forkhead box transcription factors (FoxO)* and *Fushi tarazu factor-1*. Sequences were validated by end-point PCR and Sanger sequencing. We used phylogenetic analysis to infer evolutionary relationships among contig sequences and ortholog of Halloween genes and ecdysteroid -responsive genes in other species. The results showed all the contig sequences clustered with their corresponding orthologous genes. Tissue distributions for spook and phantom genes showed significantly higher mRNA levels in the YO compared to other tissues. By contrast, the mRNA levels of *NADK*, *ALAS* and all ecdysteroid-responsive genes were not higher in the YO than those in other tissues. These data show that the YO is the primary source of ecdysteroid production and that the YO can respond to ecdysteroid, suggesting a feedback regulation of ecdysteroid synthesis and secretion

Introduction

In arthropods ecdysteroids are the important steroid hormones that play pivotal roles in many developmental and physiological process, such as molting and metamorphosis (Gilbert et al., 2002; Niwa and Niwa, 2014). Steroid hormones are synthesized through a series of enzymatic steps starting with cholesterol and ending with 20-hydroxyecdysone (20E). Halloween genes are expressed in the molting gland and encode enzymes that catalyze the synthesis of ecdysteroid hormones. Although, ecdysteroid biosynthesis genes have been intensively studied over many years, there are parts of the ecdysteroid biosynthetic pathway that are unclear. (Mykles, 2011; Saito et al., 2016).

During the molt cycle, ecdysone is synthesized in the molting gland. Cholesterol is converted to 7-dehydrocholesterol (7DC) by the Rieske oxygenase Neverland (Nvd) (Yoshiyama-Yanagawa et al., 2011). The mechanism of the conversion of 7dC to 5β-ketodiol is still unclear. The enzymes involved in theses reactions are not known. This part of the pathway is commonly referred as the 'Black Box (Mykles, 2011; Niwa et al., 2010; Saito et al., 2016). To date, we know just three enzymes that are involved in this part of the pathway. These enzymes are non-molting glossy *(nm-g)/shroud* (*sro*) (Niwa et al., 2010), *Spook/Spookier* (*Spok*) (Pondeville et al., 2013), and *CYP6T3* (Ou et al., 2011). The next step converts 5β-ketodiol to ecdysone by three P450 enzymes (Niwa et al., 2004; Niwa et al., 2014; Warren et al., 2002; Warren et al., 2004). The last step converts ecdysone to 20E in peripheral tissues and is mediated by the *Shed* gene. These four genes are known as Halloween genes because null mutations of these genes cause embryonic phenotypes in *Drosophila* due to ecdysteroid deficiency (Rewitz et al., 2006a).

Cytochrome P450 (CYP) genes constitute a large family of genes that contain heme as a cofactor. The CYP450 family is found in all the domains of life and all living organisms, which include animals, plants, fungi, and prokaryotes (Córdova et al., 2017; Werck-Reichhart and Feyereisen, 2000). These enzymes require an electron donor; the electrons are transferred from NAD(P)H to P450 through electron donor system (Paine et al., 2005). In the ecdysteroid biosynthesis pathway, there are five CYP450 enzymes involved in this pathway: *CYP307A1* (*Spo*), *CYP306A1* (*Phm*), *CYP302A1* (*Dib*), *CYP315A1* (*Sad*) and *CYP314A1* (*Shd*). These Halloween genes have been characterized in *Drosophila melanogaster* Bombyx *mori* and other insects. In crustaceans the Halloween genes have been identified in the genome of *Daphnia pulex* and phantom in *M. japonicus* (Asazuma et al., 2009; Rewitz and Gilbert, 2008) which indicates that the Halloween genes pathway is highly conserved.

Halloween genes and *CYP18a1* contain many of the important conserved domains characteristic of a P450 protein, including the Helix-K (ExLR), PERF (PxxFxPxRF) and the heme-binding domain (PFxxGxRxCxG) Helix-C, Helix-I, where 'x' means any amino acid sequence (Gilbert et al., 2005; Ono et al., 2006; Sztal et al., 2012). Microsomal P450s consist of many hydrophobic residues followed by a proline–glycine rich region in N- terminal sequence, they are located in the endoplasmic reticulum such as *phantom* and *spook*. However, disembodied and shadow are mitochondrial enzymes that have specific hydroxylase activity at C-2, and C-22 (Iga and Smagghe, 2010; Kappler et al., 1988). Mitochondrial CYP450 enzymes meditated their reactions by transferring electrons through the action of adrenodoxin reductase and adrenodoxin, while, the microsomal CYP450 is mediated by NADPH cytochrome P450 reductase (Freeman et al., 1999; Neubueser et al., 2005). The WXXXR motif is located in the C-helix which where the arginine is formed a charge pair with the propionate of the heme.

(AGxxT) motif is located in Helix-I and corresponds to a proton transfer groove on the distal side of the heme. ExxR is located in Helix-K and has a role to the stabilizes the structure of the enzyme through salt bridge interactions. PERF' motif (PxxFxPxRF) is the aromatic region, or 'The heme-binding loop (PFxxGxRxCxG), which has an axial Cys. This cysteine works as ligand to the heme iron (Feyereisen, 2005; Ono et al., 2006; Zhou et al., 2013).

20 hydroxyecdysone bind to its receptors, and these receptors were identified in many insects and crustacean species (Browning et al., 2007; Koelle et al., 1991). The ecdysone receptor (EcR) and a vertebrate retinoid-X receptor (*RXR*) ortholog, ultraspiracle (*USP*) in insects are heterodimers that bind to response elements in the promoter regions of ecdysteroid-responsive genes. The domain organization of *EcR* and *USP* (*RXR*) consists of the A/B (transactivation) domain, C domain (DNA-binding domain (DBD) composed of 2 zinc fingers D (hinge), E domain is a ligand-binding domain (LBD) formed by 12 α helices DNA binding), and F domain (Clayton et al., 2001). DNA binding domains are highly conserved among insect species, while ligand-binding domains are moderately conserved among insect (Wurtz et al., 1996). Ecdysteroids and EcR/RXR complex induce the transcription of a variety of other nuclear receptors, such as *BR-C, E75, E74, HR3*, *HR4*, and *βFTZ-F1*(Ashburner, 1972; Huet et al., 1995).

BR-C, *E75*, and *E74* are classified as early ecdysteroid-responsive genes and are involved in the regulation of secondary responses to the initial induction. The *Broad-Complex* (*BR-C*) encodes a family of zinc finger proteins (DiBello et al., 1991). Null mutations that inactivate all three essential *BR-C* sub-functions caused prolonged third instar larvae that fail pupariate (Kiss et al., 1988; Restifo and White, 1992). *BR-C* mutations also have widespread effects on early and late ecdysteroid- responsive gene expression (Guay and Guild, 1991). *E74* has an ETS DNA

binding domain (Burtis et al., 1990). *E74* mutants display a lethality effect in morphogenesis, as well as defects in transcription of ecdysteroid-regulated genes (Fletcher et al., 1995).

The *E75* encodes three protein isoforms *E75A*, *E75B*, and *E75C. E75* and was identified in insects and crustaceans as a 20E primary-response gene (Feigl et al., 1989; Kim et al., 2005b; Segraves and Hogness, 1990; Zhou et al., 2019). These proteins contain an N-terminal activation domain AF-1, the canonical DNA binding domain, a hinge region, and ligand binding domain and C-terminal activation domain AF that define members of the nuclear receptor superfamily (King-Jones and Thummel, 2005; Mangelsdorf et al., 1995; Zhou et al., 2019). *E75* mutants in *Drosophila melanogaster* caused low ecdysteroid level, leading to developmental defects, while in black tiger shrimp, *Penaeus monodon,* silencing *PmE75* prevents shrimp to molt (Bialecki et al., 2002; Zhou et al., 2019). *E75* might act as a repressor of the *HR3* gene in *Drosophila melanogaster* (Feigl et al., 1989).

HR3 and *HR4* are nuclear receptors classified as 'early-late' genes while *FTZ-F1* is consider as late gene. *HR3*and *HR4* are expressed in late third instar larvae of *D. melanogaster* (Koelle et al., 1992; MartÍn, 2010). The expression of these genes depends on the titers of 20E, which decreased the expression of these genes after withdrawal of 20E. (Horner et al., 1995; Mazina et al., 2015). *DHR3* is expressed at high levels in early prepupae, as the early genes are repressed and before *βFTZ-F1* is induced. *HR3* and *HR4* act both a repressor of the early ecdysone-induced regulatory genes and as an inducer of *βFTZ*-F1 (King-Jones and Thummel, 2005; Ruaud et al., 2010). *HR3* and *HR4* proteins contain the DNA-binding domain (DBD) and a ligand-binding domain (LBD) (Laudet and Gronemeyer, 2002; Yang et al., 2017). While *βFTZ-F1* contains a highly conserved DNA binding domain with two zinc finger motifs and show

some conservation in their C-terminal regions with other ligand-binding domains (Ohno and Petkovich, 1993; Ohno et al., 1994).

FOXO belongs to the subfamily of the Forkhead family of transcription factors, which are characterized by a fork head DNA-binding domain (Hannenhalli and Kaestner, 2009). *FOXO* protein has a vital role in the insulin/insulin-like growth factor signaling (IIS) pathway (Dong et al., 2008; Kenyon et al., 1993) Hundreds of Fox genes are identified in humans and are conserved from yeast to human, while invertebrates have just one *FOXO* gene (Genin et al., 2014; Tuteja and Kaestner, 2007a; Tuteja and Kaestner, 2007b).

In this present study, we hypothesize is that the ecdysteroid biosynthesis pathway in *G. lateralis* is conserved and similar to a previously identified pathway in model arthropods like *Drosophila melanogaster*. To identity Halloween genes and ecdysteroid-responsive genes in *G. lateralis* we used RNA-seq data from both MLA and ESA YO transcriptome libraries to identify ecdysteroid biosynthesis genes (*Gl-phantom, Gl-disembodied, Gl-shadow, Gl-spook, Gl-CYP18a1,* and *Gl-Neverland*) and ecdysteroid-responsive genes (*Gl-Broad-Complex, Gl-E75, Gl- E74, Gl- Hormone Receptor 4, Gl-Hormone Receptor 3, Gl-Forkhead box transcription factor,* and *Gl-Fushi tarazu factor-1*). We did multiple sequence alignments that showed regions of high sequence identity between them. PCR products of the above genes were sequenced to verify the contig sequences from the transcriptome. A phylogenetic tree was constructed to determine the relationship between the *G. lateralis* and sequences of ecdysteroid genes and arthropod sequences obtained from the GenBank database. The *G. lateralis* sequences for *phantom, disembodied, shadow, spook*, and *neverland* clustered with their respective orthologs in the phylogenetic tree.

Materials and Methods

Animals and experimental treatments

Adult male *Gecarcinus lateralis* arrived from the Dominican Republic and were kept at Colorado State University, CO. Upon arrival in late May, the animals were first acclimatized for one month under controlled environment of ~27 °C and 75-80% humidity and placed under a cycle of twelve hours darkness and 12 hours light. Communal plastic cages that contained aspen beddings moistened with 5 ppt Instant Ocean (Aquarium Systems, Mentor, OH). Lettuce, carrots, and raisins were used two times a week to feed the crabs (Covi et al. 2010). Animals were dissected for collecting YOs that would be utilized for make RNA-seq database and YO transcriptome.

Identification of gene sequences in the YO transcriptome

A custom tBLASTn web portal was used in the running of a BLAST with every one of the Halloween genes and ecdysteroid-responsive genes protein sequence derived from NCBI against the *G. lateralis* YO transcriptome (Shavirin, 2014). We made a compilation of the top contig hits for *phantom, cyp306a1 (phm), disembodied, cyp302a1 (dib); shadow, cyp315a1 (sad); spook*, *cyp307a1* (*spo*), non-Halloween genes *neverland* (*nvd*), and *CYP18a1*, *Broad Complex*, *E75*, *E74*, *Hormone Receptor 4*, *Hormone Receptor 3*, *forkhead box transcription factor*, and *Fushi tarazu factor-1*, based on sequence identity of e-value and amino acid sequence identity. The full nucleotide sequences of the selected contigs were obtained for all genes except *BR-C*, *FTZ-F1* and *FOXO* genes from the transcriptome, using prfectBlast (Santiago-Sotelo and Ramirez-Prado, 2012). We confirmed the contig matching at the nucleotide level with an NCBI BLASTn search. tBLASTn results are summarized in (Tables 2. 4- 7).

The protein sequences were obtained using the online ExPASy translate tool (Artimo et al., 2012). We derived from the Swiss Institute of Bioinformatics to identify a putative open reading frame (ORF) for every one of the contigs that was extracted. We selected the longest putative ORF that coinciding with the frame that the transcriptome tBLASTn returned.

Multiple sequence alignments

We made alignments with regards to the multiple sequencing of amino acid sequences to identifying conserved sequence regions. In generating and editing of the multiple sequence alignment, I used of two computer programs - Clustal X version 2.1(Larkin et al., 2007) and GeneDoc version 2.7 (Nicholas and Nicholas, 1997). The Conserved Domain Database (Marchler-Bauer et al., 2014) was used to identify the variable regions in each gene and used the results in cross referencing with those of the published information on the structure of each gene in other species.

Phylogenetic analysis

A phylogenetic tree was constructed using a BLAST-Explorer (Dereeper et al., 2010) phylogenetic tree programmer (http://www.phylogeny.fr/) to determine the relationship between the *G. lateralis* contig sequences with arthropod sequences in the GenBank database.

Tissue distribution expression of *Phantom*, *Spook* and Ecdysteroid-responsive genes:

qPCR was used to quantified assess the tissue distribution of *Gl-Phm*, *Gl-Spo* and ecdysteroid-responsive genes. RNA was purified from mid gut, hind gut, antennal gland, YO, thoracic ganglion, brain, gill, claw muscle, hepatopancreas and heart. We collected tissues from intermolt animals. A Light cycler 480 Thermocycler was used to perform Quantitative PCR (qPCR) (Roche Applied Science, Indianapolis, IN, USA). The following were the qPCR conditions: forward and reverse gene-specific primers at 0.5 µl each as displayed on (Table 2. 1,

2) 5 µl SYBR Green (Roche), 3 µl nuclease free water, and a template of 1 µl cDNA. Upon denaturation for a period of 3 minutes at a temperature of 95 °C, repetitions of 45 PCR cycle were made for 30 seconds at a temperature of 95 °C, for 30 seconds at a temperature of 62 °C, for 20 seconds at a temperature of 72 °C, and a final extension of 7 min at a temperature of 72 °C. The quantification of each gene's concentration was made by making comparison with established standard curves.

Results

cDNAs encoding Halloween and ecdysteroid-responsive genes in the *G. lateralis* YO transcriptome.

YO transcriptome was assembled from Illumina sequencing of cDNA libraries from three biological replicates of mRNA and bioinformatics analyses and denovo assembly was used to create a YO transcriptome database of *G. lateralis* (Das et al., 2016). Gene annotation and quantification of gene expression were used to characterize contigs from MLA and ESA transcriptomes. The MLA transcriptome consisted of biological replicates of mRNA collected from five stages: intermolt, premolt (early, mid, and late) and post molt (Das et al., 2016). The ESA transcriptome consisted of mRNA of from replicates of ESA animals injected with DMSO or rapamycin and collected at 0, 1, 3, and 7 days post-ESA (Shyamal et al., 2018).

Contigs were identified using BLAST with Halloween genes and Ecdysteroid-responsive gene sequences in the GenBank database. Six ecdysteroid biosynthesis genes (*Gl-phantom*, *Gl-disembodied*, *Gl-shadow*, *Gl-spook*, *Gl-CYP18a1,* and *Gl-Neverland*) were identified. transcriptomes. All these genes were full length (Table 2. 4, 5). Ecdysteroid-responsive genes (*Gl-Broad-Complex*, *Gl-E75*, *Gl- E74*, *Gl- Hormone Receptor 4*, *Gl-Hormone Receptor 3*, *Gl-Forkhead box transcription factor*, and *Gl-Fushi tarazu factor-1*) were identified. *Gl- BR-C*, *Gl-*

HR4, *Gl- FOXO* and *Gl-FTZ-F1* genes were not complete length, while *Gl-E75*, *Gl- E74* and *Gl-HR3* were full length (Table 2. 6, 7).

PCR was used to verify the contig sequences. The Halloween genes were extracted from the MLA YO transcriptome and sequences were verified using BLAST against the GenBank database. PCR was used to obtain cDNAs encoding the known Halloween genes and *nvd* to verify the contig sequences by designing primers for these genes (Table. 2. 3). The sequence of an 1146-bp PCR product using Phm primers was identical to the *Gl-Phm* contig. Using the same strategy, PCR product sequences matched the contigs encoding *Spook*, *Neverland*, *CYP44* and *Shadow* (Figs. 2. 1-5).

Sequence identification and phylogenetic analysis

To determine a similarity between *G. lateralis* sequences of Halloween genes and ecdysteroid-responsive genes with orthologous Halloween genes and ecdysteroid-responsive genes, we did multiple sequence alignments that showed regions of high sequence identity between *G. lateralis* genes with crustacean and insect orthologs. Multiple alignments of Halloween genes and *CYP18a1* gene of the deduced amino acid sequences from crustacean and insect species indicated high sequence identities in the Helix-C, Helix-I, Helix-K, PERF motif, and heme-binding domains characteristic of this gene family (Fig .2. 7, 8, 9, 10, 13).

Multiple sequence alignment of the *neverland* gene showed high sequence identities in the Rieske domain (Fig. 2. 6). Multiple sequence alignment of *Gl-BR-C* gene showed high sequence identities in the BTB/POZ domain (Fig. 2. 14). The *Gl-ALAS* gene showed high identities in 5-aminolevulinate synthase presequence and 5-aminolevulinate synthase domain (Fig. 2. 12). *E74* proteins from five arthropod species were highly conserved and showed high identity in the ETS DNA-binding domain (Fig.2. 18). *Gl-HR3* protein showed high identities in

the DNA-binding domain which is composed of two C4-type zinc fingers containing 8 conserved cysteines (Fig. 2. 19). In addition, the alignment showed conserved the "KXGRZS" sequence at the C-terminal extension (CTE) to the DBD and the ligand binding domain. The ligand binding domain (LBD) has a putative ligand-dependent activation function domain (AF-2) with a conserved "LYSETF" sequence (Fig.2. 19).

Multiple sequence alignment of crustacean and insect of *EcR* and *RXR* revealed high levels of sequence identity in the highly conserved DNA binding domain (C), a hinge region (D), and the ligand binding domain (E). The AF2 region is critical for ligand-dependent transactivation of *RXR*, and it was identical among the *RXRs* from both vertebrates and invertebrates. *RXR* in crustaceans contain identical A/B domain sequences but have differences in the T-box or in the LBD (Fig. 2. 16).

The multiple sequence alignment revealed that *Gl-FTZ-F1* proteins had the main structure of *FTZ-F1*, which showed of an A/B domain, a conserved DBD (C domain), LBD and an activation function (AF-2) (Fig. 2. 22). The multiple sequence alignment of partial *Gl-HR4* missed DBD domain, however, the alignment is showed full length of the ligand binding domain (LBD; E domain) (Fig. 2. 20).

The *Gl-E75* protein sequence aligned with *E75* orthologs from crustacean and insect species showed low sequence identity in the A/B domain and high identity in a two-zinc-finger DBD (C domain), hinge region (D domain,), LBD (E domain) with the putative ligand-dependent activation motif, and F domain (Fig. 2. 17). *Gl-FOXO* showed high identity and similarity in a conserved region, Forkhead domain (FH), or winged-helix domain and it showed three identified AKT/PKB phosphorylation sites (Fig. 2. 21).

Gl-NADK gene showed high identity in a diacylglycerol kinase catalytic domain (Fig. 2. 11). We used a phylogenetic tree to investigate the inferred evolutionary relationships among contig sequences and other orthologs of Halloween genes and Ecdysteroid-responsive genes from insects and crustaceans. The results showed all the contig sequences of these genes clustered with their corresponding orthologs in other arthropod species (Fig.2. 23, 24).

Tissue distribution expression of *Phantom*, *Spook* and ecdysteroid responsive genes

We used qPCR to quantify the expression of *phantom and spook* genes. *Phantom* mRNA was detected in all tissues examined (midgut, hindgut, antennal gland, YO, thoracic ganglion, brain, gill, claw muscle, hepatopancreas, and heart). *Gl-phm* mRNA levels showed significantly higher mRNA levels in YO compare to the other tissues (Fig 2. 25A, B). Also, the results showed *Gl-Phm* mRNA level were higher in hindgut than heart tissue. *Gl-Spo* mRNA showed a significantly higher level in YO compared to other tissues ($p<0.001$) (Fig.2. 25A-B). *Gl- NADK* and *Gl-ALAS* were expressed in all tissues examined (Fig.2. 25C-D). *Gl-HR3* mRNA level was highest in midgut, antennal gland, YO, thoracic ganglion, brain and gill, and were lowest level in hepatopancreas ($P <0.001$). *Gl-FOXO* mRNA levels were highest in antennal gland, heart, and midgut, and lowest mRNA levels in the hindgut, thoracic ganglion, and claw muscle ($P<0.050$). *Gl-E74* gene showed the highest mRNA levels in claw muscle, midgut, hindgut, antennal gland, thoracic ganglion, brain, gill, and heart and lowest level in YO and hepatopancreas ($P <0.001$). *Gl-BR-C* gene showed the highest level of mRNA in midgut and lowest level in YO ($P<0.05$). *Gl-HR4* gene showed the highest level in antennal gland, thoracic ganglion, and brain, and lowest level in the YOs ($P<0.05$) (Fig. 2. 26E-J).

DISCUSSION

Ecdysteroid biosynthesis in the YO is mediated by cytochrome p450 (CYP) enzymes encoded by the Halloween genes. These genes are necessary for normal development, molting, reproduction and homeostasis in insects (Gilbert et al., 2002; Rewitz and Gilbert, 2008). Molting and metamorphosis process are conserved and the Halloween genes which are involved in steroidogenesis are highly conserved in insect (Rewitz et al., 2007). This study is the first characterization of these important genes in *Gecarcinus lateralis*. In *Gammarus fossarum,* molting hormone (20E) binds to their receptors (EcR/RXR) and this complex activates a gene regulatory cascade that will mediate molting (Gouveia et al., 2018). RNA-Seq data was used to identify these genes in the YO. We extracted Halloween genes and ecdysteroid- responsive genes from both MLA and ESA YO transcriptomes based on insect orthologs of these candidate genes. We demonstrated the presence of Halloween genes orthologs, *Gl-Nvd* and *Gl-CYP18a1*. (Table 2 .4-7).

Gl- Nvd gene had the highly conserved of Rieske-domain which indicated that the *Gl-Nvd* was highly similar to Rieske domain oxygenase *Neverland* of other species such as, *Drosophila melanogaster*. The main structure of *Gl-Nvd* protein is distinct from other known genes expressed in YO and plays an important role in steroidogenesis (Gilbert et al., 2005). In insects knock down of *nvd* in the PG causes developmental defects that lead to arrest growth and molting in larvae of *Drosophila* (Yoshiyama-Yanagawa et al., 2011). Moreover, feeding 20E or the precursor 7-dehydrocholesterol to *Dm-nvd* RNAi larvae partially rescues the RNAi larvae (Yoshiyama-Yanagawa et al., 2011).

Gl-phm, *Gl-dib*, *Gl-sad*, and *Gl-CYP18a1* share the same structural of P450 motifs (Helix-C, Helix-I, Helix-K, PERF motif and heme-binding domain) and they are well conserved

(Fig. 2. 8, 9, 10, 13). The role of Halloween genes is well studied in insects, in which they mediate the biosynthesis of 20E from cholesterol. To date, there are four of P450 enzymes, *CYP306A1* (*Phm*), *CYP302A1* (*Dib*), *CYP315A1* (*Sad*) and *CYP314A1* (*Shd*) involved in the ecdysteroid biosynthesis pathway. These enzymes convert of ketodiol to ecdysone in the molting gland and the conversion ecdysone to 20E takes place in peripheral tissues. conversion of 7-dehydrocholesterol (7dC) to diketol is hypothetically catalyzed by series of unknown reactions, which is known as the Black Box (Marchal et al., 2010; Warren et al., 2009). Halloween genes have been identified in many insect species, such as: silkworm *Bombyx mori* (Niwa et al., 2004; Warren et al., 2004) and the tobacco hornworm, *Manduca sexta* (Rewitz et al., 2006b). Halloween genes also have identified in the crustacean Daphnia and identified just the *phantom* gene in *Marsupenaeus japonicus* (Asazuma et al., 2009; Rewitz and Gilbert, 2008).

Broad complex (*BR-C*), *E74*, and *E75* early genes and transcription factors, which are stimulated by hormone signal and activate or repress expression of many of other secondary-response late genes. *BR-C* has conserved (BTB)/Pox virus domain and Zinc finger (POZ) protein interaction motif at the N-termini identified in the silkworm and fruit fly. (Bayer et al., 1996; Uhlirova et al., 2003). *E74* encodes ETS domain transcription factor, while *E75* gene belong to the nuclear receptor superfamily (Fletcher et al., 1995; Kim et al., 2005b; Segraves and Hogness, 1990). Molecular studies on these genes are showed 20E induced directly *BR-C*, *E74A*, and *E75A* and then are repressed quickly in late third larvae. Knock down of *BR-C* in the PG results in larval arrest growth and prevent molting (Fletcher et al., 1995; Kiss et al., 1988; Lam et al., 1999; Niwa and Niwa, 2016).

ALAS and Contig *CG33156* is a predicted gene with the NAD kinase domain that is reported in playa role in ecdysteroid synthesis. Knockdown of these genes in silkworm *Bombyx*

mori PG and the fruit fly *Drosophila melanogaster* at third instar larva caused developmental arrest with a low level of ecdysteroid hormone, which that indicated that *NADK* and *ALAS* genes have essential function in ecdysone biosynthesis pathway. *CG33156* and *Gl-NADK* showed high identity to human *NADK1* by BLASTP with NAD kinase domain, which suggest that they serve as NAD kinase. Cytochrome P450 enzymes which Halloween genes require NADP(H) (electron donor) and heme (electron transfer) for their function. *Bm-NADK* is thought function as a provider of NADP(H) (Nakaoka et al., 2017). *ALAS* gene encodes δ-aminolevulinic acid synthase and has role in heme biosynthesis. Theses result suggest both *Bm-ALAS* and *Bm-NADK* are required for ecdysone synthesis and/or release in larval development. (Nakaoka et al., 2017).

Ultraspiracle (USP) the insect homologue of vertebrate retinoid X receptor and *EcR* (ecdysone nuclear receptor) form a heterodimer and mediate ecdysteroid-regulated gene expression in insects (Ghbeish et al., 2001; Tan et al., 2008; Yao et al., 1993). *EcR* and *RXR* genes have different N-terminal domain and most well conserved central site DNA binding domain (DBD) and C-terminal ligand binding domain (LBD) (Dawson and Xia, 2012; Helsen et al., 2012; Rastinejad et al., 2000). We identified *Gl-EcR* and *Gl-RXR* in YO which share the same domain with nuclear receptor proteins from other species (Fig. 2. 15,16). We obtained multiple complete isoforms from baseline transcriptome which had highest sequence identity to cDNAs encoding *Gl-RXR* isoforms obtained by RT-PCR and fiddler crab *RXR* (*Up-RXR*) and insect ultraspiracle (*USP*) (Chung et al., 1998; Durica et al., 2002; Kim et al., 2005b). *EcR* and *RXR* were first cloned from *Uca pugilator* and *G. lateralis.* In this study We found *Gl-EcR,* and *Gl-RXR* expressed in YO while in *Uca pugilator Gl-EcR* and *Gl-RXR* are expressed in other tissues, such as testis, ovary, and skeletal muscle (Durica and Hopkins, 1996; Kim et al., 2005a; Kim et al., 2005b).

Gl-HR3 and *Gl-HR4* are called early-late genes in insects and have highly-conserved DNA-binding domain (DBD), containing two C4-type zinc finger regions. and a less-conserved ligand binding domain (LBD) (Cruz et al., 2007; King-Jones and Thummel, 2005; Lam et al., 1997; Yang et al., 2017). *DHR3* and *DHR4* gene expression depends on level of molting hormone and decreased their level by reduced titers of 20E. High level of molting hormone keeps *HR3* and *HR4* at active transcription in in S2 cells for a while (Mazina et al., 2015b)

βFTZ-F1 protein is a member of the steroid receptor superfamily and has the conserved regulatory function of steroidogenesis in insects. Moreover, *βFTZ-F1* has vital role in ecdysteroid biosynthesis in the PG by increase expression of Halloween genes (Lam et al., 1999; Niwa and Niwa, 2016). Loos function of *βFTZ-F1* in *Drosophila* leads to low protein level of *phantom* and *disembodied* genes in the PG, in which low titer of 20E leads to activation of *βFTZ-F1* and more activation of Halloween genes, suggesting that *βFTZ-F1* which functions as a positive regulator of Halloween genes (Niwa and Niwa, 2016; Parvy et al., 2005). *HR3* activated β*FTZ-F1* in mid prepupae when 20E at the low level, consider that *βFTZ-F1* is dependent on *HR3* for its activation (Lam et al., 1999; Niwa and Niwa, 2016)

FOXO (*Forkhead box,* sub-group *O*) is a transcription factor in the insulin signaling cascade (Cheng et al., 2011). Activated insulin receptor stimulates downstream effectors, including phosphoinositide 3-kinase (PI3K), serine-threonine kinase *Akt*, and forkhead transcription factor *FOXO* (Barthel et al., 2005; Jünger et al., 2003; Puig et al., 2003). *FOXO* is characterized by a conserved domain DNA-binding motif that is called the "winged helix" or "Forkhead" domain. This conserved domain occurs in eukaryotic organisms from yeast to human (Tuteja and Kaestner, 2007b). In *G. lateralis* we identified a *FOXO* ortholog with its conserved Forkhead (FH) domain (Fig. 2. 21).

Gl-phm and *Gl-spo* were highly expressed in a tissue that synthesizes ecdysone, molting gland (YOs), compared to other tissues (midgut, hindgut, antennal gland, thoracic ganglion, brain, gill, claw muscle, hepatopancreas and heart) (Fig. 2. 25A-B). In insect larvae, ecdysone (E) is released from the PG into the hemolymph and is converted by the peripheral tissues to 20E. Ecdysteroid receptor (*EcR*/*RXR*) binds active molting hormone (20E), which induces ecdysteroid-responsive genes, leading to molting and metamorphosis (Ashburner et al., 1974; McBrayer et al., 2007; Ou et al., 2016; Parvy et al., 2005; Russell and Ashburner, 1996; Yamanaka et al., 2013).

In this study, we investigated the expression of ecdysteroid- responsive genes in *G. lateralis* in these tissues (midgut, hindgut, antennal gland, Y organ, thoracic ganglia, brain, gill, claw muscle, hepatopancreas and heart). The result showed high expression of these genes in most of these tissues. *Gl-E75*, *Gl-HR4* and *Gl-BR-C* have low expression in YO tissues compared with other tissues while *Gl-FTZ-F1* has high expression but were not significantly different between YO and other tissues (Fig. 2. 26E-J). In insects some ecdysteroid-responsive genes are expressed in the PG for feedback regulation on the PG. They are expressed the salivary glands, gut, Malpighian tubules, fat bodies and imaginal discs (Ashburner et al., 1974; Huet et al., 1993; Segraves, 1990).

Table 2. 1. Oligonucleotide primers used for quantitative PCR of ecdysteroidogenic genes from *G. lateralis*.

Gene	Primer Sequence (5"–3")	Product size (bp)	TM (°C)
Phantom	F1 TCTTTCACTTCACCACCACC	182	54.9
	R1 TCCTCTGTGACTCAGGTCTTA		54.4
Disembodied	F1 TCTCTTCAGTCAGTCCCTATGT	234	54.6
	R1 GCATCTCAGCTACCTCTCATTT		54.6
Shadow	F1 CGGCTGACTCCCTCATAATTT	234	54.7
	R1 GGAAGGCAGCTCGCTATAAG		55.4
Spook	F1 CCCTTCAGCACCGGAAAG	251	56.2
	R1 CTAGTGATACTCGTGATGCCTG		54.6
Neverland	F1 GTGTCCGAGGCGAGACATT	183	57.3
	R1 ACGTCGACCATCACCATTAC		54.7

Table 2. 2. Oligonucleotide primers used for quantitative PCR of ecdysteroid-responsive genes from *G. lateralis*.

Gene	Primer Sequence (5'–3')	Product size (bp)	TM (ºC)
BR-C-F1	CAAAGGACTGACTGAGCAGAA	235 bp	54.7 ºC
BR-C-R1	GAGAGTTGGACTGCTGGTT		
E75-F1	GAGTATGAGTCCTATGCAGCC	226 bp	60ºC
E75-R1	CGATGAAGACGATCTCTGGTG		
E74-F1	CAGGGAGAAGGGAGTGTTCA-	183 bp	56.3ºC
E74-R1	GGAACATCAACAAACTGGTACACG		56.4ºC
HR3-F1	TACATCCCGCAGACCACCAC-	115 bp	59.4 ºC
HR3-R1	CCGACTCCGACAGGGGCTC		60.3 ºC
HR4-F1	TGACGACTTACTTGACCACAA-	150 bp	53.8ºC
HR4-R1	TTGTGTGTGAGGAGTCTCGT		55.7ºC
FTZ-F1- F1	CTACAGCACTCTTGGTCTGACTTG-	115 bp	57.2ºC
FTZ-F1-R1	GGGACAGCAGGTCAAACTT		55.2ºC
FOXO-F1	GCCGCCCAAGAAGAATACG-	161bp	56.4 ºC
FOXO-R1	ATACTTCAAGGACAAGGGCG		54.8 ºC
RXR-F1	CTCAGGCAAGCACTATGGCGT-	164 bp	60ºC
RXR-R1	TCAAGCACTTCTGGTAGCGGCAG		61.5ºC
EcR-F1	GCGTTATGATGCCAAGACAGATTC-	117 bp	56.3 ºC
EcR-R1	CGGCAGAAACGGAAGAGTATC		55.5 ºC
NADK-F1	GCCGAATCATGCGAAACTC-	101bp	54.5 ºC
NADK-R1	CTTGTCTGTGTTGGTCATCAAG		53.9 ºC
ALAS-F1	CAAGGTCTCGGATGAACTGATAA-	129bp	54.3 ºC
ALAS-R1	CATACCAAGCCCATGATGGA		54.7 ºC

Table 2. 3. Oligonucleotide primers used to confirm contig sequences of *G. lateralis* ecdysteroidogenic genes.

Gene	Primer sequence	Product size bp	Melting Temperature °C
Phantom	F1 AGGTGCGAATAGGAGAGTGACAC R1 GCCTCTCCTTGATATAAGCGTCA	1143	58.4 56.3
	F2 AAGGGAAGTGAAGCTGATGTCCT R2 TGGTGAAGAGGAATGGTGCTTAAC	1289	58.2 57.2
CYP44	F1 AACAGTTTGCCCGACATACGT R1 CACTAGTCTTTGAGCCACGTCC	733	57.1 57.5
	F2 GAGAAGTCTCTTGGAAGCCTTGA R2 TAGCAGCAGTAGTAGCAGGAGT	1089	56.4 57.2
Shadow	R1 TCAGTGAGGAGAGGAATGATGTCA F1 CCTCTGCCAGACGCATGATTAA	1398	57.1 57.3
	F2 CATGGTCAATAGCCTCGATGAAG R2 AGTATGCAATGAGGGACAGAGCAA	1075	55.5 58.3
Spook	F1 TGCTCGCAAAGATGGTCTTC R1 CGTTGATGTAGGAAGGGAAGAG	813	55.5 54.9
	F2 GGGCACATCATCGACTTCCTT R2 CTAGTGATACTCGTGATGCCTG	884	57.3 55.5
Neverland	F1 GCATTTTATACACGCTGCACC R1 TTCTTTCTGAGCCTCTGCAC	615	54.9 54.9
	F2 CTGCTTCTCTTCTTCGTCTACC R2 GTGTAGGTGTCCAAGATGAGC	657	54.7 55.3
	F3 AGATACCCTACTCCTCCAAGG R3 ACGTCGACCATCACCATTAC	807	54.9 54.7

Table 2. 4. Halloween genes *Neverland* and *CYP18a1* sequences from the *G. lateralis* MLA YO transcriptome.

Gene	Contig Number	Contig Length (bp)	ORF (aa)	Domain	Top BLASTp Hit	Accession # to BLAST hit	E-value	Score	Positives
Phantom	c268220_g1_i1	3513	564	P450 superfamily	*Marsupenaeus japonicus*	dbj|AB455969.1	0.0	550	71%
Disembodied	lcl|c268194_g1	2452	538	P450 superfamily	*Portunus trituberculatus*	gb|KM596851.1	0.0	797	85%
Spook	c262802_g1_i1	3241	520	P450 superfamily	*Neocaridina denticulata*	gb|KJ200319.1	0.0	568	78%
Neverland	*c218937_g1_i1*	1961	515	Rieske superfamily	*Danio rerio*	dbj|AB607951.1	7e-134	405	66%
CYP18a1	c259236_g1_i1	1845	527	P450 superfamily	*Neocaridina denticulata*	gb|KJ579128.1	0.0	661	86%
Shadow	c247804_g1_i2	2980	556	P450 superfamily	*Portunus trituberculatus*	gb|KM880023.1	0.0	619	79%
ALAS	c209048_g2_i2	2696	532	5-aminolevulinate synthase presequence	*Nicrophorus vespilloides*	XM_017920669.1	0.0	714	65%
NADK	c242467_g1_i5	2109	452	Diacylglycerol kinase catalytic domain1	*Hyalella Azteca*	XM_018166947.1	0.0	703	76%

Table 2. 5. Halloween, *Neverland* and *CYP18a1* gene sequences from the *G. lateralis* ESA YO transcriptome.

Gene	Contig Number	Contig Length (bp)	ORF Length (aa)	Domain	Top BLASTp Hit	Accession # to BLAST hit	E-value	Score	Positives
Phantom	c222063_g2_i1	3052	441	P450 superfamily	*Marsupenaeus japonicus*	AB455969.1	6e-165	483	(69%)
Disembodied	c210314_g2_i1	5535	538	P450 superfamily	*Portunus trituberculatus*	KM596851.	0.0	793	(84%)
Spook	c205351_g1_i1	3536	520	P450 superfamily	*Neocaridina denticulate*	KJ200319.1	0.0	568	(78%)
Neverland	c202105_g1_i1	1661	467	Rieske superfamily	*Danio rerio*	XM_018161838.1Length	0.0	523	(73%)
CYP18a1	c212757_g1_i1	1676	527	P450 superfamily	*Neocaridina denticulate*	AIY69132.1	0.0	662	(78%)
Shadow	c206183_g1_i2	2222	497	P450 superfamily	*Portunus trituberculatus*	AJF94636.1	0.0	662	(78%)
ALAS	c266479_g1_i1	4133	564	5-aminolevulinate synthase presequence	*Nicrophorus vespilloide*s	XM_017920669	0.0	712	(61%)
NADK	c207430_g2_i3	2094	452	Diacylglycerol kinase catalytic domain	*Hyalella Azteca*	XM_018166947.1	0.0	703	(76%)

Table 2. 6. Ecdysteroid-responsive genes from the *G. lateralis* MLA YO transcriptome.

Name of the gene	The Contigs name	Length	ORF	Domain name	Top BLASTp Hit	Accession #to BLAST Hit	E-Value	Score	Positive
Broad-complex	>lcl|c107699_g1_i1	883	260 uncom	BTB/POZ domain	*Melipona quadrifasciata*	gb|KOX74790.1|	3e-54	192	60%
E75	>lcl|c251839_g1_i1	3689	823	DNA-binding domain	*Gecarcinus lateralis*	gb|DQ058409.2	0.0	1302	99%
E74	>lcl|c259511_g1_i1	2755	367	Ets-domain	*Copidosoma floridanum*	ref|XM_014362419.1	3e-67	221	99%
Hormone Receptor 4	>lcl|c217203_g1_i1	2467	286	Ligand-binding domain	*Cimex lectularius*	ref|XP_014251569.1	7e-143	428	83%
Hormone Receptor 3	c234512_g1_i5	2299	497	NR_DBD_ROR	Daphnia pulex	gb|ACY56691.1|	0.0	637	77%
Fushi tarazu factor-1	lcl|c227663_g1_	1173	194aa uncom	Ligand-binding domain	*Eriocheir sinensis*	gb|AKN52404.1|	1e-135	391	98%
forkhead box transcription factors (O class)	>lcl|c217292_g1_i2	2285	356 uncom	Forkhead (FH)	*Blattella germanica*	CCF23214	8e-104	327	78%
EcR	c264982_g1_i4	5940	577	DNA-binding domain	*Eriocheir sinensis*	AHG30901.1	0.0	1064	95%
RXR	c261204_g1_i1	6844	151	DNA-binding domain	*Gecarcinus lateralis*	DQ067280.1	9e-102	309	100%

Table 2. 7. Ecdysteroid-responsive genes sequences from the *G. lateralis* ESA YO transcriptome

Name of the gene	**The Contigs name**	**length**	**ORF**	**Domain name**	**Top BLASTp Hit**	**Accession #to BLAST Hit**	**E-Value**	**Score**	**Positive**
Broad-complex	c165757_g1_i3	1231	372 aa com	BTB/POZ domain	*Hyalella Azteca*	XM_018153012.1	6e-77	333	(98%)
E75	c164502_g1_i3	3529	823 aa com	DNA-binding domain	*Gecarcinus lateralis*	DQ058409.2	0.0	1348	(99%)
E74	c201759_g4_i1	1375	367aa com	Ets-domain	*Hyalella Azteca*	XM_018167827	7e-85	272	(97%)
Hormone Receptor 4	c210835_g1_i1	2464	286aa com	Ligand-binding domain	*Cryptotermes secundus*	XP_023718845.1	6e-129	439	(83%)
Hormone Receptor 3	c199749_g4_i2	921	222aa com	NR_DBD_ROR	*Thermobia domestica*	AB829732.1	6e-110	338	(85%)
Fushi tarazu factor-1	c114850_g1_i1	1173	195 aa com	Ligand-binding domain	*Eriocheir sinensis*	KM657205.1	2e-137	391	(98%)
forkhead box transcription factors (O class)	c193876_g1_i1	1466	438 aa uncom-	Forkhead (FH)	*Limulus polyphemus*	XM_013923372.1Length	3e-107	337	(71%)
EcR	c202041_g1_i1	5940	577 aa com	Ligand-binding domain	*Eriocheir sinensis*	KF469222.1	0.0	955	(93%)
RXR	c214921_g1_i3	4880	302 aa com	Ligand-binding domain	*Gecarcinus lateralis*	DQ067280.1	0.0	619	(99%)

Spook

F1

```
tgtcggcttggcgatctccaggtcagtcactgctccgccgggttcagaactgctcgcaaag

atggtcttcgttttggctcccgcaacaatcgtgctgatgatgatggtcctcgtggccatc
 M  V  F  V  L  A  P  A  T  I  V  L  M  M  M  V  L  V  A  I
gcggtgcaggagaccgcccgcagacgaaggaaacaccagaagcagcagtatttccaagga
 A  V  Q  E  T  A  R  R  R  R  K  H  Q  K  Q  Q  Y  F  Q  G
accgcgacaacaccaacggcgagcagtgacgacctggagatcgccaagccgacaccacca
 T  A  T  T  P  T  A  S  S  D  D  L  E  I  A  K  P  T  P  P
cccggcccgactccactcccttcgtcggcaacctactcagcctccggaagcacagcgaa
 P  G  P  T  P  L  P  F  V  G  N  L  L  S  L  R  K  H  S  E
tgcccctaccaaggcttctcggagctgaaggacaagtacggccccgtctactccctgaag
 C  P  Y  Q  G  F  S  E  L  K  D  K  Y  G  P  V  Y  S  L  K
cttggaagcagttcagccgtcatcgtcaacacttacgacaccatcaaggaggtgctcgtc
 L  G  S  S  S  A  V  I  V  N  T  Y  D  T  I  K  E  V  L  V
aacaaggcaaactccttcgatgcgcgtcctgaccttacccgcttcaagctctacttcgga
 N  K  A  N  S  F  D  A  R  P  D  L  T  R  F  K  L  Y  F  G
ggcgaccgtcagcactccctggccctgtgtgactggtccgaccaccagaagcgccgcatg
 G  D  R  Q  H  S  L  A  L  C  D  W  S  D  H  Q  K  R  R  M
actctggctcgctccttcctcatgttccgtggccaagaggaccacttcaccaagtttgag
 T  L  A  R  S  F  L  M  F  R  G  Q  E  D  H  F  T  K  F  E
gccaacgtcgtgtctgagatgccgactctgactactgagttcgacaaggtgttgggtcag
 A  N  V  V  S  E  M  P  T  L  T  T  E  F  D  K  V  L  G  Q
ccggtcgaggccaaggagatcttgtcatactgcgccttgaacatcttcaccgggtacatg
 P  V  E  A  K  E  I  L  S  Y  C  A  L  N  I  F  T  G  Y  M
tgctccaagaagttccagtacgagcagagcgacttccagaaattggtgcaaaactttgac
 C  S  K  K  F  Q  Y  E  Q  S  D  F  Q  K  L  V  Q  N  F  D
                                F1
tacatcttcagagacatcaacaccgggcacatcatcgacttcctccccagcctggagccg
 Y  I  F  R  D  I  N  T  G  H  I  I  D  F  L  P  S  L  E  P
        R1
ctcttcccttcctacatcaacgagatcaagaaaaatgcgtcggacatccgcgagcacatc
 L  F  P  S  Y  I  N  E  I  K  K  N  A  S  D  I  R  E  H  I
ctcaacaacatctgcctcgagaagtacgagaagctcaggcagaaccccaacgacgtggag
 L  N  N  I  C  L  E  K  Y  E  K  L  R  Q  N  P  N  D  V  E
gacctggtggatgcctgcttcgctaacctgctgactgagaacgaaggcgaaaagtgggac
 D  L  V  D  A  C  F  A  N  L  L  T  E  N  E  G  E  K  W  D
Tggcagacaatcctgtacatcgtggaggacctgctcgggggctccatggcaatcagcaac
 W  Q  T  I  L  Y  I  V  E  D  L  L  G  G  S  M  A  I  S  N
Atcgtgatgcgcctcctgggccacatcctccagcacccccatgtggtccaggccctccgc
 I  V  M  R  L  L  G  H  I  L  Q  H  P  H  V  V  Q  A  L  R
aacgagatcgacgagaaggttgggcgtgagcgtgccgccaccctggaggaccgccaccaa
 N  E  I  D  E  K  V  G  R  E  R  A  A  T  L  E  D  R  H  Q
atgctttacagccaggccgtgctctacgaaacgctcagactcacctcgtctcccattgtt
 M  L  Y  S  Q  A  V  L  Y  E  T  L  R  L  T  S  S  P  I  V
ccccacgtcgctaccgaggacgccactgtcggaggttactccgtcgagaagggatccatc
 P  H  V  A  T  E  D  A  T  V  G  G  Y  S  V  E  K  G  S  I
gtcttcctcaacaacttcgagatgaacacgtcgccgagtctgtgggacgagccaaccaag
 V  F  L  N  N  F  E  M  N  T  S  P  S  L  W  D  E  P  T  K
ttcatgccagaaaggttcctgaaggacggttgcctcaagaagcccgagtacttcatcccc
 F  M  P  E  R  F  L  K  D  G  C  L  K  K  P  E  Y  F  I  P
ttcagcaccggaaagcgctcctgcgtcggctccaaggtagtggccaacatcgccttcctc
 F  S  T  G  K  R  S  C  V  G  S  K  V  V  A  N  I  A  F  L
gtcgtcaccaccctcctgcagcgctacgacatctccttggctgaggggacgccggagctg
 V  V  T  T  L  L  Q  R  Y  D  I  S  L  A  E  G  T  P  E  L
cccaggggcaagatctccctcgactggaaccccttcaagctggtcttcgcgatgaggcag
 P  R  G  K  I  S  L  D  W  N  P  F  K  L  V  F  A  M  R  Q
                                                  R2
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 -
atcactagtttcgcgccgcgtgtggcggcaggcgggaagttccggcaacactccaccgac
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caaagtcatagttccgccaacactaaagcgactcttcacaacatttcggaacgccggcgg
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ggccggggccggcgctccccacgaccccgtcacaccaagtatttactgggtcaagtcaac
aaattctctcactgtgttgcacgagaaaggataattcggcccccaaggcaagccaccccct
agttggtccaacagctgccccaggggggctgtggcctcccatccaagagttagaaattg
tagcgacgcgaagcgcaagattcagctccaagacttggtcacaacctatgttattgctca
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Figure 2.1. Nucleotide and amino acid sequences of G. lateralis spook (*Gl-Spo*). The green and red boxes indicate start and stop codons, respectively. Red font indicates the conserved cytochrome P450 catalytic domain, and blue color sequence indicates Proline-rich domain. Shaded boxes and arrows indicate locations of forward (F) and reverse (R) primers used for PCR to verify contig sequence from YO transcriptome.

Neverland

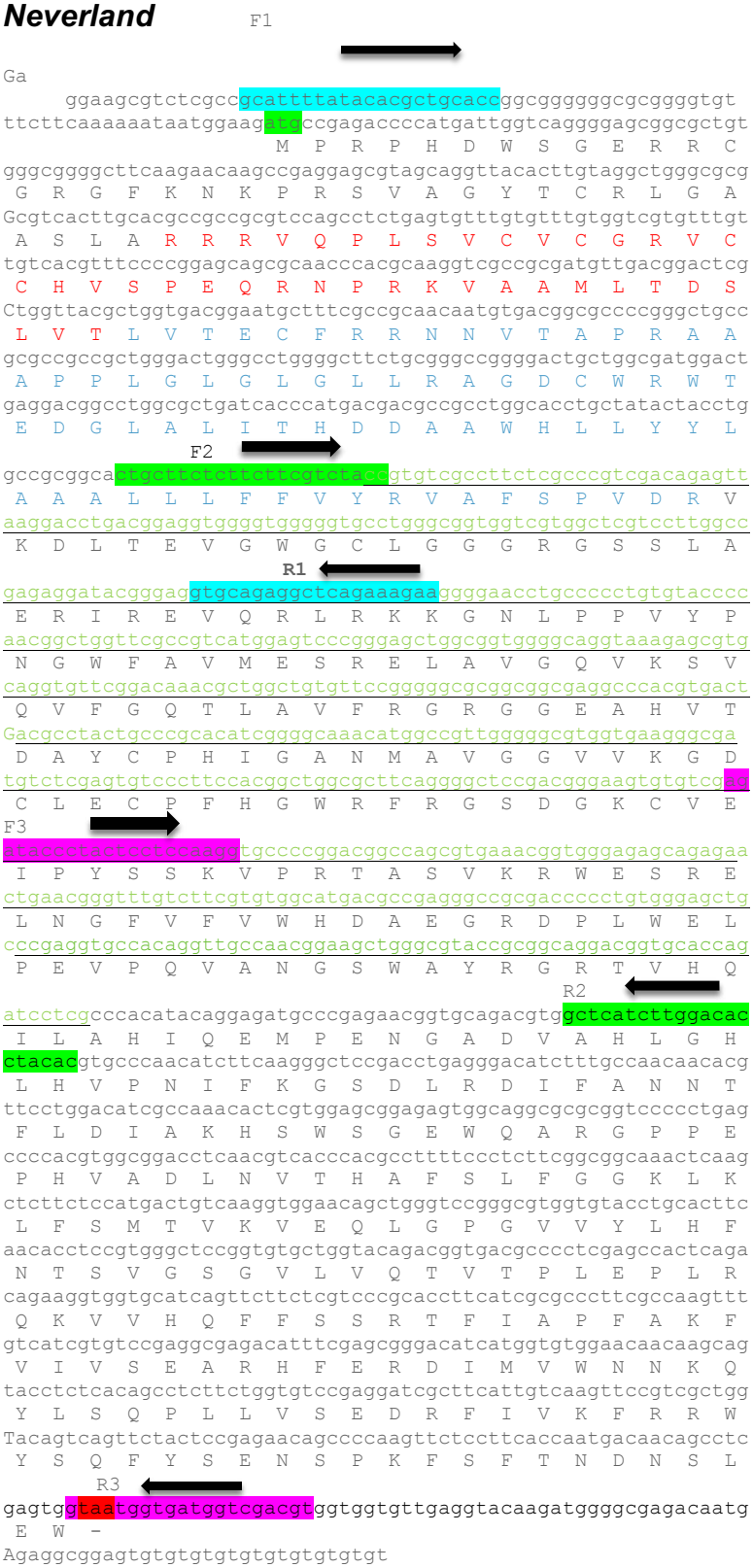
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Figure 2. 2. Nucleotide and amino acid sequences of *G. lateralis Neverland* (*Gl-Nvd*) The green and red boxes indicate start and stop codons, respectively. Red font red color indicates, Rieske domain and blue color sequence indicates HcaE domain. Shaded boxes and arrows indicate locations of forward (F) and reverse (R) primers used for PCR to verify contig sequence from YO transcriptome.

Phantom

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Figure 2. 3. Nucleotide and amino acid sequences of *G. lateralis Phantom* (*Gl-Phm*). The green and red boxes indicate start and stop codons, respectively. Red font indicates the conserved cytochrome P450 catalytic domain. Shaded boxes and arrows indicate locations of forward (F) and reverse (R) primers used for PCR to verify contig sequence from YO transcriptome. Green font color indicates the PCR product sequence.

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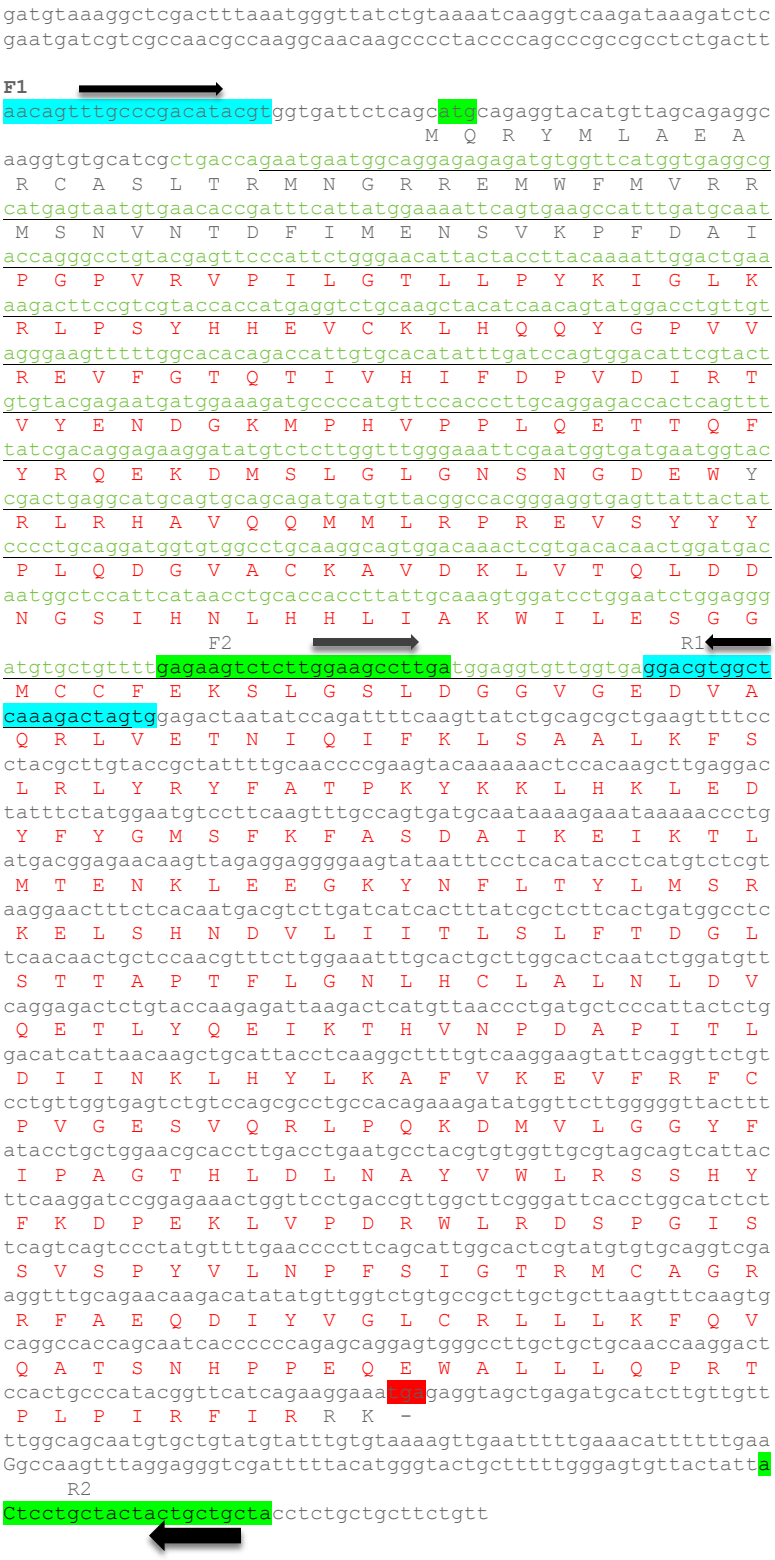

Figure 2. 4 Nucleotide and amino acid sequences of *G. lateralis CYP44* (*Gl-CYP44*). The green and red boxes indicate start and stop codons, respectively. Red font indicates the conserved cytochrome P450 catalytic domain. Shaded boxes and arrows indicate locations of forward (F)

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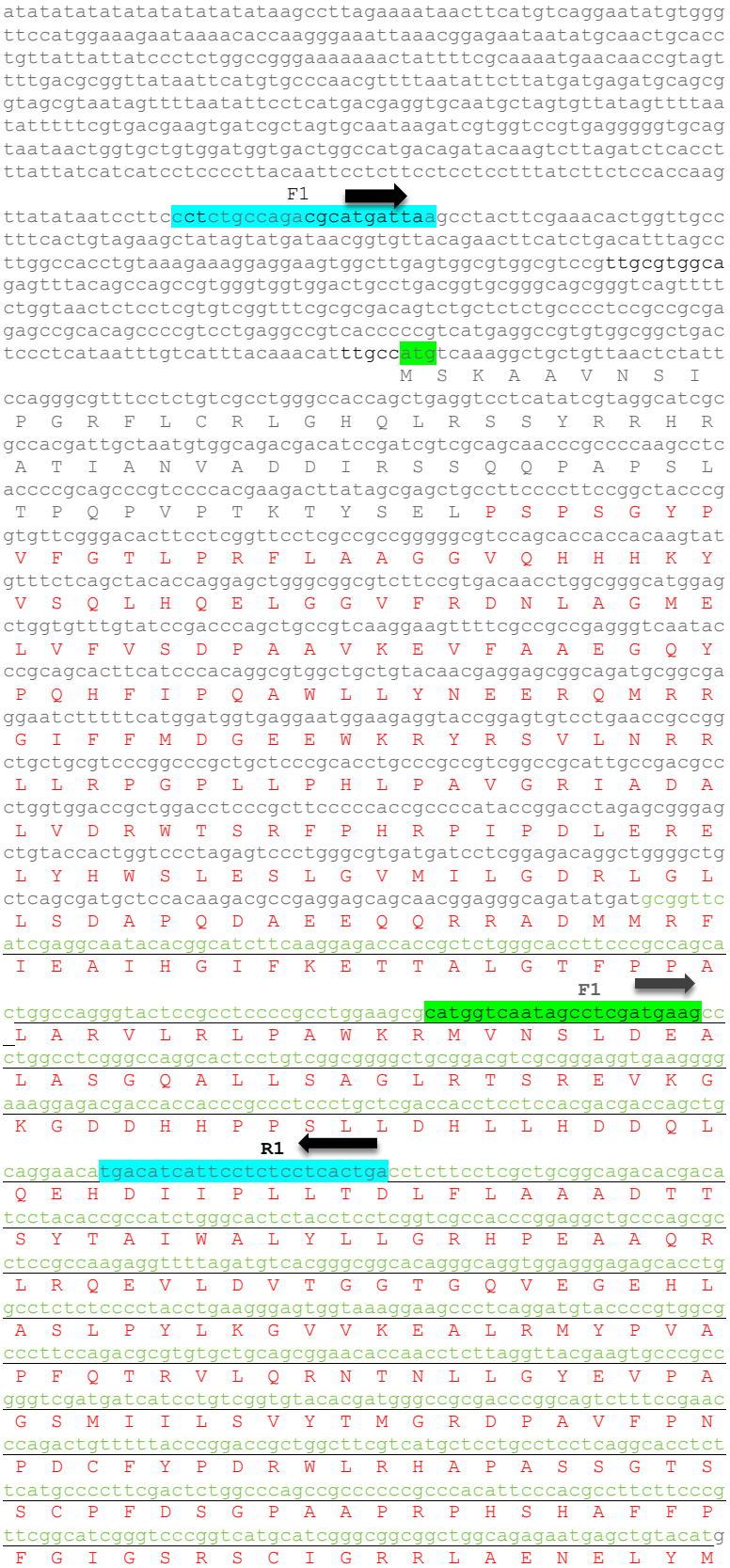


Figure 2. 5. Nucleotide and amino acid sequences of G- lateralis *Shadow* (*Gl-Sad*). The gene and red boxes indicate start and stop codons, respectively. Red font indicates the conserved cytochrome P450 catalytic domain. Shaded boxes and arrows indicate locations of forward (F) and reverse (R) primers used for PCR to verify contig sequence from YO transcriptome.

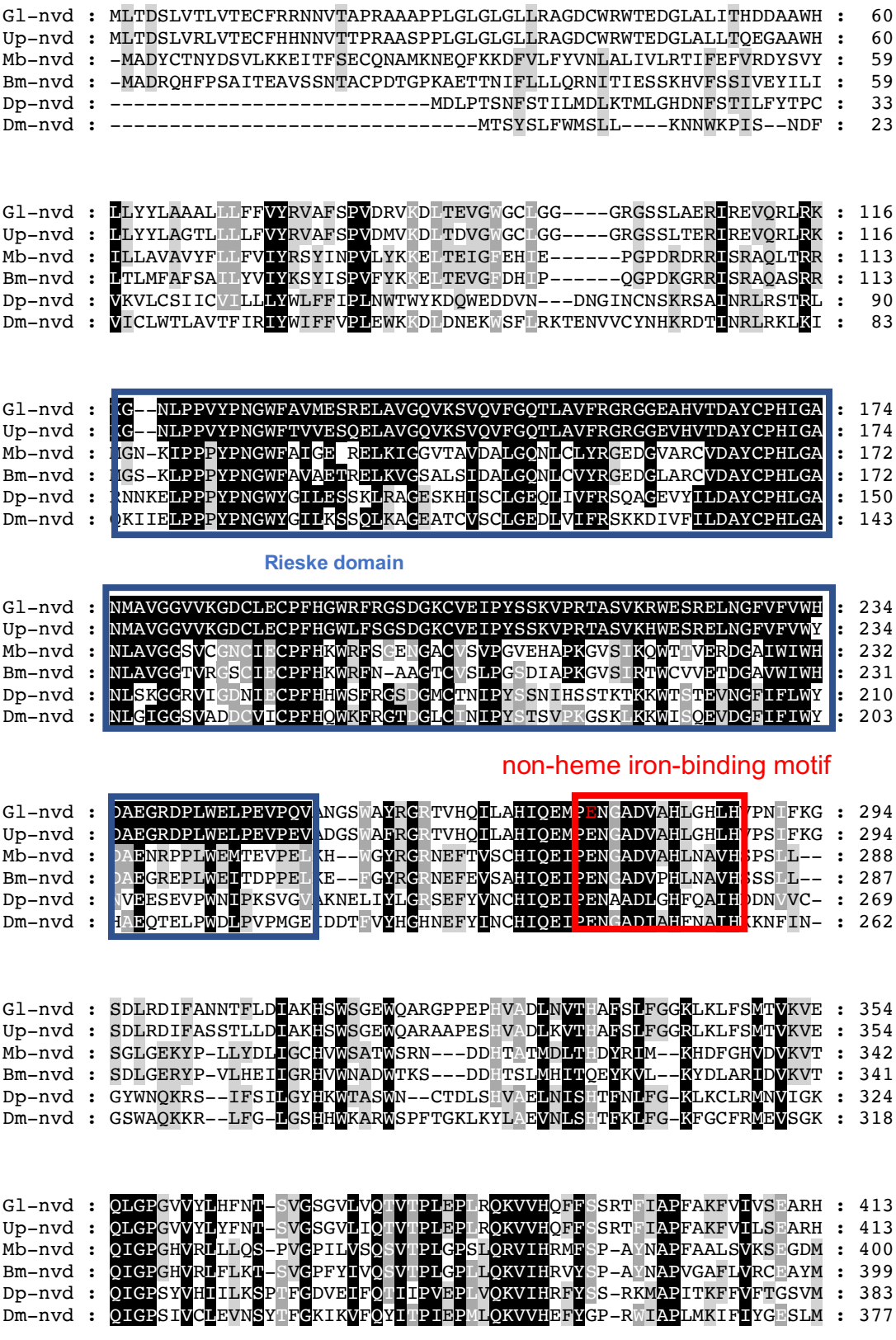
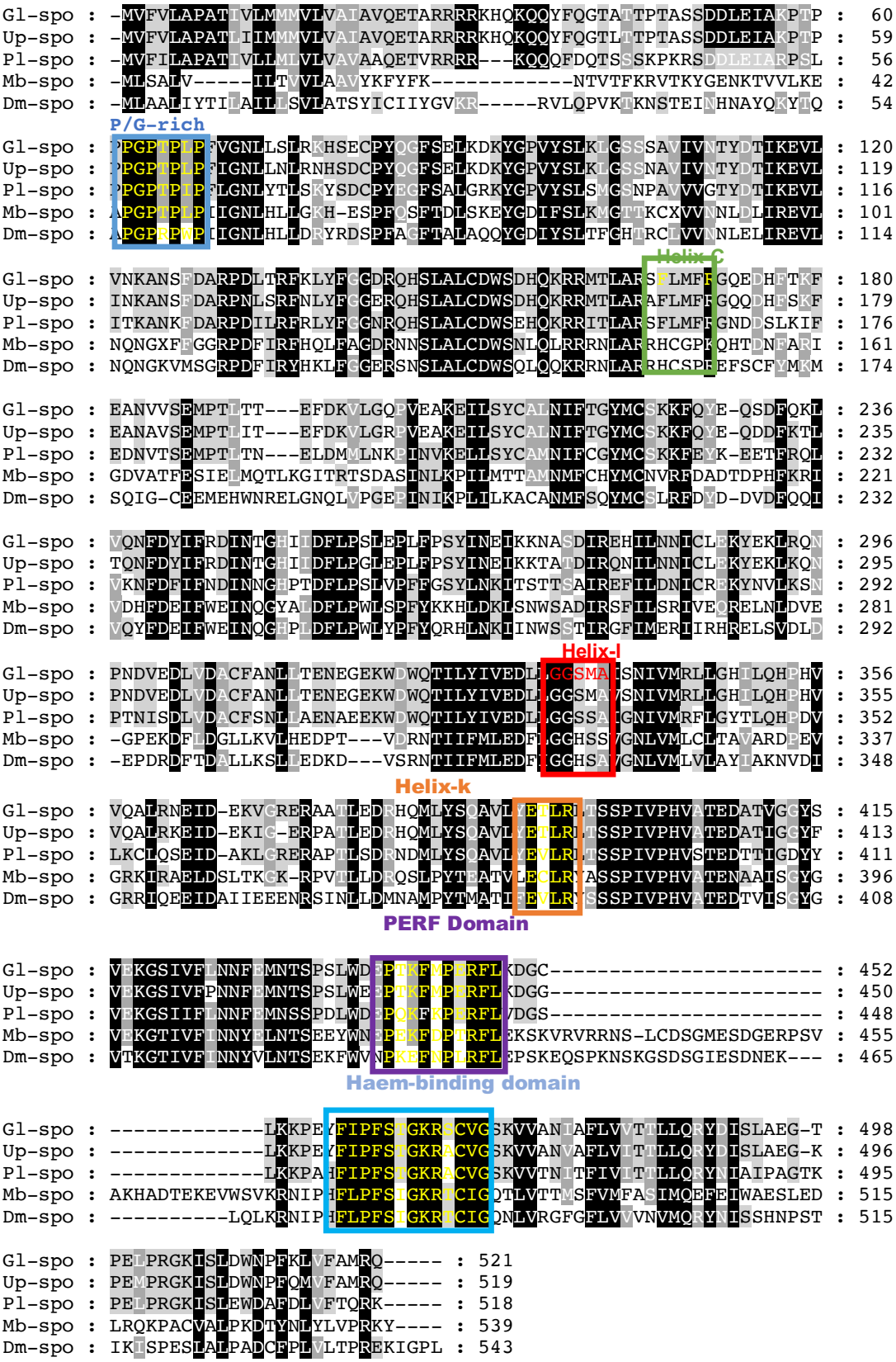

Rieske domain
non-heme iron-binding motif

Figure 2. 6. Multiple alignment of deduced amino acid sequences of *neverland* proteins in three crustacean species and two insect species. Abbreviations Dm, *Drosophila melanogaster* (NP_001097670.1); Mb, *Mamestra brassicae* (BAN66310.1); Pl, *Pontastacus leptodactylus* transcriptome; Up, *Uca pugilator*, and Bm, *Bombyx mori (NP_001037626.1);* Dp *Drosophila pachea* (AFD97329.1); Gl, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the Rieske domain.

spook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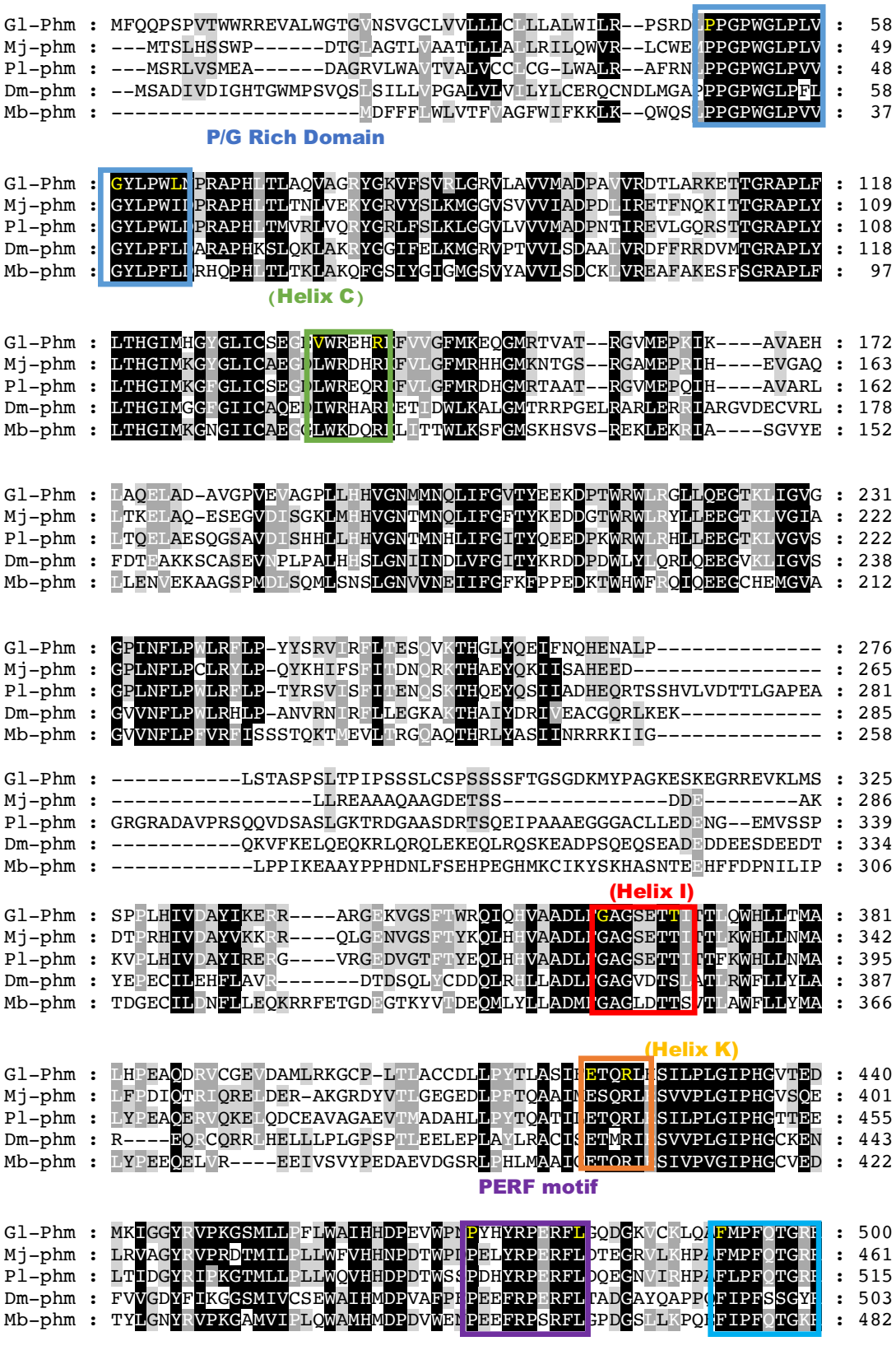
Helix-C
Helix-I
Helix-k
PERF Domain
Haem-binding domain

Figure 2. 7. Multiple alignment of deduced amino acid sequences of *spook* (*CYP307A1)* proteins in three crustacean species and two insect species. Abbreviations Dm, *Drosophila melanogaster* (NP_647975.2); Mb, *Mamestra brassicae* (BAN66314.2); Pl, *Pontastacus leptodactylus* (*PL157)* transcriptome; Up, *Uca pugilator*, and *Gl*, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the P/G Rich Domain, (Helix C), (Helix I), (Helix K), PERF motif and Haem-binding domain.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PERF motif

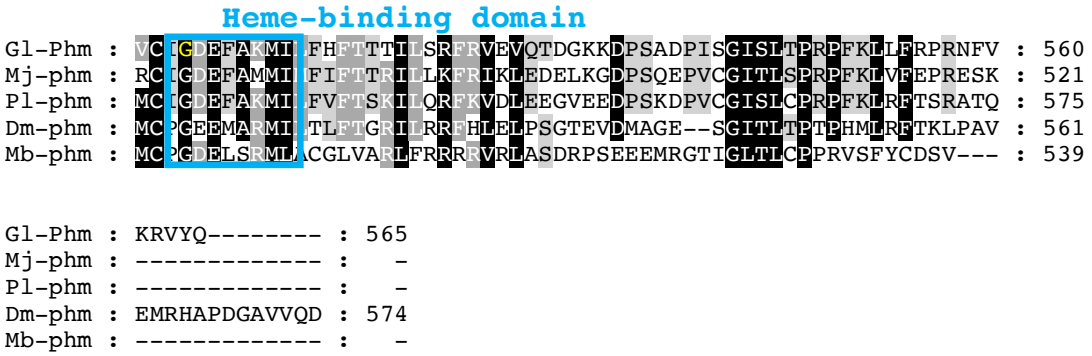

Figure 2. 8. Multiple alignment of deduced amino acid sequences of *phantom (*CYP*306A1) PL163* proteins in three crustacean species and two insect species. Abbreviations: Mj, *marsupenaeus japonicus* (BAH24005.1) Dm, Drosophila melanogaster (NP_573319.1); Mb, *Mamestra brassicae* (BAN66311.1); Pl, *Pontastacus leptodactylu*s (PL163) transcriptome; Up, *Uca pugilator* transcriptome, and Gl, G. lateralis. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the P/G Rich Domain, (Helix C), (Helix I), (Helix K), PERF motif and Heme-binding domain.

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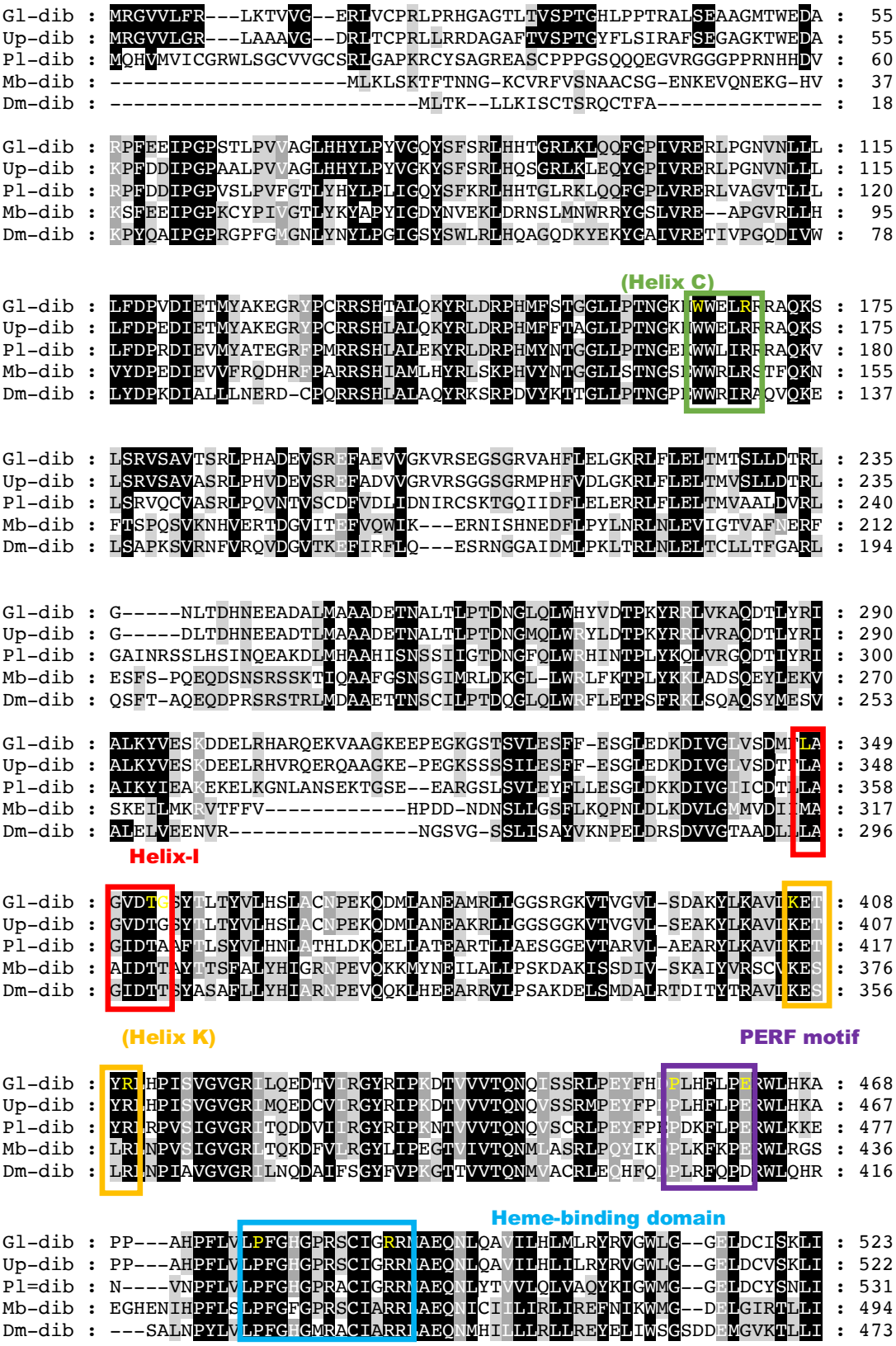

(Helix C)
Helix-I
(Helix K)
PERF motif
Heme-binding domain

Figure 2. .9. Multiple alignment of deduced amino acid sequences of disembodied (CYP302A1) proteins in three crustacean species and two insect species. Abbreviations Dm*, Drosophila melanogaster* (NP_524810.2); Mb, *Mamestra brassicae* (BAN66312.1); Pl, *Pontastacus leptodactylus* (PL158) transcriptome; Up, *Uca pugilator*, and Gl, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the P/G Rich Domain, (Helix C), (Helix I), (Helix K), PERF motif and Haem-binding domain.

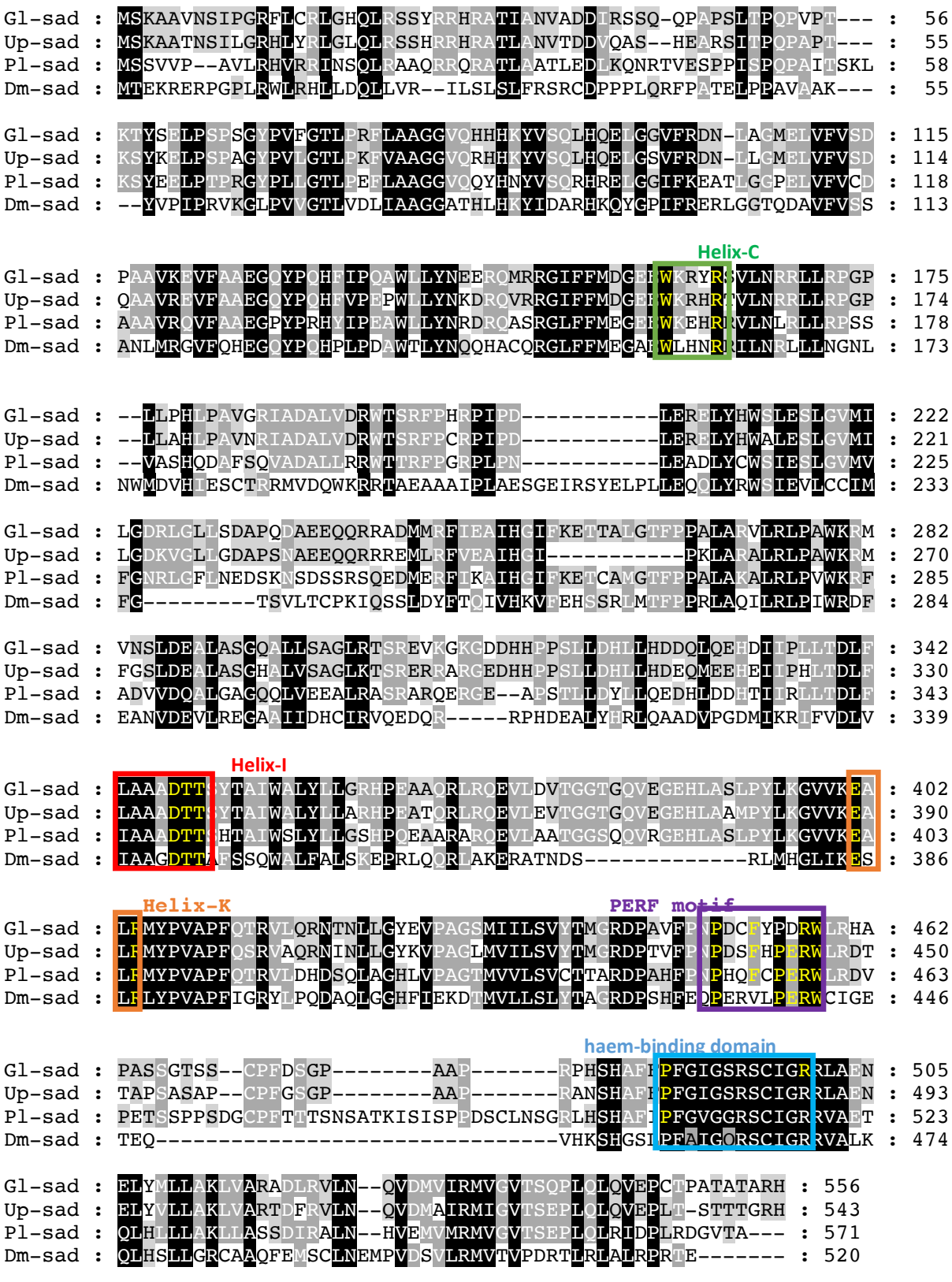

Helix-C
Helix-I
Helix-K
PERF motif
haem-binding domain

Figure 2. 10. Multiple alignment of deduced amino acid sequences of shadow (CYP315A1) proteins in three crustacean species and two insect species. Abbreviations: Dm, *Drosophila melanogaster* (NP_650123.1); Pl, *Pontastacus leptodactylus (PL182)* transcriptome; Up, *Uca pugilator* transcriptome, and Gl, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the P/G Rich Domain, (Helix C), (Helix I), (Helix K), PERF motif and Heme-binding domain.

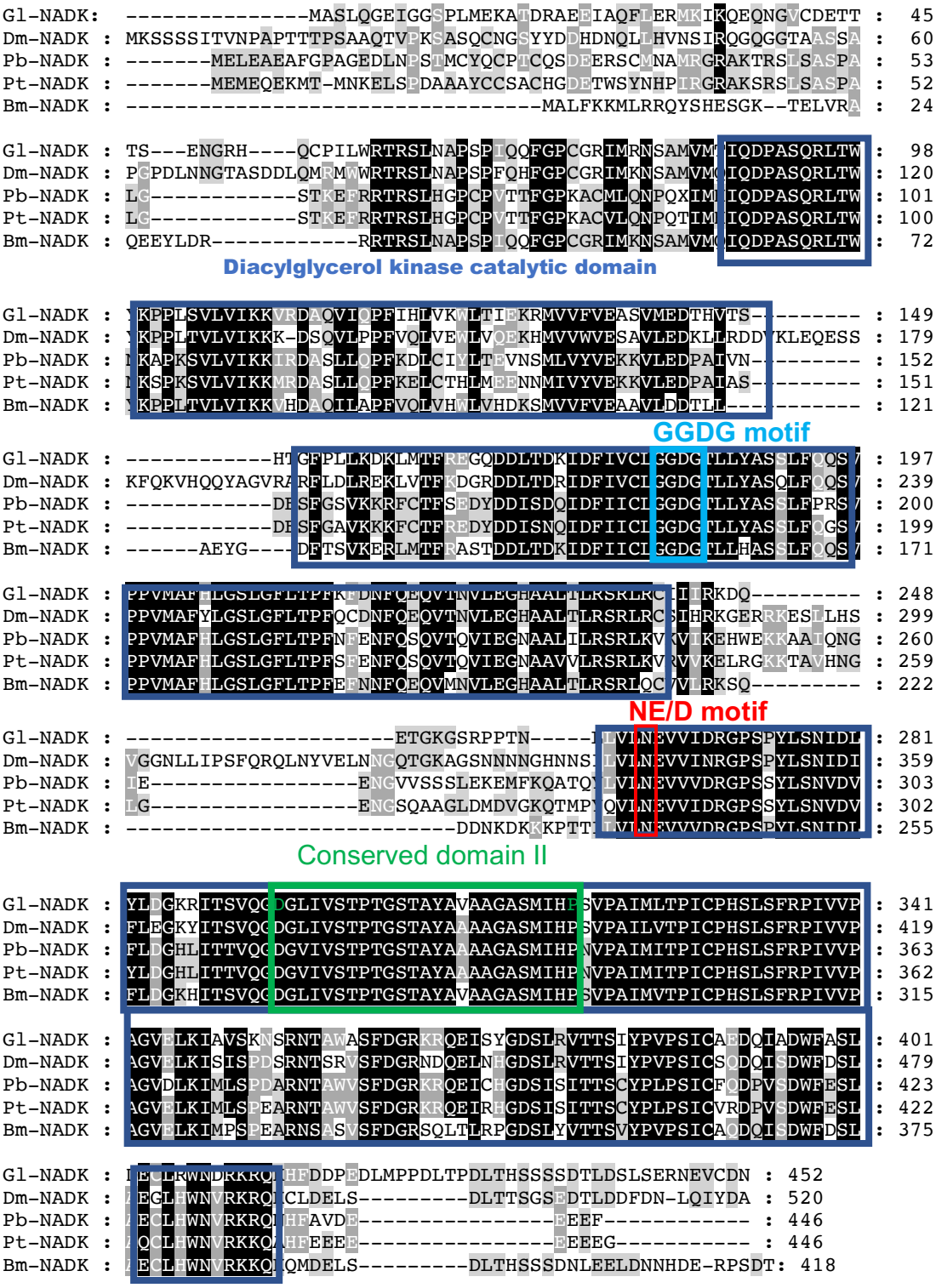
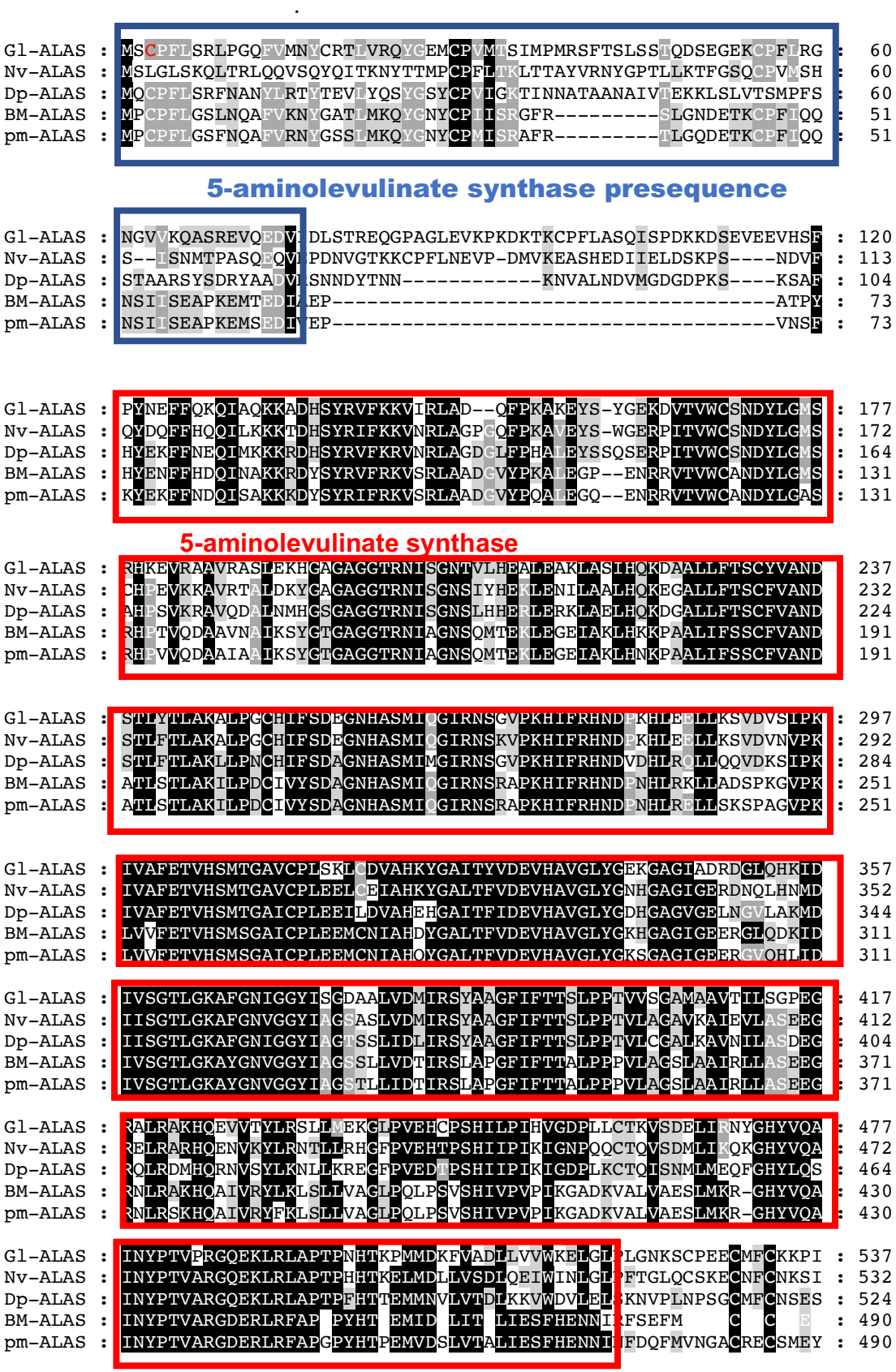


Figure 2. 11. Multiple alignment of deduced amino acid sequences of NADK proteins in one crustacean species and one insect species and one. Abbreviations: Dm, *Drosophila melanogaster* (NP_610884.1); Pb, *Python bivittatus* (XP_007441114.1); Pt, *Pan troglodytes* (XP_513722.4); Nv, *Nicrophorus vespilloides* (XP_017776158.1); Bm, *Bombyx mori* XP_004926312.1 and Gl,

G. *lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the Diacylglycerol kinase catalytic domain.


5-aminolevulinate synthase presequence
5-aminolevulinate synthase

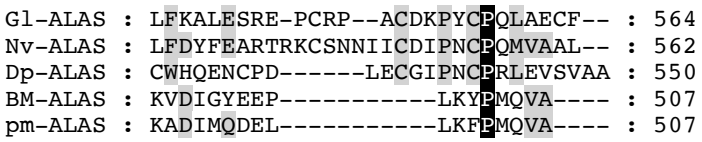
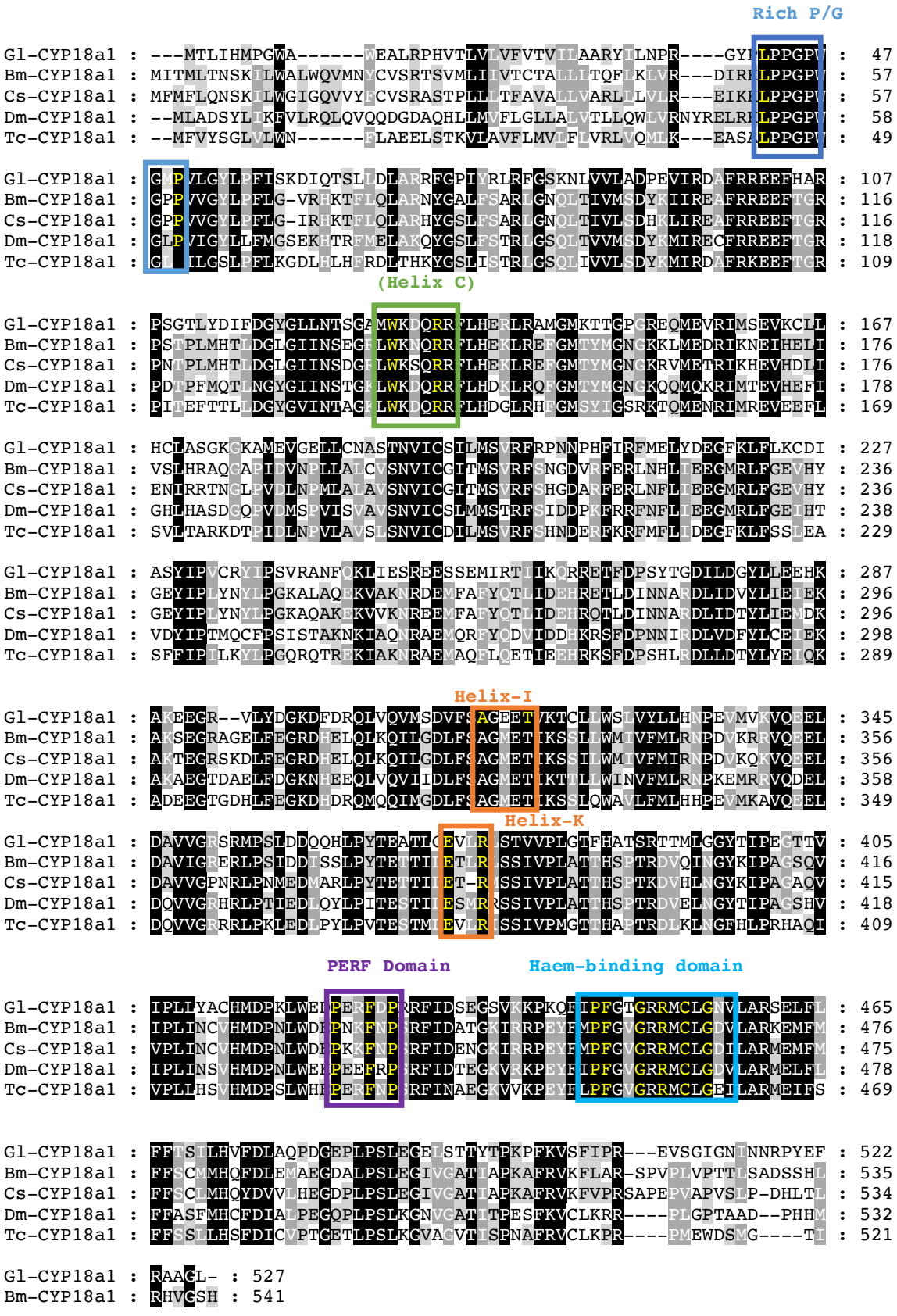


Figure 2.12. Multiple alignment of deduced amino acid sequences of *ALAS* proteins in one crustacean species and four insect species. Abbreviations: Db, *Drosophila busckii* (XP_017835807.1); Bm, *Bombyx mori* (XP_004922915.1), Pm, *Papilio machaon* (XP_014370513.1); Nv, *Nicrophorus vespilloides* (XP_017776158.1); and Gl, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the 5-aminolevulinate synthase presequence and5-aminolevulinate synthase.


Rich P/G
(Helix C)
Helix-I
Helix-K
PERF Domain
Haem-binding domain

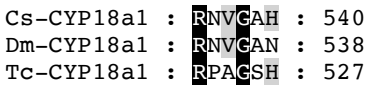
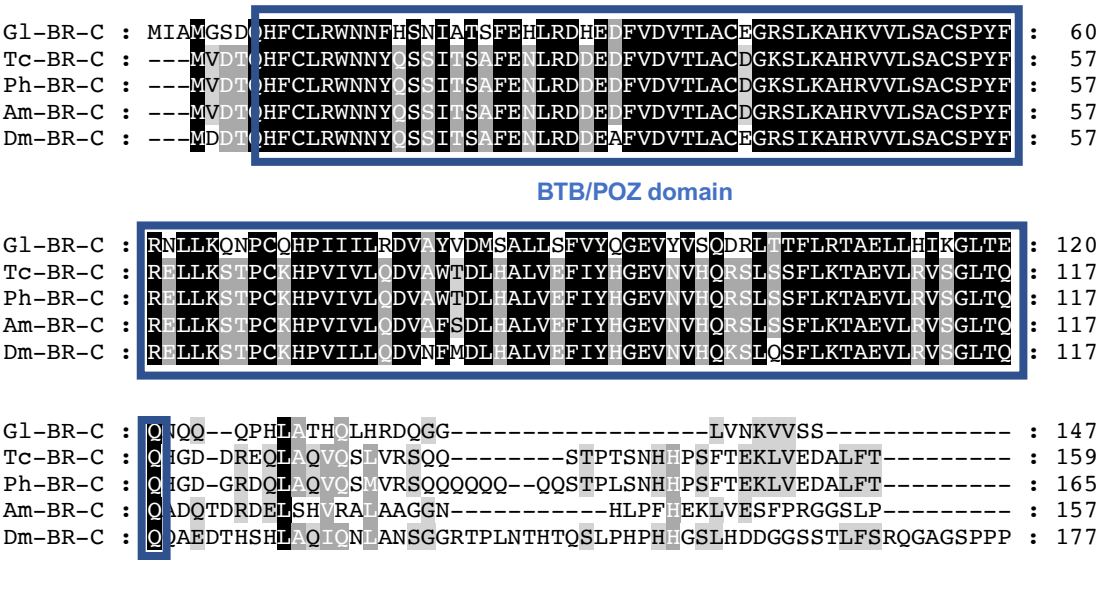


Figure 2. 13. Multiple alignment of deduced amino acid sequences of CYP18a1 proteins in one crustacean specie and four insect species. Abbreviations Dm, *Drosophila melanogaster* (NP_728191.1); Bm (NP_001077078.1); Cs, *Chilo suppressalis* (AHW57292.1); Tc, *Tribolium castaneum* (NP_001123908.1) and Gl, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the P/G Rich Domain, (Helix C), (Helix I), (Helix K), PERF motif and Heme-binding domain.


BTB/POZ domain

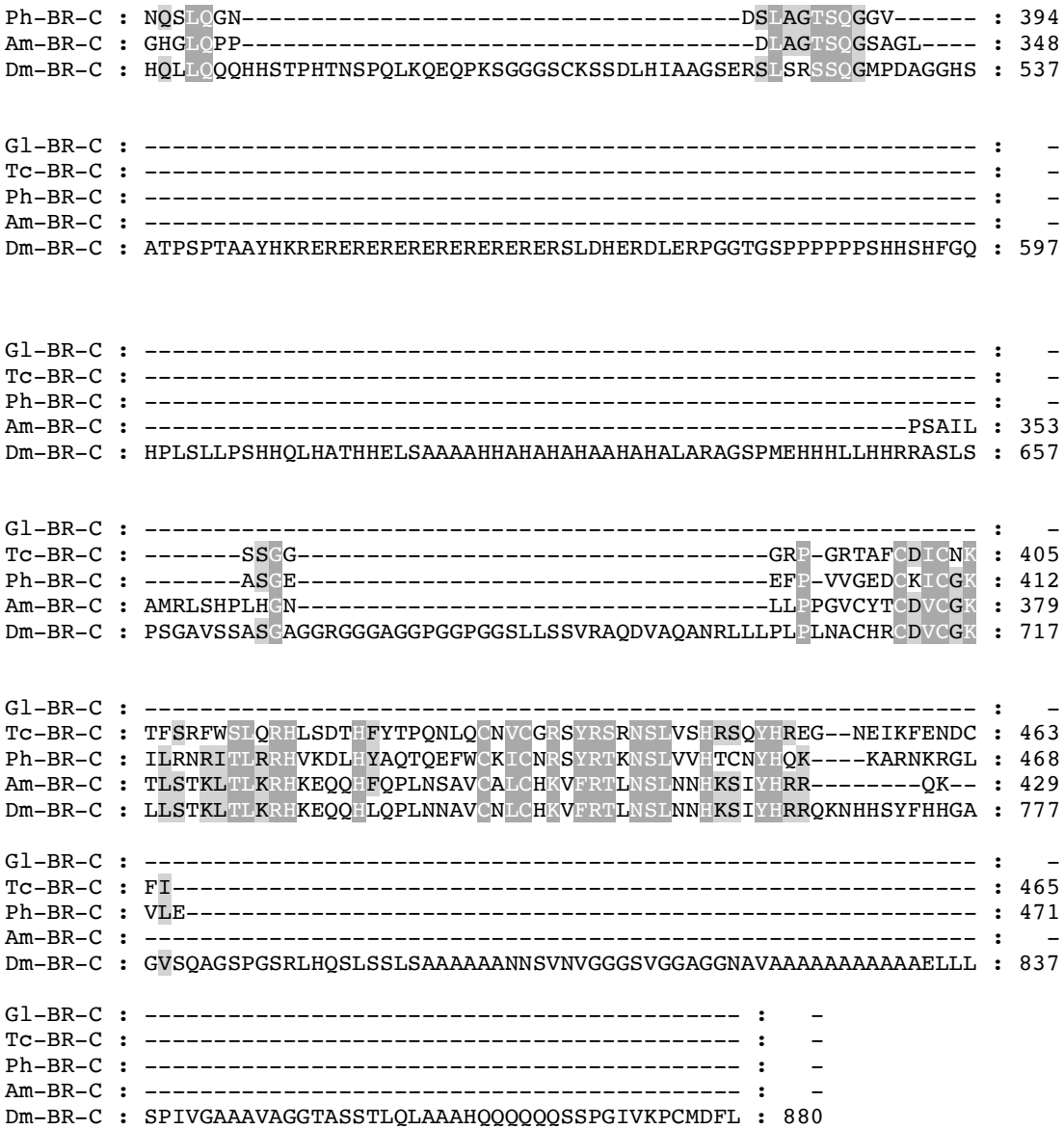
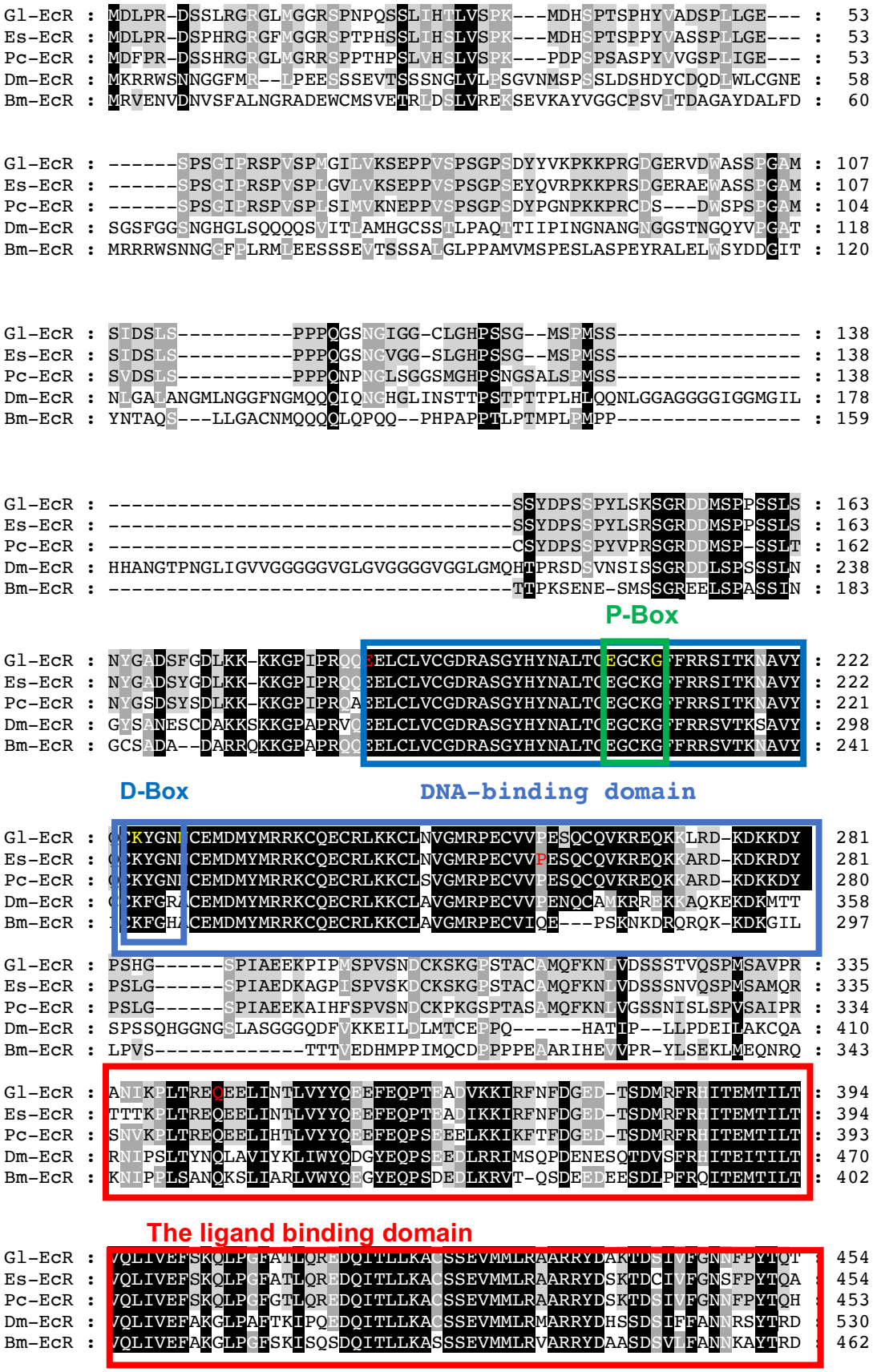


Figure 2. 14. Multiple alignment of deduced amino acid sequences of BR-C proteins in one crustacean species and four insect species. Abbreviations: Tc, *Tribolium castaneum* (NP_001104734.1); Am*, Apis mellifera* (NP_001035356.1); Ph, *Psacothea hilaris* (BAO01157.1); Dm, *Drosophila melanogaster* (NP_726752.1and Gl, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the BTB/POZ domain


P-Box
D-Box
DNA-binding domain
The ligand binding domain

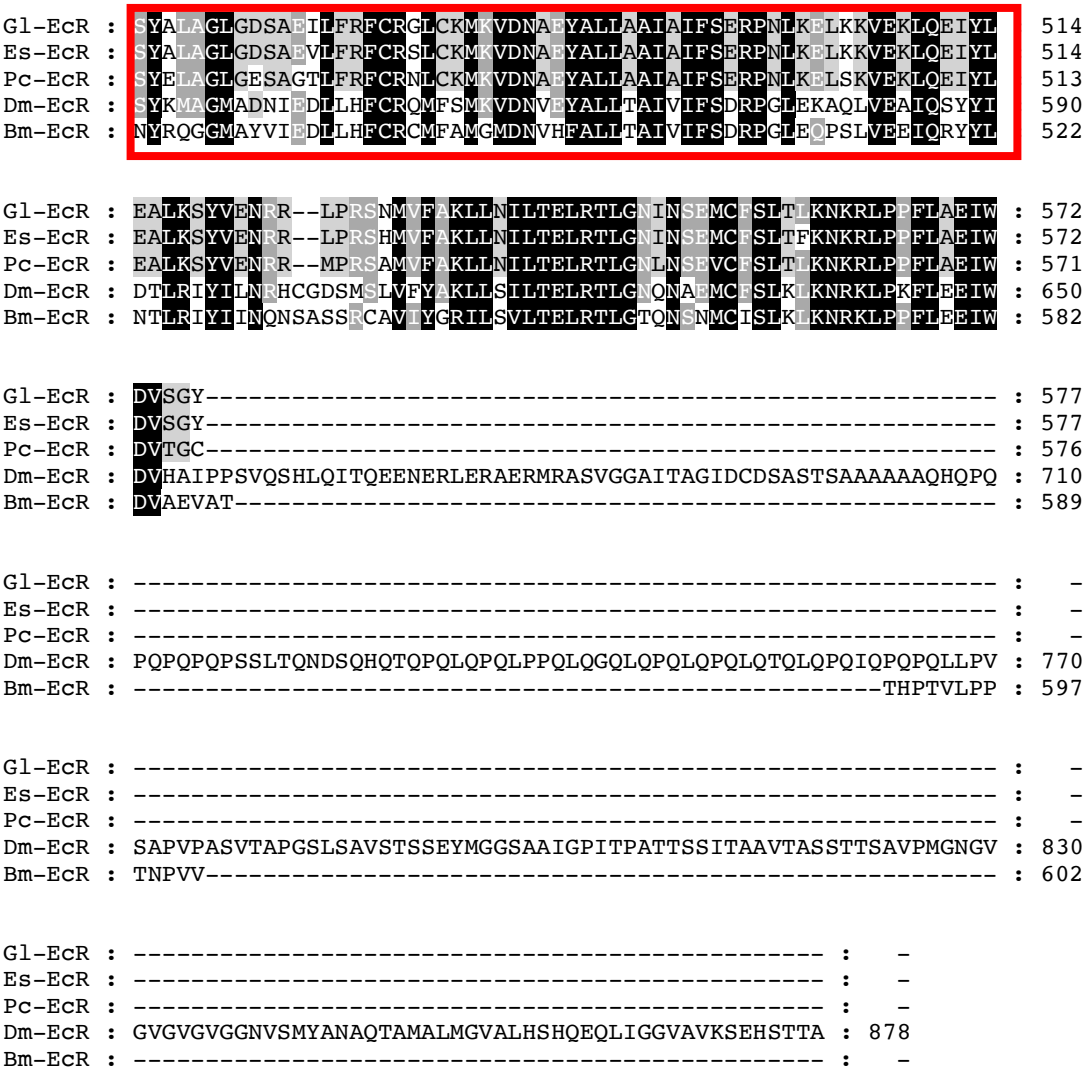


Figure 2.15. Multiple alignment of deduced amino acid sequences of EcR proteins in three crustacean species and two insect species. Abbreviations: Pc, *Procambarus clarkii* (AOZ21143.1); Bm, *Bombyx mori* (NP_001037331.2), Es, *Eriocheir sinensis* (AHG30901.1); Dm*, Drosophila melanogaster* (NP_724460.1); and Gl, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the DNA-binding domain and the cysteine residues of the zinc finger motifs in the DBD are indicated by asterisks. The P-box and the D-box residues indicated by green and yellow boxes. the ligand binding domain (LBD) indicated by red box.

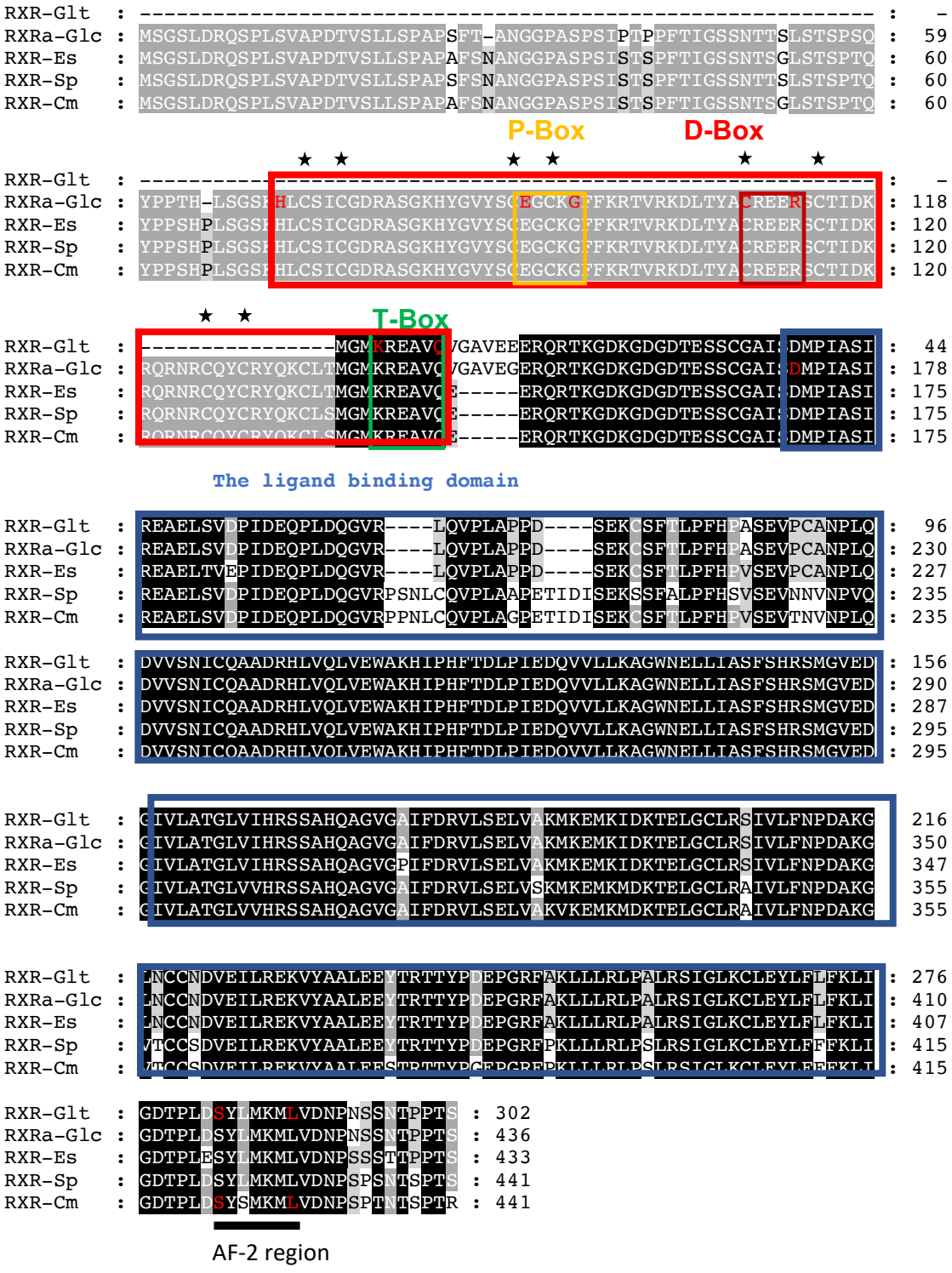
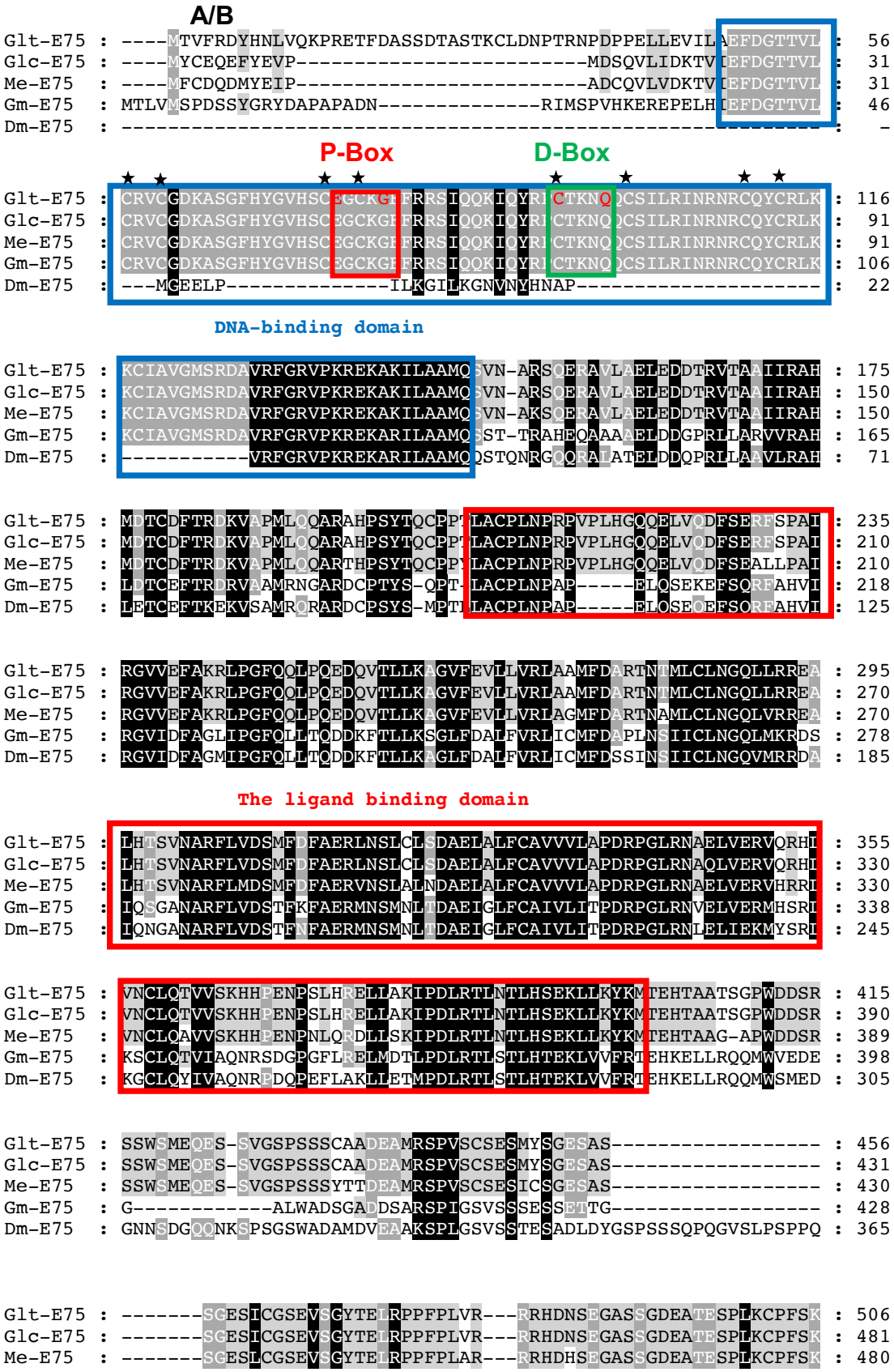


Figure 2. 16. Multiple alignment of deduced amino acid sequences of RXR proteins in one crustacean species and four insect species. Abbreviations: Glc, *Gecarcinus lateralis* (AAZ20368.1); Cm, *Carcinus maenas* (ACG63788.1), Es, *Eriocheir sinensis* (AHF65151.1); Sp,

Scylla paramamosain (ALM98949.1); and Gl, G. lateralis. The conserved DBD and LBD regions indicated by blue and red boxes. The P-box, D-box and T-box are highlighted with boxes (orange, red, green). The AF2 region is underlined. The residues known to interact with 9-cis are indicated by an asterisk above the sequence. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the ligand binding domain


A/B
P-Box
D-Box
DNA-binding domain
The ligand binding domain

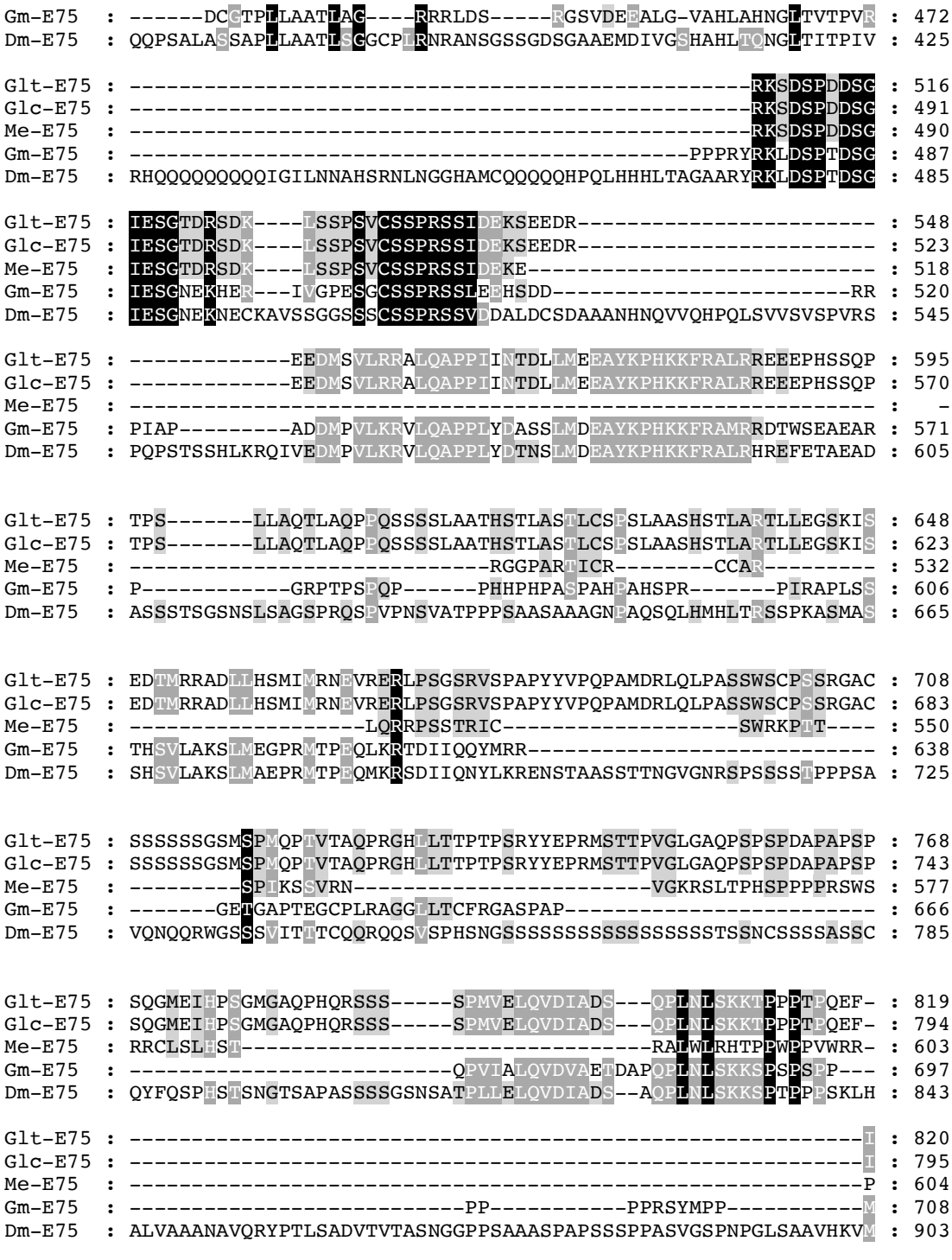
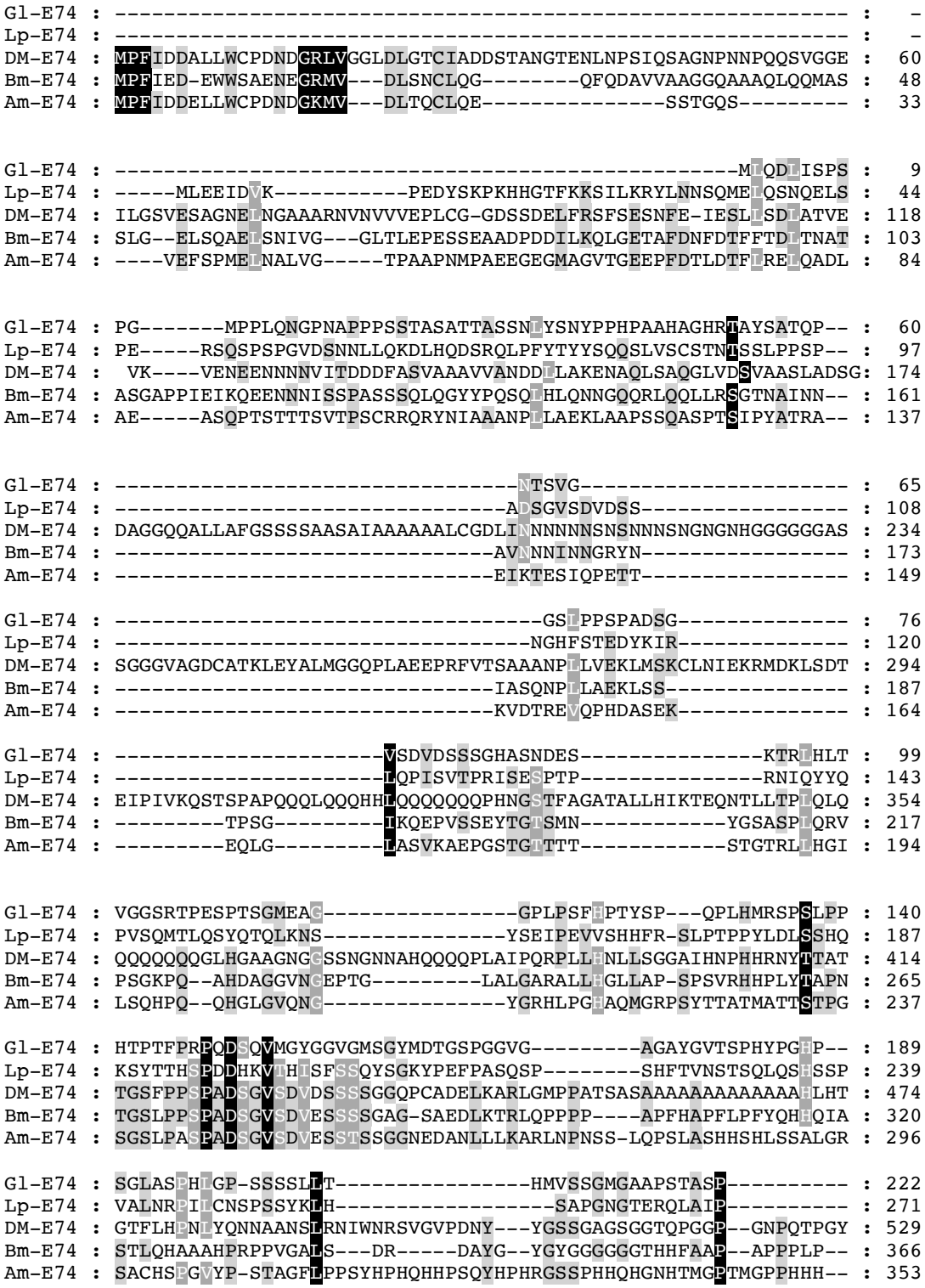

Gm-E75 : ------DCGTPLLAATLAG----RRRLDS-----RGSVDEEALG-VAHLAHNGLTVTPVR : 472
Dm-E75 : QQPSALASSAPLLAATLSGGCPLRNRANSGSSGDSGAAEMDIVGSHAHLTQNGLTITPIV : 425
Glt-E75 : ----------RKSDSPDDSG : 516
Glc-E75 : ----------RKSDSPDDSG : 491
Me-E75 : ----------RKSDSPDDSG : 490
Gm-E75 : ----------PPPRYRKLDSPTDSG : 487
Dm-E75 : RHQQQQQQQQQIGILNNAHSRNLNGGHAMCQQQQQHPQLHHHLTAGAARYRKLDSPTDSG : 485
Glt-E75 : IESGTDRSDK----LSSPSVCSSPRSSIDEKSEEDR---------- : 548
Glc-E75 : IESGTDRSDK----LSSPSVCSSPRSSIDEKSEEDR---------- : 523
Me-E75 : IESGTDRSDK----LSSPSVCSSPRSSIDEKE---------- : 518
Gm-E75 : IESGNEKHER---IVGPESGCSSPRSSLEEHSDD----------RR : 520
Dm-E75 : IESGNEKNECKAVSSGGSSSCSSPRSSVDDALDCSDAAANHNQVVQHPQLSVVSVSPVRS : 545
Glt-E75 : ----------EEDMSVLRRALQAPPIINTDLLMEEAYKPHKKFRALRREEEPHSSQP : 595
Glc-E75 : ----------EEDMSVLRRALQAPPIINTDLLMEEAYKPHKKFRALRREEEPHSSQP : 570
Me-E75 : ---------- : -
Gm-E75 : PIAP---------ADDMPVLKRVLQAPPLYDASSLMDEAYKPHKKFRAMRRDTWSEAEAR : 571
Dm-E75 : PQPSTSSHLKRQIVEDMPVLKRVLQAPPLYDTNSLMDEAYKPHKKFRALRHREFETAEAD : 605
Glt-E75 : TPS-------LLAQTLAQPPQSSSSLAATHSTLASTLCSPSLAASHSTLARTLLEGSKIS : 648
Glc-E75 : TPS-------LLAQTLAQPPQSSSSLAATHSTLASTLCSPSLAASHSTLARTLLEGSKIS : 623
Me-E75 : ----------RGGPARTICR--------CCAR---------- : 532
Gm-E75 : P----------GRPTPSPQP------PHHPHPASPAHPAHSPR-------PIRAPLSS : 606
Dm-E75 : ASSSTSGSNSLSAGSPRQSPVPNSVATPPPSAASAAAGNPAQSQLHMHLTRSSPKASMAS : 665
Glt-E75 : EDTMRRADLLHSMIMRNEVRERLPSGSRVSPAPYYVPQPAMDRLQLPASSWSCPSSRGAC : 708
Glc-E75 : EDTMRRADLLHSMIMRNEVRERLPSGSRVSPAPYYVPQPAMDRLQLPASSWSCPSSRGAC : 683
Me-E75 : ----------LQRRPSSTRIC----------SWRKPIT---- : 550
Gm-E75 : THSVLAKSLMEGPRMTPEQLKRTDIIQQYMRR---------- : 638
Dm-E75 : SHSVLAKSLMAEPRMTPEQMKRSDIIQNYLKRENSTAASSTTNGVGNRSPSSSSIPPPSA : 725
Glt-E75 : SSSSSSGSMSPMQPLVTAQPRGHLLTTPTPSRYYEPRMSTTPVGLGAQPSPSPDAPAPSP : 768
Glc-E75 : SSSSSSGSMSPMQPLVTAQPRGHLLTTPTPSRYYEPRMSTTPVGLGAQPSPSPDAPAPSP : 743
Me-E75 : ---------SPIKSSVRN----------VGKRSLTPHSPPPPRSWS : 577
Gm-E75 : -------GETGAPTEGCPLRAGGLLTCFRGASPAP---------- : 666
Dm-E75 : VQNQQRWGSSSVITITCQQRQQSVSPHSNGSSSSSSSSSSSSSSSTSSNCSSSSASSC : 785
Glt-E75 : SQGMEIHPSGMGAQPHQRSSS-----SPMVELQVDIADS---QPLNLSKKTPPPTPQEF- : 819
Glc-E75 : SQGMEIHPSGMGAQPHQRSSS-----SPMVELQVDIADS---QPLNLSKKTPPPTPQEF- : 794
Me-E75 : RRCLSLHST----------RALWLRHTPPWPPVWRR- : 603
Gm-E75 : ----------QPVIALQVDVAETDAPQPLNLSKKSPSPSPP--- : 697
Dm-E75 : QYFQSPHSTSNGTSAPASSSSGSNSATPLLELQVDIADS--AQPLNLSKKSPTPPPSKLH : 843
Glt-E75 : ----------I : 820
Glc-E75 : ----------I : 795
Me-E75 : ----------P : 604
Gm-E75 : ----------PP----------PPRSYMPP----------M : 708
Dm-E75 : ALVAAANAVQRYPTLSADVTVTASNGGPPSAAASPAPSSSPPASVGSPNPGLSAAVHKVM : 903
Glt-E75 : LEA : 823
Glc-E75 : SEA : 798
Me-E75 : LA- : 606
Gm-E75 : LPA : 711
Dm-E75 : LEA : 906

Figure 2. 17. Multiple alignment of deduced amino acid sequences of E75 proteins in three crustacean species and three insect species. Abbreviations: Gl *Gecarcinus lateralis* (AAY89587.2); Dm, Drosophila melanogaster (NP_730323.1); Gm, *Galleria mellonella*(P50239.1); Me, *Metapenaeus ensis*(O77245.1); and Gl, G. lateralis. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the DNA-binding domain annd Cysteine residues and the ligand binding domain. Cysteine residues of the zinc finger motifs in the DBD are indicated by asterisks and the P-box and D-box is indicated by red and green boxes within DNA-binding domain. LBD is indicated by red boxes. A/B and F domains are not conserved


Gl-E74 : ---------- : -
Lp-E74 : ---------- : -
DM-E74 : MPFIDDALLWCPDNDGRLVGGLDLGTCIADDSTANGTENLNPSIQSAGNPNNPQQSVGGE : 60
Bm-E74 : MPFIED-EWWSAENEGRMV---DLSNCLQG--------QFQDAVVAAGGQAAAQLQQMAS : 48
Am-E74 : MPFIDDELLWCPDNDGKMV---DLTQCLQE---------------SSTGQS--------- : 33
Gl-E74 : ----------M-QDLISPS : 9
Lp-E74 : -----MLEEIDVK-----------PEDYSKPKHHGTFKKSILKRYLNNSQMELQSNQELS : 44
DM-E74 : ILGSVESAGNETNGAAARNVNVVVEPLCG-GDSSDELFRSFSESNFE-IESLLSDLATVE : 118
Bm-E74 : SLG--ELSQAELSNIVG---GLTLEPESSEAADPDDILKQLGETAFDNFDTFFTDLTNAT : 103
Am-E74 : ----VEFSPMELNALVG-----TPAAPNMPAEEGEGMAGVTGEEPFDTLDTFFREILQADL : 84
Gl-E74 : PG-------MPPLQNGPNAPPPSSTASATTASSNLYSNYPPHPAAHAGHRLAYSATQP-- : 60
Lp-E74 : PE-----RSQSPSPGVDSNNLLQKDLHQDSRQLPFYTYYSQQSLVSCSTNLSSLPPSP-- : 97
DM-E74 : VK----VENEENNNNVITDDDFASVAAAVVANDDLLAKENAQLSAQGLVDSVAASLADSG: 174
Bm-E74 : ASGAPPIEIKQEENNNISSPASSSQLQGYYPQSQLHLQNNGQQRLQQLLRSGTNAINN-- : 161
Am-E74 : AE-----ASQPTSTTTSVTPSCRRQRYNIAAANPLLAEKLAAPSSQASPTSIPYATRA-- : 137
Gl-E74 : ----------NTSVG---------- : 65
Lp-E74 : ----------ADSGVSDVDSS---------- : 108
DM-E74 : DAGGQQALLAFGSSSSAASAIAAAAAALCGDLINNNNNNSNSNNNSNGNGNHGGGGGGAS : 234
Bm-E74 : ----------AVNNNINNGRYN---------- : 173
Am-E74 : ----------EIKTESIQPETT---------- : 149
Gl-E74 : ----------GSLPPSPADSG---------- : 76
Lp-E74 : ----------NGHFSTEDYKIR---------- : 120
DM-E74 : SGGGVAGDCATKLEYALMGGQPLAEEPRFVTSAAANPLLVEKLMSKCLNIEKRMDKLSDT : 294
Bm-E74 : ----------IASQNPLLAEKLSS---------- : 187
Am-E74 : ----------KVDTREVQPHDASEK---------- : 164
Gl-E74 : ----------VSDVDSSSGHASNDES----------KTRLHLT : 99
Lp-E74 : ----------LQPISVTPRISESPTP----------RNIQYYQ : 143
DM-E74 : EIPIVKQSTSPAPQQQLQQQHHLQQQQQQQPHNGSTFAGATALLHIKTEQNTLLTPLQLQ : 354
Bm-E74 : ---------TPSG---------IKQEPVSSEYTGTSMN----------YGSASPLQRV : 217
Am-E74 : ---------EQLG---------LASVKAEPGSTGTTTT----------STGTRLLHGI : 194
Gl-E74 : VGGSRTPESPTSGMEAG----------GPLPSFHPTYSP---QPLHMRSPSLPP : 140
Lp-E74 : PVSQMTLQSYQTQLKNS----------YSEIPEVVSHHFR-SLPTPPYLDLSSHQ : 187
DM-E74 : QQQQQQQGLHGAAGNGSSNGNNAHQQQQPLAIPQRPLLHNLLSGGAIHNPHHRNYTTAT : 414
Bm-E74 : PSGKPQ--AHDAGGVNCEPTG---------LALGARALLHGLLAP-SPSVRHHPLYTAPN : 265
Am-E74 : LSQHPQ--QHGLGVQNC----------YGRHLPGHAQMGRPSYTTATMATTSTPG : 237
Gl-E74 : HTPTFPRPQDSQVMGYGGVGMSGYMDTGSPGGVG---------AGAYGVTSPHYPGHP-- : 189
Lp-E74 : KSYTTHSPDDHKVTHHSFSSQYSGKYPEFPASQSP---------SHFTVNSTSQLQSHSSP : 239
DM-E74 : TGSFPPSPADSGVSDVDSSSSGGQPCADELKARLGMPPATSASAAAAAAAAAAAAAHLHT : 474
Bm-E74 : TGSLPPSPADSGVSDVESSSSGAG-SAEDLKTRLQPPPP----APFHAPFLPFYQHHQIA : 320
Am-E74 : SGSLPASPADSGVSDVESSTSSGGNEDANLLLKARLNPNSS-LQPSLASHHSHLSSALGR : 296
Gl-E74 : SGLASPHLGP-SSSSLLT----------HMVSSGMGAAPSTASP---------- : 222
Lp-E74 : VALNRPILCNSPSSYKLH----------SAPGNGTERQLAIP---------- : 271
DM-E74 : GTFLHPNLYQNNAANSLRNIWNRSVGVPDNY---YGSSGAGSGGTQPGGP--GNPQTPGY : 529
Bm-E74 : STLQHAAAHPRPPVGALS---DR-----DAYG--YGYGGGGGGTHHFAAP--APPPLP-- : 366
Am-E74 : SACHSPGVYP-STAGPLPPSYHPHQHHPSQYHPHRGSSPHHQHGNHTMGPTMGPPHHH-- : 353

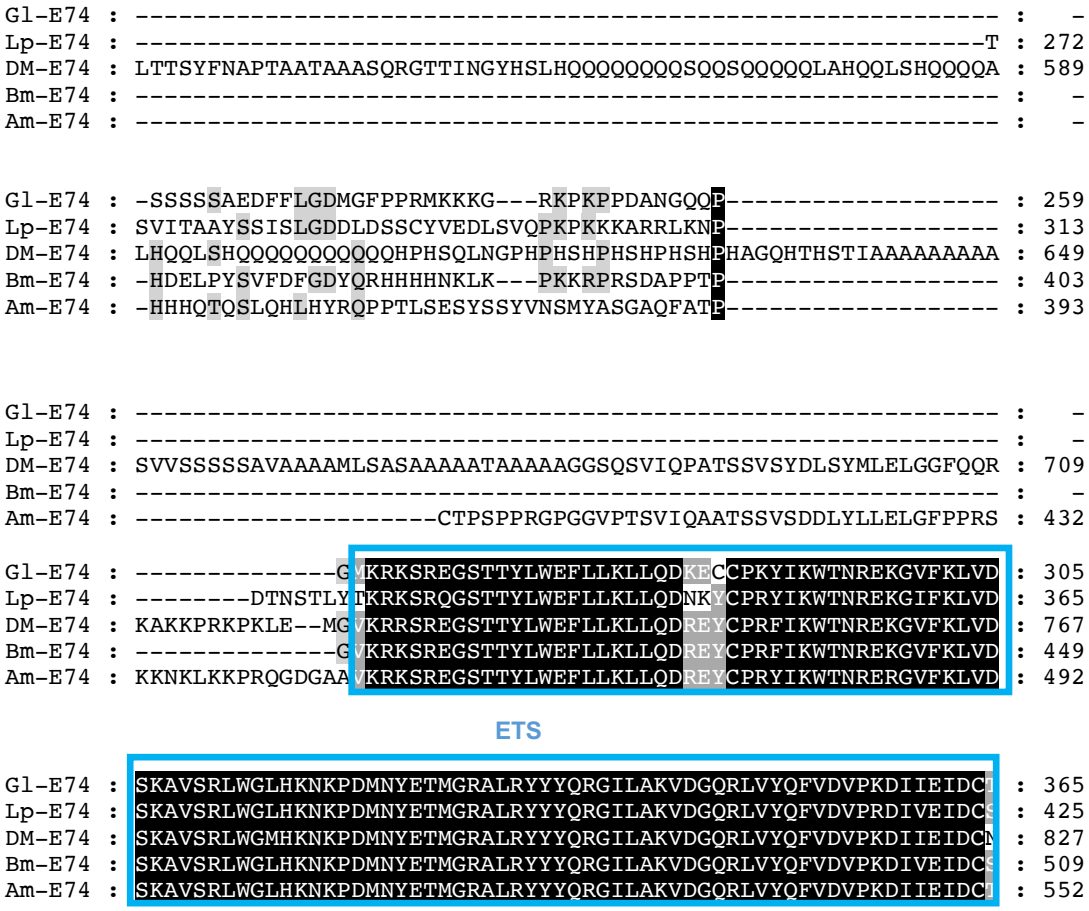
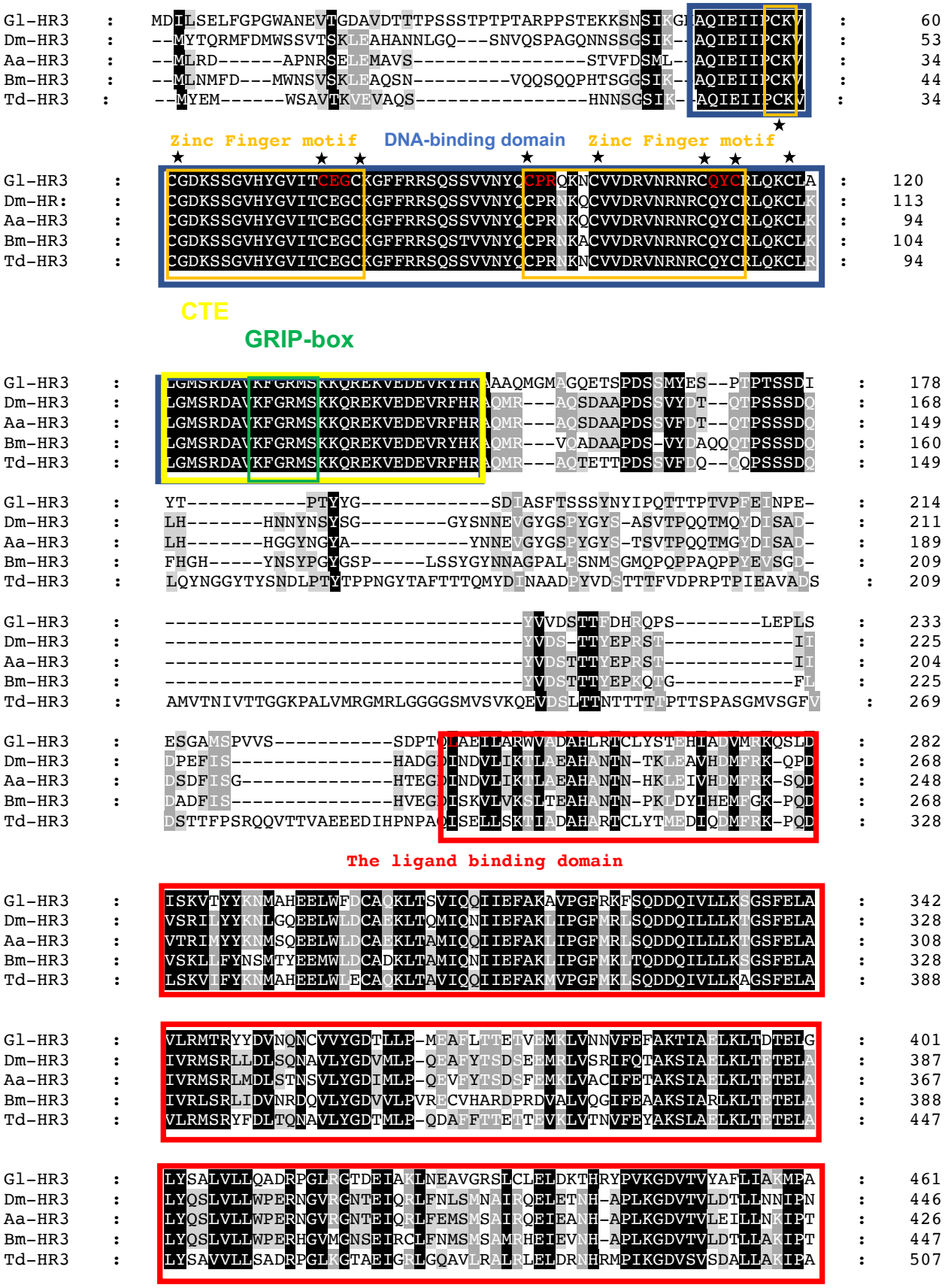


Figure 2.18. Multiple alignment of deduced amino acid sequences of E74 proteins in one crustacean species, one Chelicerate and three insect species. Abbreviations: Lp, Limulus Polyphemus (XP_013776213.1); Dm, *Drosophila melanogaster* (NP_001246816.1); Bm, Bombyx *mori* (NP_001037444.1); Am *Apis mellifera* (NP_001011631.1),and Gl*, G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the the ETS (E26 transformation-specificor E-twenty-six) domain


Zinc Finger motif
DNA-binding domain
Zinc Finger motif
CTE
GRIP-box
The ligand binding domain

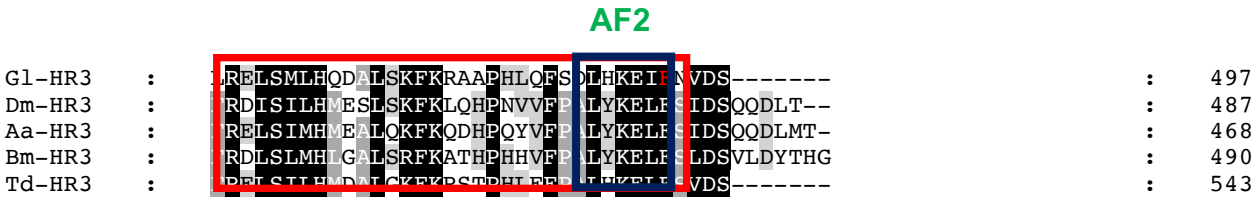


Figure 2. 19. Multiple alignment of deduced amino acid sequences of *HR3* proteins in one crustacean species and four insect species. Abbreviations: Dm, *Drosophila melanogaster* (NP_788303.1); Aa, *Aedes aegypti* (AAF36970.1); Bm, *Bombyx mori* (NP_001037012.1); Td, *Thermobia domestica* (BAP76394.1) and Gl, *G. lateralis*. Blue boxes indicated to DNA- binding domain, orange boxes amino acids correspond to the zinc fingers within the DBD domain. The GRIP-box is indicated by green color. Red boxes indicated to ligand binding domain. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment.

A/B domain

```
Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : MTLSRGPYSELDKMSLFQDLKLKRRKIDSRCSSDGESIADTSTSSPDLLAPMSPKLCDSG :   60
Ms-HR4 : MTLSRGP-CDLDNMSLFQDLKLKRRKVDSRCSSDGESAADTSTSSP-----------DPG :   48
Tc-HR4 : MTLTRAP-CELDKMSLFQDLKLKRRKVDSRCSSDGESVADTSTSSPDLVSPSSPKMSEAV :   59
Tm-HR4 : MTLTRAP-CELDKMSLFQDLKLKRRKVDSRCSSDGESVADTSTSSPDLVSPSSPKMSEAV :   59

Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : SAGASLGASLPLPLALPLPMALPLPMSLPLPLTAASSAVTVSLAAVVAAVAETGGAGAGG :  120
Ms-HR4 : PP---------------------------------------------------------- :   50
Tc-HR4 : VSPPS------------------------------------------------------- :   64
Tm-HR4 : LNPPS------------------------------------------------------- :   64

Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : AGTAVTASGAGPCVSTSSTTAAAATSSTSSLSSSSSSSSSTSSSTSSASPTAGASSTATC :  180
Ms-HR4 : ------------------------------------------------------------ :    -
Tc-HR4 : ------------------------------------------------------------ :    -
Tm-HR4 : ------------------------------------------------------------ :    -

Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : PASSSSSSGNGSGGKSGSIKQEHTEIHSSSSAISAAAASTVMSPPPAPATRSSPATPEGG :  240
Ms-HR4 : ------------------------------------------SPRMAPAGCSTPPHPP-- :   66
Tc-HR4 : ----------------------------------PDSTP--HHPPPPTLDSVSSRLED :   86
Tm-HR4 : ----------------------------------PDSTPIQIHPPPPTLDKISSRLED :   88

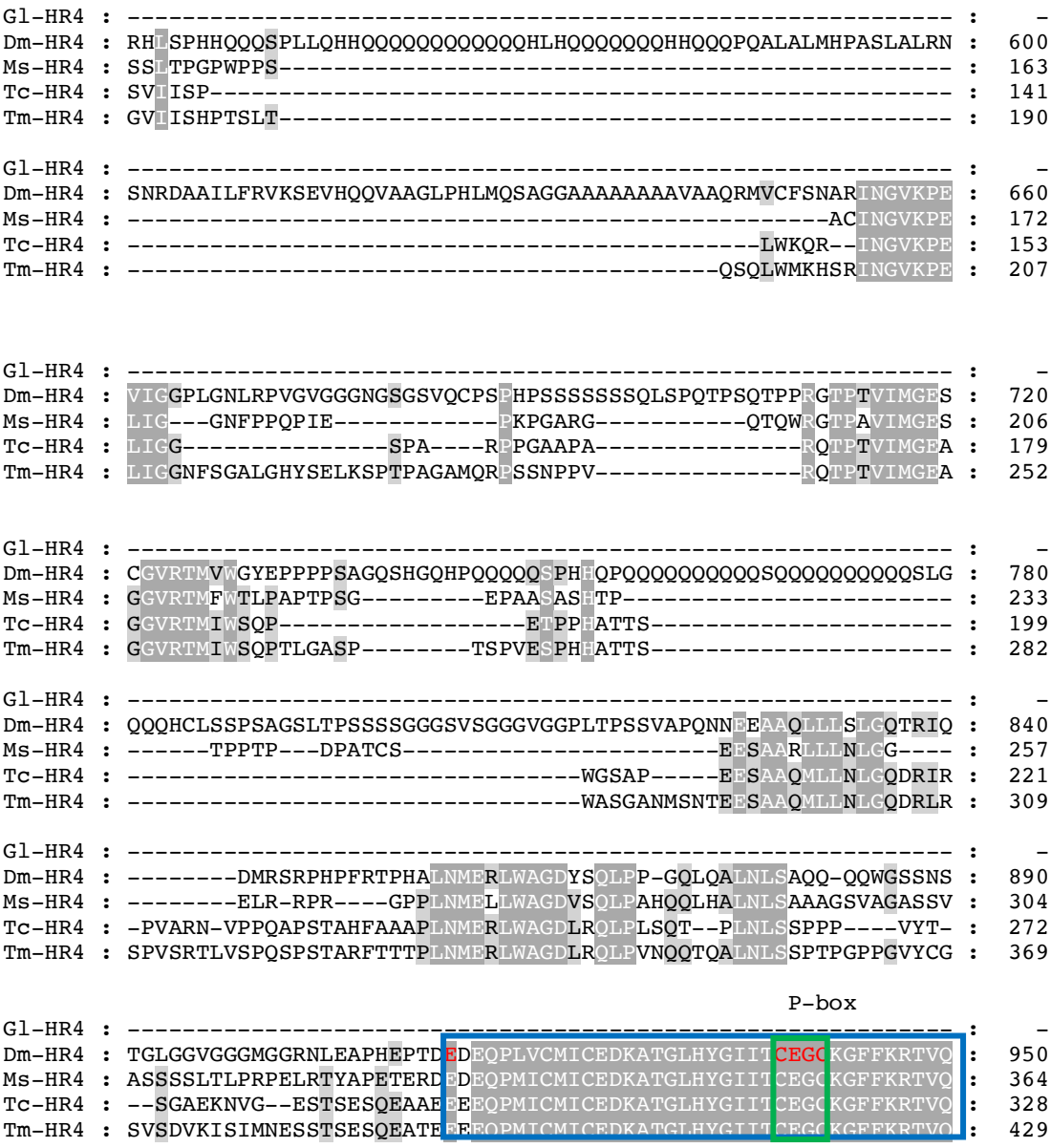
Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : GPAGDGSGATGGGNTSGGSTAGVAINEHQNNGNGSGGSSRASPDSLEEKPSTTTTTGRPT :  300
Ms-HR4 : -PVFDG-----------------------------GGSPSPSP----------------A :   80
Tc-HR4 : SGVFDGGG---------------------------------------------------- :   94
Tm-HR4 : SGVFDGGGTA-------------------------------------------------- :   98

Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : LTPTNGVLSSASAGTGISTGSSAKLSEAGMSVIRSVKEERLLNVSSKMLVFHQQREQETK :  360
Ms-HR4 : CHPT---------------------------VIRSAP-------SYSVIKFEG------- :   99
Tc-HR4 : -------------------------------VIRPLP----------------------- :  100
Tm-HR4 : -----------------------------SVIRSLPG----------------------- :  106

Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : AVAAAAAAAAAGHVTVLVTPSRIKSEPPPPASPSSTSSTQRERERERDRERDRERERERD :  420
Ms-HR4 : ------------------APGSVKAESSPASS---------------------------- :  113
Tc-HR4 : ------------------RSQSPPL-PAPAAGP--------------------------- :  114
Tm-HR4 : ----------------VRSQSPPLRPAPTSSP---------------------------- :  122

Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : RDRERERERQSISSSQQHLSRVSASPPTQLSHGSLGPNIVQTHHLHQQLTQPLTLRKSSPP :  480
Ms-HR4 : -------------------KHGAAPPSQMSS-------VKLEGTQQESPQPYRSRTIAPP :  147
Tc-HR4 : ----------------------RQPPRPHSSP-----------HSPGMAPP---------- :  132
Tm-HR4 : ----------------------CQPPRPHSSPGRPANTSPVIIHNPTIAQPTSSRAKLST :  160

Gl-HR4 : ------------------------------------------------------------ :    -
Dm-HR4 : TEHLLSQSMQHLTQQQAIHLHHLLGQQQQQQQQASHPQQQQQQQQHSPHSLVRVKKEPNVGQ :  540
Ms-HR4 : ------------------------------------------------------PPGSH :  152
Tc-HR4 : ------------------------------------------------------EPR :  135
Tm-HR4 : MVTCQEPTSSSPS-------------------------------------------AVSKSR :  179
```


P-box
DNA-binding domain
CTE

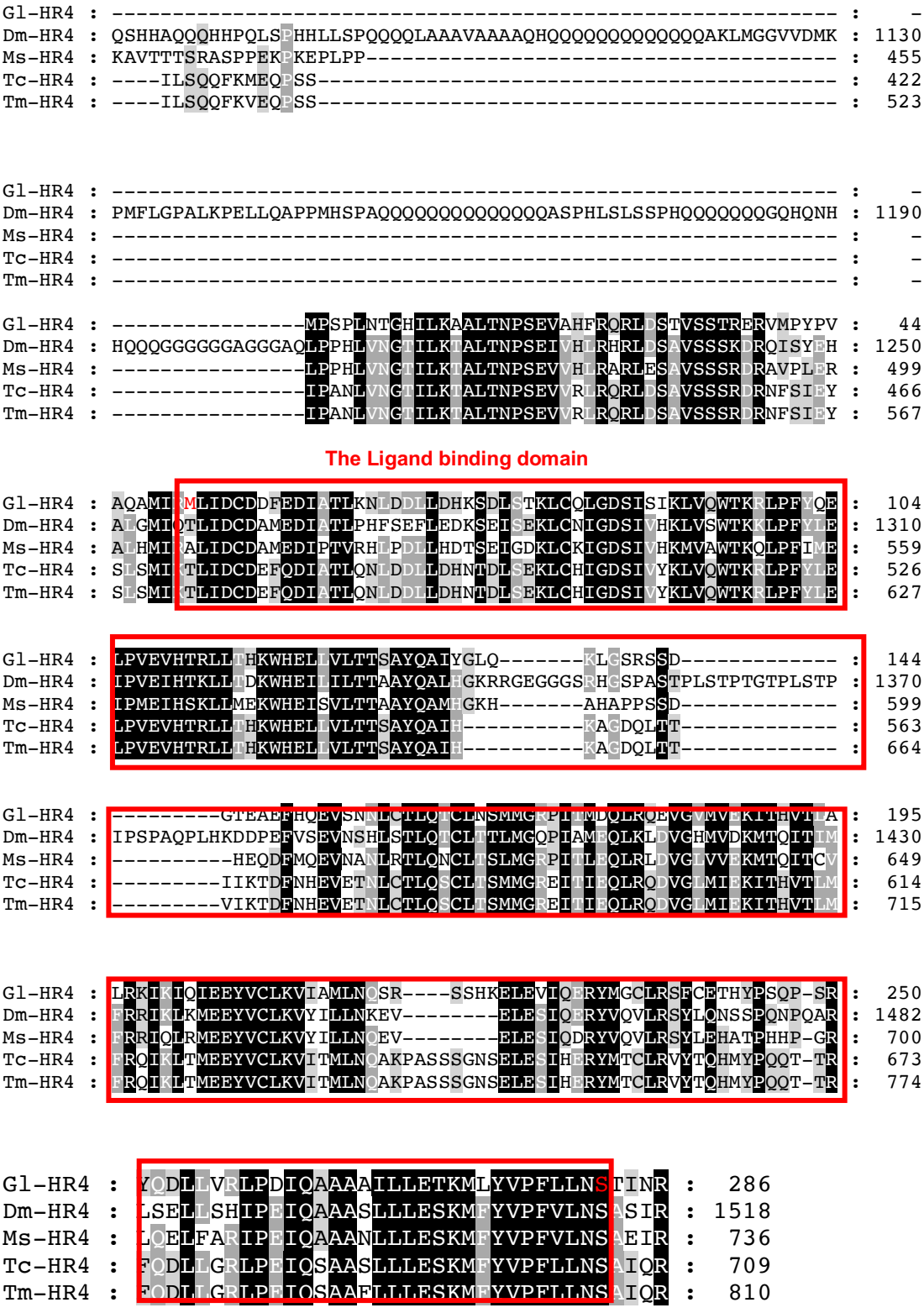

The Ligand binding domain

Figure 2. 20. Multiple alignment of deduced amino acid sequences of *HR4* proteins in one crustacean species and four insect species. Abbreviations: Dm, *Drosophila melanogaster* (NP_001259161.1); Tc, *Tribolium castaneum* (XP_974320.3); Tm Tenebrio molitor (CAA06670.1); Tm, *Manduca sexta* (AAL50350.1) and Gl, *G. lateralis*. Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment.the green boxes indicate p boa ant CTE within the DNA-binding domain and the red boxes indicated to ligand binding domain. A/B domain is conserved in *HR4* protein

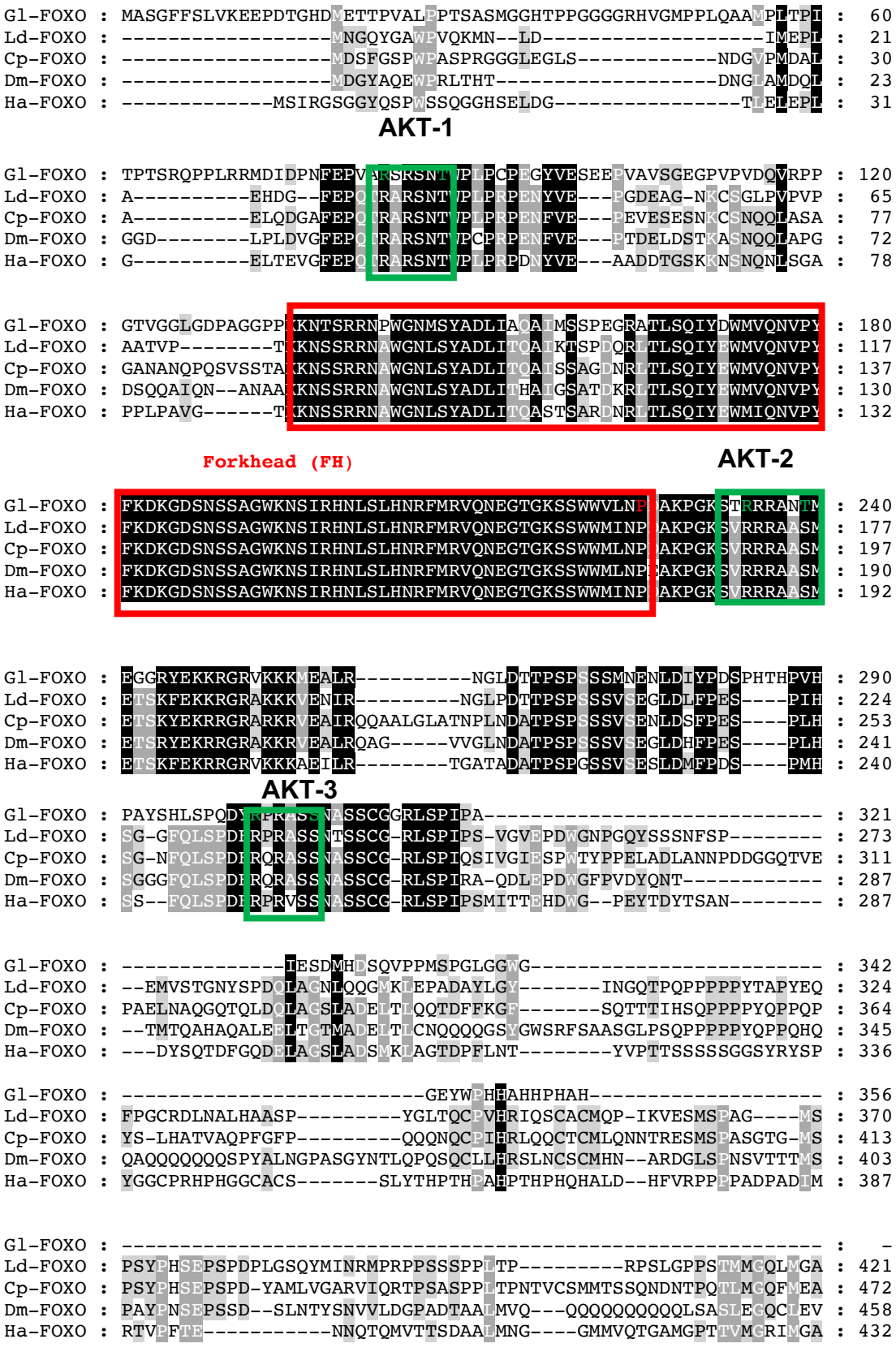
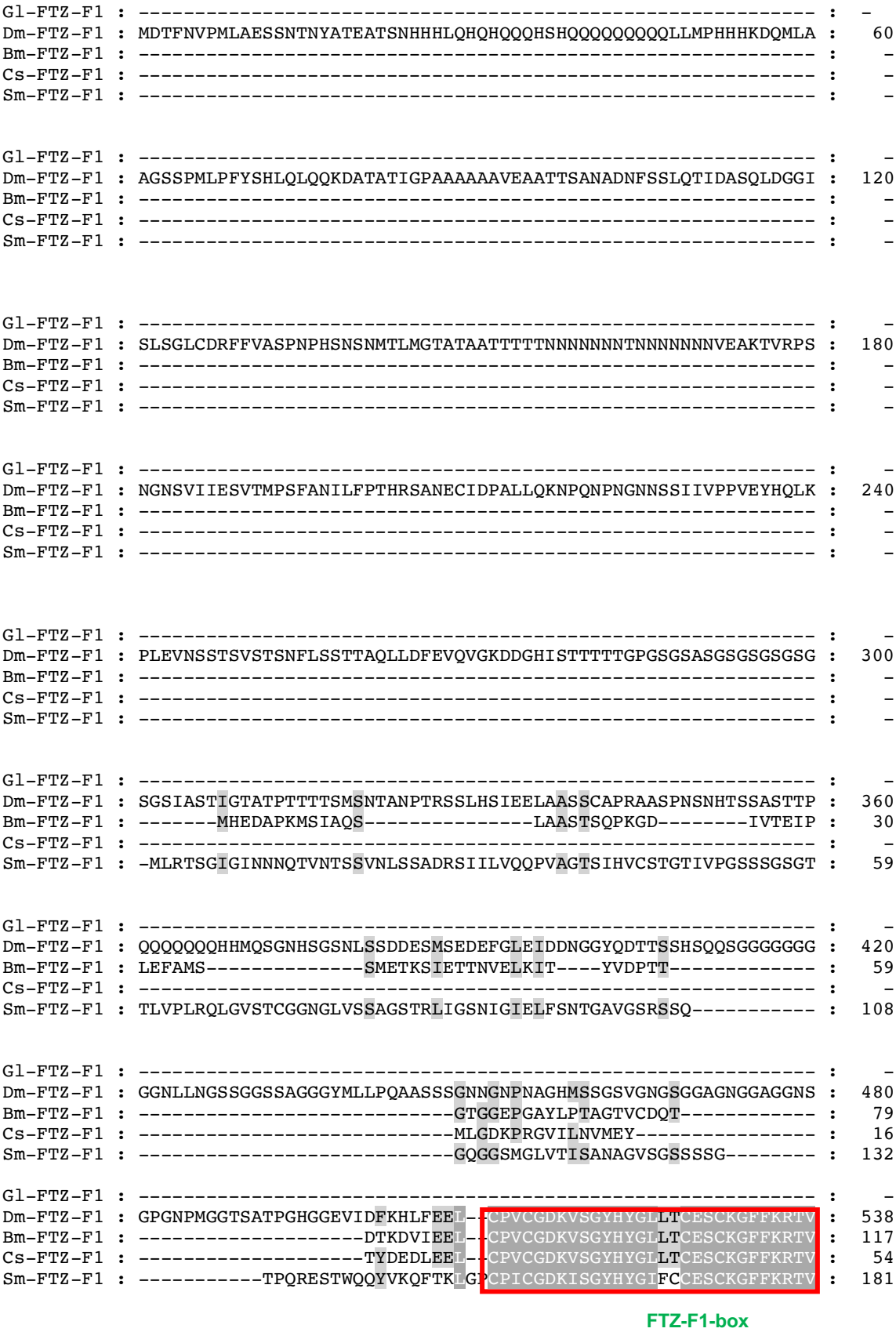
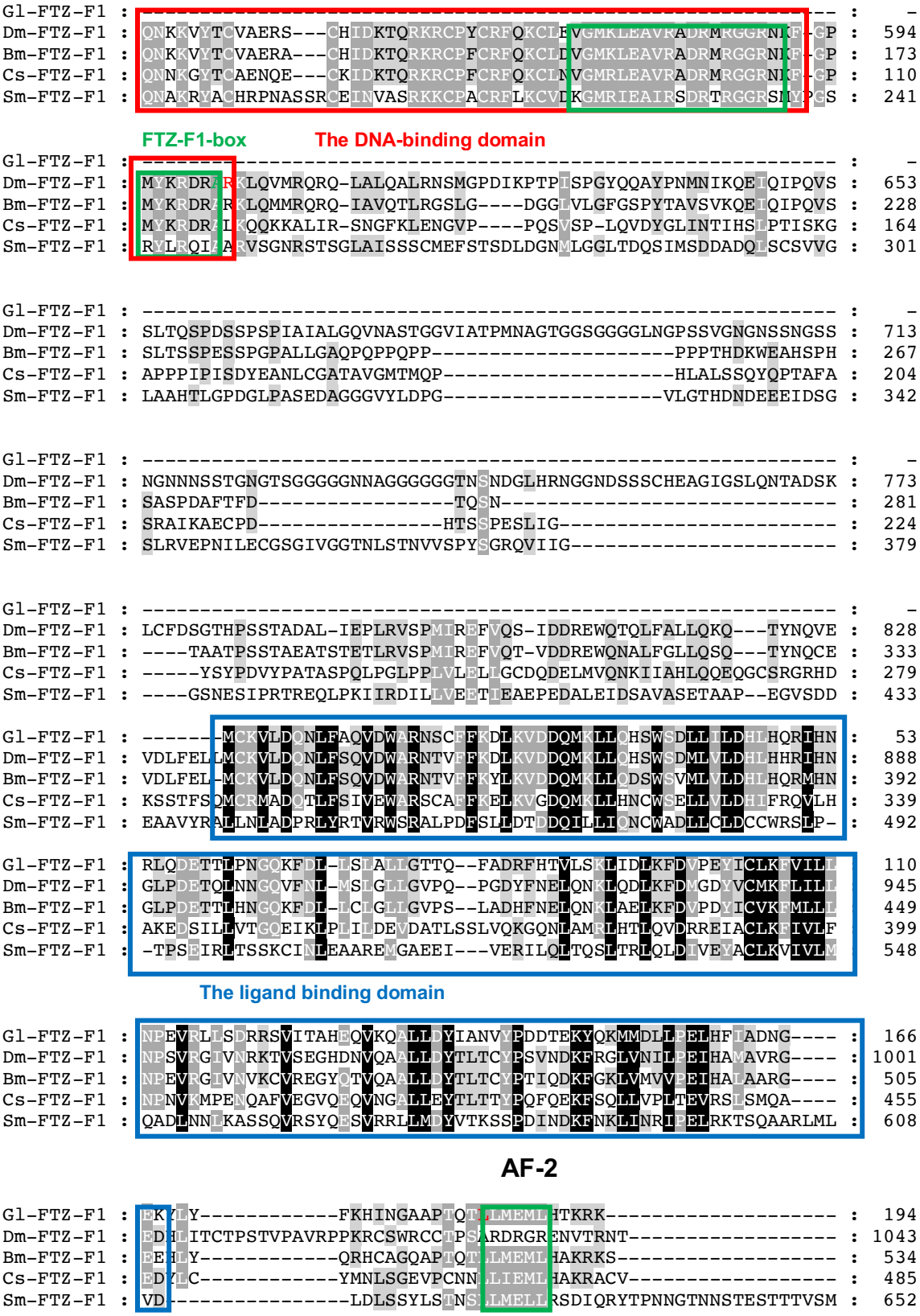

AKT-1
Forkhead (FH)
AKT-2
AKT-3

Figure 2. 21 Multiple alignment of deduced amino acid sequences of FOXO proteins in one crustacean species and four insect species. Abbreviations: Dm, *Drosophila melanogaster* (NP_001303426.1); Ha, *Helicoverpa armigera* (AKQ99123.1), Ld, *Leptinotarsa decemlineata* (XP_023017134.1); Cp, *Culex pipiens* (AEI86721.1); and Gl, *G. lateralis.* Amino acid residues that are identical or similar in all sequences are shaded in black; gray shading indicates identical or similar amino acids in most of the sequences. Dashes indicate gaps introduced to optimize the alignment and the boxes indicate the Forkhead domain (FH).
Green boxes indicate three identified AKT/PKB phosphorylation sites (conserved regulatory motifs).

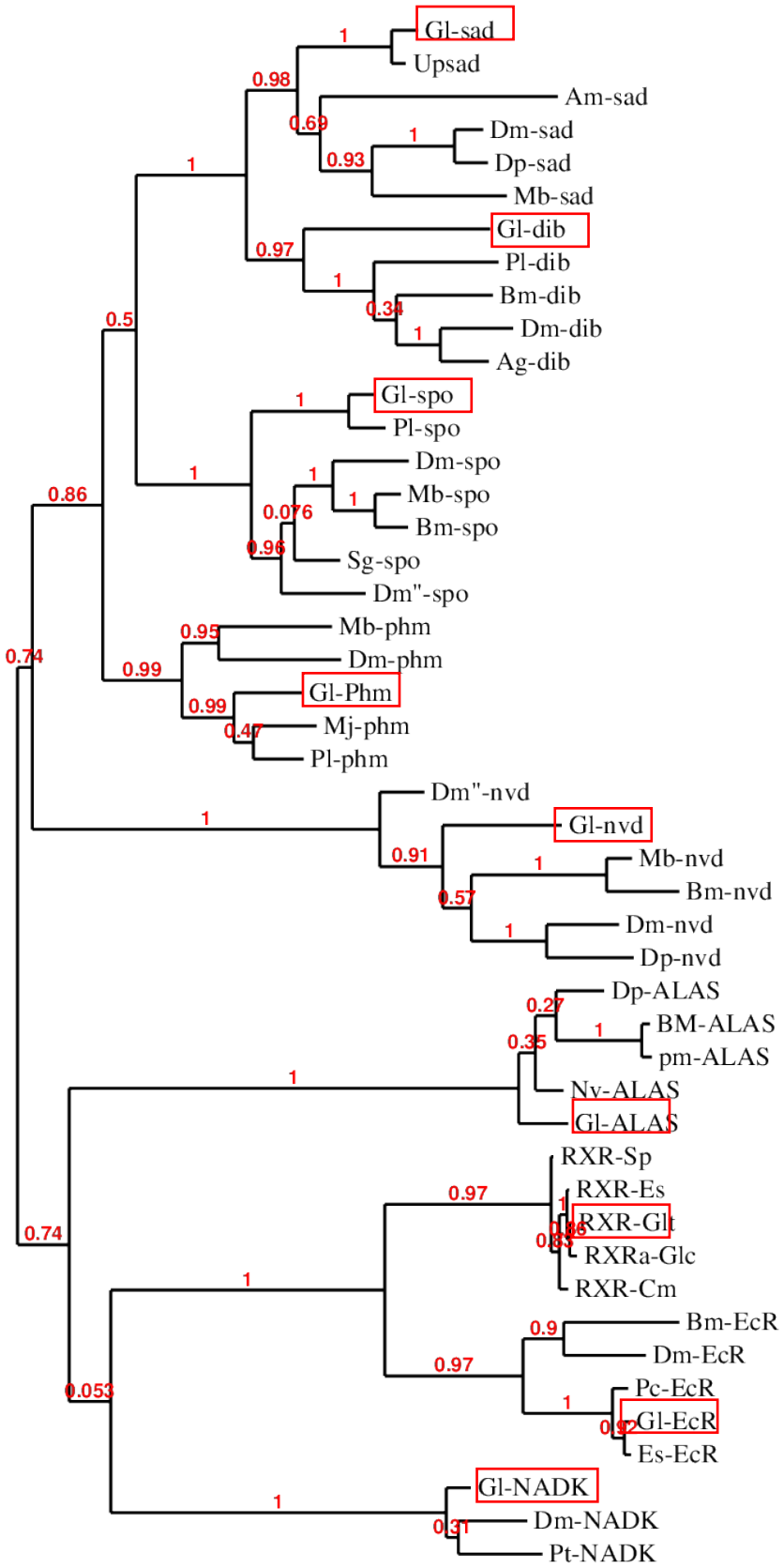

Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : MDTFNVPMLAESSNTNYATEATSNHHHLQHQHQQQHSHQQQQQQQQQQLLMPHHHKDQMLA : 60
Bm-FTZ-F1 : ---------- : -
Cs-FTZ-F1 : ---------- : -
Sm-FTZ-F1 : ---------- : -
Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : AGSSPMLPFYSHLQLQQKDATATIGPAAAAAAVEAATTSANADNFSSLQTIDASQLDGGI : 120
Bm-FTZ-F1 : ---------- : -
Cs-FTZ-F1 : ---------- : -
Sm-FTZ-F1 : ---------- : -
Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : SLSGLCDRFFVASPNPHSNSNMTLMGTATAATTTTTNNNNNNNTNNNNNNNVEAKTVRPS : 180
Bm-FTZ-F1 : ---------- : -
Cs-FTZ-F1 : ---------- : -
Sm-FTZ-F1 : ---------- : -
Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : NGNSVIIESVTMPSFANILFPTHRSANECIDPALLQKNPQNPNGNNSSIIVPPVEYHQLK : 240
Bm-FTZ-F1 : ---------- : -
Cs-FTZ-F1 : ---------- : -
Sm-FTZ-F1 : ---------- : -
Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : PLEVNSSTSVSTSNFLSSTTAQLLDFEVQVGKDDGHISTTTTTGPGSGSASGSGSGSGSG : 300
Bm-FTZ-F1 : ---------- : -
Cs-FTZ-F1 : ---------- : -
Sm-FTZ-F1 : ---------- : -
Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : SGSIASTIGTATPTTTTSMSNTANPTRSSLHSIEELAASSCAPRAASPNSNHTSSASTTP : 360
Bm-FTZ-F1 : -------MHEDAPKMSIAQS---------------LAASTSQPKGD--------IVTEIP : 30
Cs-FTZ-F1 : ---------- : -
Sm-FTZ-F1 : -MLRTSGIGINNNQTVNTSSVNLSSADRSIILVQQPVAGTSIHVCSTGTIVPGSSSGSGT : 59
Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : QQQQQQQHHMQSGNHSGSNLSSDDESMSEDEFGLEIDDNGGYQDTTSSHSQQSGGGGGGG : 420
Bm-FTZ-F1 : LEFAMS--------------SMETKSIETTNVELKIT----YVDPTT------------- : 59
Cs-FTZ-F1 : ---------- : -
Sm-FTZ-F1 : TLVPLRQLGVSTCGGNGLVSSAGSTRLIGSNIGIELFSNTGAVGSRSSQ----------- : 108
Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : GGNLLNGSSGGSSAGGGYMLLPQAASSSGNNGNPNAGHMSSGSVGNGSGGAGNGGAGGNS : 480
Bm-FTZ-F1 : ----------------------------GTGGEPGAYLPTAGTVCDQT------------ : 79
Cs-FTZ-F1 : ----------------------------MLGDKPRGVILNVMEY---------------- : 16
Sm-FTZ-F1 : ----------------------------GQGGSMGLVTISANAGVSGSSSSG-------- : 132
Gl-FTZ-F1 : ---------- : -
Dm-FTZ-F1 : GPGNPMGGTSATPGHGGEVIDFKHLFEEL--CPVCGDKVSGYHYGLLTCESCKGFFKRTV : 538
Bm-FTZ-F1 : --------------------DTKDVIEEL--CPVCGDKVSGYHYGLLTCESCKGFFKRTV : 117
Cs-FTZ-F1 : --------------------TYDEDLEEL--CPVCGDKVSGYHYGLLTCESCKGFFKRTV : 54
Sm-FTZ-F1 : -----------TPQRESTWQQYVKQFTKLGPCPICGDKISGYHYGIFCCESCKGFFKRTV : 181
FTZ-F1-box


FTZ-F1-box
The DNA-binding domain
The ligand binding domain
AF-2

```
Gl-FTZ-F1 : ------------------------------------------------------------ :    -
Dm-FTZ-F1 : ------------------------------------------------------------ :    -
Bm-FTZ-F1 : ------------------------------------------------------------ :    -
Cs-FTZ-F1 : ------------------------------------------------------------ :    -
Sm-FTZ-F1 : QGSANSGLKTVSVTLGADTAENQEENTTVTIVPSSGVTDLLSLPNHTTITTVTESRLDET :  712


Gl-FTZ-F1 : ------------------- :    -
Dm-FTZ-F1 : ------------------- :    -
Bm-FTZ-F1 : ------------------- :    -
Cs-FTZ-F1 : ------------------- :    -
Sm-FTZ-F1 : ITSNSNNSLTTSGVDVISK :  731
```

Figure 2 22. Multiple alignment of deduced amino acid sequences of *FTZ-F1* proteins in one crustacean species and four insect species. Abbreviations: Bm, Bombyx mori (NP_001037528.2); Dm, Drosophila melanogaster (AAA28542.1); Cs, Cynoglossus semilaevis (NP_001281131.1); Sm, Schistosoma mansoni (AAG49449.1). DBD (C domain) is indicated by red boxes and the Ftz-F1 box (boxed in green color), LBD (E region) indicated by blue boxes and AF-2 motif (boxed in green color).


Gl-sad
Upsad
Am-sad
Dm-sad
Dp-sad
Mb-sad
Gl-dib
Pl-dib
Bm-dib
Dm-dib
Ag-dib
Gl-spo
Pl-spo
Dm-spo
Mb-spo
Bm-spo
Sg-spo
Dm"-spo
Mb-phm
Dm-phm
Gl-Phm
Mj-phm
Pl-phm
Dm"-nvd
Gl-nvd
Mb-nvd
Bm-nvd
Dm-nvd
Dp-nvd
Dp-ALAS
BM-ALAS
pm-ALAS
Nv-ALAS
Gl-ALAS
RXR-Sp
RXR-Es
RXR-Glt
RXRa-Glc
RXR-Cm
Bm-EcR
Dm-EcR
Pc-EcR
Gl-EcR
Es-EcR
Gl-NADK
Dm-NADK
Pt-NADK

2.

Figure 2. 23. Phylogenetic tree showing the relationship between the six Halloween, *Neverland, CYP18a1, NADK, ALAS, EcR and RXR,* protein sequences of *Gecarcinus lateralis* with other arthropod species. Abbreviations: Am *(Apis mellifera*), Mj (*Marsupenaeus japonicas*), Cm, Carcinus maenas, Es, Eriocheir sinensis Sp, *Scylla paramamosain* Sg (*Schistocerca gregaria*), Dm, *Drosophila melanogaster* Mb, *Mamestra brassicae* Pl, *Pontastacus leptodactylus* transcriptome; Up, *Uca pugilator*, Mb (*Mamestra brassicae*), Bm (*Bombyx mori*), Dp (*Drosophila pachea*), Bd (*Bactrocera dorsalis*), Ls (*Lepeophtheirus salmonis*), Ag (*anopheles gambiae*), Dm" (*Daphnia magna*), and Ms (*Manduca sexta*) Pm, *Papilio machaon* Nv, *Nicrophorus vespilloides* Pb, *Python bivittatus* Pt, *Pan troglodytes*

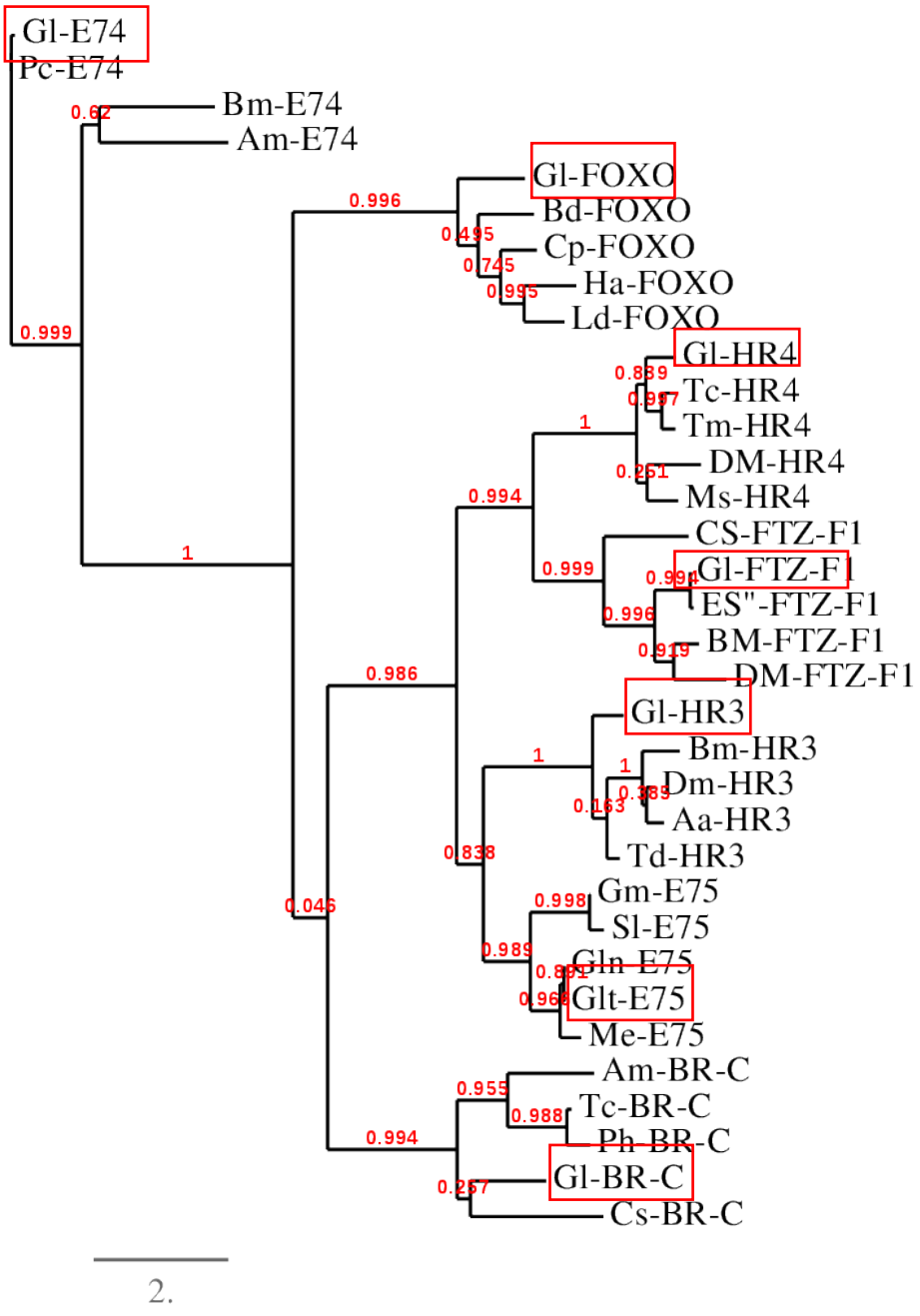


Figure 2. 24. Phylogenetic tree showing the relationship between the seven ecdysone-responsive genes (BR-C, E74, E75, HR3, HR4.FOXO and FTZ-F1) protein sequences of Gecarcinus lateralis with other arthropod species. Abbreviations: Am (*Apis mellifera*),Ha(*Helicoverpa armigera*), Ld (*Leptinotarsa decemlineata*),Sg (*Schistocerca gregaria*), Mb (*Mamestra* brassicae), Dm (*Drosophila melanogaster*), Bm (*Bombyx mori*), Dp (Drosophila pachea), Bd (bactrocera dorsalis), Ls (*Lepeophtheirus salmonis*),Cp (*Culex pipiens*), Ag (*anopheles gambiae*), Dm" (*Daphnia magna*), and Ms (*Manduca sexta*) Gm (*Galleria mellonella*),Sl

(*Spodoptera littoralis*),Td (*Thermobia domestica*),Es (*Eriocheir sinensis*),Cs (*Cynoglossus semilaevis*), Aa (*Aedes aegypti*),Tc(*Tribolium castaneum*)Tm(*Tenebrio molitor*),Ms (*Manduca sexta*) Gm, (*Galleria mellonella*),Me, (*Metapenaeus ensis*) Lp, (*Limulus Polyphemus*), Am (*Apis mellifera*).

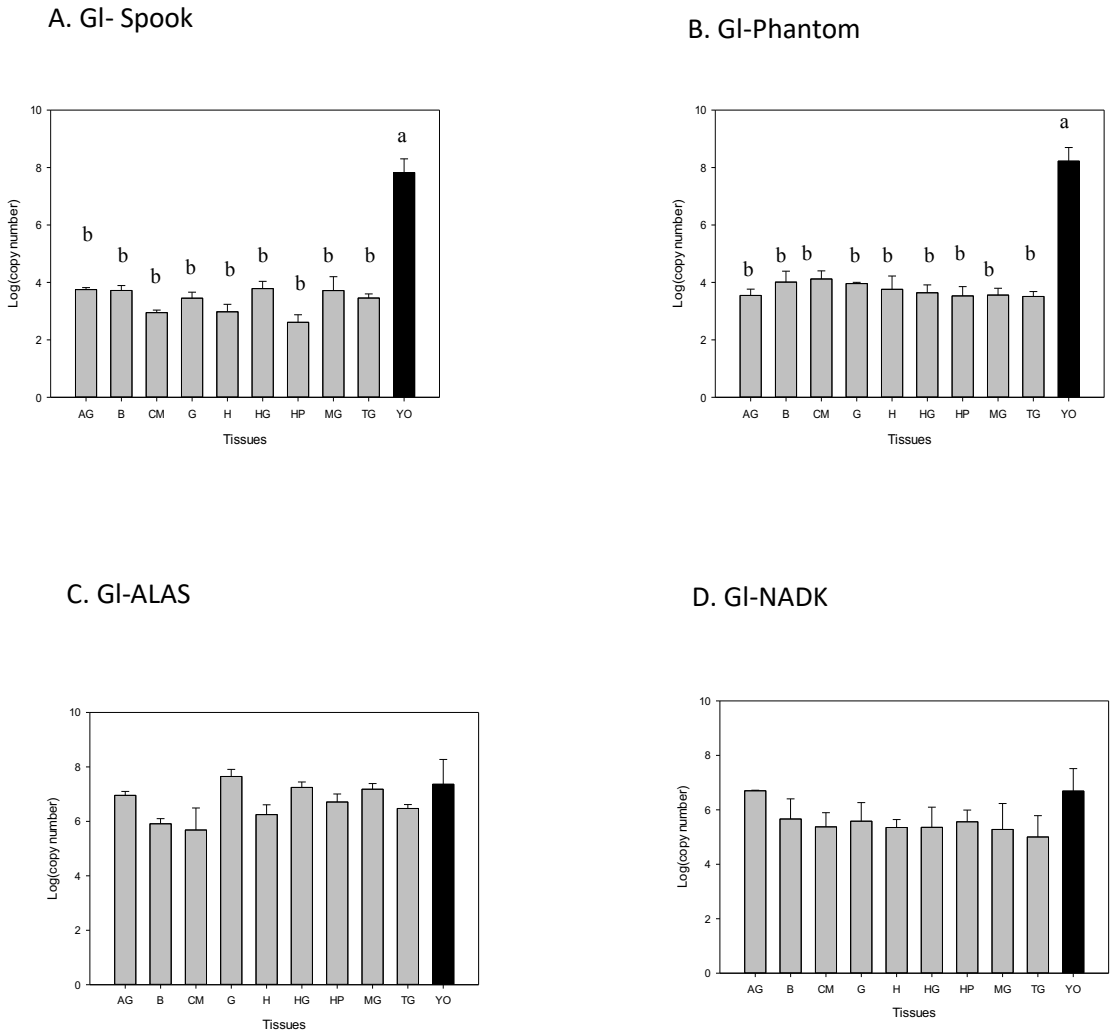
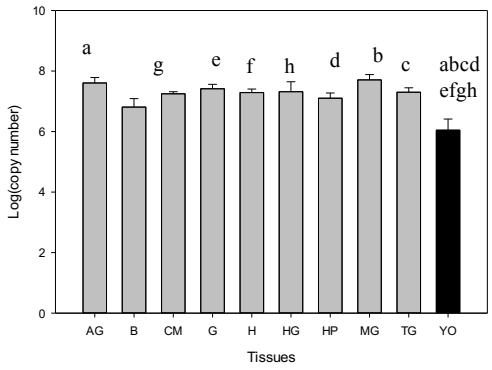
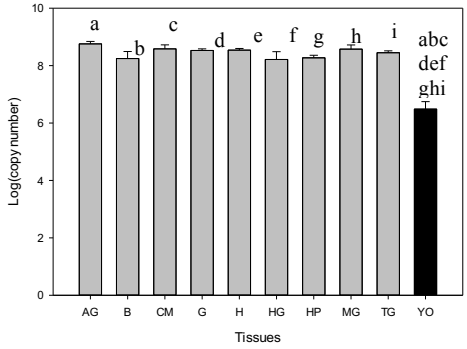


Figure 2. 25. Expression of the ecdysteroid biosynthesis genes in different tissues from intermolt animals in G. lateralis. Data are presented as mean + S.E. (MG n=3;HG n=3; AG n=3;YO n=3;TG n=5;B n=5;G n=4;CM n=5;HP n=4;H n=6) Abbreviations: MG, midgut; HG, hindgut; AG, Antennal ganglia; YO, Y-organ; TG, thoracic ganglia; B, Brain; G, gill; CM, claw muscle; HP, hepatopancreas; H, hart. *Gl-spook*, *Gl-Phantom*, *Gl-ALAS*, and *Gl-NADK*, mRNA levels were quantified in different tissues from intermolt animals. Different letters indicate significant difference at $P < 0.001$.

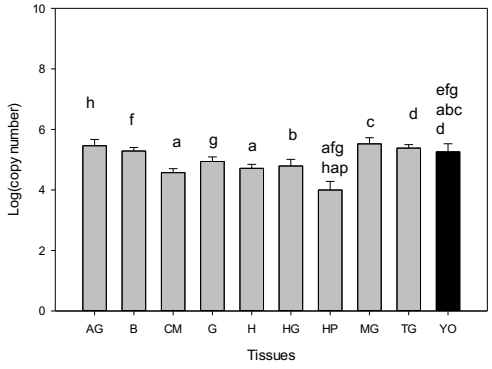
E. Gl-BR-C



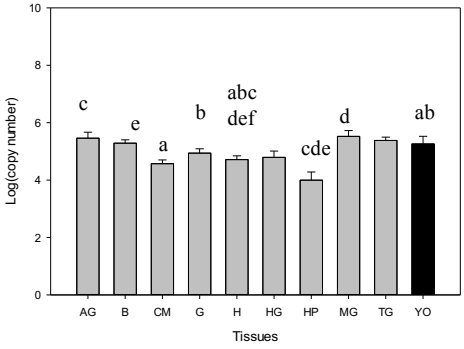
F. Gl-E75



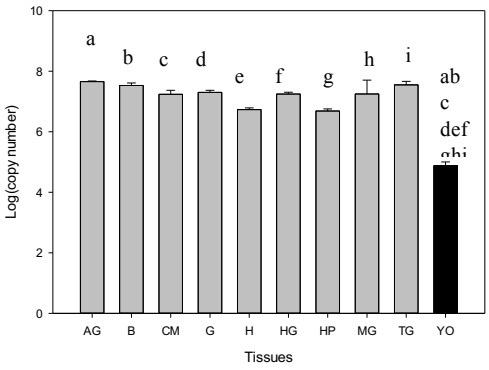
G. Gl-E74



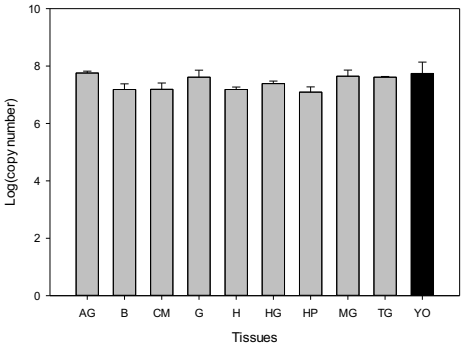
H. Gl-HR3



I. Gl-HR4



J. Gl-FTZ-F1



K. G-FOXO

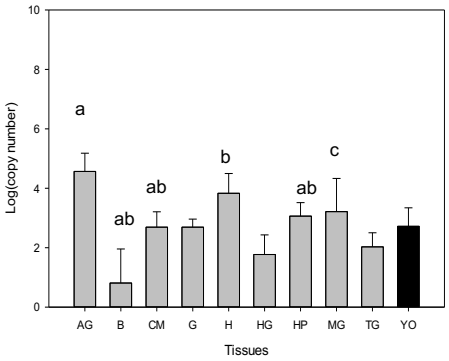


Figure 2. 26. Expression of the ecdysteroid-responsive genes in different tissues from intermolt animals in *G. lateralis*. Data are presented as mean + S.E. (MG n=3;HG n=3; AG n=3;YO n=3;TG n=5;B n=5;G n=4;CM n=5;HP n=4;H n=6) Abbreviations: MG, midgut; HG, hindgut; AG, Antennal gland; YO, Y-organ; TG, thoracic ganglia; B, Brain; G, gill; CM, claw muscle; HP, hepatopancreas; H, hart. *Gl-BR-C*, *Gl-E75*, *Gl-E74*, *Gl-HR3*, *Gl-HR4*, *Gl- FTZ-F1* and *Gl-FOXO* mRNA levels were quantified in different tissues from intermolt animals. Same letters indicate significant difference at $P < 0.05$ and $P<0.001$.

CHAPTER 3

The effect of molting on Halloween and ecdysteroid-responsive genes expression in the YO of *Gecarcinus lateralis*

Summary

Molting is necessary for growth and development in all arthropods. Halloween genes are expressed in the molting gland (Y-organ or YO) and encode enzymes that catalyze the synthesis of ecdysteroid hormones that coordinate molting processes during the premolt stage. mTOR activity is required for YO activation and entry into premolt. qPCR was used to quantify gene expression of Halloween and ecdysteroid-responsive gene expression in the YO of *Gecarcinus lateralis* induced to molt by multiple limb autotomy (MLA) or eyestalk ablation (ESA). ESA decreased mRNA levels of *Gl-Phm, Gl-E75* and *Gl-RXR* at 3 days post ESA. *Gl-Dib* and *Gl-sad* increased at 3 days post ESA and decreased at; 7 and 14 days post-ESA. *Gl-Cyp18a1*, *Gl-BR-C*, *Gl-NADK* and *Gl-ALAS* mRNA were higher at Day 0 and 1 post ESA and lower at Day14 post ESA. *Gl-HR3*, *Gl-HR4*, and *Gl-E74* were expressed at low levels. MLA decreased mRNA levels of Halloween genes, *Gl-Nev,* and *Gl-E75,* except *Gl-dib*, at premolt and postmolt stages. *Gl-Dib*, *Gl-NADK*, *Gl-ALAS,* and *Gl-BR-C* mRNA levels were not affected by molt stage. *Gl-EcR, Gl-HR4* and *Gl-HR3* mRNA levels were highest in premolt and lowest in postmolt. *Gl- FOXO* mRNA levels were highest in premolt and lowest in intermolt. This data suggests molting maybe have an indirect effect on regulate Halloween genes and directly regulates *HR3, HR4, RXR* and *FOXO* to increase synthesis of ecdysteroid. The presence of *EcR*/*RXR* and ecdysteroid-responsive genes suggest that elevated ecdysteroid represses the YO at the end of premolt.

Introduction

Molting is required for development and growth in arthropods. All arthropods have an outer skeleton (exoskeleton) that is shed in order to grow or metamorphose into other stages. Arthropods molt through their lifetime based on environmental conditions such as temperature and photoperiod (Caddy, 1987). The Y-organ (YO) in the crustacean is molting gland that secretes ecdysteroids (molting hormones), particularly ecdysone. The molt cycle in crustaceans is regulated by two different endocrine glands, the X-organ/sinus gland (XO/SG) complex and YO (Chang and Mykles, 2011; Hopkins, 2012). The X-organ, located at the base of eyestalks, secretes a neuropeptide called molt-inhibiting hormone (MIH) although secondary locations of MIH in other nervous tissues such as the brain and thoracic ganglion in *Carcinus maenas* (Abuhagr et al., 2014a; Huang et al., 2015; Pitts and Mykles, 2017).

MIH inhibits ecdysteroid synthesized by the YO. The pair of YOs, located in the cephalothorax, secretes ecdysteroid hormones (molting hormones). Interplay of the levels of these hormones in hemolymph control the molting process (Mykles, 2011). The molt cycle can be manipulated in crabs by two ways, eyestalk ablation (ESA) or by multiple leg autonomy (MLA). ESA removes the gland that secretes MIH, which in turn reduces the inhibition of MIH on YO. Following this, the YO is activated and the ecdysteroid level increases in the hemolymph. MLA is autotomy of 5-8 walking legs, which leads to molt induction via activation of YO, which this method mimics a natural mode of molting (Skinner and Graham, 1972).

The molt cycle consists of four major stages. The first stage of the molt cycle is premolt or proecdysis ($D_{1–4}$). Premolt is where the highest levels of ecdysteroids are observed and new exoskeleton is deposited, and limb regenerates grow (Chang and Mykles, 2011; Skinner, 1985). The second stage is ecdysis (E) which is the active shedding of exoskeleton. The third stage is

postmolt (A, B, and C_{1-3}), in which the freshly molted crab completes the synthesis and calcification of the new exoskeleton. The fourth stage is intermolt; during this stage the animal feeds and reproduces. Intermolt is the longest stage and YO is in the basal state (Skinner, 1985). Regenerating limbs in MLA provide measure of the progress events in the crabs. YO goes through different physiological states. Low level of MIH transitions the YOs from the basal state in intermolt to the activated state in early premolt and this transition requires mTOR activity for the synthesis ecdysteroids (Chang and Mykles, 2011). TGFβ is required for the transition from the activated state to the committed state in mid premolt and high ecdysteroids trigger the transition from committed state to the repressed state in late premolt. The measure is defined as the R index (calculated as the length of the regenerate x 100/carapace width), which increases from 0 to ~23 prior to ecdysis. ESA is an effective and convenient method, as the XO/SG complex is the primary source of MIH (Chang and Mykles, 2011; Skinner and Graham, 1972b; Yu et al., 2002).

Ecdysone (E) and 20-hydroxyecdysone (20E) are important hormone for molting in both insects and crustaceans. Theses hormones are synthesized from cholesterol, which these animals, obtain from their food (Niwa and Niwa, 2014a; Rewitz et al., 2007). Halloween genes encode enzymes that synthesize these hormones, and this pathway is known as the ecdysteroid biosynthesis pathway. The gene *Neverland* encodes 7,8-dehydrogenase, the enzyme that converts cholesterol to 7 dehydrocholesterol. *Phantom*, *Disembodied*, *Shadow*, and *Shade* encode enzymes that convert 5B-diketol to ecdysone. In peripheral tissues the enzyme encoded by the *shade* gene (*Shed* in crustaceans) converts ecdysone to 20E. Knockdown of these genes leads to larval lethality and delayed development (Chang and Mykles, 2011; Iga and Smagghe, 2010; Luan et al., 2013; Rewitz and Gilbert, 2008; Rewitz et al., 2007; Warren et al., 2004).

Steroid molting hormone 20E binds to its nuclear receptor (EcR/USP). This complex regulates ecdysteroid-responsive gene cascade that regulates many physiological aspects including metamorphosis, egg development, embryogenesis and molting (Li et al., 2016; Liu et al., 2015; Warren et al., 2006). In insects, *EcR*/*USP* binding to hormone response elements in the DNA induces the transcription three sets of ecdysteroid responsive genes: early (primary) genes *BR-C*, *E75* and *E74*, and early-late genes (*HR3*, *HR4, βFTZ-F1*). E75 is a negative regulator of *HR3* transcriptional activation at high levels of molting hormone. Moreover, *DHR3* work as both a repressor of *E75* by *HR4* and as an inducer of *βFTZ-F1* (Kageyama et al., 1997; King-Jones et al., 2005; Lam et al., 1999; Pierceall et al., 1999).

The goal of this project is to examine the effects of molt stage on Halloween gene and ecdysteroid responsive gene expression in the YO. We hypothesize that increased hemolymph ecdysteroid levels as a result of ESA and MLA prevents repression of ecdysteroidogenesis by MIH. We expected that the expression of Halloween genes would be up regulated during premolt when ecdysteroid synthesis is stimulated. In the YO of *Marsupenaeus japonicas*, an increase in *Phm* expression at premolt is inhibited by MIH *in vitro* (Asazuma et al., 2009). A transcriptomics study showed that ESA increased the relative expression of ecdysteroid biosynthesis genes (*Nvd*, *Spo*, *Phm*, *Dib, and Sad*) *CYP18a1*, *EcR* and *RXR*; (Shyamal et al., 2018). Transcriptomics and qPCR were used to quantify the mRNA levels of Halloween genes and ecdysteroid-responsive genes in animals induced to molt by MLA or ESA. The genes that were quantified were *Spo*, *Phm*, *Dib, Sad*, *Cyp18a1*, *Neverland*, *Broad Complex*, *E75*, *E74*, *Hormone Receptor 4*, *Hormone Receptor 3*, *forkhead box transcription factor*, and *Fushi tarazu factor-1*. An enzyme-linked immunosorbent assay (ELISA) was used to quantify the effects of MLA and ESA on hemolymph ecdysteroid titers.

Materials and Methods

Animals and experimental treatments

Adult male *G. lateralis* were collected from the Dominican Republic and kept at Colorado State University, CO. Animals were acclimatized for one month under controlled environment of ~27 °C and 75-90% humidity and placed under a cycle of twelve hours darkness and 12 hours light. Communal plastic cages that contained aspen bedding moistened with 5 ppt Instant Ocean was used to keep intermolt individuals (Aquarium Systems, Mentor, OH). Animals were fed lettuce, carrots, and raisins two times a week (Covi et al., 2010). Molting was induced by autotomy 5-8 of walking legs (MLA) and animals were kept in individual containers with a quartz sand substrate moistened with 10 ppt Instant Ocean (Covi et al., 2010; Skinner, 1985; Yu et al., 2002). Eyestalk ablation (ESA) induced molting by eliminating the primary source of MIH (Skinner, 1985). There was immediate activation of YO and an increase of ecdysteroid titers by 1 day post- ESA (Lee et al., 2007). The limb bud (LB) growth was used to measure premolt stages. The regenerate index (R index) is the LB length percentage in comparison to the size of the body and it utilizes the following equation: regenerate length x 100)/carapace width. There is growth of the regenerate during premolt in response to ecdysteroids while reaching 23- 25 R index before ecdysis (Hopkins et al., 1979; Skinner and Graham, 1972; Yu et al., 2002). The hemolymph ecdysteroid titers and integumentary structure confirmed molt stage.

RNA isolation and cDNA synthesis

Trizol and chloroform/phenol was used in the isolation of RNA based on the description of (Covi et al., 2010). YOs were placed in 350 µl RNAlater (Ambion) over night at a 4°C and subsequently stored at 20°C until additional processing. Tissue was homogenized in TriZol in an

RNase/DNase/protein free tube for 5 minutes, with the use of motorized tissue grinder (Fisher). The supernatant fraction underwent extraction of phenol-chloroform, while 22 µl of RNase/DNase/protease free water was used to dissolve RNA pellets. DNase I treatment was given to every sample based on the instructions of the manufacturer (Thermo Scientific, Grand Island, NY, SA). There was inclusion of Ribolock (Thermo Scientific) ten units (0.75 µl) in the DNase I treatment for the prevention of degradation of RNA. 24:1 chloroform isoamyl alcohol was utilized for the second phenol-chloroform extraction, and the precipitation of RNA was accomplished by the addition of 0.5 volume of 3 M sodium acetate (pH 5.2) to 1.5 volume of isopropanol. The pellets were dissolved in 22 µl RNase/DNase/protease free water, and a Nanodrop 1000 (Thermo Scientific) used to determine the concentration of RNA. SuperScript IV Reverse Transcriptase was used for a reverse-transcription of 4 µl of RNA (Thermos Fisher) in accordance with the instructions of the manufacturer. The cDNA was stored at -20°C.

A Lightcycler 480 Thermocycler was used to perform quantitative PCR (qPCR) (Roche Applied Science, Indianapolis, IN, USA). The following were the qPCR conditions: forward and reverse gene-specific primers at 0.5 µl each (Table 3.1, 2), 5 µl SYBR Green (Roche), 3 µl nuclease free water, and a template of 1 µl cDNA. Upon denaturation for a period of 3 minutes at a temperature of 95 °C, repetitions of 45 cycles were made for 30 seconds at a temperature of 95 °C, for 30 seconds at a temperature of 62 °C, for 20 seconds at a temperature of 72 °C, and a final extension of 7 min at a temperature of 72 °C. The quantification of each gene's concentration was made by making comparison with 72 established standard curves.

Differential gene expression analysis

Transcriptomics was used to quantify gene expression in *G. lateralis* induced to molt by (MLA) and (ESA) ± mTOR inhibitor rapamycin. Using the differentially expressed gene (DEG)

file of the RNA-seq data to calculate the, FPKM (Fragments Per Kilobase of transcript per Million mapped reads). For the ESA± rapamycin experiment animals were ES-ablated and injected with rapamycin in DMSO (10 μM final concentration) or vehicle (DMSO, ~1% final concentration) at Day 0. We assumed that the hemolymph volume is 30% of the wet weight in grams, to calculate the volume to inject we used the equation: mass (g) x 0.3 μl = volume of a 10 mM stock solution. Hemolymph samples were taken and YOs were harvested at 0, 1, 3, and 7 days post-injection.

Statistical analysis

One-Way ANOVA in Sigma Plot for titers of ecdysteroid and transcript levels of gene for every one of the genes determined the statistical implication of ($p<0.05$) for the tissues gathered at different stages and days after ESA. The performance of a paired t-test in determining whether significance between experimental and control at a given time point.

Results

The effects of MLA on hemolymph ecdysteroid and gene expression in YOs

Animals were induced molt by autotomizing walking legs and YOs were collected at different stages as determined by the R-index (intermolt: R=7-9; early premolt: R=11-13; mid premolt: R=16-18; late premolt: R>19). qPCR is used to quantify mRNA levels. Hemolymph ecdysteroid levels were statistically higher in mid premolt and late premolt compared to intermolt. The postmolt stage showed a low ecdysteroid titer (Fig. 3. 1). *Gl-phm*, *Gl-dib*, *Gl-spo*, *Gl-Nvd*, and *Gl-CYP18a1* mRNA levels were higher during intermolt and mid premolt with the means at intermolt and mid premolt significantly higher than the mean at post molt ($p<0.05$) (Fig. 3. 2). *Gl-Sad*, *Gl-EcR*, and *Gl-FOXO* mRNA levels were higher during mid premolt with the means at midpremolt significantly higher than the mean at post molt. *Gl-NADK* and *Gl-ALAS*

mRNA levels were not affected by MLA. *Gl-HR3* mRNA level increased during premolt with the means at mid premolt and late premolt significantly higher than the means at intermolt postmolt. *Gl-E75* and *Gl-RXR* mRNA levels were significantly lower in premolt when compared to intermolt and showed the lowest level in postmolt ($p<0.05$). *Gl-BR-C*, *Gl-FTZ-F1*, *Gl-HR4*, and *Gl-E74* mRNA levels were not affected by MLA (Fig. 3. 3)

Effect ESA on hemolymph ecdysteroid and gene expression in YOs

Intermolt animals were eyestalk ablated at Day 0. YOs were harvested from intact (intermolt) animals and from animals at 1, 3, 7, and 14 days post-ESA. There was a significant increase in hemolymph ecdysteroid titers (Fig. 3.4A). *Gl-Nvd*, showed no significant change while *Gl-spo* and *Gl-phm* mRNA levels showed a decrease at 3 days compared to 1 day post ESA ($P<0.05$) (Fig. 3.4C-F). *Gl-sad* mRNA level was significantly higher at 1, 3- and 7-days post-ESA compared to 14 days post ESA (Fig. 3.4H). *Gl-dib* mRNA level showed a significant decrease at 14 days post-ESA, compared to 0,1,3, and 7 days post-ESA ($P<0.001$) (Fig. 3.4G). *Gl-CYP18a1* mRNA level showed a significant decrease at 14 days post-ESA compared to 0 day and 1 day post-ESA ($P<0.050$) (Fig. 3.4I). *Gl-FOXO*, *Gl- FTZ-F1*, and *Gl- EcR* mRNA levels showed no significant change. *Gl-RXR* mRNA level showed a significantly decrease at 3 days post ESA compared to 0 and 1 day post-ESA ($P<0.05$) (Fig. 3.5Q-R). *Gl-NADK* and *Gl-ALAS* mRNA levels decreased at 14 days post-ESA ($P<0.05$) (Fig. 3.4D-E), while ESA lowered *Gl-E75* mRNA level at 3 days post ESA ($P<0.050$) (Fig. 3.5M). *Gl-BR-C* mRNA level decreased at 3 and 14 days post- ESA ($P<0.05$) (Fig. 3.5L). G*l- HR3* and *Gl-HR4* mRNA levels showed no significant change (Fig. 3.5O-P).

Effects of MLA on transcriptomics of Halloween and ecdysteroid-responsive gene expression in YO

Transcriptomics was used to quantify relative gene expression in *G. lateralis* induced to molt by MLA. Relative mRNA levels of the Halloween genes *spook, phantom, disembodied, shadow,* and *neverland* were statistically higher in intermolt and early premolt and then decreased during mid and late premolt to their lowest levels 10 days post molt (Fig. 3.6). *Gl-ALAS* FPKM value was significantly higher at early premolt and lower at mid premolt, late premolt, and postmolt. *Gl-NADK* and *Gl-CYP18a1* FPKM values were not significantly affected by MLA (Fig. 3.6). Ecdysteroid-responsive genes showed high FPKM at premolt stage compared to intermolt and postmolt for *Gl-EcR, Gl-E75, Gl-FOXO, Gl-HR3* and *Gl-HR4* genes, while *Gl-BR-C* and *Gl-E74* showed no significant difference between the stages (Fig. 3.7). *Gl-FTZ-F1* was significantly higher at intermolt, then decreased during premolt and was at its lowest levels 10 days postmolt (Fig. 3.7).

Effects of ESA ± rapamycin on transcriptomics of Halloween and ecdysteroid-responsive gene expression in YO

Injection of rapamycin into ES-ablated *G. lateralis* significantly decreased hemolymph ecdysteroid levels at 1 day to 7 days post-injection (Fig. 3.9A). The ecdysteroid titer in the hemolymph was increased by 1 day post-ESA. ESA increased mRNA levels of ecdysteroid biosynthesis genes in Day 7 control. The relative expression of Halloween genes *ALAS*, and ecdysteroid receptor heterodimer (*EcR*/*RXR*) increased significantly from 1 day to 7 days post-ESA (Fig.3.8B-K). Rapamycin reduced mRNA levels of ecdysteroid biosynthesis genes *Gl-Nvd*, *Gl-ALAS*, *Gl-Spo*, *Gl-Sad*, *Gl- Dib*, *Gl-Phm* and *Gl-CYP18a1* (Fig. 3.8B-I). Also, the ecdysteroid receptor heterodimer (*EcR/RXR*) showed the same result. There was no significant difference between control groups and experimental groups for *Gl- Gl-HR3, Gl-HR4, Gl-E74, Gl-E75, Gl-FTZ-F1* and *Gl-FOXO* (Fig 3.9M-R*)*.

Discussion

Ecdysteroids are important arthropod hormones involved in molting, development and metamorphosis (Techa and Chung, 2015). In this study we investigated the expression of Halloween genes and ecdysteroid-responsive genes in the YO over the molt cycle. MLA increased hemolymph ecdysteroids during premolt, peaked at late premolt, and decreased to low levels in postmolt (Fig. 3.1). *Gl-Nvd*, *Gl-Spo*, *Gl-Phm* and *Gl-CYP18a1* were expressed at their highest levels in intermolt animals and decreased at premolt and postmolt, whereas *Gl-Sad* was expressed at high levels in premolt and low level in intermolt and postmolt (Fig. 3.2). MLA had no effect on mRNA levels of *Gl-Dib*, *Gl-NADK* and *Gl-ALAS* (Fig. 3.2). These data suggest that many of the Halloween genes, *Gl-Nvd* and *CYP18a1* are regulated post-transcriptionally. By contrast, in insects the expression of Halloween genes and *Nvd* are correlated with 20E titers during larval development. This is consistent with kuruma prawn, in which the *phantom* gene is up-regulated during premolt. These results indicate Halloween genes are upregulated to support ecdysteroid synthesis (Asazuma et al., 2009; Iga and Smagghe, 2010; Niwa and Niwa, 2016). MLA YO transcriptome RNA-seq data support the qPCR data. All the Halloween genes showed higher relative expression at intermolt, decreased during premolt and low expression at postmolt. *Gl -ALAS* showed higher expression at early premolt and lower expression during mid and late premolt and postmolt while Gl-*NADK* expression was unaffected. *Neverland* gene along with Halloween genes play important role in the regulation of molting in insects and crustaceans. *Nvd* in *Penaeus monodon* is mainly expressed in the YO and also showed high expression in premolt stage which is similar *phantom* in *M. japonicus* (Asazuma et al., 2009; Sathapondecha et al., 2017). In addition, *Nvd* is expressed at high levels before ecdysis in *D. melanogaster* and *B.*

mori. Our results are not consistence to these results, but our RNA-Seq and qPCR data Halloween genes and *Nvd* gene were expressed at their highest levels at intermolt.

qPCR showed that MLA affected *Gl-EcR* mRNA level by increasing during premolt, while *Gl-RXR* had high mRNA level at intermolt and premolt and low level at postmolt. A previous study reported ecdysteroids regulate the transcriptional of *EcR,* which binds to *USP* (*RXR* in crustacean) to mediate ecdysteroid signals (Antoniewski et al., 1996; Tarrant et al., 2011). *EcR* in *U. pugilator* limb bud is upregulated by 20E and the same result is found in *M. sexta* (Durica and Hopkins, 1996; Gilbert et al., 2002). Our current results of *Gl-EcR* are similar to the findings reported in which expression of *EcR* increased with an increase in hemolymph ecdysteroid level. MLA does not affect mRNA level of *Gl-FTZ-F1.* In a previous study on insect *FTZ-F1* is induced by a pulse of 20E during the molt. Induction of *FTZ-F1* requires activation of *HR3* when the ecdysteroid titer is low (Hiruma and Riddiford, 2001; Weller et al., 2001). This scenario seems different in this study as qPCR of *FTZ-F1* showed high level of mRNA during all molt stages without any effect on expression by MLA

Gl-FOXO by qPCR showed increased level of mRNA during premolt stages, which is associated with higher ecdysteroid titers. However, the MLA transcriptome showed high level of *Gl-FOXO* in intermolt. Previous research showed 20E upregulates the *FOXO* gene. 20E induces a change in the localization of *FOXO* from the cytoplasm to nucleus, which induces lipolysis in fat body cells during molting and pupation (Hossain et al., 2013; Hou et al., 2012). Insulin induces phosphorylation of *FOXO* by *AKT*; phosphorylation of *FOXO* in the cytoplasm promotes larval growth and produces more 20E. High level of 20E represses *AKT* phosphorylation via PTEN; the dephosphorylated *FOXO* translocate into the nucleus to allow the larvae to molt (Cai et al., 2016; Garelli et al., 2012; Koyama et al., 2014).

qPCR of *Gl-E75* showed higher mRNA at intermolt and lower level at premolt and postmolt, whereas *Gl-BR-C*, and *Gl-E74* are unaffected by MLA. The MLA transcriptome showed the same result for *Gl-BR-C* and *Gl-E74,* while *Gl-E75* showed higher FPKM values during premolt and lower values at intermolt and postmolt. In the fruit fly, *E75A* expression coincides with the peak of ecdysteroid during embryogenesis (Cruz et al., 2007). *E75* is an early ecdysteroid responsive gene that is regulated by low 20E levels (Sullivan and Thummel, 2003). Analysis of the MLA transcriptome for *Gl-HR3* and *Gl-HR4* showed highest FPKM value at latepremolt, which is consistent with the function of early-late response genes. In insects, *HR3* and *HR4* have important roles during metamorphosis, which act together to inhibit *BR-C*, *E74A*, and *E75A* early genes and induce the *FTZ-F1* early-late gene (Kageyama et al., 1997; King-Jones et al., 2005; White et al., 1997). There were significant effects of molting on *Gl-FTZ-F1,* which showed high FPKM values in intermolt and premolt. These data support the model in which early ecdysteroid induced transcription factors directly control the expression of secondary and late-response target genes (Burtis et al., 1990; Riddiford et al., 2003; Thummel, 1990).

Eyestalk ablation (ESA) was used to examine the effect acute withdrawal of MIH on the expression of Halloween genes and ecdysteroid-responsive genes in *G. lateralis*. Hemolymph ecdysteroid titers increased indicating that ESA was effective in inducing molting (Fig. 3. 4A). *Gl-phm* and *Gl-spo* mRNA levels were decreased by 3 days post-ESA (Fig. 3.4C, F). *Gl-Sad* and *Gl- Dib* showed a strong decline in the expression at Day 14 compared to Days 0,3, and 7 post-ESA. *CYP18A1* is involved in 20E inactivation by metabolizing 20E to 26-hydroxylated product (Guittard et al., 2011; Rewitz et al., 2010). In *Drosophila* it has been reported that the high level of expression of *CYP18a1* at the end of the third instar corresponds with high titers of 20E (Bassett et al., 1997; Rewitz et al., 2010). Our result of *Gl-CYP18a1* is not consistent with these

results. *Gl-CYP18a1* showed high level of mRNA at Day 0 and decreased by Day 14 post-ESA. *Gl -NADK*, *Gl-ALAS*, *Gl-E74*, *Gl-HR3*, *Gl-HR4*, *Gl-FTZ-F1,* and *Gl-FOXO* mRNA levels were not affected by ESA. *Gl-E75* and *Gl-BR-C* have high expression at 0 and 1 day post ESA then dramatically deceased at 3 days post-ESA. All these genes showed high level of expression at Day 0 (Fig. 3.4J-R). These data suggest that these genes are regulated post-transcriptionally by mTOR, which is activated by ESA. mTOR controls global translation of mRNA into protein and is necessary for ecdysteroidogenesis (Abuhagr et al., 2016; Lamming, 2016; Weichhart, 2018). Rapamycin inhibits ecdysteroid secretion by the YO *in vitro* and *in vivo*, indicating that mTOR-dependent protein synthesis is required for ecdysteroid secretion (Abuhagr et al., 2014b). In insects, rapamycin inhibits phosphorylation of S6K and reduced secretion of ecdysteroid synthesis by PTTH in the prothoracic gland (Gu et al., 2012; Song and Gilbert, 1994).

We used RNA-Seq to quantify the effects of ESA ± rapamycin on Halloween and ecdysteroid-responsive genes mRNA levels in the YO. Ecdysteroid biosynthesis genes showed decreases levels of mRNA experimental group compare with control group. All Halloween genes (*Gl-Spo*, *Gl-Phm*, *Gl- Dib*, and *Gl-Sad*) and *Gl-Nvd* reached highest of mRNA levels at 3 days post-ESA, which suggest that mTOR is involved in YO activation in early premolt and rapamycin inhibits YO ecdysteroidogenesis. However, all ecdysteroid-responsive genes were not affected by rapamycin except *BR-C* gene. *Gl- BR*-C mRNA levels decreased by 3 days post-ESA.

Both qPCR and transcriptome data from the MLA animals showed highest expression of *Phm*, *Spo*, *Nvd*, and *CYP18a1*, at intermolt animals, while *Gl-EcR*, *Gl-HR3*, *Gl-HR4*, and *FOXO* showed high mRNA levels of mRNA at premolt. Molting up-regulates *HR3*, *HR4*, *EcR* and *FOXO*. These data suggest molting has as indirect effect on the regulation of Halloween genes,

Nvd, and *CYP18a1* regulated post transcriptionally. ESA rapamycin showed mTOR required to up regulate for ecdysteroid biosynthesis genes while ecdysteroid-responsive genes does not require mTOR. We conclude rapamycin blocked or delayed the effects of molting in *G. lateralis* by suppressing Halloween genes expression during premolt.

Table 3. 1. Oligonucleotide primers used for quantitative PCR of ecdysteroidogenic genes from *G. lateralis*.

Gene	Primer Sequence (5"–3")	Product size (bp)	TM (ºC)
Phantom	F1 TCTTTCACTTCACCACCACC R1 TCCTCTGTGACTCAGGTCTTA	182	54.9 54.4
Disembodied	F1 TCTCTTCAGTCAGTCCCTATGT R1 GCATCTCAGCTACCTCTCATTT	234	54.6 54.6
Shadow	F1 CGGCTGACTCCCTCATAATTT R1 GGAAGGCAGCTCGCTATAAG	234	54.7 55.4
Spook	F1 CCCTTCAGCACCGGAAAG R1 CTAGTGATACTCGTGATGCCTG	251	56.2 54.6
Neverland	F1 GTGTCCGAGGCGAGACATT R1 ACGTCGACCATCACCATTAC	183	57.3 54.7

Table 3. 2. Oligonucleotide primers used for quantitative PCR of Ecdysteroid-responsive genes from *G. lateralis*.

Gene	Primer Sequence (5'–3')	Product size (bp)	TM (ºC)
BR-C-F1	CAAAGGACTGACTGAGCAGAA	235 bp	54.7 ºC
BR-C-R1	GAGAGTTGGACTGCTGGTT		
E75-F1	GAGTATGAGTCCTATGCAGCC	226 bp	60ºC
E75-R1	CGATGAAGACGATCTCTGGTG		
E74-F1	CAGGGAGAAGGGAGTGTTCA-	183 bp	56.3ºC
E74-R1	GGAACATCAACAAACTGGTACACG		56.4ºC
HR3-F1	TACATCCCGCAGACCACCAC-	115 bp	59.4 ºC
HR3-R1	CCGACTCCGACAGGGGCTC		60.3 ºC
HR4-F1	TGACGACTTACTTGACCACAA-	150 bp	53.8ºC
HR4-R1	TTGTGTGTGAGGAGTCTCGT		55.7ºC
FTZ-F1- F1	CTACAGCACTCTTGGTCTGACTTG-	115 bp	57.2ºC
FTZ-F1-R1	GGGACAGCAGGTCAAACTT		55.2ºC
FOXO-F1	GCCGCCCAAGAAGAATACG-	161bp	56.4 ºC
FOXO-R1	ATACTTCAAGGACAAGGGCG		54.8 ºC
RXR-F1	CTCAGGCAAGCACTATGGCGT-	164 bp	60ºC
RXR-R1	TCAAGCACTTCTGGTAGCGGCAG		61.5ºC
EcR-F1	GCGTTATGATGCCAAGACAGATTC-	117 bp	56.3 ºC
EcR-R1	CGGCAGAAACGGAAGAGTATC		55.5 ºC
NADK-F1	GCCGAATCATGCGAAACTC-	101bp	54.5 ºC
NADK-R1	CTTGTCTGTGTTGGTCATCAAG		53.9 ºC
ALAS-F1	CAAGGTCTCGGATGAACTGATAA-	129bp	54.3 ºC
ALAS-R1	CATACCAAGCCCATGATGGA		54.7 ºC

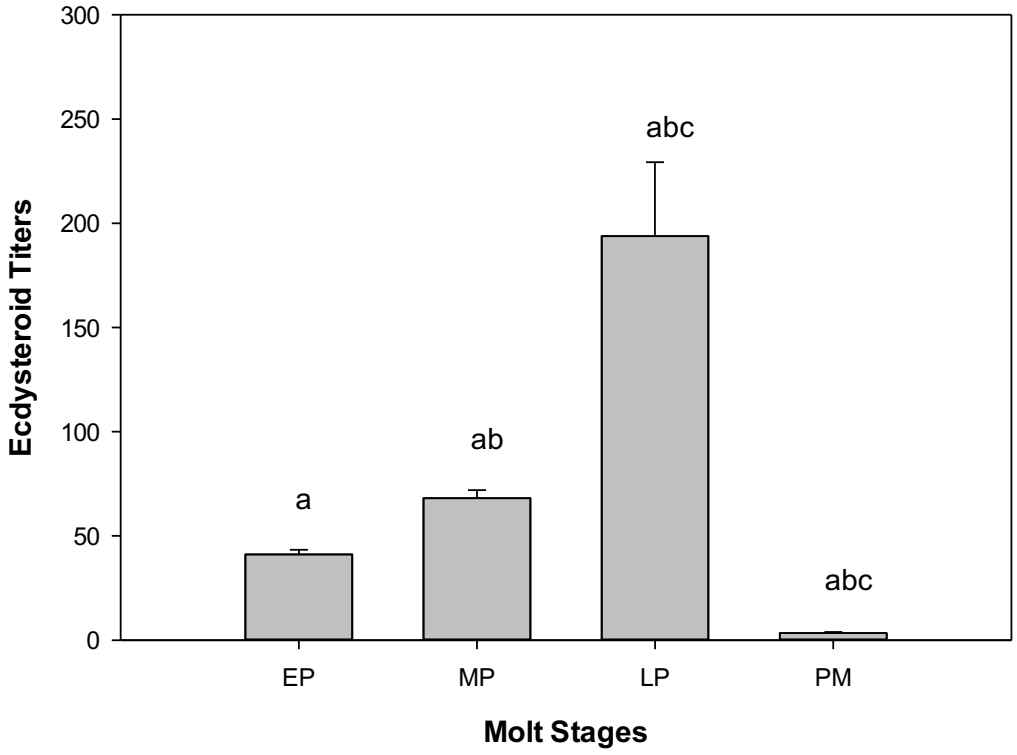


Fig. 3-1 Effects of molting on hemolymph ecdysteroid titers. Hemolymph collected from molting land crab at 4 molt stages: Earlypremolt, midpremolt, latepremolt and postmolt. Data are presented as mean ± 1 S.E. (Earlypremolt n = 6, midpremolt n= 8, Latepremolt n=10 and postmolt n = 10). Same letters indicate means that were significantly different between stages.

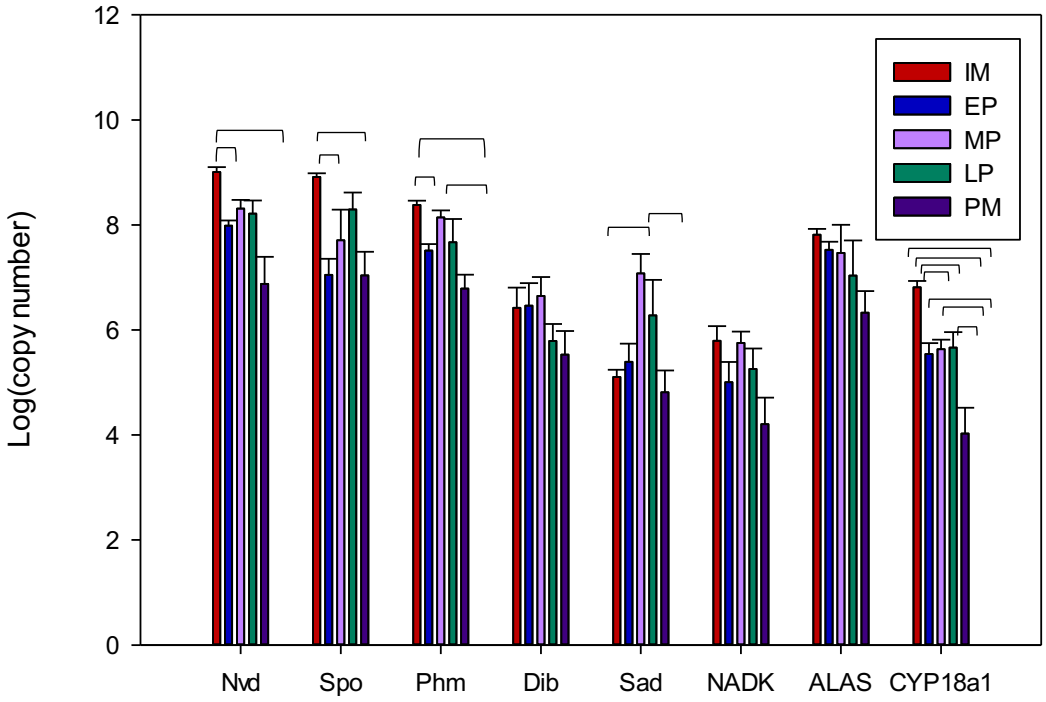


Figure. 3. 2. Effects of molt induction by MLA on YO expression of Gl-*ALAS*, Gl-*CYP18A1*, Gl-*Dib*, Gl- *NADK*, Gl-Nvd, Gl-*Phm*, Gl-*Sad* and Gl-*Spo* in *G. lateralis*. mRNA levels at early premolt, mid premolt, late premolt and 10 days post molt were quantified by qPCR. mean ± 1 S.E. (EP n =6; MP n=8; LP n=10; PM n=10) brackets showed means that are significantly different from each other have.

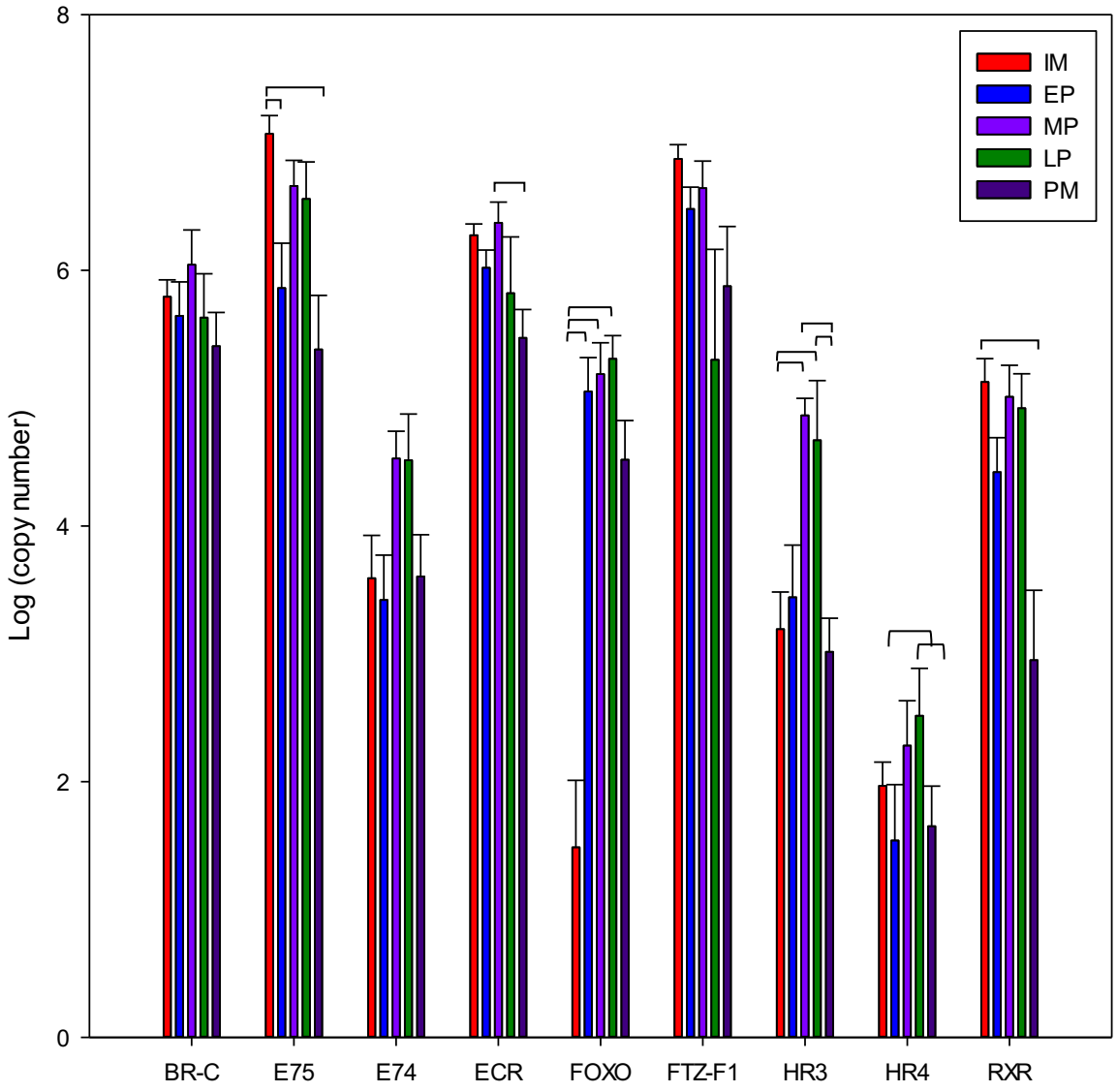
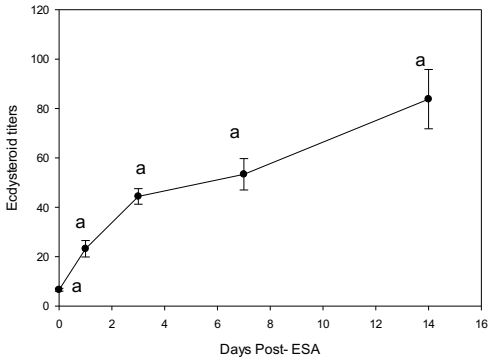
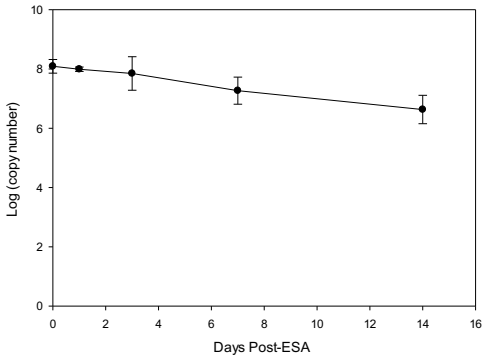


Figure. 3. 3. Effects of molt induction by MLA on YO expression of *Gl-BR-C*, *Gl-E75*, *Gl-Dib*, *Gl- E74*, *Gl-EcR*, *Gl-FOXO*, *Gl-FTZ-F1*, *Gl-HR3*, *Gl-HR4*, and *Gl-RXR* in *G. lateralis*. mRNA levels at early premolt, mid premolt, late premolt and 10 days post molt were quantified by qPCR. mean ± 1 S.E. (EP n =6; MP n=8; LP n=10; PM n=10) brackets showed means that are significantly different from each other have.

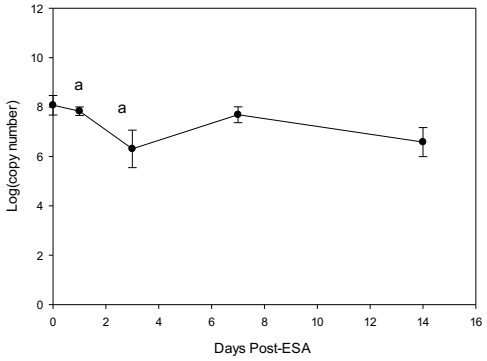
A. Ecdysteroid level



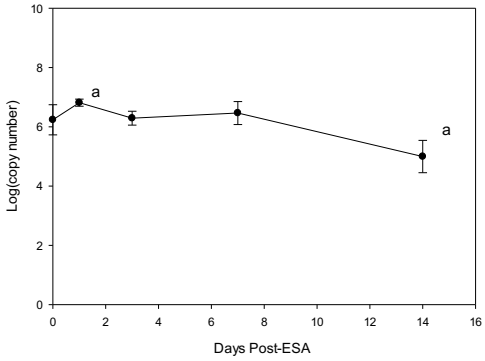
B. Gl-NEVERLAND



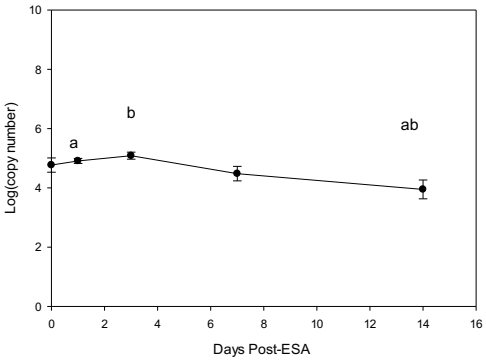
C. Gl-SPOOK



D. Gl-ALAS



E. Gl-NADK



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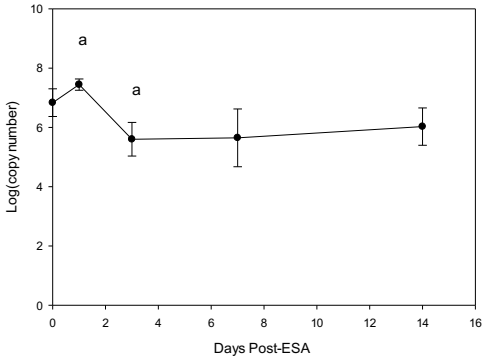
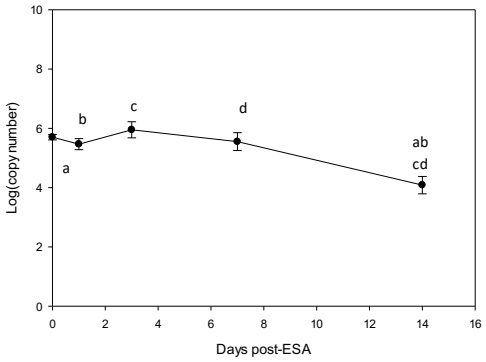


G. Gl-Disembodied



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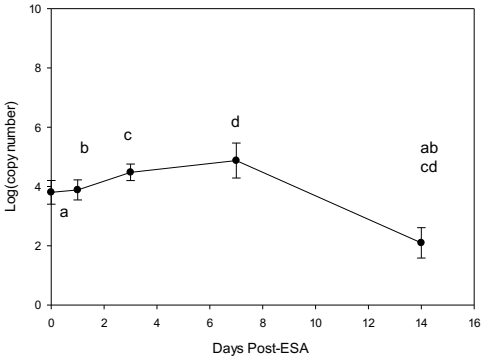

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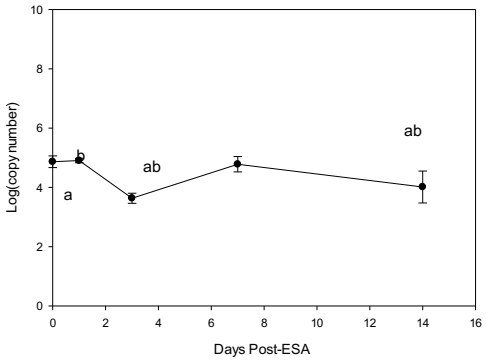
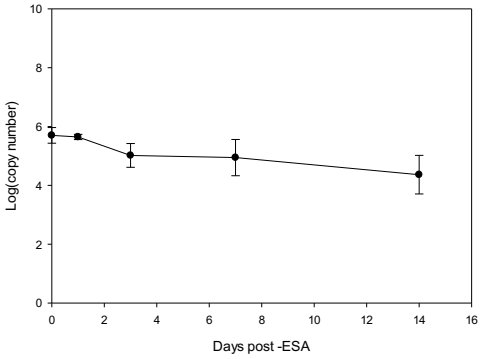


Figure. 3. 4. Effects of ESA on hemolymph ecdysteroid titer (A) and YO expression of Ecdysteroid biosynthesis genes in *G. lateralis* (B-I). Hemolymph and YOs tissues collected from intact (Day 0), 1-day,3-day, 7-day and 14-day ESA). Ecdysteroid levels were quantified by ELISA. mRNA levels were quantified by qPCR. Data presented as mean ± 1. S.E. (day 0 n = 8, day 1 n = 6, day 3 n=8, day 7 n=8, day 14 n=7); Same letters indicate means that were significantly different between the treatment at the same time point.

J. Gl-EcR



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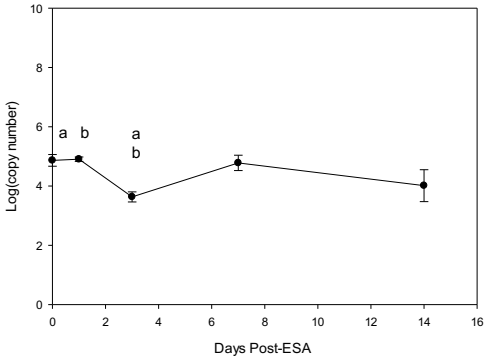

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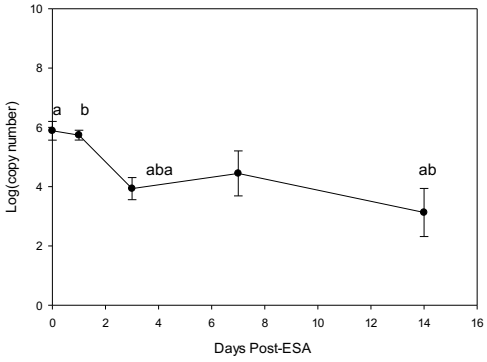

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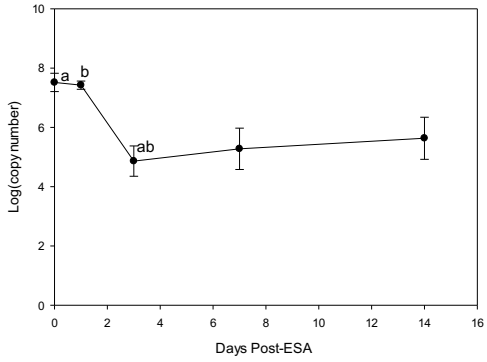

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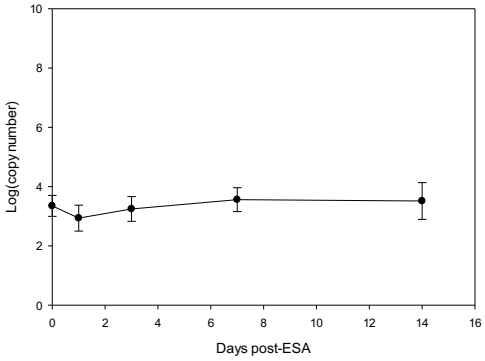

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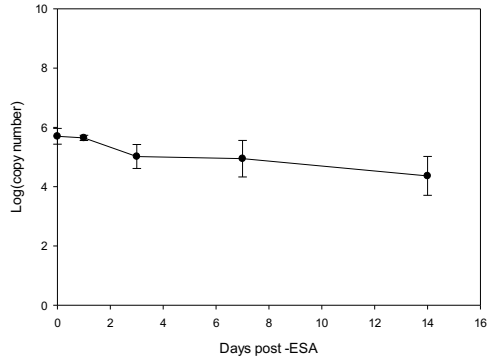

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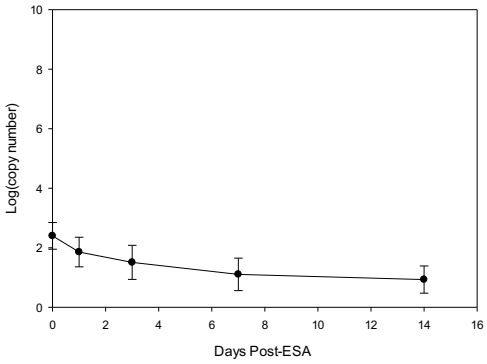
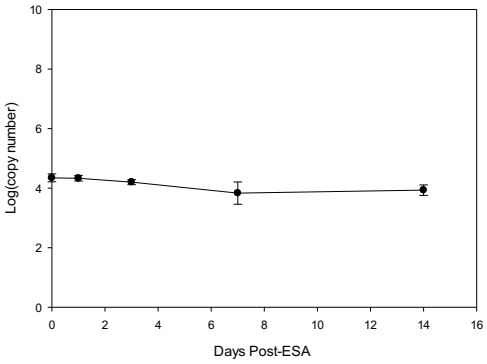


Q. Gl-FTZ-F1



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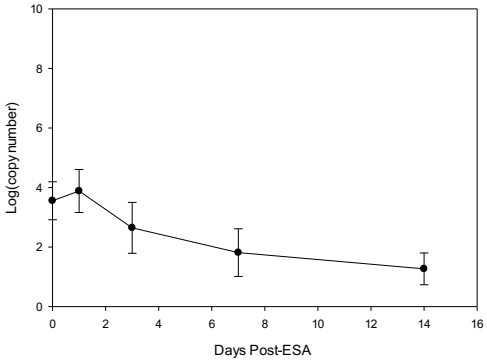


Figure. 3. 5. Effects of ESA on YO expression of ecdysteroid receptors and ecdysone responsive genes in *G. lateralis.* YOs tissues collected from intact (Day 0), 1-day,3-day, 7-day and 14-day ESA). mRNA levels were quantified by qPCR. Data presented as mean ± 1. S.E. (day 0 n = 8, day 1 n = 6, day 3 n=8, day 7 n=8, day 14 n=7); Same letters indicate means that were significantly different between the treatment at the same time point.

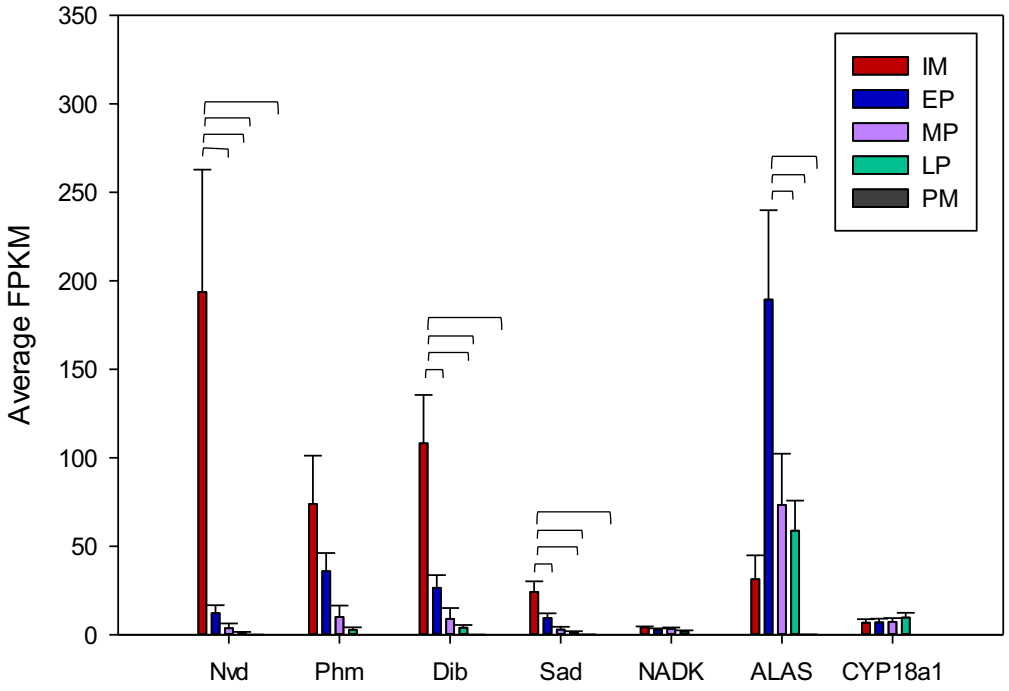


Figure. 3.6. Relative expression of *ALAS, CYP18A1, Disembodied, NADK, Neverland, Phantom and Shadow* genes in *G. lateralis*. RNA-Seq data from three biological replicates, expression profiles (FPKM as mean ± 1 S.E.). IM, EP, MP, LP, PM different stages of molting cycle. (n = 3, except PM n = 2). brackets showed means that are significantly different from each other have. Statistical significance was detected at $P<0.05$

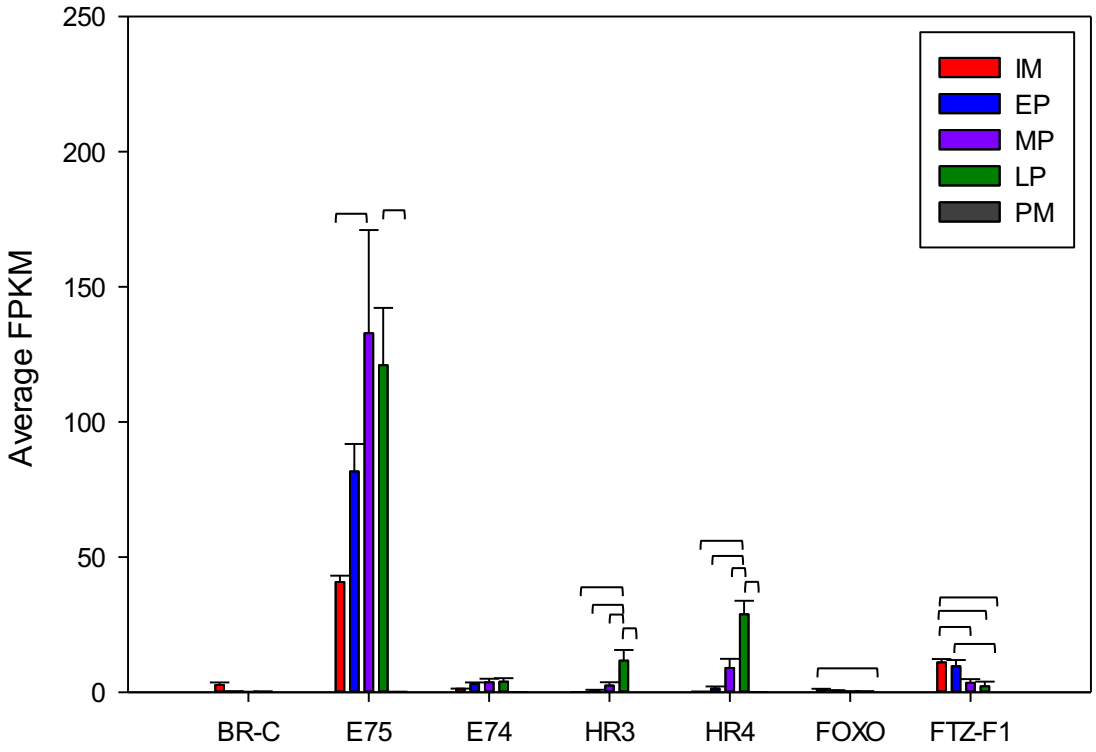
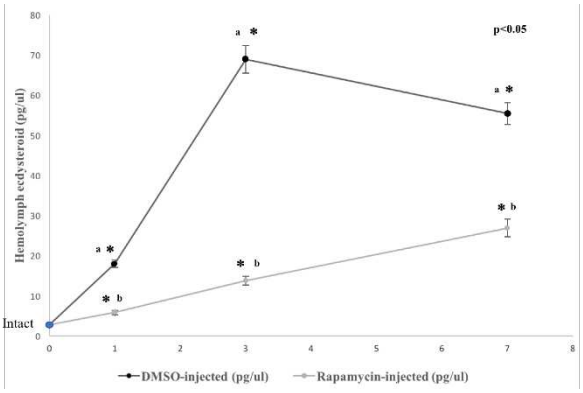


Figure. 3.7 Relative expression of *BR-C, E75, E74, HR3, HR4, FOXo* and *FTZ-F1* genes in *G. lateralis.* RNA-Seq data from three biological replicates, expression profiles (FPKM as mean ± 1 S.E.). IM, EP, MP, LP, PM different stages of molting cycle. (n = 3, except n = 2 for PM). Brackets showed means that are significantly different from each other have. Statistical significance was detected at $P<0.05$

A. Ecdysteroid level



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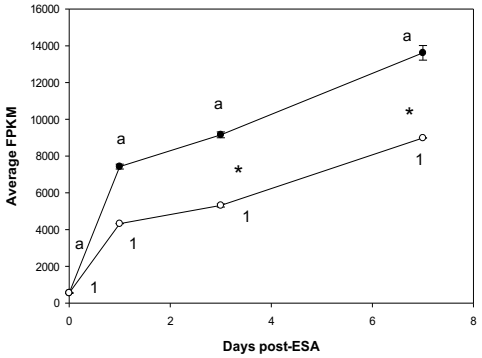
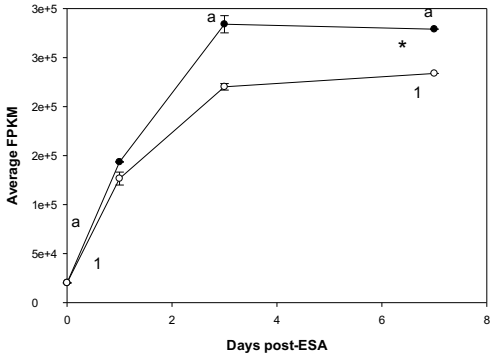


C. Gl-Spook



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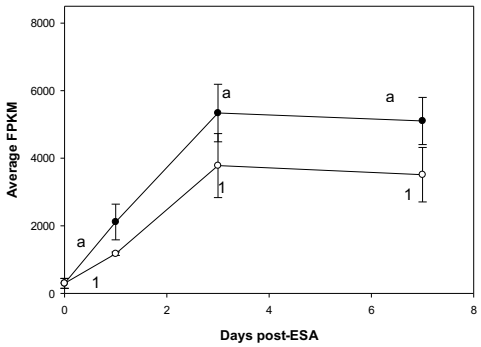

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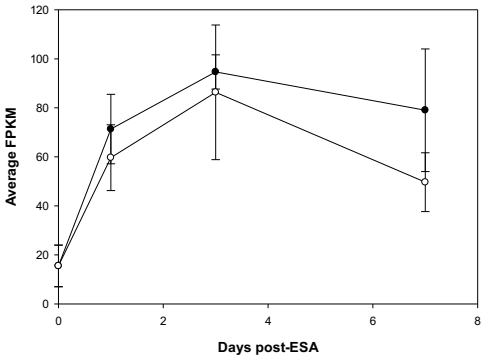

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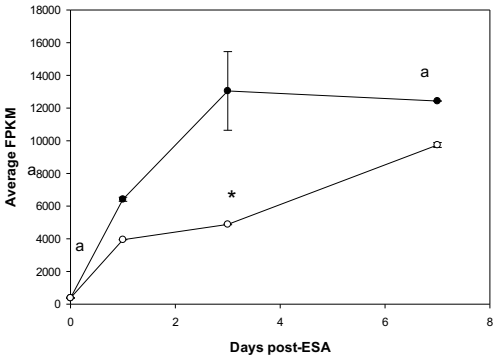
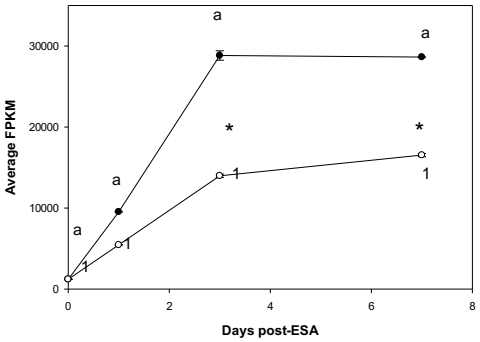
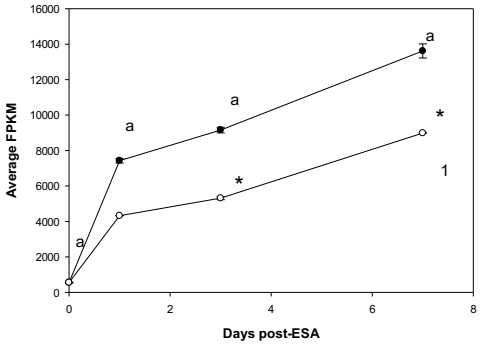


G. Gl-Disembodied



H. Gl-Shadow



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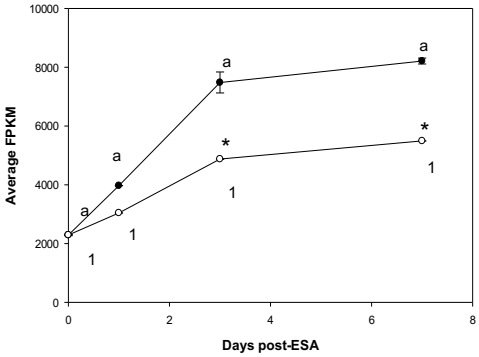
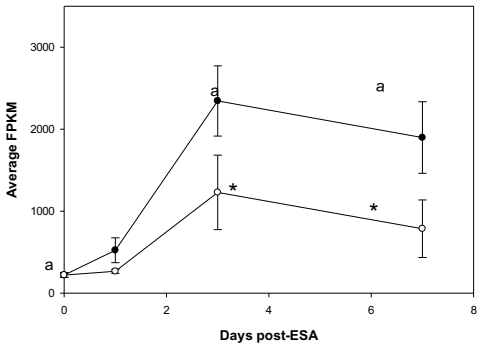
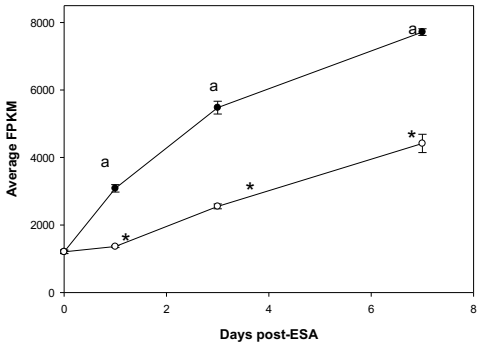


Figure. 3.8. Effects of mTOR inhibitor rapamycin inhibits on of hemolymph ecdysteroid titer (Shyamal et al; 2018) (A) and YO expression of ecdysteroid biosynthesis genes(B-I) in G. lateralis after ESA and injection with rapamycin. Animals were ES-ablated at Day 0 and injected with a single dose of rapamycin in DMSO (10 μM) or 1% DMSO. Same letters indicate means were significantly different between control at the same time point. Numbers indicate means were significantly different between rapamycin at same time point. Asterisk indicate means were significantly different between rapamycin and control at same time point. RNA-Seq data from three biological replicates, expression profiles (FPKM as mean ± 1 S.E.). (n = 3, except n = 2 for Day0).

J. Gl-EcR



K. Gl-RXR



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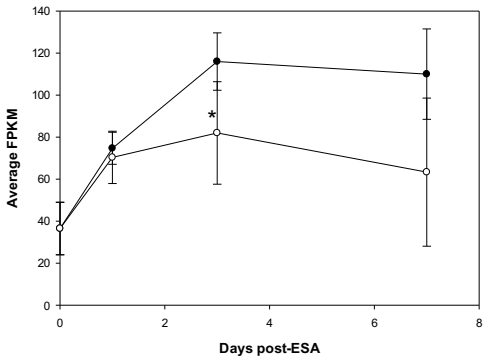

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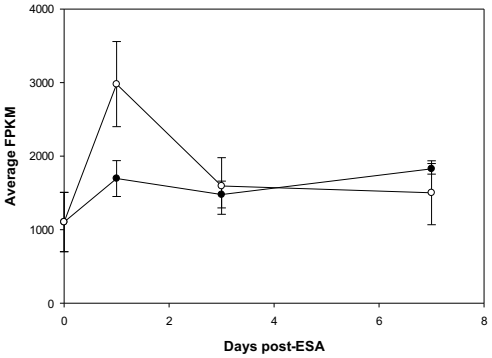
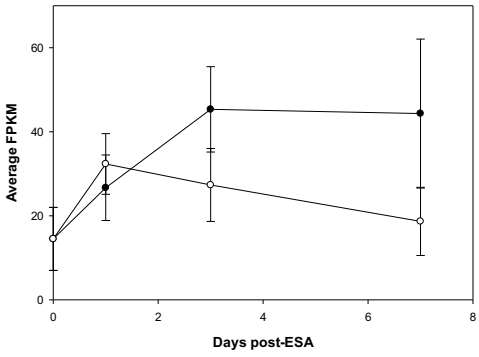


N. Gl-E74



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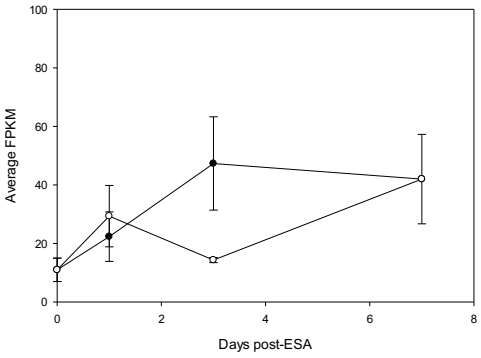
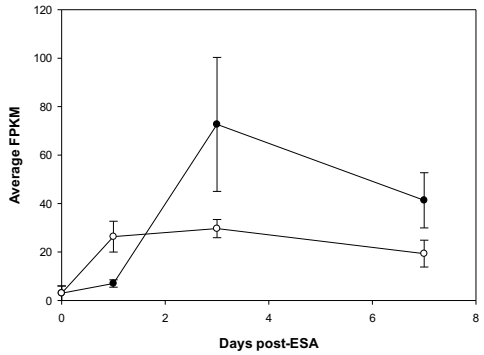
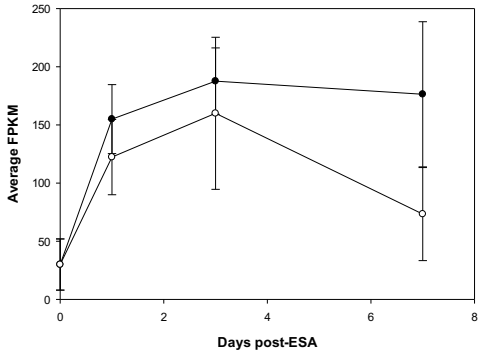


P. Gl-HR4



Q. Gl-FTZ-F1



R. Gl-FOXO

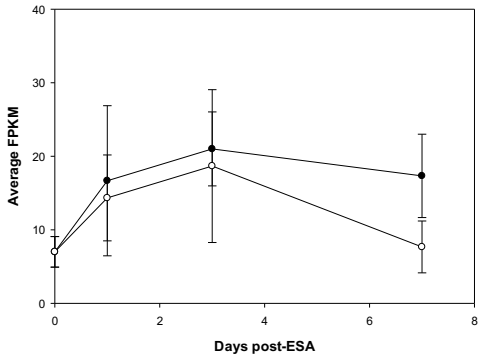


Figure. 3.9. Effects of mTOR inhibitor rapamycin inhibits on YO expression of ecdysteroid receptors and ecdysteroid-responsive genes (J-R) in G. lateralis after ESA and injection with rapamycin. Animals were ES-ablated at Day 0 and injected with a single dose of rapamycin in DMSO (10 μM) or 1% DMSO. Same letters indicate means were significantly different between control at the same time point. Numbers indicate means were significantly different between rapamycin at the same time point. Asterisks indicate means were significantly different between control and rapamycin at different time point RNA-Seq data from three biological replicates, expression profiles (FPKM as mean ± 1 S.E.). (n = 3, except n = 2 for Day0).

Chapter 4

Effect of blocking TGFβ/activin signaling on Halloween and ecdysteroid-responsive genes expression in the Y-organ in *Gecarcinus lateralis*

Summary

Molting is controlled by ecdysteroids synthesized and secreted by the molting gland, or Y-organ (YO). Halloween genes encode enzymes that catalyze the synthesis of ecdysteroid hormones. Ecdysteroid receptor (EcR/RXR) binds active molting hormone, which induces serial activation of ecdysteroid-responsive genes. During premolt, TGFβ/activin signaling mediates the transition of the YO from the activated to the committed state, as SB431542 blocks this transition. *G. lateralis* were eyestalk-ablated to induce molting and injected with vehicle (DMSO) or SB431542 at Day 0. In controls, ESA increased hemolymph ecdysteroid titers at 3, 7 and 14 days post-ESA. There were significant increases in the mRNA levels of *Gl-Nvd* at 7 and 14 days post-ESA and other Halloween genes (*Gl-Spo, Gl-Phm, Gl-Dib, Gl-Sad*), as well as *Gl-CYP18a1, Gl-ALAS, Gl-NADK*, *Gl-BR-C, Gl- FOXO, Gl-EcR*, and *Gl-RXR*, at 14 days post-ESA. SB431542 reduced hemolymph ecdysteroid titers at 7 and 14 days post-ESA compared to control animals, but titers were no different from controls at 1, 3, and 5 days post-ESA, indicating that SB431542 had no effect on YO activation. SB431542 blocked the increases in mRNA levels of *Gl-Nvd*, *Gl-Spo, Gl-Phm, Gl-Dib, Gl-Sad*, *Gl-CYP18a1*, *Gl-ALAS, Gl-NADK*, *Gl-BR-C, Gl-EcR*, and *Gl-RXR* by ESA. SB431542 had no effect on mRNA levels of the ecdysteroid-responsive genes *Gl-HR3 Gl-HR4, Gl-E74, Gl-E75* and *Gl-FTZ-F1*. These data suggest that an activin-like TGFβ factor stimulates YO ecdysteroidogenesis in the committed YO by up-regulating Halloween genes and the *Gl-BR-C* and *Gl-FOXO* genes.

Introduction

Decapod crustaceans must molt in order to grow. In crustaceans, molting is controlled by two different endocrine glands. The X-organ/sinus gland (XO/SG) complex, which is located within the eyestalks and a pair Y-organs (YOs), which are located in the cephalothorax. The XO secretes the neuropeptide molt-inhibiting hormone (MIH) that inhibits ecdysteroid production by YO. MIH binds receptors on the YO to inhibit synthesis of ecdysteroids (Mykles, 2011).
The surgical extirpation of the eyestalk ablation removes the major source of MIH and resultes in an immediate induction of molting. MIH and crustacean hyperglycemic hormone (CHH) have important roles in regulating ecdysteroid pathway by changes in the activity of YOs during the molt cycle. MIH binds to its receptor on the YO membrane and represses the conversion of ketodiol and 25 deoxyecdysone (Gäde and Marco, 2006; Techa and Chung, 2015). MIH induces an increase in cAMP and cGMP and subsequently represses YO ecdysteroidogenesis (Covi et al., 2009). The effect of MIH on YOs has been extensively studied in many crustacean species, such as *Carcinus maenas*, *Gecarcinus lateralis*, and spiny lobster (Lee et al., 2007; Mykles et al., 2010; Skinner, 1985). The use of ESA allows researchers precise timing for molt initiation.

The YO progresses through four physiological states during the molt cycle that are mediated by various signaling molecules and isolated pathways. A reduction in MIH triggers the transition from the basal state in intermolt (C_4) to the activated state in early premolt (D_0). This transition requires mechanistic target of rapamycin (mTOR) activity. TGFβ has an important role in the transition of the YO from the activated to the committed state at mid premolt (D_{1-2}); and high levels of ecdysteroids trigger the transition from the committed state to the repressed state in late premolt (D_{3-4}) (Chang and Mykles, 2011). YOs secrete inactive ecdysteroid products ecdysone, and 25-deoxyecdysone (25-dE), which are hydroxylated in peripheral tissues to active

hormone 20-hydroxyecdysone (20-HE) and ponasterone A (PoA), respectively (Mykles, 2011a). mTOR and TGFβ and other signaling pathways are upregulated during premolt in the MLA and ESA YO transcriptome (Das et al., 2016; Shyamal et al., 2018). In *Drosophila* the TGFβ signaling pathway has an important role for ecdysteroidogenesis, which are upregulated by of *torso* and *insulin receptor* (*InR*) in the molting gland (prothoracic gland). Blocking TGFβ/Activin signaling prevents development prior to metamorphosis (Warren et al., 2011). Loss of PTTH and insulin signal reception in molting gland lead to reduce synthesis of ecdysone and 20E (Warren et al., 2011).

MIH regulates ecdysteroid biosynthesis in the YO, but the mechanism of action is still not clear. Ecdysteroid biosynthesis is mediated by cytochrome p450 (CYP) enzymes encoded by the Halloween genes *Phantom* (*phm*), *Disembodied* (*dib*), *Shadow* (*sad*), and *Shade* (*shd*). They hydroxylate the substrates at C25→C22→C2→C20 in cholesterol in that order (Truman, 2005). Crustacean orthologs of Halloween genes have been identified in *Daphnia, Pontastacus leptodactylus Portunus trituberculatus, Neocaridina denticulata*, and *phantom* (Member of Halloween gene family) in *Marsupenaeus japonicus* (Asazuma et al., 2009; Rewitz and Gilbert, 2008; Sin et al., 2015; Tom et al., 2013; Xie et al., 2016). *βFTZ-F1* regulates Halloween genes in *Drosophila* (Parvy et al., 2005). The orthologs of other ecdysteroid responsive genes such as *RXR* and *EcR* are found in crustaceans but their function in molting is poorly understood (Mykles, 2011). None of the crustacean Halloween enzymes have been characterized biochemically.

The transforming growth factor-β (TGFβ) is family of potent multifunctional cytokines that modulates a wide variety of cellular activities, such as wound healing, cellular differentiation, and deposition of extracellular matrix proteins (Robers, 1990). Binding of TGFβ

to its receptor kinases activates Smad signaling proteins that regulate genes through transcriptional activation or repression (Ellenrieder et al., 2001; Heldin and Moustakas, 2012)). A TGFβ-like factor in the activated YO commits the animal to proceed through premolt and molt and increase in ecdysteroid synthesis. The activated YO remains sensitive to MIH. However, the committed YO is less sensitive to MIH (Skinner, 1985). We hypothesize that YO commitment requires a TGFβ factor acting through Activin receptor/Smad signaling, resulting in sustained mTOR activation, up-regulation of ecdysteroid biosynthetic enzymes, and down-regulation of MIH signaling.

In this present study, we hypothesize is that YO ecdysteroidogenesis requires up-regulation of mTOR signaling. MIH suppresses the mTOR pathway. YO commitment requires a TGFβ factor acting through Activin receptor/Smad signaling, resulting in sustained mTOR activation, up-regulation of ecdysteroid biosynthetic enzymes, and down-regulation of MIH signaling. We eyestalk ablated animals and injected with inhibitor (SB431542) or DMSO vehicle (control). We determined the effects of Activin receptor antagonist SB431542 on hemolymph ecdysteroid levels and expression of Halloween genes and ecdysteroid-responsive genes *G. lateralis* YO using qPCR.

Materials and Methods

Animals and Experimental Treatments

Adult male *Gecarcinus lateralis* arrived from the Dominican Republic and were kept at Colorado State University, CO. The animals were first acclimatized for one month under controlled environment at conditions of ~27 °C and 75-90% humidity and placed under a cycle of twelve hours darkness and 12 hours light. Communal plastic cages that contained aspen

bedding moistened with 5 ppt Instant Ocean were used to keep intermolt individuals (Aquarium Systems, Mentor, OH). Crab were fed lettuce, carrots, and raisins twice a week (Covi et al., 2010). Eyestalk ablation (ESA) induced molting by eliminating the primary source MIH (Skinner, 1985a). There was immediate activation of the YO, as indicated by an increase of ecdysteroid titers by 1 day post-ESA (Lee et al., 2007). The regenerate index (R index) of the growing limb bud was used to measure progress through premolt (Yu et al., 2002)

The *in vivo* experiments determined the effect of SB 431542 on YO gene expression. On Day 0, intact and ESA crab were injected with compound (10 µM final concentration) or vehicle (DMSO, ~1% final concentration). The following equation was used to calculate the volume to inject: mass (g) x 0.3 µl of 10 mM SB431542 in DMSO, assuming a hemolymph volume of 30% of wet weight. Hemolymph samples (100 µl) were combined with 300 µl methanol. The harvesting of YOs took place at 0, 1, 3, 5,7, and 14 days post-ESA.

RNA isolation and cDNA synthesis

Trizol and chloroform/phenol was used in the isolation of RNA as described in (Covi et al., 2010). YOs were placed in 350 µl RNAlater overnight (Ambion) at 4°C, and then stored at 20°C. Tissue was homogenized for 5 minutes in Trizol with a motorized tissue grinder (Fisher). The supernatant fraction was extracted with phenol-chloroform. RNA pellets were dissolved in 22 µl of RNase/DNase/protease free water. DNase I treatment was given to every sample based on the instructions of the manufacturer (Thermo Scientific, Grand Island, NY, SA). Ribolock (Thermo Scientific) ten units (0.75 µl) was included in the DNase I treatment to prevent degradation of RNA. 24:1 chloroform isoamyl alcohol was utilized for the extraction of a second phenol-chloroform; precipitation of RNA was accomplished by the addition of 0.5 volume of 3 M sodium acetate (pH 5.2) to 1.5 volume of isopropanol. The pellets were dissoled in 22 µl

RNase/DNase/protease free water. A Nanodrop 1000 (Thermo Scientific) was used to quantify the concentration of RNA. SuperScript IV Reverse Transcriptase was used for a reverse-transcription of 4 µl of RNA (Thermos Fisher) in accordance with the instructions of the manufacturer. cDNA samples were stored at -20°C.

qPCR and gene expression

Quantitative PCR (qPCR) was performed using a Light cycler 480 Thermocycler (Roche Applied Science, Indianapolis, IN, USA). qPCR conditions were as follows: 0.5 µl each of forward and reverse gene-specific primers (Table 1), 5 µl SyBr Green (Roche), 3 µl nuclease free water, and 1 µl cDNA template were combined. After denaturation at 95 °C for 3 min, 45 PCR cycles were repeated at 95 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 20 sec, with a final extension time of 7 min at 72 °C.

Ecdysteroid ELISA

Ecdysteroid were quantified ELISA by (Kingan, 1989; Von Gliscynski et al., 1995). Polystyrene microtiter plate (96 plates Costar 3366, Corning, NY, USA) were used for the coating step with Affini Pure goat anti-rabbit IgG Fc fragment antiserum (Jackson Immuno Research Labs 111-005-008, West Grove, PA, USA; 0.5 µg in 90 µl per well). The coating buffers was: Coat buffer: phosphate-buffered saline (PBS) (10 mM sodium phosphate, 83 0.15 M NaCl, pH 7.5). Incubation time for the coating step was 2 hours at 23 ˚C. Assay buffer 300 µl (AB; 25 mM sodium phosphate, pH 7.5; 150 mM NaCl; 1 mM EDTA disodium dihydrate and containing 0.1% bovine serum albumin) (BSA, Fraction V; Sigma A-9647, St. Louis, MO, USA) was added per well for 2 hours at 23 ˚C. The washing buffer: phosphate buffered contained PBS and 0.05% Tween 20. Wells were washed three times with 300 µl of PBS. Standards were 0 to 120 pg 20-hydroxyecdysone (20E) in AB (50 µl per well). Hemolymph samples in methanol

were centrifuged for 10 min at 20,000 xg at 4 ˚C to remove precipitated protein. Supernatant aliquots (2 µl) were dried under vacuum in a Speed Vac centrifuge and dissolved in 150 µl AB. We loaded 50 µl of samples in duplicate, followed by 50 µl of 20E conjugated to horseradish peroxidase (HRP) reagent (1:64,000 dilution in AB) to all wells and incubated and mixed on shaker for 5 min at 23 ˚C. 50 µl of a rabbit anti-ecdysteroid primary antibody (1:100,000 dilution in AB with 0.1% BSA) was added to all wells, except for the first two wells containing NSB. Plates were covered with Parafilm and incubated overnight at 4 ˚C. We mixed equal amounts of Solutions A and B of a tetramethylbenzidine-peroxidase (TMB) kit (KPL, catalog 50-76-03, Gaithersburg, MD, USA) and added 100 µl to each well. The plates were incubated for 15 min at 23 ˚C on shaker in the dark place. Finally, we added 100 µl 1 M phosphoric acid to stop the reaction. Gen5 Microplate Reader at 450 nm**.** The data were analyzed with https://elisaanalysis.com/ software

Statistical analyses and software

Statistical analysis was performed using One-Way ANOVA, Tukey or Dunn's test in Sigma Plot (Sigma Plot 12.0, Systat 27 Software, San Jose, CA USA) for both ecdysteroid titers and gene expression. All data not plotted as individual points are represented as mean ± 1 S.E. and the level of significance for the all the data analyses was set at $\alpha = 0.05$. All qPCR data was log transformed to reduce the variance of the mean. Mean copy number was calculated and log transformed. Statistical significance ($p<0.05$) between time points were evaluated using a one-way ANOA.

Results

Effects SB431542 on YO ecdysteroidogenesis and gene expression on ESA animals

Intact (Day0) and ES-ablated animals were injected with SB431542 dissolved in

DMSO or DMSO alone at Day 0. ESA control animals showed a significant increase in hemolymph ecdysteroid titers (Fig.4.1A). ESA animals injected with SB431542 showed an increase in ecdysteroid titer with the control animals at Day 1 and Day 3 post-ESA and a decrease in ecdysteroid titer of both groups at day 5 post ESA. However, SB431542 significantly decreased the hemolymph ecdysteroid titers at Day 7 and Day 14 post-ESA, while there was a significant increase in the control group at these intervals (Fig. 4.1A).

SB431542 lowered the mRNA levels of Gl-*Phm*, *Gl- Spo*, *Gl-Sad*, *Gl-Dib* and *Gl-RXR*, *Gl-EcR*, *Gl-CYP18a1*, *Gl-ALAS*, *Gl-NADK, Gl-BR-C* and *Gl-OXO* by 14 days post-ESA (Fig. 4.1B-I, J, K, L, R). SB431542 reduced *Gl-Nvd* mRNA level at 7 and 14 days post- ESA compared to the control group. The means of the control animals were significantly greater than the means of the experimental animals at 7 and 14 days post-ESA (Fig. 4.1B-I, J, K L, R). *Gl-spo*, *Gl-phm*, *Gl-Dib*, *Gl-BR-C*, *Gl-EcR,* and *Gl-RXR* mRNA levels increased significantly by 14 days post-ESA in controls when compared at Day 0 post-ESA (Fig. 4.1C, F, G, L, J). *Gl-ALAS* mRNA level increased significantly at 1, 3, and 14 days post-ESA in controls when compared to Day 0. *Gl-HR3 Gl-HR4*, *Gl-E74*, Gl-E75, and *Gl-FTZ-F1* mRNA levels were not significantly different between the two treatments indicating that SB431542 had no effect on expression of these ecdysteroid-responsive genes (Fig. 4. 1M, N, G, P, Q, R).

Discussion

ESA was used to examine the effects of SB431542, inhibitor of Activin receptors signaling on the ecdysteroid pathway of land crab *G. lateralis*. The treatment group showed reduced hemolymph ecdysteroid titers at 7 and 14 days post-ESA, and no effect at 1,3, and 5 days post-ESA. On the other hand, ESA animals in the control group increased in hemolymph

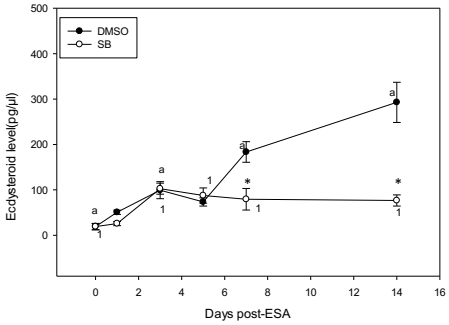
ecdysteroid concentrations (Fig. 4. 1A). SB431542 has no effect on YO activation and blocks YO commitment in ESA animals (Abuhagr et al., 2016). Previous researches reported increased hemolymph ecdysteroid titers by ESA (Covi et al., 2010). Activation of YO requires mTOR activity and stimulates secretion of a TGFβ-like factor, which is required for the mid-premolt transition to the committed state. mTOR increases ecdysteroid synthesis at early-premolt, while SB431542 shows a delay effect on ecdysteroid synthesis SB431542 is sparingly soluble in an aqueous solution which probably leads to the prolonged effect of SB431542. (Abuhagr et al., 2014b; Abuhagr et al., 2016).

We used qPCR to investigate the effect of SB431542 on the expression of Halloween genes and ecdysteroid- responsive genes. All Halloween genes (*Gl-spo*, *Gl-phm*, *Gl-dib*, *Gl-sad*), *Gl-CYP18a1*, *Gl-NADK*, *Gl-ALAS*, *Gl-RXR Gl- EcR*, *Gl-BR-C and Gl-FOXO* showed a significant decrease of mRNA level at day 14 days post-ESA, while in the control group all these genes showed increased level of mRNA at 14 days post-ESA (Fig.4.1B-I, J, K, L, R). *Gl- Nvd* was reduced at 7 and 14 days post-ESA and increased in control animals at these days (Fig.4.1B). By contrast, *Gl-E74*, *Gl- E75*, *Gl-FTZ-F1*, *Gl-HR3* and *Gl-HR4* mRNA levels were not affected by SB431542 (Fig 4.2M, N, O, P, Q). We conclude that an Activin-like TGFβ factor is involved in regulating YO ecdysteroidogenic genes in *G. lateralis*. In insects, knockdown of TGF-β signaling factor smox/Smad2 by RNAi, Type II receptor causes developmental arrest and death of the instar (Ishimaru et al., 2016; Warren et al., 2011). Moreover, loss of Activin signaling prevents the prothoracic gland to produce ecdysteroid that triggers metamorphosis in *Drosophila* (Warren et al., 2011).

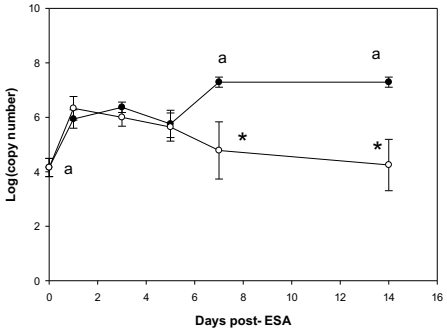
During molting processes, mTOR controls translation of mRNA into protein. mTOR activity is required for YO activation at early premolt. YO transition from the activated to

committed state by stimulation of TGFβ, which the crab becomes committed to molt, as the YO is less sensitive to MIH. We have shown that many of the ecdysteroidogenic genes and the ecdysteroid-responsive genes were upregulated in the committed YO by 14 days post-ESA SB431542 blocks these increases. We conclude that TGFβ signaling is required for the increased expression of Halloween genes, which are necessary to support the increased synthesis of ecdysteroids by the committed YO.

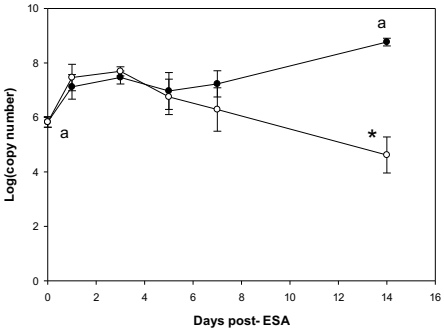
A. Ecdysteroid



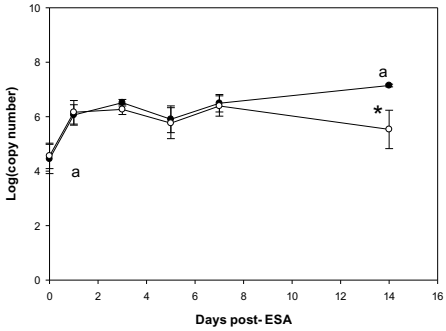
B. Gl-Neverland



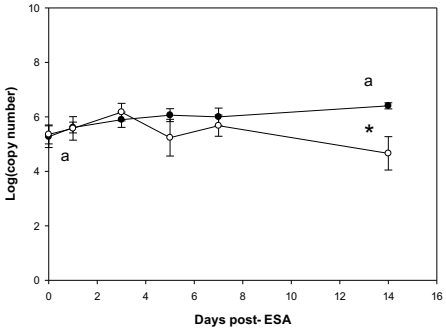
C. Gl-Spook



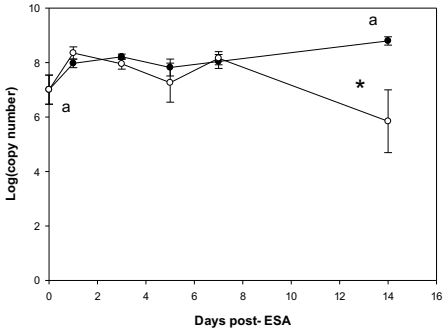
D. Gl- ALAS



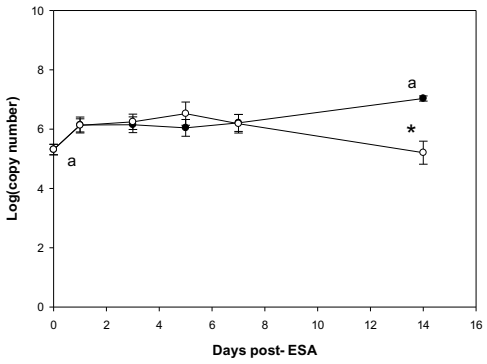
E. Gl-NADK



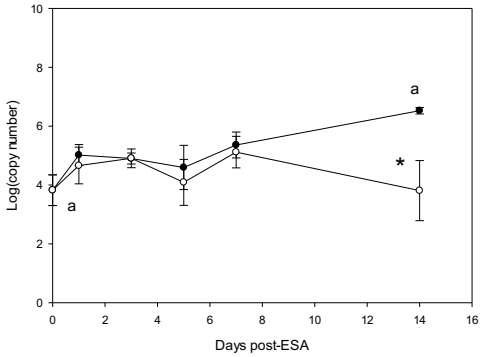
F. Gl-Phantom



G. Gl-Disembodied



H. Gl-Shadow



I. Gl-CYP18a1

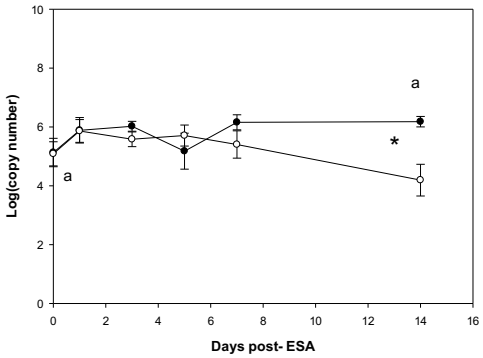
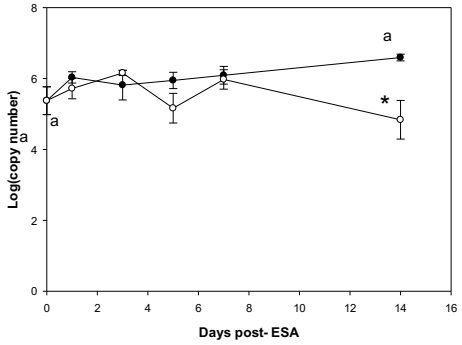
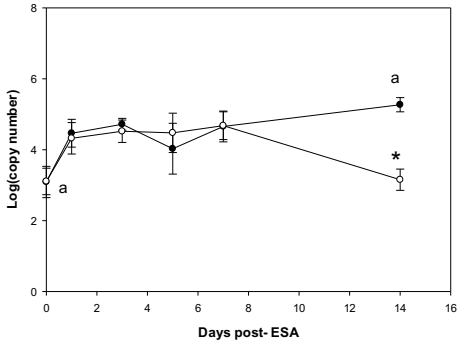


Figure 4. 1. Effects of Activin receptor antagonist SB431542 on hemolymph ecdysteroid titers (A) and YO expression of Ecdysteroid biosynthesis genes (B-I) in *G. lateralis*. Intact and ES-ablated animals were injected with a single dose of DMSO (~1%) final hemolymph concentration or SB431542 in DMSO (~20 μM final hemolymph concentration) at Day 0. Data are presented as mean ± 1 S.E. (sample size for each treatment: Day 0, n = 8; Days 1, n=8; 3, ,5 and 7, n = 7; Day 14, n = 6). Means within control that were significantly different from each other have the same letters for the DMSO. Same number indicate means that were significantly different between treatments at the same time point. Asterisks indicate means that were significantly different between control and treatment at the same time point.

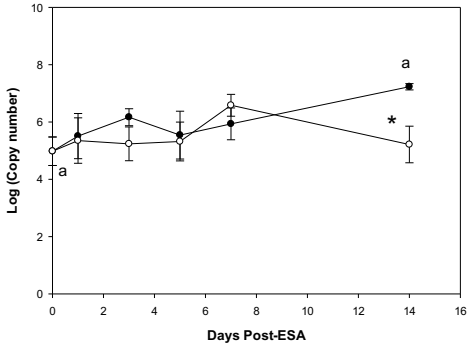
J. Gl-EcR



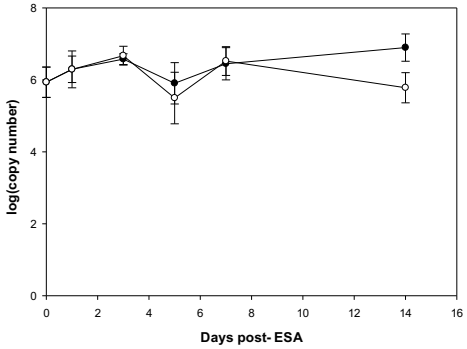
K. Gl-RXR



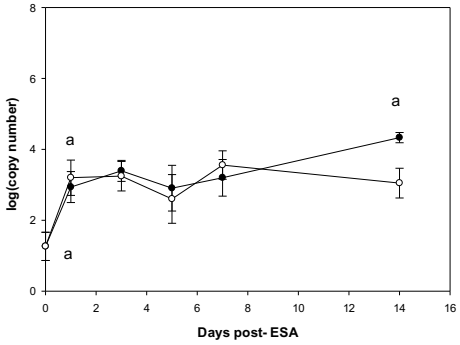
L.Gl-BR-C



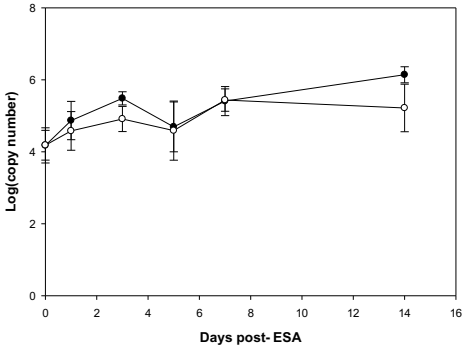
M. Gl-E75



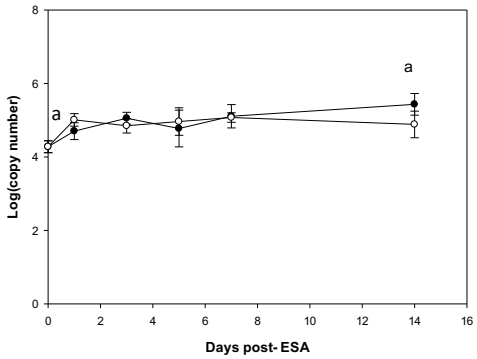
N. Gl-E74



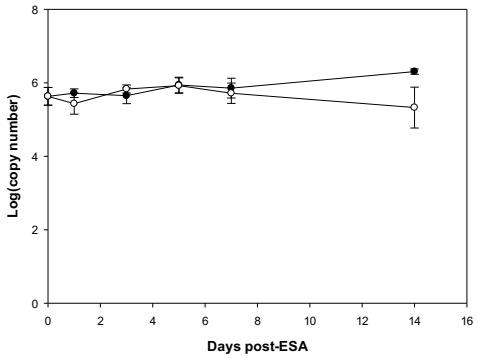
O. Gl-HR3



P. Gl-HR4



Q. Gl-FTZ-F1



R. Gl-FOXO

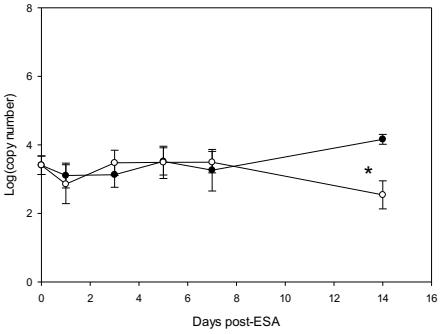


Figure 4 .2 Effects of Activin receptor antagonist SB431542 on YO expression of ecdysteroid responsive genes (J-R) in *G. lateralis*. Intact and ES-ablated animals were injected with a single dose of DMSO (~1%) final hemolymph concentration or SB431542 in DMSO (~20 μM final hemolymph concentration) at Day 0. Data are presented as mean ± 1 S.E. (sample size for each treatment: Day 0, n = 8; Days 1, n=8; 3, ,5 and 7, n = 7; Day 14, n = 6). Means within control that were significantly different from each other have the same letters for the DMSO. Same number indicate means that were significantly different between treatments at the same time point. Asterisks indicate means that were significantly different between control and treatment at the same time point.

SUMMARY AND FUTURE DIRECTIONS

Ecdysteroids play pivotal functions in all arthropods. Ecdysteroid hormone is responsible for coordinating many aspects of biological processes associated with molting. Halloween genes encode cytochrome P450 enzymes that mediate the synthesis 20-hydroxyecdysone (20E) (molting hormone). We successfully identified and characterized full-length contigs of Halloween genes, *neverland*, *CYP18a1*, *EcR*, *RXR* and ecdysteroid-responsive genes (*broad complex*, *E75*, *E74*, *Hormone receptor 4*, *Hormone receptor 3*, f*orkhead box* (FOXO), and *Fushi tarazu factor-1*) from the YO transcriptome. Multiple sequence alignments showed high sequence identities with orthologs from other species. The phylogenetic analysis showed that all the contig sequences clustered with their corresponding orthologs in other arthropod species. These results show that Halloween genes and ecdysteroid-responsive genes in crustaceans and insects are highly conserved and evolutionarily originated from a common lineage. Tissue distribution experiments showed Halloween genes are specific tissue genes expression, while expression ecdysteroid-responsive genes are distributed in all tissues. This indicates that a variety of tissues can potentially respond to 20E.

Quantitative PCR was used to determine the effects of molting on expression of Halloween and ecdysteroid-responsive genes in the YO. Molting has little or no effect on the expression of *phantom*, *disembodied*, *spook*, *neverland*, *CYP18a1*, *E75*, NADK and *ALAS*, as these gene showed high mRNA levels at the intermolt stage before entered premolt. However, *FOXO*, *RXR*, *HR3* and *HR4* were affected by molting, as they had increased mRNA levels at premolt stages. These data suggest that 20E directs control the expression of *HR3*, *HR4, FOXO* and *EcR* We conclude that ecdysteroids may control gene expression via an autoregulatory feedback.

The ESA transcriptome ± rapamycin experiment showed that ecdysteroidogenesis genes are up regulated by stimulating mTOR activity. These results suggest that mTOR activity is required for the regulation ecdysteroidogenesis genes at the activation of the YO. qPCR analysis in the ESA ± SB431542 experiment showed that all ecdysteroid biosynthesis genes were up regulated at 7 or 14 days post-ESA. This data indicates that an Activin-like TGFβ is involved in regulating YO ecdysteroidogenesis and required for the committed YO state.

In this study, characterization the Halloween genes in land crab, *Gecarcinus lateralis*, is consider as the first step for future work on the function and diversity of Halloween genes in crustaceans. We know very little about the players that regulate YO transitions. Both mTOR and TGFβ are important players for the activated and committed YO states. Little is known about the signaling mechanisms in the transition of the YO from the committed to the repressed state at the end of premolt that extends into postmolt. mRNA levels of all genes in this study were at their lowest in postmolt. We hypothesize that the ecdysteroid peak in late premolt initiates YO repression via a negative feedback on the Halloween genes mediated by ecdysteroid-responsive signaling. Thus, an important future direction is to investigate how ecdysteroid hormones regulate this transition by knocking down the 20E receptor (*EcR* or *RXR*). Also, we recommend that the future work determine the interplay between ecdysteroid-responsive genes, such as *HR3* and *HR4*, and their roles in regulating ecdysteroidogenesis throughout molt cycle.

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