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MOLECULAR BASIS OF ECDYSTEROID MEDIATED LIMB REGENERATION IN THE FIDDLER CRAB, *UCA PUGILATOR*

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MOLECULAR BASIS OF ECDYSTEROID MEDIATED LIMB REGENERATION IN THE FIDDLER CRAB, *UCA PUGILATOR*

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

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Dedicated

to my

mother

Anita Das

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"I was taught that the way to progress is neither swift nor easy."

Marie Curie, 1923

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Abstract

Regeneration is a biological phenomenon that provides an organism the ability to recapitulate part of embryonic development as an adult. The capacity to regenerate is not limited to any particular group of animals; however, invertebrates have greater potential to regenerate lost body parts than vertebrates. The first scientific report of regeneration was a study on crayfish claw and leg regeneration published by the French scientist, René-Antoine Ferchault de Réaumur, in 1712. Efforts to delineate the cellular, molecular, genetic and hormonal basis of regeneration have subsequently been advanced by using model systems like hydra, planaria, salamanders and zebrafish. This dissertation focuses on understanding the hormonal and molecular basis of limb regeneration in a non-model crustacean, the Atlantic sand fiddler crab, *Uca pugilator*.

Chapter I of this dissertation provides a review of research on limb regeneration in crustaceans and outlines the rationale and focus of the dissertation. The review describes the process of crustacean limb regeneration and its relationship to arthropod growth and molting, and the cyclic changes in circulating hormone (ecdysteroid) titers accompanying this process. Changes in circulating ecdysteroid titers are tightly correlated with the two phases of limb regeneration (basal and proecdysial growth). For the purpose of this dissertation, two different approaches have been taken to study the potential role of ecdysteroid signaling during limb regeneration: a) RNA interference (RNAi) mediated gene knockdown of hormone receptor expression, to investigate any phenotypic consequences of blocking

ecdysteroid signaling; and b) a study of global gene expression profiles via next generation sequencing technology (Illumina).

Chapter II addresses the function of ecdysteroid signaling during blastema (basal growth) formation via RNAi-mediated knockdown of ecdysteroid receptors. Knockdown of ecdysteroid receptors resulted in a block in cellular proliferation in the experimental blastemas, absence of the cuticular ingrowths characteristic of the beginnings of limb segmentation, and excessive cuticle deposition. The phenotypic effects on blastemal regenerates induced by RNAi were bilateral and also observed in uninjected limbs in experimental animals. Circulating ecdysteroid levels were also observed to be initially lowered in experimental animals suggesting a systemic effect of receptor knockdown and potential feedback to the Y-organ, the site of ecdysteroid synthesis. These results suggest that although circulating ecdysteroid titers are normally low during basal limb bud growth, signaling via the ecdysteroid receptor pathway is necessary for establishment of blastemal cell proliferation. The receptors may be functioning as repressors in a context of low circulating ecdysteroid; removal of repression could trigger a block in cell proliferation as well as aberrant cuticle synthesis.

Chapter III describes a next generation sequencing approach to study global gene expression profiles in three morphologically distinct stages of limb regeneration as well as in blastemas with disrupted and intact ecdysteroid signaling. RNA-seq analysis generated 848 million short reads produced via the Illumina HiSeq 2000 platform. Following assembly of clean short reads and annotation of contigs, 61,373 non-redundant accession numbers were generated. This represents a major new

database resource for crustacean biologists. The dataset has been placed on a web site (http://www.genome.ou.edu/crab_Illumina.html) that is both sequence and keyword searchable.

This database was used to further explore the putative role of ecdysteroid signaling during the regeneration process. As proof of principle, the depth of the sequence analysis has led to the identification of alternate nuclear receptor A/B domain isoforms, which were not identified through previous genomic or cDNA library screenings. Surprisingly, putative orthologs of ecdysteroid biosynthesis enzymes and a putative ortholog of molt inhibiting hormone (MIH), a negative regulator of Y-organ ecdysteroidogenesis, were also identified in the limb regenerate libraries.

The RNAi knockdown phenotypes prompted an examination of genes that could potentially be contributing to the block in cell proliferation. Examination of the relative expression levels of cell division candidate genes between receptor knockdown and control libraries revealed a significant down-regulation of proliferation marker genes like *PCNA*, *MCM2* and the cell cycle regulatory gene *CycB* in the receptor knockdown library. This was accompanied by a significant up-regulation of the nuclear receptor *HR3*, which has been shown in insect systems to inhibit *CycB* expression. Hence, I hypothesize that the block in blastemal cell proliferation in RNAi-treated animals is due to a significant up-regulation of *HR3*, leading to a lowering of *CycB* expression and consequent G2/M arrest of blastemal cells.

The excessive cuticle deposition seen in animals with disrupted ecdysteroid signaling suggested an aberrant regulation of cuticular protein synthesis. There was a significant up-regulation of cuticular protein transcripts in the ecdysteroid receptor knockdown libraries when compared to the controls. Interestingly, many of these show an aberrant developmental profile of synthesis; cuticular proteins normally expressed at later stages of the molt cycle are aberrantly expressed in the early blastema.

The lowering of circulating ecdysteroid titers observed as a consequence of receptor knockdown is correlated with a significant increase in *MIH* mRNA steady state transcripts in experimental animals. We hypothesize that this increase in *MIH* expression down-regulates ecdysteroid biosynthesis from Y-organs, thereby, further lowering circulating ecdysteroid titers below the critical level needed to maintain blastemal cell proliferation.

To summarize, putative candidate genes that may be mechanistically involved in ecdysteroid-mediated control of cell proliferation, circulating ecdysteroid titer and cuticle deposition during early limb regeneration have been identified. Gene knockdown and global gene expression analysis technologies provided the tools for candidate gene identification in this 'non-model' system; these technologies can now also be employed to examine the mechanistic role of these candidate genes in the postulated model.

CHAPTER I

A general introduction to the molecular basis of limb regeneration in crustaceans and focus of the dissertation

"Nature gives back to the animal precisely and only that which it has lost, and she gives back to it all that it has lost."

René-Antoine Ferchault Réaumur, 1712

1. Introduction to regeneration

Regeneration is a developmental process that allows an organism to re-grow a bodily part, often as a consequence of loss or injury. Documentation of regeneration goes as far back as the work of Aristotle (384-322 BC), when he observed re-growth of tails in tadpoles, lizards and snakes as well as re-growth of eyes in swallow-chicks (see Odelberg, 2004, for review). The first study on regeneration, presented to the French Academy by Réaumur in 1712, described crayfish leg and claw regeneration (see Dinsmore, 2007, for review). Since then, both the extent and the process of regeneration across various phyla have been widely studied.

Regenerative abilities have been observed in several genera belonging to phyla ranging from Cnidarians to Chordates (see Brockes and Kumar, 2008, for review). However, regenerative potential varies within each phylum (Sánchez Alvarado, 2000; Bely, 2010). In addition, regeneration strategies differ among organisms. Historically, two modes of regeneration have been described: morphallaxis and epimorphosis (Morgan, 1901). During morphallaxis, the animal regenerates by reorganizing the remaining tissues following injury or loss of body parts along with little or no cell division. Regeneration in hydra is an example of this phenomenon, where a small, dissected part of a hydra has the potential to grow into a new hydra (see

Galliot, 2012, for review). Cell division is not necessary for this mode of regeneration, and a smaller-sized hydra is obtained. On the other hand, epimorphosis occurs when the lost parts are regenerated with accompanying cellular proliferation. Epimorphosis is sub-divided into two broad groups: non-blastemal and blastemal based regeneration (see Sánchez Alvarado, 2000, for review). Non-blastemal based regeneration is observed in the following cases:

1. Re-growth of missing tissues via transdetermination (change of cell fate), e.g., change in cell fate during imaginal disc regeneration in *Drosophila* (Maves and Schubiger, 1998; 2003).
2. Re-growth of missing tissues via dedifferentiation and proliferation of remaining tissues following an injury, e.g., liver regeneration in humans (Diehl and Chute, 2013).
3. Re-growth of missing tissues via differentiation (formation of specialized cells) and cell division of a reserve population of adult stem cells present with the injured tissues, e.g., bone and muscle regeneration in humans (Dimitriou et al., 2011).

The other mode of epimorphic regeneration involves the formation of a specialized and transient structure called a blastema. The regenerating blastema is a mass of dedifferentiated cells (obtained through loss of cellular specialization) with the ability to proliferate and re-differentiate into all cellular components of the lost structure (Gilbert, 2010). Among vertebrates, salamanders and tadpoles are capable of limb regeneration via blastema formation (Nacu and Tanaka, 2011). The cellular organization of this blastema resembles limb buds formed during embryogenesis. In

invertebrates, blastema formation occurs in planarians (Wagner et al., 2011), crustaceans (Hopkins et al., 1999; Hopkins, 2001), and echinoderms (see Sánchez Alvarado and Tsonis, 2006, for review). In the phylum Arthropoda, 35 genera of the sub-phylum Crustacea and 38 genera of the Class Insecta are capable of limb regeneration (Maginnis, 2006). Among crustaceans, limb regeneration is well documented in the decapod crabs (Adiyodi, 1972; Skinner, 1985; Hopkins, 2001). In addition to limb regeneration, crustaceans are also capable of regenerating their antennae (Maruzzo et al., 2007).

Uca pugilator (Bosc 1802), the Atlantic sand fiddler crab, is the focus of this dissertation's investigation on limb regeneration. This brachyuran belongs to Class Malacostraca and Order Decapoda. *Uca pugilator* are found along the brackish to saltwater shores from Massachusetts to Florida (Kaplan, 1988), the Gulf of Mexico from Florida to Texas, and the Bahamas (Crane, 1975). These crabs are small in size with carapace length ranging from 14 mm to 21 mm (Crane, 1975). They are sexually dimorphic; one conspicuous trait subject to sexual selection is the presence of a large cheliped in males used for combat and mate attraction while females have bilaterally symmetrical feeding claws (Dennenmoser and Christy, 2013; Swanson et al., 2013). Like several brachyuran crabs, molting and limb regeneration occur in tandem in adult fiddler crabs. The major focus of this chapter will be on hormonal and molecular aspects of crustacean limb regeneration, with emphasis on *Uca pugilator*, but will include relevant parallels to insect limb regeneration.

2. The crustacean molt cycle

The members of phylum Arthropoda have a hard exoskeleton that prevents them from growing continuously. To overcome this barrier they periodically shed their hard exoskeleton (ecdysis) and grow a new one, that is usually larger, which allows for an increase in body mass. As adults, crabs are capable of alternating molting and reproduction (see Chang, 1993, for review). This is unlike the strategy used by holometabolous insects where metamorphosis results in a non-growing adult that only reproduces. The first comprehensive documentation of the crustacean molt cycle was performed by Drach in 1939 by studying *Cancer* and *Maia* species (Drach, 1939). Drach sub-divided the molt cycle into four distinct stages: intermolt (Stage C), pre-molt (Stage D), molt (Stage E) and post-molt (Stage A/B), based on changes in the exoskeleton, the cuticle. Two layers of cuticle have been used in analyzing the exoskeleton changes corresponding to the molt cycle: the exocuticle (distal layer) and the endocuticle (medial layer). Both these layers are comprised of chitin proteins and the exocuticle is hardened via mineral deposition and sclerotization (see Dillaman et al., 2013, for review). Skinner (1962) used cytological changes in the epidermis to further define the stages of the molt cycle using the *blackback land* crab, *Gecarcinus lateralis*. *Uca pugilator* molt cycle stages were defined based on morphology and histology of epidermis and setae development by Vigh and Fingerman (1985). An overview of the molt cycle stages is provided in Table 1.

The ecdysis, or E stage, where the old exoskeleton is shed, is followed by postmolt stages A and B, which are marked by a hardening of the new exoskeleton.

Stage C, the anecdysis or intermolt stage, is defined by a rigid exoskeleton, and is the stage during which the animal feeds and reproduces. During stage D, the premolt or proecdysis stage, the animal prepares for shedding of the old exoskeleton. This stage is further divided into four sub-stages ($D_1 – D_4$) based on the changes in the cuticle and epidermal cells of the crab (Skinner, 1985). The D_1 sub-stage is characterized by apolysis (separation of the old cuticle from epidermis), reabsorption of old cuticle and enlargement of epidermal cells. Formation of new cuticle occurs during the D_2 sub-stage and is followed by a decrease in length of epidermal cells during D_3. The D_4 substage, preceding ecdysis, is marked by a change in hemolymph color from bluish to pink, due to resorption of astaxantin from the old exocuticle (Skinner, 1962). Following ecdysis, crabs enter postmolt stages and the cycle is repeated. In conjunction with periodic molting, blastemal-based epimorphic limb regeneration is integrated with the life cycle of brachyuran crabs like *Uca pugilator*.

3. Crustacean limb regeneration

The ability to facilitate limb loss with minimum damage and to regenerate limbs provides crabs with a survival mechanism against predation. When attacked by predators or injured, the fiddler crab can reflexively cast off its leg at a predetermined breakage plane within the basi-ischiopodite, the second limb segment, by a process called autotomy (A) (Findlay and McVean, 1977; Hopkins, 1993). Under laboratory conditions, it is possible to induce crabs, as a consequence of limb injury, to reflexively lose their limbs at the same pre-determined point (Hopkins, 1993). The

wound left by the autotomized limb is sealed by a connective tissue membrane called the autotomy membrane (AM). In an intact limb, the AM is a double membrane that splits after limb loss. Following autotomy the distal membrane balloons out to reduce blood loss while the proximal membrane remains in direct contact with the pedal nerve and blood vessels. The AM also protects against bacterial infection (Hopkins, 1993).

The first step in the process of regeneration is wound healing. Following limb autotomy, blood cells, mainly granulocytes and blastocytes, migrate towards the wound site. The granulocytes degranulate to form a clot and seal the wound (Adiyodi, 1972; Hopkins, 1993). In addition to the blood cells, epidermal cells migrate to occupy the space underneath the scab. These epidermal cells become the precursors for the regenerating limb.

Limb regeneration in these crabs occurs in two phases, termed basal and proecdysial growth, and is superimposed on the molt cycle (Adiyodi, 1972; Hopkins, 1993; 2001). These two phases are monitored by measuring R-values: the normalized size of the regenerating limb bud (a description of R-values is provided in the Figure 1 legend). Figure 1 represents progression of limb regeneration phases through the molt cycle. The two phases are part of an adaptive feature of crabs that allows them to regenerate their limbs in coordination with their molt cycle stages and other activities like reproduction and feeding (Skinner, 1985). The basal growth phase is comprised of two general events: blastema formation and differentiation of the blastema into a basal limb bud that contains all the limb segments in folded position and secured in a cuticular sac. The blastema develops through cellular proliferation of the epidermal

cells and starts as early as two days after autotomy (A+2) (Emmel, 1910; Hopkins, 1993). Four to seven days following autotomy, the blastema is comprised of dividing epidermal cells that start secreting a thin layer of cuticle underneath the scab. The first signs of limb segmentation are invaginations caused by inward migration and division of epidermal cells and cuticle secretion by epidermal cells. This occurs prior to emergence of the blastema. Around seven to nine days after autotomy, the continuous proliferation of epidermal cells leads to the emergence of a blastema from the coxal segment. This protuberance is called a papilla and has an R-value of less than five (Hopkins, 1993). The period of growth leading to and following papilla formation is called basal growth (R-values range from 5 – 10). Basal growth may occur anytime during the molt cycle and most often occurs during the intermolt (stage C). Cross-sections of papilla show incomplete cuticular ingrowths. At the end of the basal growth phase (A+10), cellular proliferation ceases. Histological preparations of basal limb buds show the presence of myofibril clusters attached to the apodeme (Adiyodi, 1972; Hopkins, 1993). The basal limb bud contains all the segments (basio-ischium, merus, carpus, propodus and dactylus) folded onto one another, and it is encased in a cuticular sac (Hopkins et al., 1999). The ability to encase limb buds is an adaptive feature that allows the animal to move and forage without hindrance during the intermolt stage. Depending on the duration of the intermolt, the limb bud growth may cease (basal plateau phase) until the crab enters the pre-molt stage.

The second growth phase following the basal plateau is sub-divided into early and late proecdysial phases. The early proecdysial growth phase occurs during the early pre-molt (D_0) substage of the molt cycle. This phase of limb bud is marked by

hypertrophic growth of muscles via protein synthesis and water uptake (Hopkins, 1993). The R-values of early proecdysial limb buds range from 12-15. Following rapid growth, the proecdysial limb bud can increase in size by almost three fold in comparison to the basal limb bud. When the maximum size of the proecdysial limb bud is obtained, the crab enters terminal plateau. This corresponds to the late pre-molt (D_1D_4) stage (R-value 19-21; Table 1), and the regenerated bud in terminal plateau is called a late proecdysial limb bud. Following ecdysis, the late proecdysial limb bud is detached from its cuticular sac and a functional limb is deployed as the blood rushes into the bud unfolding the segments. Newly regenerated limbs are often smaller in size than the non-regenerated limbs (Maginnis, 2006). Given the evidence for tight coordination between molt cycle and regeneration, the limb bud R-values can be used as an indicator to identify molt cycle stages (Table 1).

Although much has been written and researched about the coordination of molt cycle stages with limb regeneration phases, little is known about the cellular and genetic basis of crustacean limb regeneration. Histology and electron microscopy have been used to determine the cellular types associated with the blastema. Although histological studies of regenerating arthropod limbs have shown that the blastema is formed via dedifferentiation and proliferation of epidermal cells (Needham, 1965; Adiyodi, 1972; Truby, 1985; Hopkins et al., 1999), validation of this phenomenon has not been performed using molecular markers for dedifferentiation (see Maruzzo and Bortolin, 2013, for review).

The regeneration of limb and wing imaginal discs in *Drosophila* larvae, following irradiation or ablation, involves dedifferentiation of an epidermis-derived

blastema and has been well studied (see Repiso et al., 2011, for review). The Wnt (Wingless) signaling pathway gene, *wnt-1*, and *dmyc* are up-regulated during the proliferation phase of imaginal disc regeneration (Smith-Bolton et al., 2009). Both crustacean limb regeneration and insect leg imaginal disc regeneration are comparable. In both instances, the cells that give rise to limbs must proliferate, be assigned a specific positional coordinate and following specification differentiate into appropriate morphological structures. Wnt signaling establishes the proximo-distal (PD) axis of limbs during leg imaginal disc development in *Drosophila* larvae. It has been reported that expression of both *wingless* (*wg*) and Decapentaplegic (*Dpp*) initiate (PD) axis formation by inducing expression of *distal-less* (*dll*) (see Estella et al., 2012, for review). Dll then induces transcription of *dachshund* (*dac*), and the boundaries of the limb segments are established via cross-regulation of *dll* and *dac* expression. Recent work in a phylogenetically basal insect, the cricket, has shown that *wg, dpp* and *hedgehog* are expressed during leg regeneration and are involved in establishment of the PD axis (Mito et al., 2002). Further studies have shown that limb regeneration does not occur following knockdown of the β-catenin *armadillo* gene, suggesting a functional role of the canonical Wnt signaling pathway (MacDonald et al., 2009; Nakamura et al., 2007). Although the Wnt signaling pathway has not been implicated during crustacean limb regeneration, it is probable that this conserved pathway is required for the establishment of the PD axis during the blastema phase.

4. Hormonal control of molting and regeneration

In arthropods, polyhydroxylated steroid hormones called ecdysteroids play a major role in growth, development and reproduction by regulating gene expression (Thummel, 1996; Riddiford et al., 2000; Schwedes and Carney, 2012). In 1954, the first ecdysteroid and also the first insect hormone, called ecdysone or molting hormone, was isolated from 500 kg of moth pupae (see Karlson, 1996, for review). The active form of ecdysone was identified as 20-hydroxyecdysone (20E) in both insects and crustaceans in 1966 (see Chang, 1993, for review). In *Uca pugilator*, at least four different kinds of ecdysteroids have been identified in the hemolymph: 25-deoxyecdysone, ecdysone, Ponasterone A (PonA; 25-deoxy-20-hydroxyecdysone) and 20E (Hopkins, 1986). The titers of these hormones, detected by radioimmunoassay (RIA), vary during the molt cycle stages and limb regeneration phases (Hopkins, 1992). Two hormones, 25-deoxyecdysone and ecdysone, are synthesized from cholesterol in a pair of bilateral Y-organs (YO), located anteriorly in the cephalothorax (see Chang and Mykles, 2011, for review), then released into the circulating hemolymph and converted to PonA and 20E, respectively, in the peripheral tissues. Circulating ecdysteroid titers are low during the intermolt stage of the molt cycle (Hopkins, 1989). On entering pre-molt, ecdysteroids in the hemolymph increase in concentration and reach a maximum at late pre-molt. The levels of endogenous circulating ecdysteroids can also be correlated with the two phases of limb regeneration (Figure 2; Hopkins, 2001). Circulating ecdysteroid titers remain low

during the basal (<20 pg/µl) and early proecdysial (D_0) growth phases (<35 pg/µl) (Hopkins, 1989). Transition from basal growth to early proecdysial growth of the limb bud is associated with a small peak of ecdysteroids (40 pg/µl) (Hopkins, 1989; 2001). The competency of limb buds to grow following the small peak during stage D_0 depends on the reduction of circulating ecdysteroid titers. On entering the late proecdysial growth (D_{1-4}) phase, ecdysteroids in the hemolymph begin to increase in concentration (50-100 pg/µl) (Hopkins, 1983; 1989). Various physiological processes like apolysis (detachment of old cuticle from the exoskeleton) and synthesis of new cuticle occur during this time, which implies that the increase in ecdysteroid titers may play a role in mediating these physiological processes during pre-molt.

4.1. Regulation of circulating ecdysteroids via neuropeptides

Ecdysteroid biosynthesis in the crustacean Y-organs (YOs) is negatively regulated by peptide hormones, such as molt inhibiting hormone (MIH), crustacean hyperglycemic hormone (CHH) and gonad inhibiting hormone (GIH) (see Chang, 1993, for review; Chan et al., 2003; Nakatsuji et al., 2009; Webster et al., 2012). These peptide hormones are secreted from the medulla terminalis X-organ (MTXO) and are transported and secreted from an aggregation of neurohemal axon terminals called the sinus gland (SG) (Chung and Webster, 2003; Webster et al., 2012, for review). The MTXO and SG structures are located at the base of the eyestalks. Removal of eyestalks from crustaceans leads to rapid elevation of circulating ecdysteroids, resulting in precocious molts. This negative control of ecdysteroid synthesis is unlike

that of the insects, where ecdysteroid synthesis is induced by a peptide hormone called prothoracicotropic hormone, PTTH (Gilbert et al., 1997).

The YO is sensitive to the inhibitory neuropeptides MIH and CHH early in the normal molt cycle, but it loses sensitivity to these neuropeptides at mid pre-molt (see Chang and Mykles, 2011, for review). The loss of the sensitivity of YO to MIH causes the rise in ecdysteroid hormone titers during pre-molt. Furthermore, successful molting occurs when the ecdysteroid titers decline sharply right before ecdysis (Hopkins, 1992). This decline is believed to result from a negative feedback effect of ecdysteroids on the YO (see Chang and Mykles, 2011, for review).

As mentioned above, molting is an absolute necessity for successful limb regeneration in crabs. The lost limbs are regenerated within one molt cycle, provided that autotomy was performed when the animal was in intermolt or early pre-molt stage (Hopkins, 1993). In *Uca pugilator* and other decapod crabs, the timing and extent of limb loss determines the length of the molt cycle. When five or more limbs are autotomized in decapod crabs (mainly during the intermolt stage), a precocious molt is induced (Skinner and Graham, 1972). Autotomy of less than five limbs does not shorten the molt cycle peroid. When an animal is in basal or early proecdysial growth phase and a primary limb bud is autotomized, the growth of the remaining primary limb buds slows down to accommodate the growth of the secondary limb regenerate (Holland and Skinner, 1976). In this case, the molt cycle length increases. If autotomy is performed during the late proecdysial phase of regeneration, however, then the secondary limb regenerate does not have adequate time to re-grow during the same molt cycle and regenerates in the next molt cycle. These two observations – multiple

limb autotomy during the anecdysial stage results in accelerated molts, and primary limb bud autotomy during early proecdysial growth phase results in increased molt cycle length – suggest that cross-talk among the limb buds, YOs and X-organs may act in a feedback loop on the regulation of ecdysteroid synthesis and release. This in turn either shortens or lengthens the molt cycle period (see Mykles, 2001, for review). Skinner (1985) hypothesized that primary limb buds produce limb autotomy factor - anecdysis (LAF_{an}) that may negatively feedback on X-organ MIH synthesis, promoting YO ecdysteroid production. Skinner further proposed that the secondary limb buds produce limb autotomy factor-proecdysis (LAF_{pro}) that negatively acts on YOs and lowers ecdysteroid production in order to delay molting. The extensive work on coordination of limb regeneration and molting suggests the presence of feedback factors. Although these factors have not been isolated, biochemical and physiological studies of extracts from secondary limb buds revealed Laf_{pro} activity with physical characteristics similar to a peptide. Introduction of this putative MIH-like peptide in pre-molt crabs lowers ecdysteroid titers and delays molting (Yu et al., 2002).

4.2. Ecdysteroid receptors and limb regeneration

Like the vertebrate steroid hormones, ecdysteroids mediate their effect by binding to their cognate nuclear receptors and promoting gene transcription (Segraves, 1994). Nuclear receptors bind to a specific region on DNA called the Hormone Response Element (HRE) and then regulate the gene downstream of the HRE (see Tsai and O'Malley, 1994; Hill et al., 2013, for review). It has also been observed that

many nuclear receptors, including the thyroid hormone receptor, retinoic acid receptor and insect ecdysone receptor, can act as strong repressors of gene expression in the absence of ligand (Hörlein et al., 1995; Chen and Evans, 1995; Hu et al., 2003).

Ecdysteroid binds to the ecdysone receptor, EcR, a nuclear receptor that functions as a ligand-dependent transcription factor (Koelle, 1991; Hill et al., 2013, for review). In insects, the functional ecdysteroid receptor is a heterodimer formed by the EcR and USP (ultraspiracle, a vertebrate RXR homolog) proteins (Yao et al., 1993). The structures of both EcR and USP are similar to a typical nuclear receptor, consisting of five domains: A/B, C, D, E and F (see Manglesdorf et al., 1995; Aranda and Pascual, 2001; Fahrbach et al., 2012, for review). The variable N-terminal domain or the A/B region contains a ligand independent hypervariable AF-1 transactivation domain that interacts with transcriptional factors and co-activators. The C or the DNA binding domain (DBD) is the most conserved region among nuclear receptors, and it contains zinc fingers that are responsible for HRE recognition and receptor dimerization. The linker or hinge region is the D domain that provides flexibility to the receptor to mediate proper DNA binding, nuclear localization, and subunit pairing of the receptors. The E or ligand binding domain (LBD) is functionally complex, with regions responsible for ligand binding, nuclear localization and intermolecular silencing or activation, via a ligand-dependent activation domain (AF-2), and a second dimerization region. Some hormone receptors contain an additional C-terminal domain, known as the F domain. Its function is still unknown, and it is absent in certain steroid hormone receptors (Aranda and Pascual, 2001).

Ecdysteroid-mediated gene expression during larval and pupal metamorphosis has been widely studied in insects (Riddiford et al., 2000). Molting and metamorphosis in insects are associated with changes in circulating 20E titers (see Karlson, 1996, for review). Along with the changes in 20E, an ecdysteroid-regulated cascade of transcription factors has been characterized in *Drosophila melanogaster* and *Manduca sexta* (Thummel, 1996; Riddiford et al., 2000; Riddiford et al., 2003). The effects of ecdysone signaling have not been as widely studied in adult *Drosophila* as in larval and pupal development. However, disruption of ecdysone signaling in adult *Drosophila* affects various physiological processes, such as behavior, reproduction, stress resistance, and lifespan (see Schwedes and Carney, 2012, for review). In addition, ecdysteroid receptors are localized in a variety of adult tissues including the central nervous system, fat body, gut, and male and female reproductive tissues.

In *Drosophila melanogaster*, there are three isoforms of EcR (EcR-A, EcR-B1 and EcR-B2) that are expressed in different target tissues during metamorphosis (Talbot et al., 1993). These three isoforms differ in the N-terminal A/B domain and have a 'common' ligand- and DNA-binding domain. Receptors with N-terminal domains homologous to EcR-A and EcR-B1 have been identified in at least 45 and 76 species of Phylum Arthropoda, respectively (Watanabe et al., 2010). Among them, A and B isoforms of EcR have been cloned from several insects including the tobacco hornworm, *Manduca sexta*, (Fujiwara et al., 1995; Jindra et al., 1996), the silkworm *Bombyx mori* (Kamimura et al., 1996) and the yellow mealworm, *Tenebrio molitor* (Mouillet et al., 1997). Apart from insects, three A and B isoforms of EcR have also

been characterized in the ixodid tick, *Amblyomma americanum* (Guo et al., 1997), and the crustacean *Daphnia magna* (Kato et al., 2007).

Although only one USP variant has been observed in *Drosophila* (Oro et al., 1990), multiple USP variants have been cloned in *Manduca sexta* (Jindra, 1997), *Aedes aegypti* (Kapitskaya et al., 1996), and *Amblyomma americanum* (Guo et al., 1998) as well as in crustaceans.

In contrast to most arthropods, ligand binding domain (LBD) isoforms for ecdysteroid receptors were first reported in *Uca pugilator,* where distinct LBD as well as hinge domain isoforms have been identified for both RXR and EcR (Durica et al., 2002; Wu et al., 2004; Durica et al. submitted). There are two RXR LBD domain isoforms, distinguished by presence or absence of a 33 amino acid insert in the H1-H3 region of the LBD (Wu et al., 2004). Similar RXR LBD isoforms have subsequently been identified in another crab, *Gecarcinus lateralis* (Kim et al., 2005). In addition, through screening of cDNA libraries, two EcR LBD and two hinge region isoforms have been identified in *Uca*. Recently, two different EcR A/B domain variants have been discovered through transcriptome analysis of the limb regenerates (see chapter III) (Durica et al., submitted).

The *EcR* and *RXR* transcripts are expressed in hypodermis, eyestalks, gills, hepatopancreas, muscle of non-regenerating limbs and limb regenerates (Chung et al., 1998). Both EcR and RXR mRNAs and proteins are present in all phases of limb regeneration (Hopkins, 1999; Wu et al., 2004; Wu et al., unpublished). *In vitro,* the insect receptor heterodimer can bind to DNA, but binding is greatly enhanced by interaction with ecdysteroid (Lezzi et al., 2002). In contrast, the *Uca* cognate receptor

heterodimer shows no increase in DNA binding on hormone exposure (Wu et al., 2004, Durica et al., submitted). *In vitro* binding studies indicate that the affinity of EcR to PonA varies depending on the RXR isoform type in the heterodimer receptor complex (Hopkins et al., 2008). The presence of these various isoforms, their variable expression profiles, and different physiological properties *in vitro* imply differential physiological roles, both with regard to ligand and DNA interactions.

5. Focus of the Dissertation

The above summary describes extensive circumstantial evidence that ecdysteroid signaling is required for normal progression of limb regeneration. My dissertation focuses on providing molecular evidence for a direct role of ecdysteroid signaling during limb regeneration as well as a characterization of the genes expressed during three morphologically variable developmental time points during the limb regeneration process. This catalog of gene expression represents a first step in defining genes linked to the regeneration process that are downstream of ecdysteroid signaling. One of the major hurdles in this project has been the successful development and utilization of molecular genetic tools to study a non-model organism. Crabs are not a classical model species for studying genetics (see chapter II for details), but with the advent of new technologies to manipulate and monitor gene expression profiles, we have identified a role for ecdysteroid signaling during early blastemal development, and we have produced one of the most detailed transcriptomic analyses in a brachyuran.

In chapter II of this dissertation, I have tested the hypothesis that ecdysteroid signaling directly mediates limb regeneration. We have used RNA interference (RNAi) (Fire et al., 1998; Kurreck, 2009; Wilson and Doudna, 2013, for review) to disrupt ecdysteroid receptor signaling during the earliest stages of limb regeneration (Das and Durica, 2013). We are the first to report a successful use of RNAi as a tool to down-regulate genes in brachyuran crabs. A series of experiments were conducted, firstly, to confirm knockdown of receptor transcripts (*EcR* and *RXR*) and, secondly, to delineate the effects of lowering receptor transcript levels on limb regeneration. These effects are evident as early as 48 hrs following RNAi injections. *In vivo* knockdown of ecdysteroid receptors resulted in obstruction of blastemal cell proliferation, and failure to generate a functional limb. In addition to inhibition of blastemal cell proliferation, animals injected with RNAi also exhibited abnormal cuticle deposition associated with epidermal cell secretion. We also observed that disruption of ecdysteroid receptors in limb regenerates affected normal circulating ecdysteroid titers at the blastema phase and the ability of the crabs to molt successfully. This result provides further indirect evidence that molting and regeneration are tightly coupled phenomena and are regulated by ecdysteroids.

In the chapter III, I have taken a global transcriptomic approach to study the gene expression profiles during limb regeneration. Although this approach is not hypothesis driven, it allows us to gather information on a heterogeneous population of mRNAs that are expressed at a certain developmental time point of limb regeneration. Crustaceans in general have very limited genomic sequence data, although they are ecologically important species and are a major food source. *Daphnia pulex* is the only

crustacean that has an annotated genome (Colbourne et al., 2011; see chapter III for details). With the advent of cost effective next generation sequencing (NGS) technologies, it is now feasible to generate temporal and spatial transcriptomes from non-model organisms without using a reference genome (Liu et al., 2012; Dheilly et al., 2014). However, the annotation of the transcriptomes adds another layer of complexity to metadata analysis. Currently there are multiple databases combined under the INSCD (The International Nucleotide Sequence Database Collaboration) that are updated regularly for annotation purposes (Nakamura et al., 2013). Thus, it is possible to generate and annotate a collective dataset of transcripts that are spatially and temporally expressed from a non-model organism, like *Uca*, and use mathematical models to test biological questions.

Using Illumina sequencing technology, we have generated one of the largest annotated transcriptome databases for brachyuran crabs. We have examined the transcriptome profiles of limb regenerates at three critical stages of limb regeneration: early blastemal, and early and late proecdysial growth stages (two biological replicates). In addition, we have obtained transcriptomes of blastemas in which either ecdysteroid receptor signaling was disrupted or remained unperturbed. There are very distinct developmental, morphological, and physiological events associated with these phases, which we predicted would differentially mobilize a large segment of the organism's genetic repertoire. Using eight separate assemblies of these stage-specific transcriptome libraries, we developed a website (http://www.genome.ou.edu/crab.html) that is now easily accessible to the scientific community. Screening of these stage-specific datasets is both BLAST-enabled and

keyword searchable, providing outside investigators the ability to retrieve *Uca* orthologs/homologs of their query sequences as FASTA assemblies.

The amount of information obtained from eight transcriptome datasets (848 million short reads) is colossal. Hence, parsing out concise and biologically relevant end points becomes a bottleneck for both molecular and computational biologists. Given the vast dataset accumulated via assembly and annotation of the eight libraries, I have chosen to focus on certain genes related to ecdysteroid signaling. This led to a surprising discovery of transcripts that encode enzymes required for synthesis of ecdysteroids from cholesterol (details in chapter III). In addition, we have recovered new isoforms for ecdysteroid responsive nuclear receptors, previously not identified via cDNA library screening. Further we were able to explain probable reasons for two RNAi phenotypes (increased cuticle secretion and decreased cell proliferation) via gene expression levels.

In conclusion, this dissertation provides information on the genetic and molecular basis of limb regeneration in *Uca pugilator*. Transcriptomic studies have provided detailed expression data, helpful for formulating hypotheses on candidate genes that are involved in the regulation of limb regeneration via ecdysteroid signaling. The successful implementation of the RNAi technique suggests that it can be used to examine these hypotheses via artificial genetic manipulation of candidate gene expression. This is the first step towards characterizing the ecdysteroid-mediated gene networks that play a role during limb regeneration in crustaceans.

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Table 1. General morphological criteria used to classify crustacean molt cycle stages along with corresponding circulating ecdysteroid titers and R-values of limb regenerates (adapted and modified from Skinner, 1962; Hopkins, 1983; Vigh and Fingerman, 1985).

Molt cycle stages	Alternate stage name	Morphological characteristics of epidermis, cuticle and setae	Ecdysteroid titers	R-value of limb regenerates
A	Postmolt	Newly molted inactive crab; Water resorption continues (following ecdysis); mineralization of exocuticle begins; shrinkage of epidermal cells	Low (<20 pg/µl)	1-10
B		Deposition and mineralization of endocuticle begins; exoskeleton begins to harden		
C	Intermolt/ Anecdysis	Carapace hardens completely; endocuticle formation continues towards completion; tissue growth occurs, epidermis remains attached to cuticle; regeneration of limbs		
D_0	Early Premolt/ Proecdysis	Formation of gastrolith (only in freshwater crustaceans) and regeneration of limbs	Low (<35 pg/µl)	12-15
D_1	Late Premolt/ Proecdysis	Resorption of old exoskeleton; enlargement of epidermal cells; atrophy of somatic muscles	High and rising (50-100 pg/µl)	19-22
D_2		Secretion of new exocuticle		
D_{3-4}		Epidermis is retracted from the cuticle; water uptake begins		
E	Ecdysis	Shedding of rigid exoskeleton in one piece to expose newly laid soft exoskeleton	Sharp drop (<10 pg/µl)	NA

Figure legends

Figure 1. Graphical representation of crustacean Regenerative index or R-value versus time. R-value represents the growth of limb buds normalized to size of the crab (length of limb bud divided by carapace width X 100, Bliss, 1956). The limb regeneration phases are overlaid on molt cycle sub-stages (Drach, 1939): Stage C4 - anecdysis; D_0 – early proecdysial stage; D_1-D_4 – Late early proecdysial stage. Auto – Time when autotomy of right third walking leg was carried out (adapted from Hopkins, 1993).

Figure 2. Graphical representation of fluctuations in circulating ecdysteroid titers in crabs (measured via RIA). The hormone levels are correlated with four molt cycle stages and the two phases of limb regeneration. The titers remain low throughout the basal and early proecdysial growth phases followed by a rise in the late proecdysial growth phase and an abrupt fall in titers just before ecdysis (E) (modified and adapted from Hopkins, 2001).

Figure 1.

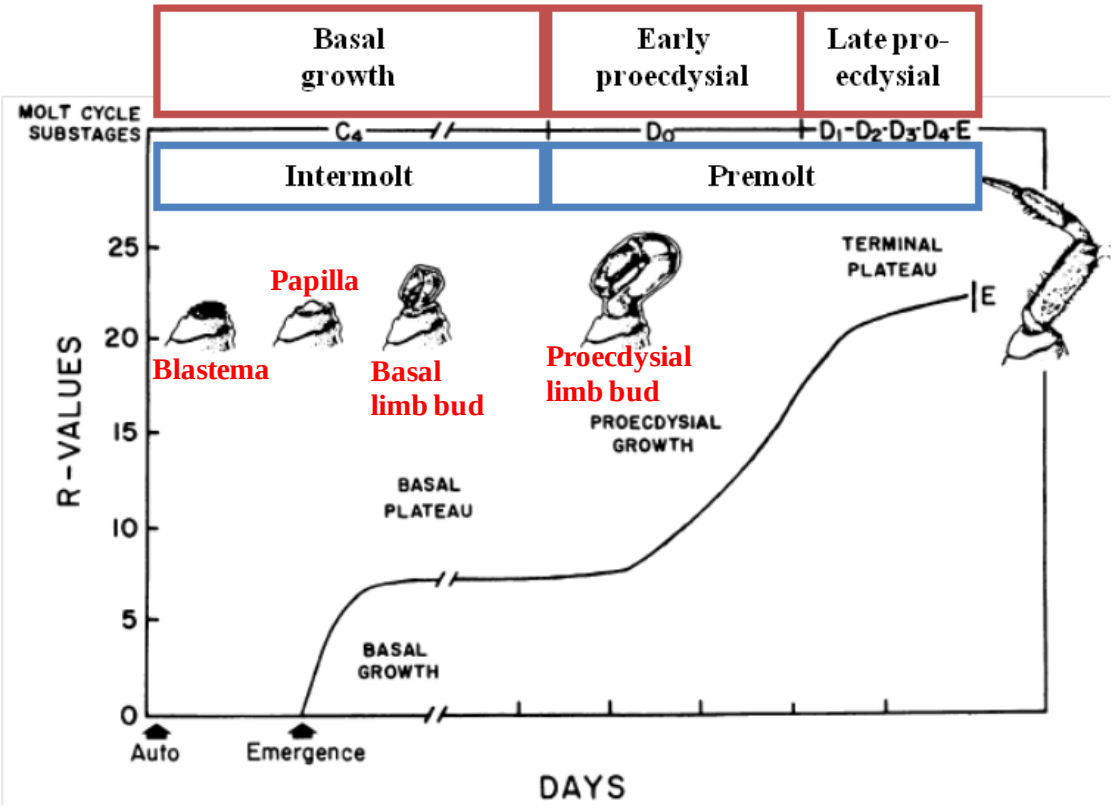
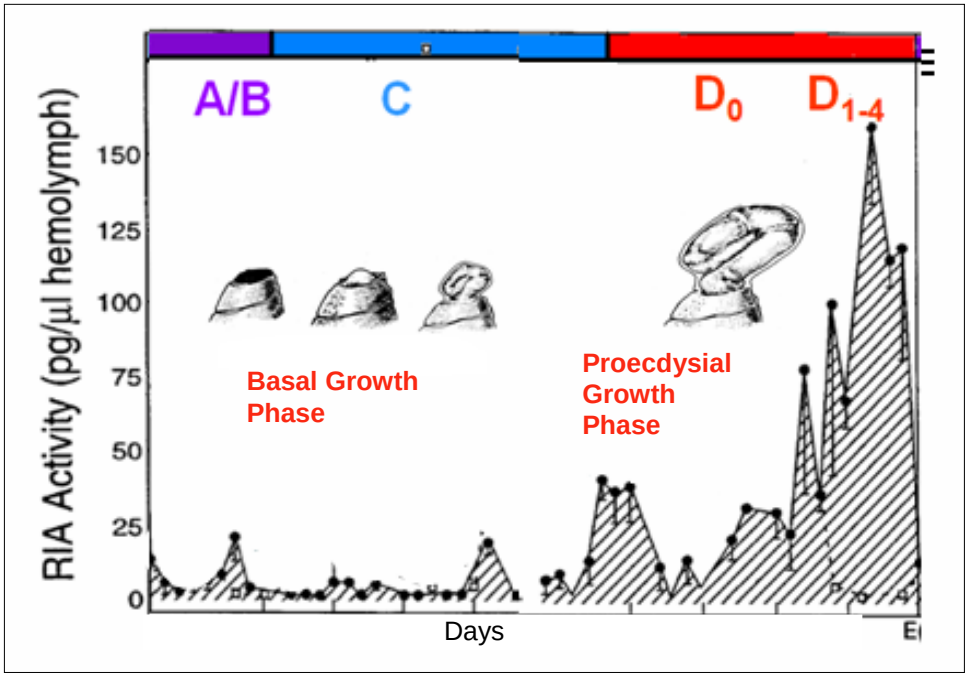

Basal growth
Early proecdysial
Late pro-ecdysial
MOLT CYCLE SUBSTAGES
C4
Do
D1-D2-D3-D4-E
Intermolt
Premolt
25
20
15
10
5
0
R-VALUES
Blastema
Papilla
Basal limb bud
Proecdysial limb bud
TERMINAL PLATEAU
E
PROECDYSIAL GROWTH
BASAL PLATEAU
BASAL GROWTH
Auto
Emergence
DAYS

Figure 2.


A/B
C
D_0
D_{1-4}
RIA Activity (pg/µl hemolymph)
150
125
100
75
50
25
0
Basal Growth Phase
Proecdysial Growth Phase
Days

CHAPTER II

Ecdysteroid receptor signaling disruption obstructs blastemal cell proliferation during limb regeneration in the fiddler crab, *Uca pugilator*

Ecdysteroid receptor signaling disruption obstructs blastemal cell proliferation during limb regeneration in the fiddler crab, *Uca pugilator*

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Keywords: *RXR*; *EcR*; dsRNA; *Uca pugilator*; limb regeneration; cell proliferation

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Abstract

To study ecdysteroid signaling during limb regeneration, we have applied RNAi (dsRNA) mediated silencing to *EcR/RXR*, the genes encoding the ecdysteroid receptor heterodimer, in the fiddler crab *Uca pugilator*. We injected RNAi into the blastemal chamber during early limb regeneration. Silencing was evaluated by knockdown in receptor transcript abundance, and disruption was evaluated by changes in growth rate and morphology of limb regenerates. q-PCR results indicated a 50% drop in transcript abundance 48 hours post injection in both RNAi (ds*EcR*/ds*RXR)* injected ipsilateral and uninjected contralateral blastemas in experimental animals relative to controls. *EcR/RXR* transcript levels further decreased over time. Several phenotypes were associated with knockdown. The experimental blastema failed to develop; microscopic examination of the arrested blastema revealed an absence of the cuticular ingrowths characteristic of the beginnings of limb segmentation and cell proliferation assays revealed that the arrested blastema had few dividing cells. Ecdysteroid levels were also lowered in experimental animals; given the bilateral effects of RNAi on limb buds in experimental animals, these results suggest RNAi had a systemic effect. Although hormone titers in experimental animals rose to comparable control levels during the late proecdysial phase of limb regeneration, most experimental crabs failed to molt and died. The overall failure to molt indicates that RNAi receptor knockdown has long-term effects. The combined effects of receptor knockdown indicate that, although circulating ecdysteroid titers are normally low during basal limb bud growth, signaling via the ecdysteroid receptor pathway is

necessary for establishment of blastemal cell proliferation and development in the regenerating limbs of *Uca pugilator*.

"What's really terrific about RNAi is that once it's inside the cell, it enters very efficiently into the cellular machinery."

Craig C Mello, 2013

1. Introduction

Regeneration in invertebrates has been widely studied (see Giudice et al., 2008; Galliot and Chera 2010; Repiso et al., 2011, for review). Among arthropods, reports of crustacean limb regeneration date to the 18th century, and 35 genera under the subphylum *Crustacea* have been demonstrated to possess limb regeneration capabilities (see Maginnis, 2006, for review). Arthropod growth and development, particularly the process of metamorphosis in holometabolous insects, have served as important model systems to study the molecular basis of steroid hormone signaling (Swevers and Iatrou, 2003; King-Jones and Thummel, 2005; De Loof, 2008). Arthropod steroid hormones, ecdysteroids, regulate various developmental phenomena like reproduction, limb regeneration, and growth (via molting) in crabs, including the fiddler crab, *Uca pugilator* (Gunamalai et al., 2004; Hopkins, 1989; Hopkins et al., 1999).

The members of Phylum Arthropoda have a hard exoskeleton that prevents them from growing continuously. To overcome this barrier they periodically separate hard exoskeleton from underlying epidermis (apolysis), grow a new larger epidermis under the old exoskeleton, shed the old one and increase in body mass. Unlike holometabolous insects, as adults most crustaceans grow throughout their life cycle,

alternating growth with reproduction. The molt cycle of crustaceans can be divided into five distinct stages based on changes in the exoskeleton: A - E (Drach, 1939). The Ecdysis, or E stage, is followed by postmolt stages A and B, which are marked by hardening of the newly secreted exoskeleton. Stage C, anecdysis or intermolt stage, is marked by a rigid exoskeleton that is tightly affixed to the epidermis, and is the stage during which the animal feeds and reproduces. Stages D_{1-4}, comprising premolt or proecdysis, precede stage E where the animal prepares for apolysis and shedding of the old exoskeleton (Vigh and Fingerman, 1985).

In concert with periodic molting, another form of growth, limb regeneration, is associated with the life cycle of *Uca pugilator*. Limb regeneration in these crabs occurs in two phases, basal and proecdysial growth, and is superimposed on the molt cycle (Hopkins, 1993). The two phases are part of an adaptive feature of crabs that allows them to regenerate their limbs in coordination with their molt cycle stages and other activities like reproduction and feeding (Skinner, 1985). When attacked by predators or injured, the fiddler crab can reflexively cast off its leg at a predetermined breakage plane within the second limb segment, the basi-ischiopodite, by a process known as autotomy (Findley and McVean, 1977; Hopkins, 1993). The wound left by the autotomized limb is sealed by a connective tissue membrane called the autotomy membrane (AM). Autotomy (A) involves minimal tissue damage, with the exception of severance of the pedal nerve and some blood sinuses. The AM ensures negligible blood loss and protects against bacterial infection (Hopkins, 1993). After autotomy, there is a rapid invasion of granulocytes and blastocyte cells under the AM. The granulocytes degranulate and a scab is formed. Three different cell types, composed of

granulocytes, blastocytes and epidermal cells migrate from the coxa wall and along the pedal nerve to occupy the space underneath the scab. Two days following autotomy (A+2), the epidermal cells underlying the scab start dividing mitotically to form the blastema (Emmel, 1910; Hopkins, 1993). As the cells divide, cuticular invaginations within the blastema occur to produce the first indication of segment formation of the regenerating limb. Seven to nine days following autotomy, continuous mitosis of epidermal cells results in emergence of the blastema from the coxa. This protuberance is called a papilla (Hopkins, 1993). The period of growth leading to and following papilla formation is called basal growth. Basal growth may occur anytime during the molt cycle and most often occurs during the intermolt (stage C) stage. During this period, the papilla grows via cell division and differentiates into limb segments. This basal limb bud with its folded segments is encased in a cuticular sac (Hopkins, 1993). The folding of segments allows the animal to move freely and does not become an obstruction while searching for food and engaging in reproductive behavior, activities associated with intermolt animals. Basal growth is followed by a second growth phase, associated with preparation for molt, termed proecdysial growth, which is marked by hypertrophic growth of muscles via protein synthesis and water uptake (Hopkins, 1993). Early proecdysial growth occurs during an additional sub-stage of proecdysis called the D_0 substage. Both basal and proecdysial growth phases are followed by a basal plateau and a terminal plateau respectively, where the growth of the limb bud effectively stops (Hopkins, 1993). When the animal is ready to molt, following terminal plateau, the limb bud is detached from its cuticular sac and a functional limb is deployed when the blood rushes into the bud as it unfolds at ecdysis.

Ecdysteroids are polyhydroxylated steroid hormones that play a major role in arthropod growth, development and reproduction by regulating gene expression (Thummel, 1996; Riddiford et al., 2000). In brachyuran crustaceans ecdysteroids are synthesized in a pair of bilateral Y organs (Chang and O'Connor 1977) located ventrally and anteriorly in the cephalothorax, within the branchial chamber (see Chang and Mykles, 2011, for review). In *Uca pugilator*, at least four different kinds of ecdysteroids have been identified in the hemolymph: Ecdysone (E), 25 deoxyecdysone (25dE), Ponasterone A (PonA) and 20 hydroxyecdysone (20E) (Hopkins, 1986). The titers of these hormones, detected by radioimmunoassay (RIA), vary during the molt cycle (Chang et al. 1976; Hopkins, 1992). Circulating ecdysteroid titers are low during the intermolt stage of the molt cycle and during D_0 stage of proecdysial growth (<30 pg/µl) (Hopkins, 1989). On entering the $D_{1\text{-}4}$ stages of proecdysis, ecdysteroids in the hemolymph begin to increase in concentration (50-100 pg/µl). The levels of endogenous circulating ecdysteroids can also be correlated with the two phases of limb regeneration. The basal phase of limb regeneration occurs only when circulating ecdysteroid titers are low (Hopkins, 1983; 1989). Transition from basal growth to proecdysial growth of the limb bud is associated with a small peak of ecdysteroids (40 pg/µl) (Hopkins, 1989, 2001). The competency of limb buds to grow following the small peak during stage D_0 depends on the reduction of circulating ecdysteroid titers. When the crab is preparing for ecdysis, the ecdysteroid titers are at a maximum level. Various physiological processes like apolysis and synthesis of new cuticle occur during this time, which implies that the increase in ecdysteroid titers plays an important role in mediating these physiological processes during pre-molt ($D_{1\text{-}4}$). In

some brachyuran crabs, it has been observed that multiple autotomy (loss of five or more limbs) during the intermolt stage of the molt cycle results in precocious molts (Skinner and Graham, 1972; Hopkins, 1982). This implies that there is feedback from the regenerating limb buds leading to changes in circulating ecdysteroids, thereby shortening the molt cycle period (see Mykles, 2001, for review). The requirement for low ecdysteroid titers during basal growth phase (Hopkins, 1986) and the fluctuations in titer at early and late proecdysial growth phase suggest that steroid hormones regulate limb regeneration, but the consequence of ecdysteroid receptor signaling disruption has not been studied in this model system.

Like the vertebrate steroid hormones, ecdysteroids mediate their effect by binding to their cognate nuclear receptors (Segraves, 1994; Riddiford et al., 2000; Nakagawa and Henrich, 2009; Fahrbach et al., 2012, for review). Nuclear receptors are transcription factors that bind to specific paired six base recognition sites within gene promoters called the Hormone Response Element (HRE) and then regulate gene expression (Tsai and O'Malley, 1994) through recruitment of nuclear proteins modifying chromatin architecture (see Carlberg and Seuter 2010; Kato and Fujiki 2011, for review). It has also been observed that many nuclear receptors, like thyroid hormone and retinoic acid receptors, act as strong repressors of gene expression in the absence of ligand (Hörlein et al., 1995; Chen and Evans, 1995). In *Uca,* both *EcR* and *RXR* mRNAs are present in blastema and proecdysial limb buds during limb regeneration as detected by Northern blot and ribonuclease protection assay (Durica et al., 2002). Immunohistochemical staining of blastema and regenerating limb have shown that EcR and RXR are co-localized in nuclei of epidermal, blood and muscle

cells (Hopkins et al. 1999). The presence of both receptor mRNA and protein in the blastema suggest that ecdysteroids or their receptors may regulate blastema formation.

The use of classical genetic approaches in crab models is impractical given the life history of the organism. Raising crabs to sexual maturity has had limited success (Shelley and Lovatelli, 2011) and crabs have a longer life cycle relative to established genetic models (Berrill, 1982). To overcome these difficulties, RNA interference (RNAi) can be used to silence specific genes post-transcriptionally (Meister and Tuschl, 2004; Tijsterman and Plasterk, 2004). Over the past few years RNAi, or double stranded (ds) RNA, has been used to knockdown target gene expression in various metazoans ranging from nematodes to mice (Fraser et al., 2000; Musatov et al., 2006). Although the use of RNAi to study effects of target gene silencing has not been reported in brachyuran crabs, crustaceans like *Artemia*, shrimp and crayfish have recently proven to be successful models to knockdown genes by using RNA interference (Copf et al., 2006; Tiu and Chan, 2007; Rijiravanich et al. 2008; Shechter et al.; 2008; Hui et al., 2008, Priya et al., 2010; Kato et al., 2011).

We report here that RNAi can be successfully used in brachyuran crabs to investigate the morphological and physiological consequences of target *EcR* and *RXR* gene knockdown. We developed a dsRNA microinjection protocol to knockdown *EcR*/*RXR* transcript levels in the developing blastema to investigate the role of ecdysteroid receptor signaling during limb regeneration. Disrupting *EcR/RXR* mRNA levels resulted in developmental 'arrest' of growth during early blastemal development, although a small fraction of the blastemas that were injected with ds*EcR*/ds*RXR* progressed towards later phases of limb regeneration. Examination of

the ‘arrested’ blastema phenotype revealed that the epidermal cells were not actively dividing. In addition, the ds*EcR*/ds*RXR* injected blastemas that emerged and formed papilla progressed significantly slower towards later phases of limb regeneration than control injected limb buds. RNAi has a long term silencing effect (up to 24 days post final injection) on the ds*EcR*/ds*RXR* injected blastemas, compared to injected blastemas of control crabs. Three observations also indicated that local RNAi injections also produced systemic phenotypic effects: 1) contralateral uninjected limbs in experimental animals also showed blocked blastemal differentiation; 2) we noticed a significant decrease in the ecdysteroid titers in experimental relative to control animals during emergence and basal growth, although proecdysial titers in ds*EcR*/ds*RXR* injected groups returned to control levels; 3) the ds*EcR*/ds*RXR* treated crabs failed to molt. In summary, local RNAi treatment generates receptor transcript knockdown, resulting in obstruction of early blastemal cell proliferation during basal growth, at a critical period of normally low circulating ecdysteroid titers. This is accompanied by a systemic signal evidenced by contralateral limb bud involvement, initial drop in circulating titer, subsequent titer recovery, but general failure to molt. Taken together these results indicate that *EcR* and *RXR* receptor signaling is essential during early blastemal development, mediating cellular proliferation and limb bud growth.

2. Materials and methods

2.1 Animal preparation

Male fiddler crabs were obtained from the Gulf Specimen Co., Panacea, Florida. They were acclimatized for a week in the laboratory under a constant temperature of 25°C and a 14:10 hour light:dark cycle. Crabs were kept separate from one another in plastic boxes containing artificial sea water (Instant Ocean, specific gravity: 1.02). Eyestalks were ablated (ESA) prior to multiple autotomy (MA) by cutting the articulating membrane at the base of the eyestalk with a pair of dissecting scissors. Although MA of five limbs results in precocious molt (Skinner and Graham, 1972**),** ESA removes the X organ, a source of molt inhibiting neuropeptides (see Lachaise et al. 1993; Chang and Mykles, 2011, for review), further accelerating the molt cycle and providing the synchrony necessary for collecting appropriately staged tissues. The eyestalk ablations were performed a day before MA to reduce mortality. Four walking legs and the large cheliped were autotomized from each crab by pinching the merus with a forcep. Data are expressed in number of days after autotomy (A + number of days).

2.2 Synthesis of dsRNAs and injection

Single stranded RNA was synthesized from full length cDNA clones of *EcR* (1557 bp) and *RXR* (1398 bp), that were inserted in opposite orientations in pBluescript plasmids, by *in vitro* transcription with T7 polymerase (Promega). These

clones share sequence overlap with all known receptor isoforms (Durica et al., 2002). Following transcription, plasmid DNA was removed by treating with RQI DNase (Qiagen). The quality of dsRNA was monitored by agarose gel electrophoresis. Equimolar amounts of complementary RNA (ds*EcR*, ds*RXR*), as quantified spectrophotometrically, were annealed by heating at 70°C and gradually cooling to room temperature. The final concentration of both ds*EcR* and ds*RXR* was adjusted to 2 µg/µl.

Following limb autotomy, a total of 420 ng of dsRNA mixture, containing equal concentration of ds*EcR/*ds*RXR,* was injected two times by using a Nanoject II auto nanoliter injector (Drummond scientific Co) into the cavity underneath the autotomy membrane of the claw and the third ipsilateral walking leg. Two different injection protocols were carried out depending on timing of injections: either on day one (A+1) and day four (A+4) (= 'A1/4 injection') or on day four (A+4) and day seven (A+7) (= 'A4/7 injection') following autotomy (A). Injections consisted of nuclear receptor RNAi, RNase free water or a control RNAi complementary to a full-length green fluorescent protein encoding transcript (ds*EGFP;* 720 bp; Life Technologies) that was transcribed as described above and was injected (420 ng) in two sessions into the blastemal chamber.

2.3 Analysis of RNAi phenotypes

The relative ecdysteroid receptor (*EcR* and *RXR*) transcript abundance was examined from control and experimental limb regenerates via q-PCR. The limb

regenerates were dissected and stored in RNAlater. The injected claw and third walking limb blastemas constituted a single pooled sample, and for this study are designated the ipsilateral side. The uninjected contralateral second and third walking limb regenerates were also pooled to analyze contralateral transcript abundance in both experimental and control crabs. Previous reports on limb bud growth rate were generated using the third walking leg in *Uca*. This gave us platform to compare the growth rate of limb buds following RNAi injection. The large cheliped blastema was injected to ensure sufficient RNA can be extracted for q-PCR. The feeding claw was not used as contralateral control, since it might interfere with the feeding ability of the crab. The methodology for relative RNA quantification for q-PCR is described in Durica et al. (2006). 18S ribosomal transcript was used to standardize for RNA input. Primers for *EcR*, *RXR* and 18S, as well as controls for non-specific RNAi effect (*E75* and *GAPDH*) are given in Table 1.

Hemolymph was collected at several different stages of the molt cycle to measure the ecdysteroid titers via radioimmunoassay (RIA; Hopkins, 1983). Crabs were bled using a syringe with 26.5 gauge needle. The needle was inserted into the blood sinus at the base of the feeding claw through the arthrodial membrane. Ecdysteroids were detected using antibodies raised against 20 hydroxyecdysone (Cocalico Biologicals, CA). Radio-labeled [^{3}H]-Ponasterone A (Perkin Elmer, Boston, MA) was used to generate a standard curve. Ecdysteroid titer was expressed in 20E equivalents per µl of hemolymph.

Limb regenerates at different stages, including blastema, papilla, and proecdysial limb buds were harvested from the experimental and control groups and

fixed in Lillie's fixative (picric acid:formaldehyde:formic acid – 17:2:1, by volume) for histological analysis. Hematoxylin and Eosin staining were performed to identify phenotypic effects accompanying receptor knockdown (Hopkins and Durica, 1995). Cuticle width was measured from digital images using tpsdig 2.12 software (Rohlf, 2008). Two landmarks were placed at the thickest area of the cuticle in both unemerged control and experimental blastema sections. To correct for varying sizes of crabs, the cuticle width measurement was divided by the distance between the coxa edges, i.e., the diameter of the coxa. Coxa diameter was measured using two landmarks placed at the coxa edges. Digital images used for measuring cuticle thickness were taken at 40X magnification.

Limb bud growth was monitored throughout the molt cycle for statistical differences between the control and experimental groups. Limb bud growth was quantified by measuring a regenerate's R-value (length of limb bud divided by carapace width X 100; Bliss, 1956).

To examine the proliferation of epidermal cells during blastema and papilla formation, a BrdU proliferation assay was used. Blastemas from four control and four experimental crabs were sampled for this assay. BrdU was purchased from Sigma Aldrich and a mouse antibody to BrdU was obtained from Life Technologies. Goat anti-mouse Alexa 488 conjugated IgG (Life Technologies) was used as secondary antibody to detect BrdU. BrdU was dissolved in saline and 50 µl (30 µg/g body weight) was injected into the hemolymph through the arthrodial membrane of the second walking leg. The limb regenerates from the A1/4 injection protocol were collected 24 hours later and fixed in Lillie's fixative for 24 hours. Following paraffin

embedding and sectioning (6-10 µm thickness), the sections were mounted on slides. The sections were processed for immunocytochemical detection of BrdU. The sections were hydrated through a graded ethanol series and incubated in phosphate buffered saline (PBS; NaCl – 8 g, KCl – 0.2 g, Na_2HPO_4 – 1.44 g, KH_2PO_4 – 0.24 g in 1liter of water, pH 7.4) for an hour. The DNA was then denatured for 30 min using 2N HCl at 37^0C followed by neutralizing the sections in 0.1M borate buffer, pH 8.5. To avoid tissue section loss, we mounted the sections on positively charged slides (Fisher Scientific) or gelatin subbed slides. Non–specific staining was blocked by incubating the sections in 5% goat serum and 1% milk for 45 min at room temperature (RT). The sections were then incubated with mouse anti-BrdU overnight at 4^0C. The next day, the sections were washed in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with Alexa 488 conjugated secondary antibody for two hours (RT). Following TBST washes, the sections were mounted using Vectashield (Promega) and were examined under a fluorescent microscope.

3. Results

3.1. Knockdown of EcR and RXR receptors via RNAi

RNAi uses endogenous cellular machinery to down-regulate complimentary mRNA, degrading homolog mRNAs that are perfectly matched, thereby reducing concomitant mRNA translation in cells (Srivastava and Srivastava, 2008). Using q-PCR we monitored the *EcR* and *RXR* transcript abundance at 48 hours post final injection in the blastemas collected from two injection protocols, (see experimental

methods) at 6 days and 9 days following autotomy, respectively. In both protocols, the receptor transcript abundance in ds*EcR*/ds*RXR* injected blastemas were significantly knocked-down to 34% - 50% of that in control injected blastemas (Figure 1A and 1B). Upon examining receptor abundance in the uninjected contralateral limb regenerates in experimental animals, we also observed statistically significant knockdown of receptors in both A1/4 (data not shown) and A4/7 protocol (Figure 1C), indicating the transcript knockdown was not localized to the injection site but was systemic in effect. At A+18 (14 days post final injection, A1/4 injection protocol), q-PCR results showed that in both injected and uninjected contralateral limb regenerates, the receptors remained significantly down-regulated relative to controls, indicating a prolonged effect of RNAi (Figure 2A, B). The A+18 limbs buds collected from the controls were in basal growth phase. The average R-value, a normalized measure of limb bud growth, for control limb bud papillae was 4.9 (±0.8), whereas for this experimental cohort the ds*EcR*/ds*RXR* injected limb regenerates were 'arrested' (i.e., R-value = 0) and still located internally beneath the scab at the wound site. We further tested whether the RNAi that was injected in blastema stage resulted in extended knockdown of receptors in proecdysial phase. For this purpose we collected RNA from arrested experimental limb regenerates and control ds*EGFP* injected third walking legs with an average R-value of 18.3 (±0.8). Both *EcR* and *RXR* mRNA levels from unemerged buds remained lowered even at A+30 (26 days post final injection from A1/4 injection protocol, Figure 2C). To analyze whether ds*EcR*/ds*RXR* RNAi may have a non-specific inhibitory effect on transcription, we examined transcript abundance of the housekeeping gene, *GAPDH* (A+9, A+18, A+30; Figure

3A) and another related nuclear receptor which in insects is an early response gene in the ecdysteroid cascade pathway, *E75* (Figure 3B). Following injection of RNAi, we did not observe any significant differences in *GAPDH* and *E75* transcript abundance in injected limb regenerates relative to controls.

3.2. Fluctuating ecdysteroid titers during molt cycle

We examined circulating hormone levels 48 hours post final injection. Ecdysteroid levels remained low in ds*EGFP* injected controls (Table 2A and C) or water injected control crabs (Table 2B). Circulating ecdysteroid titers in the experimental crabs injected with ds*EcR*/ds*RXR*, however, were significantly further lowered from both the A1/4 injection schedule (Table 2A) and A4/7 injection schedule (Table 2B, C). The hormone levels from A1/4 protocol animals were also examined later in the molt cycle, when the crabs were in the late proecdysial phase (A+28). In these premolt animals, the ecdysteroid levels in the experimental animals were now comparable to the controls at 24 days post second injection (A+28) (Table 2D).

3.3. Phenotype associated with receptor transcript knockdown: failure of blastema to emerge as papilla

Emergence of the limb buds as papilla is associated with mitotic division of epidermal cells in the blastema and differentiation of the limb primordium into limb segments. Table 3A and 3B documents the number of emerged limb regenerates

following injection of RNAi in both injection regimens. Emergence of control blastemas was greater than 96% of those injected. In the A1/4 injection protocol, however, the emergence of ds*EcR*/ds*RXR* injected claw and third walking leg blastemas were 19% and 6% respectively. In experimental animals from the A4/7 injection protocol, 41% of the ds*EcR*/ds*RXR* injected blastemas failed to emerge. Importantly, the uninjected contralateral limb blastema in crabs injected with ds*EcR*/ds*RXR* also showed a decreased percentage of emergence relative to the contralateral control counterpart. These results suggest that the lack of limb bud progression results from a systemic knockdown of receptor transcripts in the experimental animals (Figure 1B, 2B) and RNAi may not be confined in the injected blastema.

Two additional observations were made regarding the penetrance of RNAi. Firstly, 87% of ds*EcR*/ds*RXR* injected limb regenerates (combined claw and ipsilateral walking leg) did not emerge following the A1/4 injection protocol compared to 59% ipsilateral limb regenerates from the A4/7 injection protocol, suggesting that earlier receptor knockdown increased penetrance. Secondly, in the A1/4 injection protocol, there was a difference in growth rate of dsRNA injected limb regenerates relative to the uninjected contralateral limbs that 'escaped' the effects of receptor knockdown and emerged. The ds*EcR*/ds*RXR* injected ipsilateral limb buds that emerged had a slower growth rate compared to control ds*EGFP* injected ipsilateral limb buds and at A+32, the former reached a plateau at an average R-value of 10.5 (±1.4), significantly smaller than controls [R-value 19.3 (±0.5)] ($p<0.001$ at A+32; tested via two-tailed, unpaired t-test, Figure 4A**).** The emerged uninjected contralateral limb buds, however,

had a similar growth rate in both ds*EcR* and ds*RXR* and ds*EGFP* injected crabs (Figure 4B). These results suggest that higher penetrance of RNAi occurred when it was injected earlier during limb regeneration and that higher penetrance in growth inhibition was correlated with its proximity to the site of injection.

3.4 Histology of injected limb regenerates

To analyze the histological properties of the arrested blastema from ds*EcR/*ds*RXR* injected crabs, we stained histological sections of the blastemas from the A4/7 injection protocol with Hematoxylin and Eosin. As mentioned above, following autotomy, the epidermal cells from the coxal wall start migrating underneath the scab and begin mitosis. These dividing cells secrete a very thin cuticle underneath the scab. The first segment of the limb is formed by the invagination of this cuticle. Cuticular invagination occurs as early as seven days following autotomy in the control limb regenerates (Figure 5A) (Hopkins et al., 1999). Cellular proliferation of the control limb regenerates led to formation of papilla by A+11 (Figure 5B). Histological analysis revealed that there was a lack of cuticular invaginations in the arrested ds*EcR/*ds*RXR* injected limb regenerates (Figure 5D, E and F). Prior to emergence (samples collected before A+11), epidermal cells of unemerged control limb blastema secreted a thin layer of cuticle (labeled as C in Figure 5A). A significantly thicker cuticle deposition was observed in arrested experimental limb regenerates indicated by a double-headed arrow in figures 5D-F. The average ratio of cuticle width relative to coxal diameter in control (N=13) and

experimental (N=17) blastemas were 0.03 (±0.006) and 0.18 (±0.016) respectively ($p=<0.001$; two-tailed, unpaired t-test). The arrested ds*EcR*/ds*RXR* injected blastema population showed no development and even at the late proecdysial stage, characterized by rising ecdysteroid titers, remained undifferentiated (A+39, Figure 5F).

As noted above, the A4/7 injection protocol showed lower penetrance, where approximately 41% of the emerged ds*EcR*/ds*RXR* injected claws and third walking legs continued to grow. The ds*EcR*/ds*RXR* injected blastemas that progressed towards papilla stage were histologically similar to their control counterparts. In emerged buds in both injection protocols we observed segmentation, cuticle sac and muscle tissue formation (data not shown). This suggests that limbs that begin to emerge are capable of proximal/distal segment specification, differentiation and growth.

3.5 Cellular proliferation assay

The lack of papilla formation in the arrested blastema could be due to cell loss, lack of cellular proliferation or both. Previously, mitotic figures have been reported in normally developing blastemas (Hopkins, 1993), suggesting that the epidermal cells divide leading to the formation of a basal limb bud. The cellular proliferation assay (Figure 6) indicated that there was a lack of cell division in the ds*EcR*/ds*RXR* injected arrested blastemas (N=4 animals) when compared to the control emerged blastemas (N=4 animals). In the controls (ds*EGFP* injected blastemas) we observed cell division in the epidermal cells underneath the scab as well as in cells along the nerve (Figure

6A and C). Although we have observed the presence of immigrant epidermal cells underneath the scab in the experimental animals, suggesting normal migration, these cells failed to divide and form the differentiating blastema. We cannot at this point rule out the possibility of cell death contributing to the experimental arrested blastema phenotype. The results of the proliferation assay, however, suggests that down-regulation of the ecdysteroid receptor transcripts in the experimental blastemas caused a block in cell division that was initiated early in the developmental process of limb regeneration.

3.6. Molt cycle period and molting success

It has been reported previously that the average molt cycle length in fiddler crabs following multiple autotomy is 32.4 (±0.9) days and following ESA is 22.7 (±0.9) (Hopkins, 1982). The molt cycle period for control ds*EGFP* or water injected animals averaged 33.9 (±0.9) and 36.7(±1.1) days respectively. The ds*EcR*/ds*RXR* injected animals from A1/4 and A4/7, however, survived an average period of 43.4 (±2.5) and 48.35 (±2.4) days respectively and >97% failed to molt and died (Table 4A and 4B). This is a statistically significant prolongation in the duration of the cycle but without a successful molt ($p=<0.001$; two-tailed, unpaired t-test). We consider the period between multiple autotomy and death of ds*EcR*/ds*RXR* injected crabs as a defect in molting, since their cohorts have undergone ecdysis and the experimental crabs, measured at A+28, have late proecdysial levels of hormone titers comparable to controls. The failure of ds*EcR*/ds*RXR* injected crabs to molt again suggests a sustained

systemic RNAi effect relating to an inability to correctly respond to hormonal signaling at the end of the molt cycle.

4. Discussion

This study, using RNAi as a tool to knockdown ecdysteroid receptor function *in vivo*, examines the effects of disrupting the ecdysteroid signaling pathway during crustacean limb regeneration. Previous studies have reported the presence of both *EcR* and *RXR* mRNA at all phases of limb regeneration, including the earliest stages of the basal growth phase (Chung et al., 1998; Durica et al., 1999; Durica et al., 2002). Northern blot and ribonuclease protection assay revealed that during blastema formation both *EcR* and *RXR* transcripts are expressed at low levels and their relative expression increase during the early proecdysial phase, followed by a drop at late proecdysial phase (Durica et al., 2002). Circulating ecdysteroid titers, measured via RIA, are low during the basal and early proecdysial growth phase when the blastema proliferates and undergoes segmentation, and increase during the late proecdysial growth phase of limb regeneration, when the animal prepares for molt (Hopkins, 1989; 2001). The presence of both receptors and hormones, albeit at low levels during basal growth phase, suggests that functional ecdysteroid receptor is necessary for successful regeneration of limbs, and may be required at all stages of the regeneration process. In this paper we have established that a critical steady-state level of ecdysteroid receptor transcripts is required for the proliferation and subsequent differentiation of blastema cells. Following knockdown of the ecdysteroid receptor transcripts at a very early

phase of regeneration, a majority of animals were blocked at the earliest stages of blastemal proliferation and limb bud morphological development, thus failing to generate a functional limb.

This is the first report of successful utilization of RNAi to knockdown endogenous gene expression in a brachyuran crab. We have demonstrated that the limb blastema of *Uca pugilator* is responsive to ds*EcR*/ds*RXR* mediated knockdown and results in a disruption of blastemal development. One of the components of this study was to examine the timeline of ecdysteroid receptor down-regulation. Our results showed that both *EcR* and *RXR* transcript abundance were significantly lowered as early as 48 hours post treatment and the genes remained silenced as late as A+30 in post-treatment blastemas. In *C. elegans*, the silencing effects of a single injection of RNAi may be inherited indefinitely in about 30% of the progeny and this effect is not completely penetrant (Vastenhouw et al., 2006). These authors identified histone modifying proteins as mediators of gene silencing, and the silencing is relieved in the presence of a histone deacetylase inhibitor. *C. elegans* data suggest that RNAi induced silencing is due to chromatin remodeling and silencing is effected at the transcriptional level. We observe a long-term depression in receptor transcript levels relative to controls; this effect may be due to either translational control via RNAi induced activation of the internal RISC machinery (Paddison and Hannon, 2002), and/or the *EcR* and *RXR* genes undergoing chromatin remodeling (Vastenhouw et al., 2006). Additionally, down-regulation of *GAPDH* and *E75* transcripts were not observed in experimental crabs, supporting sequence specificity of RNAi to down-regulate *EcR* and *RXR*. Although *E75* is an ecdysteroid inducible gene in *Uca* (Durica

et al., in preparation) measurements were conducted in experimental animals when circulating ecdysteroid titers were low [15.7 pg/µl (±4.4)]. The lack of down-regulation of a house-keeping gene and a related nuclear receptor in experimental crabs, together with the ds*EGFP* controls, indicate target specificity for the ecdysteroid receptor knockdowns.

In this study we noted an early systemic effect of RNAi (48 hours post final injection) based on two observations: receptor transcript down-regulation in uninjected contralateral limb regenerates and lowering of ecdysteroid titers following RNAi injection. The *EcR* and *RXR* transcript levels in the uninjected contralateral limb regenerates were monitored at 48 hours and 14 days post final RNAi treatment. Similar to the results from the injected sites, receptor transcript levels were significantly down-regulated in the contralateral limbs of ds*EcR*/ds*RXR* injected crabs compared to their control counterparts. Correlated with a 50% reduction in the receptor transcript abundance, in both injection protocols, we also observed a significant decrease in circulating ecdysteroid titers in dsEcR/dsRXR injected crabs, at 48 hours post final injection. The injection site for introducing ds*EcR*/ds*RXR* is a cavity in the limb coxa that is surrounded by a connective tissue membrane called the autotomy membrane (Hopkins, 1993; 2001). Initially we reasoned that the presence of this autotomy membrane might restrict RNAi within the cavity. One explanation for a systemic, environmental effect is RNAi leakage from the injected blastema into the open circulatory system and uptake by other tissues, such as the contralateral limb and Y organ, the site of ecdysteroid biosynthesis. Alternatively, injection may trigger the

production of a secondary, secreted factor responsible for systemic regulation, discussed below.

Three lines of evidence suggest that a low ecdysteroid titer is required for blastema formation. First, it has been reported that exogenous infusion of ecdysteroids in the crab *Gecarcinus lateralis* during the basal growth phase results in the hindrance of limb regeneration (Hopkins et al., 1979). Second, if a limb is lost or autotomized during the late proecdysial stage of the molt cycle (when the hormone titers are high), it will fail to form a blastema and only regenerate in the next molt cycle (Hopkins, 1983). Third, in *Gecarcinus lateralis*, autotomy of a partially regenerated limb bud in multiply autotomized animals during the early proecdysial phase (before the critical rise of high ecdysteroid titers) results in delayed molting and a lowering of ecdysteroid titers. This facilitates blastema formation in the secondary autotomized limb and a coordination of growth with the primary autotomized limbs, allowing the crab to regenerate all its limbs within a single molt cycle (Yu et al., 2002). Although ecdysteroid titers are normally low during basal growth, the further lowering of circulating ecdysteroid titers that accompanies RNAi treatment could possibly contribute to problems with blastemal outgrowth, suggesting some critical concentration required for blastemal proliferation. Lowering of ecdysteroid titers has also been observed in *Blattella germanica* nymphs, where injection of either ds*EcR* or ds*RXR* resulted in reduced ecdysteroid titers (Cruz et al., 2006 and Martin et al., 2006). As noted above, two explanations can be postulated regarding lowering of ecdysteroid titers following ds*EcR*/ds*RXR* injection. First, systemic RNAi released from the injection site might silence the receptors at the site of ecdysteroid synthesis,

the Y organs, resulting in lowered production of ecdysteroids and decreased hormone release into the circulation. Second, RNAi induced down-regulation of receptors in limb regenerates might secondarily cross-talk with a signaling pathway that facilitates reduction of hormone titers. Insulin-like peptide 8 (dilp8), released from damaged or regenerating imaginal discs of *Drosophila* larvae, was recently shown to impair ecdysteroidogenic enzyme synthesis, thereby delaying 20E production (Garelli et al., 2012; Colombani et al., 2012). Dilp8 is involved in the coordination of growth and allows the damaged tissues to recover and mature before the larvae undergo metamorphosis and molting, a situation paralleling the effect of secondary limb bud autotomy in crabs (see Mykles, 2001, for review).

The initial lowering of ecdysteroid titers following RNAi injection was not sustained throughout the molt cycle. In ds*EcR*/ds*RXR* injected crabs, at 28 days post autotomy, we observed a recovery of blood hormone titers to the control late proecdysial phase ecdysteroid titers, although receptor transcript levels remained depressed relative to controls and the experimental animals failed to molt and died. There are several lines of evidence that suggest molt cycle-dependent changes occur in Y organ regulation in brachyuran crabs. For example, the Y-organ is sensitive to inhibitory neuropeptides like molt inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH) early in the normal molt cycle, but loses sensitivity to these neuropeptides at mid proecdysis (see Chang and Mykles, 2011, for review). Losing sensitivity to MIH causes the rise in hormone titers during proecdysis. Furthermore, successful molting occurs when the titers decline sharply right before ecdysis (Hopkins, 1992). This decline is believed to result from a negative feedback

effect of ecdysteroids on the Y-organ (see Chang and Mykles, 2011, for review). In ds*EcR*/ds*RXR* injected crabs it is possible that the rise in ecdysteroid titers to levels comparable to controls is associated with Y organ loss of sensitivity to MIH. These animals, however, do not progress to molt, and subsequently die without undergoing apolysis of the old cuticle. The inability of experimental crabs to molt suggests that hormone titers might not decline to levels necessary for successful ecdysis, and the ability to enter molt is compromised by disruption of the ecdysteroid signaling pathway.

In *C. elegans*, the inheritance of long term RNAi-silencing in the progeny was not fully penetrant (Vastenhouw et al., 2006). Similarly, a block in blastemal proliferation was not observed in 100% of the experimental animals in these experiments, although penetrance was higher in blastema receiving the injection, relative to the contralateral uninjected blastema. Although 13% of ds*EcR*/ds*RXR* injected blastemas from the A1/4 injection protocol progressed to form limb buds, the growth rate of these emerged injected limb regenerates was significantly slower than growth in the ds*EGFP* injected control counterpart. Correspondingly, 55% of uninjected contralateral blastemas emerged and formed a limb bud, which had similar growth rates to its uninjected control counterpart. These results indicate that penetrance is associated with the proximity of the injection site for both the proliferation and growth rate phenotypes.

Histological analyses of the arrested blastema phenotype demonstrated the presence of epidermal cells underneath the scab but a lack of cuticular invaginations. This suggests that the ability of epidermal cells to migrate from the coxal walls and

localize under the scab occurs independently of receptor complex signaling, but the ability to divide and differentiate is compromised by receptor knockdown. The blastema in *Uca* is formed by migratory epidermal and muscle cells and proliferation of the cells (Hopkins, 1993). The epidermal cells dedifferentiate and form the regenerating limb tissues, however, the extent of dedifferentiation is still unknown (Hopkins, 1993). The origin of muscle cells is also debatable. Some authors suggest that muscle tissue arises from a pool of reserve cells, while others suggest that dedifferentiation of immigrant cells gives rise to muscle (Adiyodi, 1972; Mittenthal, 1981). In axolotl, the blastema is considered to be a heterogeneous population of progenitor cells that have restricted dedifferentiation capability (Kragl et al., 2009). In *Drosophila*, the ablated imaginal disc regenerates via proliferation and regenerating imaginal discs express *wingless (wg)* and *d-myc* (see Repiso et al., 2011, for review). An ecdysone pulse drives cell cycle division during larval development in *Drosophila* (see Cranna and Quinn, 2009, for review). It has been proposed that in the wing imaginal disc, the ecdysone signaling pathway, via EcR and RXR, drives cell proliferation by inducing transcription factor CroI (Mitchell et. al, 2008; Cranna and Quinn, 2009, for review). CroI in turn inhibits the Wingless (Wg) pathway and increases expression of *d-myc* leading to G_1- S cell cycle transition. We are currently screening for *Uca* candidate gene homologs implicated in ecdysteroid-controlled cell proliferation to examine if this signaling pathway may be conserved.

Retinoids also play an important role in patterning of regenerating limbs in vertebrates as well as in fiddler crabs (Hopkins and Durica, 1995; Brockes, 1997, for review; Hopkins, 2001). In *Uca*, endogenous all-trans retinoic acid and 9-cis retinoic

acid have been isolated from A+4 blastemas and exogenous application of retinoids disrupts normal limb patterning and does not allow differentiation of blastemal cells (Hopkins and Durica, 1995; Hopkins et al., 2008). In regenerating zebrafish fin blastema, retinoic acid maintains proliferating cells via regulation of anti-apoptotic factor *bcl2* (Blum and Begemann, 2011). Whether *Uca* RXR may bind a putative retinoid ligand *in vivo* is unclear, but *in vitro* 9-cis retinoic acid binds to monomeric RXR and the presence of this ligand affects the binding of EcR to PonA (Hopkins et al., 2008). If the RXR is capable of mediating a retinoid signal, down-regulation of *RXR* transcripts in the blastema might also result in blockage of cell proliferation and differentiation. Thus, it is possible that both retinoic acid and ecdysteroid signaling pathways are important in maintaining cellular proliferation in blastema. Future research involving transcriptome comparisons of control and experimentally manipulated limb regenerates affecting ecdysteroid signaling will provide us with information regarding genes and signaling pathways downstream of these receptors.

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Table 1. Primer sequences for q-PCR.

Name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
EcR	CCAAGCAACTACCAGGGTTCG	TCGGATGAGCAAGCCTTGA
RXR	AACGAGTTGCTTATTGCCTCATT	CCAGCACGATGCCATCCT
18S	GCAGCAGGCACGCAAATTA	GGATGAGTCTCGCATCGTTATTTT
GAPDH	GGAGCCAAGAAGGTGGTCATC	TTGACGCCACACACAAACATT
E75	CAACTGCCGCAGGAGGAT	CACCAGCAACACCTCGAACA

Table 2. Ecdysteroid titers at **48 hours** post second injection.

2A. Injection protocol – A1/4

	Ecdysteroid titers (A+6)	**N**	**p-value (t-test)**
Control (ds*EGFP*)	11.4 (±1.3) pg/µl	62	<0.001
Experimental (ds*EcR*/ds*RXR*)	3.0 (±0.6) pg/µl	59	

2B. Injection protocol – A4/7

	Ecdysteroid titers (A+9)	**N**	**p-value (t-test)**
Control (RNAse-free water)	35.75 (±2.0) pg/µl	65	<0.001
Experimental (ds*EcR*/ds*RXR*)	15.20 (±1.9) pg/µl	68	

2C. Injection protocol – A4/7

	Ecdysteroid titers (A+9)	**N**	**p-value (t-test)**
Control (ds*EGFP*)	20.13 (±1.03) pg/µl	35	<0.001
Experimental (ds*EcR*/ds*RXR*)	8.91 (±0.83) pg/µl	35	

2D. **24 days** post second injection - A1/4 injection protocol

	Ecdysteroid titers (A+28)	**N**	**p-value (t-test)**
Control (ds*EGFP*)	52.08 (±5.64) pg/µl	9	0.72
Experimental (ds*EcR/RXR*)	49.02(±7.44) pg/µl	13	

Table 3A. Quantification of blastema emergence phenotype (A1/4 injection).

Limbs	Emergence criteria	Control (ds*EGFP*)	Experimental (ds*EcR*/ds*RXR*)	p-value (Chi-square)
Injected claw	Emerged	28	6	<0.001
	Unemerged	0	25	
Injected third walking leg	Emerged	27	2	<0.001
	Unemerged	1[a]	29	
Uninjected contralateral walking leg	Emerged	28	17	<0.001
	Unemerged	0	14	

[a]One ds*EGFP* injected crab failed to emerge one blastema.

Table 3B. Quantification of blastema emergence phenotype (A4/7 injection).

Limbs	Emergence Criteria	Control (water)	Control (ds*EGFP*)	Experimental (ds*EcR*/ds*RXR*)	p-value (Fisher's exact test)
Injected claw	Emerged	84	19	50	<0.001
	Unemerged	3[b]	0	52	
Injected third walking leg	Emerged	84	19	35	<0.001
	Unemerged	3[b]	0	67	
Uninjected contralateral walking leg	Emerged	84	19	54	<0.001
	Unemerged	3[b]	0	48	

[b]Seven RNase-free water injected crabs failed to emerge at least one blastema.

Table 4A. Quantification of crabs that molted following A1/4 injection.

Crabs	**Control (ds*EGFP*)**	**Experimental *(*ds*EcR*/ds*RXR)***	**p-value (Chi-square)**
Molted	20	0	
Failed to molt	0	31	<0.001
Total	20	31	

Table 4B. Quantification of crabs that molted following A4/7 injection.

Crabs	**Control (water)**	**Control (ds*EGFP*)**	**Experimental *(*ds*EcR*/ds*RXR)***	**p-value (Fisher's exact test)**
Molted	26	18	2	
Failed to molt	2	1	39	<0.001
Total	28	19	41	

Figure legends

Figure 1. Relative transcript abundance of *RXR* and *EcR* in blastema, 48 hours post second injection with both ds*EcR*/ds*RXR* (420 ng). 1A. In the A1/4 injection protocol we observed that at A+6, both *RXR* ($p<0.001$) and *EcR* ($p=0.02$) in injected blastemas (pooled claw and third walking limb) were significantly down-regulated when compared to the controls (N=8). 1B. In A4/7 injection protocol, quantification of *RXR* and *EcR* transcript abundance in injected blastemas (N=14) show significant knockdown of both *RXR* ($p<0.001$) and *EcR* ($p<0.001$) compared to its control group. 1C**.** The contralateral (pooled second and third walking limbs) uninjected blastemas from experimental animals (N=14) also show significant knockdown of both *RXR* ($p <0.001$) and *EcR* ($p <0.001$) compared to its control group indicating knockdown is not localized at point of RNAi injection. Two-tailed, unpaired t-test was used to perform all statistical analyses. The error bars indicate standard error.

Figure 2. Sustained down-regulation of *RXR* and *EcR* transcript abundance following RNAi treatment. At A+18 (2A, B) following A1/4 injection, receptor transcript abundance in both injected and contralateral uninjected limb blastemas (N=8) of experimental animals was 22% -40% of their respective control blastemas ($p<0.001$). 2C. Receptor knockdown was sustained in the ipsilateral proecdysial limb regenerates (A+30); both *RXR* (N=6, $p=0.002$) and *EcR* (N=6, $p<0.001$) transcript abundance were lowered in experimental limb regenerates. Two-tailed, unpaired t-test was used to perform all statistical analyses. The error bars indicate standard error.

Figure 3. Transcript abundance of *GAPDH* and *E75* in ipsilateral limb regenerates. 3A. At A+9 (N=7, p=0.67), A+18 (N=8, p=0.99) and A+30 (N=6, p=0.256) no significant difference in *GAPDH* mRNA levels were observed between arrested ds*EcR/*ds*RXR* injected and ds*EGFP* injected crabs. 3B. Relative *E75* transcript levels (N=8, p=0.84) at A+18 and did not show any significant difference between arrested experimental and control limb regenerates. Two-tailed, unpaired t-test was used to perform all statistical analyses. The error bars indicate standard error.

Figure 4. Regenerating limb bud growth (R-values) in experimental and control crabs which escaped basal growth arrest following A1/4 injection protocol. 4A. Growth pattern of injected ipsilateral limb buds following ds*EcR/*ds*RXR* (N=6) and ds*EGFP* (N=19) injection ($p<0.001$ at A+32, two-tailed, unpaired t-test). 4B. Growth pattern of contralateral (uninjected) limb buds following ds*EcR/*ds*RXR* (N=12) and ds*EGFP* (N=19) injection (p=0.54 at A+32, two-tailed, unpaired t-test). The error bars indicate standard error.

Figure 5. Histological paraffin sections of blastema stained with Hematoxylin and Eosin from A4/7 injection protocol. 5A and 5B are RNase-free water injected control blastema sections (10µm thick). 5D, 5E and 5F are experimental blastema sections injected with ds*EcR/*ds*RXR* (10µm thick). The epidermal cells (E) underneath the scab (S), fail to form cuticular invaginations (I) in the experimental arrested blastemas. Further, the blastemal cells (B) also fail to form papilla and emergence is not observed even after 30+ days post-autotomy (A). The experimental limb regenerates have a

significantly thicker cuticle (C) deposition underneath the scab (see text), indicated by a double-headed arrow in figures D, E and F. Scale bar: 100µm**.** Figure 5C represents a section of a late proecdysial fully grown limb bud (4 mm in width) that will give rise to a functional limb.

Figure 6. Cell proliferation assay of limb regenerates from A1/4 injection protocol. BrdU staining shows that in the ds*EGFP* injected blastemas (6A and 6C), the cells divide (N) along the nerve (Ne) and underneath the scab (S) in the area where the blastema (B) organizes. The ds*EcR*/ds*RXR* (6B and 6D) injected blastemas however, fail to proliferate. Figure 6A and 6D insets: Panoramic view of entire section of ds*EGFP* and ds*EcR*/ds*RXR* injected blastema sections reduced 5 and 8 fold respectively in comparison to figures 6A and 6D. Sections were examined using a Zeiss AxioImager fluorescent microscope.

Figure 1.

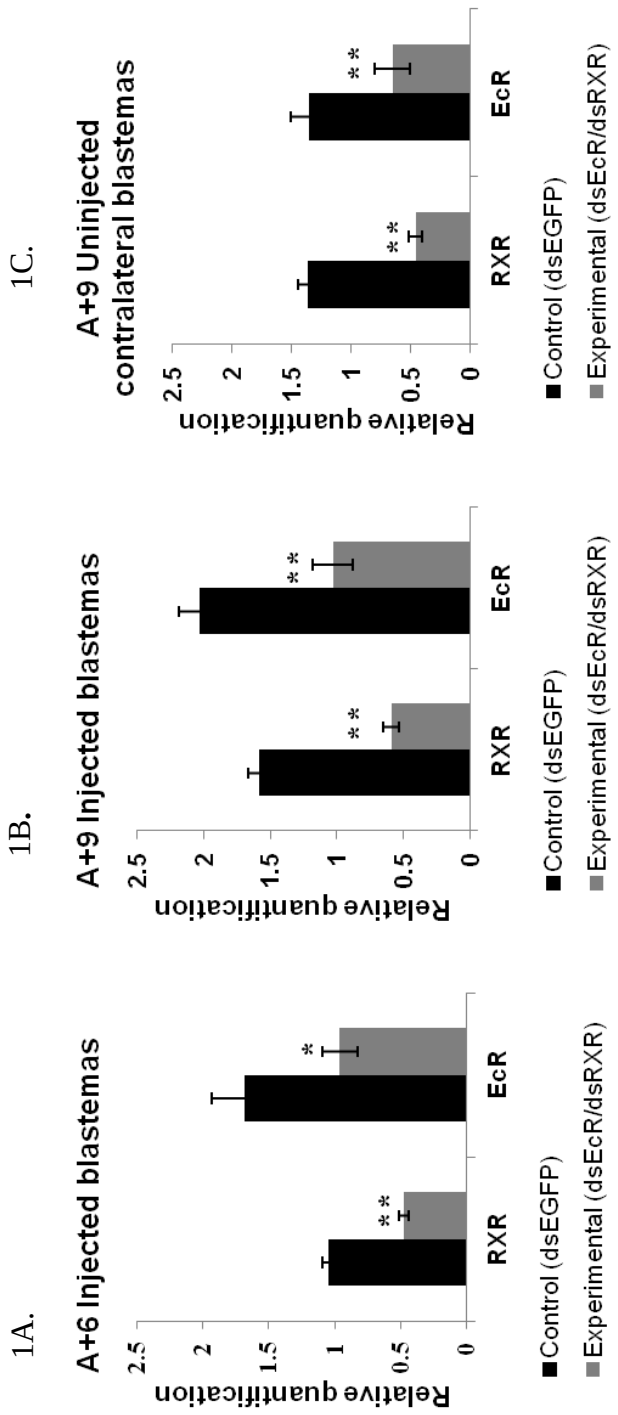

1A.
A+6 Injected blastemas
Relative quantification
2.5
2
1.5
1
0.5
0
**
*
RXR
EcR
Control (dsEGFP)
Experimental (dsEcR/dsRXR)
1B.
A+9 Injected blastemas
Relative quantification
2.5
2
1.5
1
0.5
0
**
**
RXR
EcR
Control (dsEGFP)
Experimental (dsEcR/dsRXR)
1C.
A+9 Uninjected contralateral blastemas
Relative quantification
2.5
2
1.5
1
0.5
0
**
**
RXR
EcR
Control (dsEGFP)
Experimental (dsEcR/dsRXR)

Figure 2.

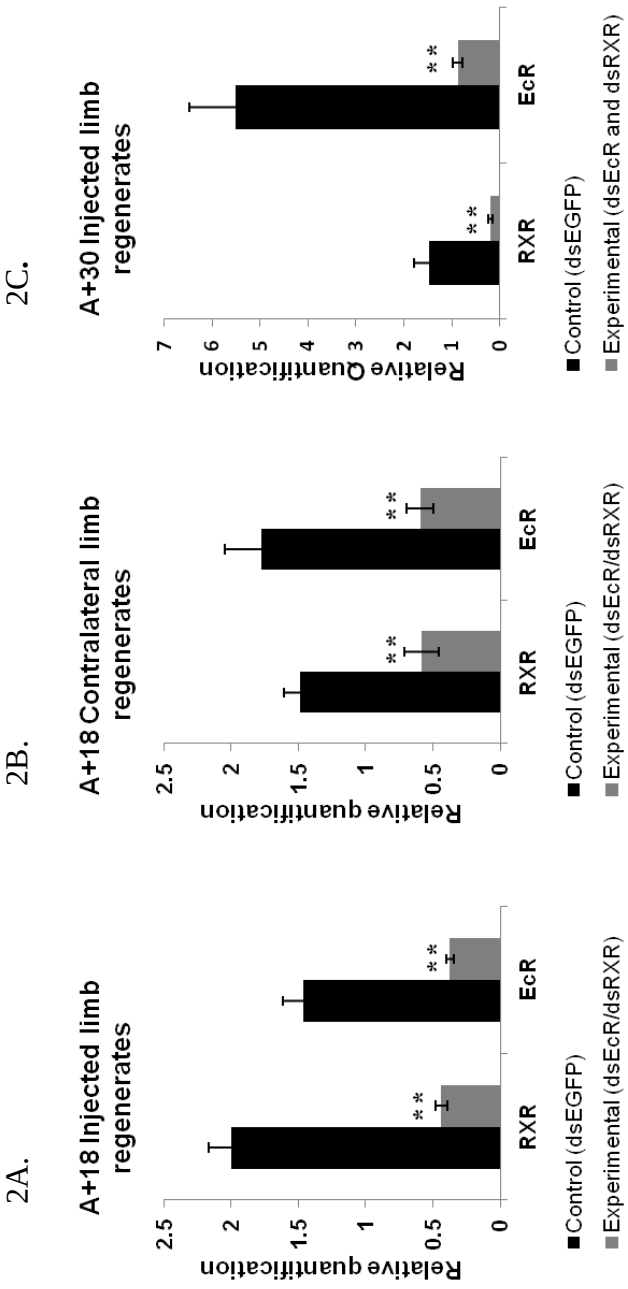
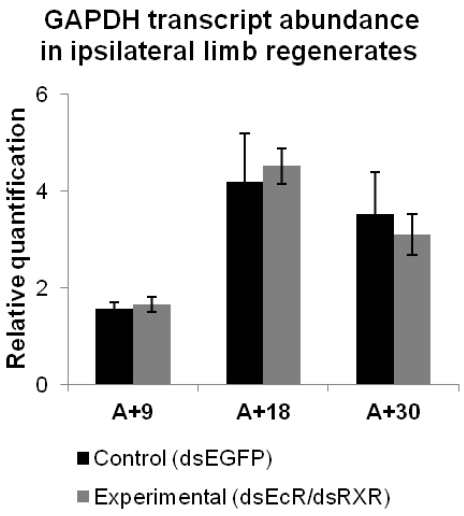

2A.
A+18 Injected limb regenerates
Relative quantification
2.5
2
1.5
1
0.5
0
**
**
RXR
EcR
Control (dsEGFP)
Experimental (dsEcR/dsRXR)
2B.
A+18 Contralateral limb regenerates
Relative quantification
2.5
2
1.5
1
0.5
0
**
**
RXR
EcR
Control (dsEGFP)
Experimental (dsEcR/dsRXR)
2C.
A+30 Injected limb regenerates
Relative Quantification
7
6
5
4
3
2
1
0
**
**
RXR
EcR
Control (dsEGFP)
Experimental (dsEcR and dsRXR)

Figure 3.

3A.



3B.

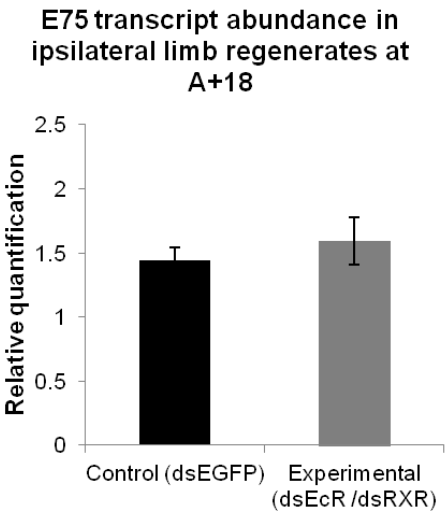


Figure 4.

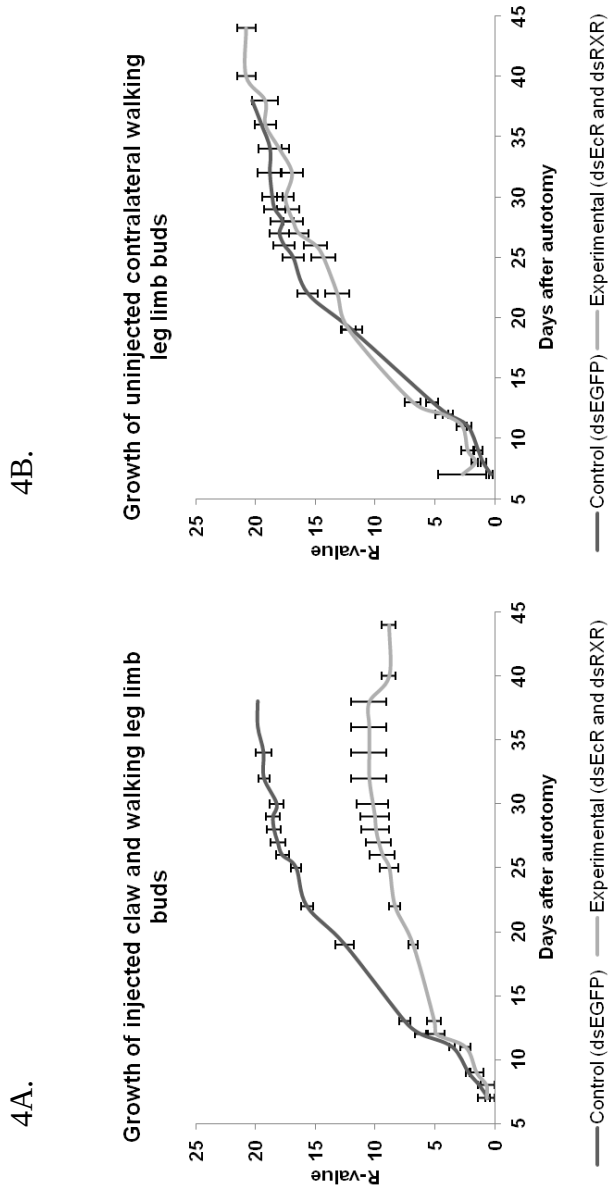
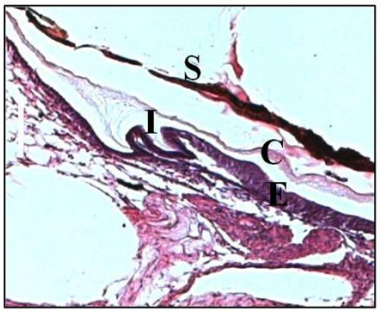
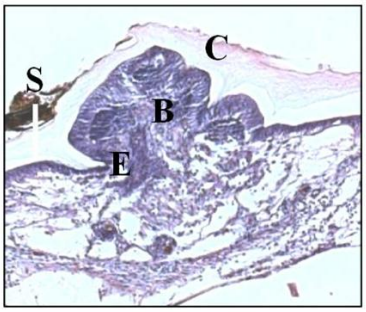

4A.
Growth of injected claw and walking leg limb buds
R-value
Days after autotomy
Control (dsEGFP)
Experimental (dsEcR and dsRXR)
4B.
Growth of uninjected contralateral walking leg limb buds
R-value
Days after autotomy
Control (dsEGFP)
Experimental (dsEcR and dsRXR)

Figure 5.

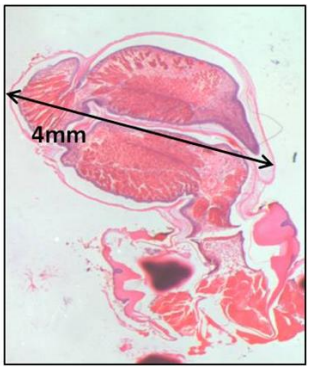
5A – A+7



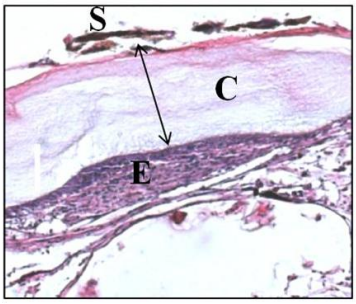
5B – A+11



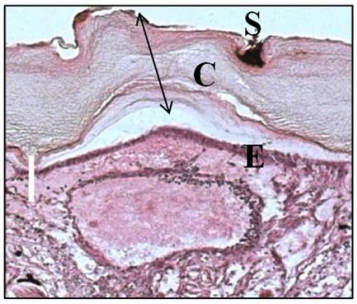
5C – A+40



5D – A+11



5E – A+30



5F – A+39

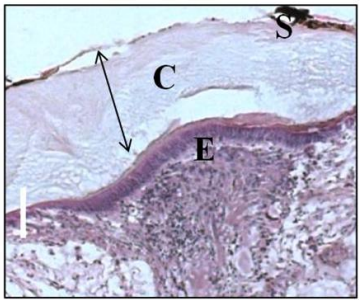
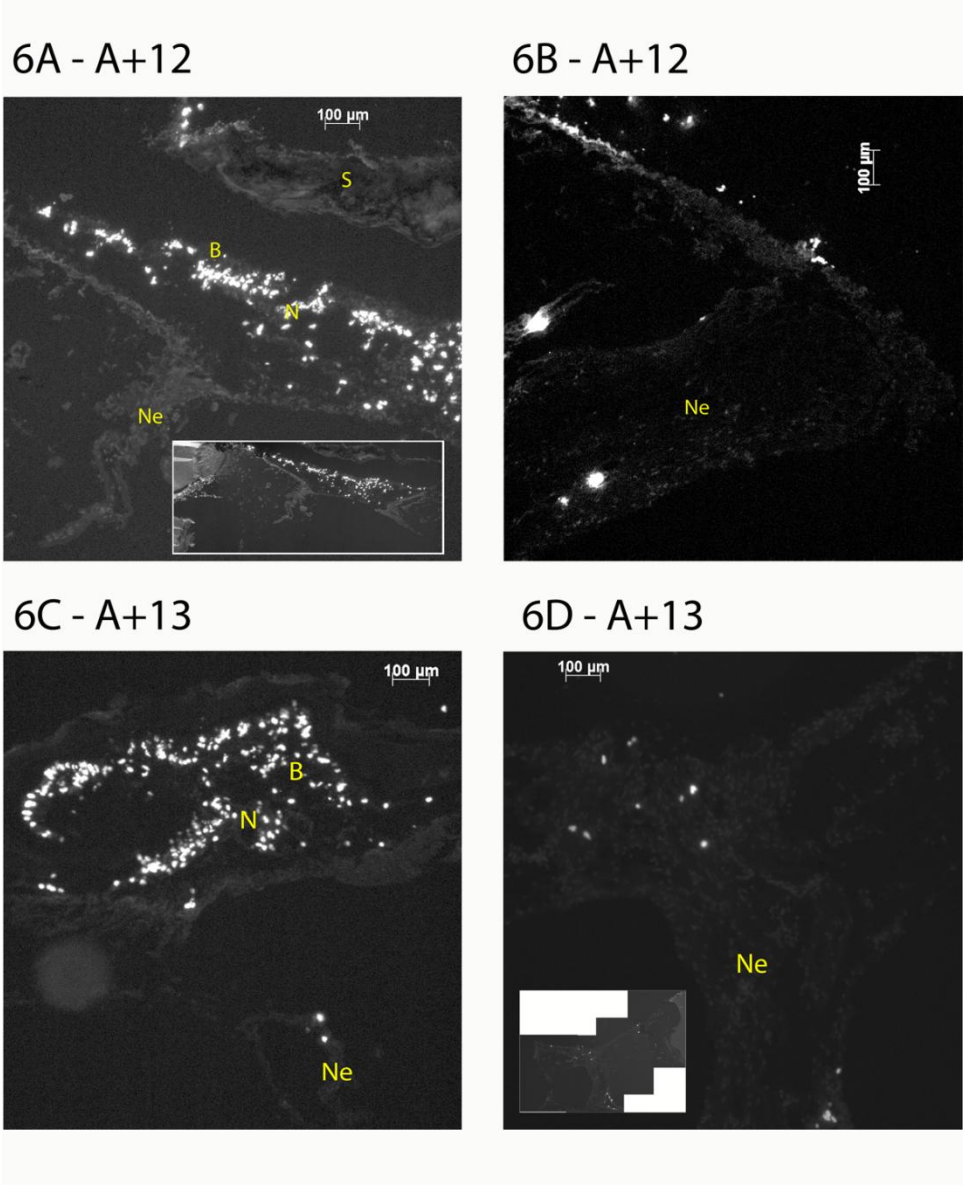


Figure 6.


6A - A+12
100 µm
S
B
N
Ne
6B - A+12
100 µm
Ne
6C - A+13
100 µm
B
N
Ne
6D - A+13
100 µm
Ne

CHAPTER III

Transcriptome profiling of crustacean stage-specific limb regenerates: alterations in gene expression linked to aberrant cell proliferation and cuticle phenotypes associated with ecdysteroid receptor signaling disruption

Abstract

We study the hormonal basis of limb regeneration in *Uca pugilator* and have produced limb bud transcriptomes for three developmental stages using Illumina sequencing: 1) early blastemal stage associated with cellular proliferation and differentiation; 2) early proecdysial stage associated with rapid limb bud growth and low hormone titers; 3) late proecdysial stage associated with the cessation of growth seen just before molt and high circulating ecdysteroid titers. We have also conducted transcriptome comparisons between early regenerates with disrupted ecdysteroid signaling (obtained via RNAi-mediated receptor knockdown) and undisrupted early regenerates (ds*EGFP*-injected controls). Sequencing the cDNA libraries yielded 848 million short reads and *de novo* assembly of quality reads generated 3,034,309 contigs (all libraries combined). Following BLASTx assignments, we filtered out 648,288 sequences that were ≥200 bp long, and depending on the library, 21% – 34% of these contigs were annotated via BLASTx (e-value cut-off 10^{-3}). We generated a set of 61,373 non-redundant accession numbers by combining BLASTx data from all libraries. Our initial focus on this data set involved genes involved in, or influenced by, the ecdysteroid signaling pathway. This included identification of alternate EcR A/B domain isoforms not identified in previous cDNA library screens, and identification of putative ecdysteroid biosynthetic enzyme orthologs and a putative ortholog of MIH. The expression profiles of genes in blastemas with disrupted ecdysteroid signaling revealed significant down-regulation of proliferation marker genes such as *PCNA* and *MCM2.* The block in cell proliferation observed following

receptor knockdown correlated with an up-regulation of *HR3*: in insect systems, HR3 has been shown to inhibit *CycB*, resulting in G2/M arrest. In addition, there was significant up-regulation of cuticular proteins in the ecdysteroid receptor knockdown libraries compared to the controls. Surprisingly, several of these cuticular proteins that are normally expressed in late pre-molt were mis-expressed in blastemas with disrupted ecdysteroid signaling. Based on this study we propose that the systemic effect of RNAi injection may be due to significant up-regulation of an *MIH* ortholog following down-regulation of *EcR* and *RXR* in experimental blastemas. This increase in *MIH* expression might in turn lower circulating ecdysteroid titers below a critical level resulting in obstruction of proliferation.

"Conducting data analysis is like drinking a fine wine. It is important to swirl and sniff the wine, to unpack the complex bouquet and to appreciate the experience. Gulping the wine doesn't work."

Daniel B. Wright, 2003

1. Introduction

The sub-phylum Crustacea comprises one of the largest globally distributed groups of animals with more than 68,000 extant species (Martin and Davis, 2001). The diversity of crustaceans in terms of number, habitat and physiological variation, and their economic importance as a major commercial food sources, make this group of animals an important model system for scientific research. Although numerous decapod species are used in endocrinology, molecular biology, ecology, and developmental biology studies, only one crustacean genome, *Daphnia pulex*, has been sequenced and annotated (Colbourne et al., 2011), with the genome of a related species, *Daphnia magna*, in draft form. We have taken the approach of transcriptome sequencing to explore gene expression profiles in limb regenerates. Sequence divergence between branchiopods and decapods, however, even when comparing highly conserved gene families, indicates significant evolutionary distance. Phylogenetic analyses using the complete mitochondrial genome have revealed that Malacostraca, the crustacean class containing *Uca*, group more closely to Insecta than to Branchiopoda (Wilson et al., 2000). In addition, sampling of nuclear genes suggests that the subphylum Crustacea is a paraphyletic group where phylogenetic relationships are not clear (Regier et al., 2005; Regier et al., 2010; Giribet and Edgecombe, 2012).

This limits the efficacy of the *Daphnia pulex* genome as a scaffold for decapod transcriptome assembly.

With the advent of next generation sequencing technologies (NGS), together with reductions in sequencing costs, transcriptome databases for several crustaceans, including three brachyuran crab species, have been successfully generated. Transcriptome study via NGS technology is a discovery-based tool and is useful in identifying new orthologs, novel genes and splice variants. Transcriptome resources are also used to map molecular interaction pathways, which may be applied to study questions related to physiological and cellular changes, stage-specific changes in the morphology of an organ, and/or responses to physiological or genetic perturbation leading to changes in gene expression profiles. In recent years, transcriptomes of porcelain crab (*Petrolisthes cinctipes*), Chinese mitten crab (*Eriocheir sinensis*) and boreal spider crab (*Hyas araneus)* have been created (Tagmount et al., 2010; Zhang et al., 2011; He et al., 2012; Harms et al., 2013). In one of the studies, transcriptome sequencing has led to the development of an open source database the porcelain crab array database (PCAD) to store EST sequence and homology information (Tagmount et al., 2010). In another study, genes relevant to crab spermatogenesis were identified by studying the transcriptome of testes from Chinese mitten crabs (He et al., 2012).

We are exploring the genetic and molecular basis of limb regeneration in conjunction with molting and circulating hormone fluctuations in a non-model decapod crustacean, *Uca pugilator*. Voluntary reflexive limb loss (called autotomy) and subsequent limb regeneration is a defensive mechanism in the life cycle of certain crustaceans used to evade predation and is associated with the molt cycle (Adiyodi,

1972; Hopkins, 1993). This process is reviewed in detail in Chapter I. We have chosen three morphologically distinguishable stages of limb regeneration (blastema, D_0 and D_1D_4) for studying changes in gene expression profiles via NGS. The blastema develops through cellular proliferation of the epidermal cells and starts as early as two days after autotomy (A+2) (Emmel, 1910; Hopkins, 1993). Four to seven days following autotomy, the blastema is comprised of dividing epidermal cells that secrete a thin layer of cuticle underneath the scab (see Chapter II, Figure 5A). During the D_0 stage, the basal limb bud undergoes a predominantly hypertrophic (cell size increase) growth at a comparatively higher rate than during basal growth. The cuticular sac is flexible enough to accommodate a three fold increase in the size over the basal limb bud (Hopkins et al., 1999). This increase in size is mainly due to muscle protein synthesis, which results in increases in myofibril size and water intake. After the early proecdysial limb buds attain their final and maximum size they become D_1D_4 buds as the crab enters terminal plateau or late pre-molt stage (see Chapter II, Figure 5C). At this point the crab is ready to molt, and following ecdysis, the functional limbs are deployed.

In addition to the aforementioned differences in morphology and growth rate, circulating ecdysteroid hormone levels differ between these three limb regeneration stages (Hopkins, 1989; Hopkins, 2001). Circulating ecdysteroid titers are low during the intermolt stage of the molt cycle, corresponding to the blastema stage (<25 pg/µl). Following a transient peak (40 pg/µl) in titer, the limb regenerate enters the D_0 stage during which titers return to low levels (<35 pg/µl) (Hopkins, 1989; Chapter I, Figure 2). Upon entering the D_1D_4 stage, ecdysteroids in the hemolymph begin to increase in

concentration (50-100 pg/µl). In summary, low levels of circulating hormone titers are observed during blastema formation, hypertrophic (D_0) limb bud growth, while ecdysteroid titers reach their maximum level during the late proecdysial stage (D_1D_4) of regeneration, and fall just prior to molt, when the new limb is deployed.

The ecdysteroid hormones mediate their effect by binding to a heterodimer nuclear receptor (NR) complex, EcR and RXR (Durica and Hopkins, 1996; Durica et al., 2002, Wu et al., 2004; Riddiford et al., 2000; Hill et al., 2013). The domain architecture of both *Uca EcR* and *RXR* is consistent with that of typical nuclear receptors (see Fahrbach et al., 2012, for review) and the EcR/RXR heterodimer complex has been shown to bind to ecdysone response elements (EcRE) like IRper-1, DR-4, eip28/29 and IRhsp-1 in electrophoretic mobility shift assays (Wu et al., 2004). *In vitro,* binding of the EcR/RXR heterodimer to EcREs occurs in the absence of ecdysteroid hormones like 20E and PonA (Wu et al., 2004) and *in vivo,* immunolocalization has shown that both EcR and RXR are located in the nucleus, at all stages of limb regeneration, even when circulating hormone titers are low (Hopkins et al., 1999). Both EcR and RXR have multiple isoforms. Initial screenings of cDNA libraries constructed from late proecdysial limb bud mRNA identified hinge region and ligand binding domain (LBD) isoforms for *RXR* and hinge region variants of *EcR* (Chung et al. 1998; Durica et al., 2002). Recent screens of *Uca* genomic libraries have verified that both hinge and LBD EcR protein isoforms arise from differential splicing from a single gene (Durica et al. submitted; discussed in next section). Similarly, for RXR there are two LBD domain isoforms where the larger protein variant contains an additional 33 amino acid (aa) insert and a +/- 5 amino acid variant in the "T" box

region of the hinge domain (Durica et al. 2002; Wu et al., 2004). Immunohistochemical staining of blastema and regenerating limb buds has shown that EcR and RXR are co-localized in nuclei of epidermal, blood and muscle cells (Hopkins et al., 1999). In addition, both *EcR* and *RXR* mRNAs are present in blastema, D_0, and D_1D_4 limb buds during limb regeneration as detected by Northern blot, ribonuclease protection assay and qPCR (Durica et al., 2002, Wu et al., 2004, Das and Durica, 2013).

We have used RNAi [mediated via double stranded (ds) RNA] as a tool to study the functional significance of ecdysteroid receptors in early blastema. Injection of ds*EcR*/ds*RXR* led to significant knockdown of ecdysteroid receptors and obstruction of cellular proliferation during the early blastema stage, along with a further lowering of circulating ecdysteroid titers from the already low level seen during basal growth (Das and Durica, 2013; see Chapter II). The lowering of ecdysteroid titers along with down-regulation of ecdysteroid receptors in the contralateral uninjected blastemas and failure of the experimental crabs to molt suggest a systemic effect due to RNAi injection. Another phenotype associated with knockdown of *EcR* and *RXR* was a significant increase (60%) in cuticle deposition in comparison to control blastemas (injected with ds*EGFP*) (see Chapter II, Figure 5). Taken together these observations suggest that in *Uca* ecdysteroid signaling directly mediates the limb regeneration process, primarily blastema proliferation and cuticle deposition, even under conditions when the circulating hormone titers are low.

A major objective of the experiments described in this work was to generate transcriptome databases via Illumina sequencing for three distinct stages of limb bud

regeneration encompassing cell proliferation, segment development and growth/differentiation of that structure. In theory this provides us with a set of genes and their relative expression profiles that are temporally expressed corresponding to an organ that recapitulates part of a metamorphic developmental program during an endocrine cycle of normal growth. We have produced transcriptomes from blastema (A+4), early proecdysial (D_0), and late proecdysial (D_1D_4) stages. In addition, we have attempted to identify putative genes subject to disruption of ecdysteroid signaling by comparing control (A+6, ds*EGFP* injected) and RNAi-treated (A+6, ds*EcR*/ds*RXR* injected) limb regenerate transcriptomes. We have generated eight transcriptome libraries which include two biological replicates each for the A+4, D_0, and D_1D_4 stages and two A+6 libraries from the RNAi experiment.

Experiments leading to generation of transcriptome data are not hypothesis driven, but provide us with a database to study gene expression profiles and generate hypotheses. Due to lack of a reference genome and limited crustacean gene and protein sequence data available through current resources, it is impossible to assemble and annotate sequence data into a finalized or complete transcriptome. Nevertheless, this process has generated a tremendous amount of validated sequence information and has allowed us to assign on the basis of sequence homology putative ortholog status to large numbers of previously unidentified crustacean genes. For the purpose of this paper we have concentrated on:

1. Profiling characteristic features of transcriptome libraries.
2. Annotation of contigs using BLASTx.
3. Correlation of annotated contig expression levels between replicates.

4. BLASTx hit assignments to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.
5. Validation of library depth through examination of previously well-characterized genes in the library, such as nuclear receptors. This has led to the identification of alternatively spliced isoforms not previously recovered using other technologies.
6. Investigation and identification of genes related to ecdysteroid signaling previously uncharacterized in decapods or brachyuran crabs, including orthologous genes involved in ecdysteroid biosynthesis.
7. Examination of relevant genes that are differentially expressed in blastemas where ecdysteroid signaling had been disrupted. Abnormal up-regulation of specific cuticle genes was also observed in experimental animals; thus both the block in proliferation and the thickened cuticle phenotype may be a consequence of mis-regulation of ecdysteroid signaling through receptor knockdown and the concomitant lowering of circulating ecdysteroid titer. We have focused on putative genes that correspond to the abnormal phenotype obtained following RNAi injections, specifically effects on cell growth and proliferation (*PCNA*, *MCM2*, cyclins) and excessive cuticle synthesis (cuticle proteins). Results of ecdysteroid receptor knockdown suggest a down-regulation of that *cyclinB* may be mediated via an increase in NR *HR3* expression.

8. Exploration of hypotheses to explain the systemic effect mechanism as a result of RNAi (ds*EcR*/ds*RXR*) injection in blastema stage. This led to the identification of a molt inhibiting hormone (*MIH*) ortholog expressed primarily in the blastema stage that was significantly up-regulated in the 'receptor knockdown' A+6-E transcriptome (see Chapter I for details on negative regulation of ecdysteroid production via MIH). We propose that up-regulation and secretion of limb bud MIH leads to inhibition of ecdysteroid synthesis, and a lowering of ecdysteroid levels below a level critical to maintain blastemal cell proliferation.

2. Materials and methods

2.1. Animal preparation and tissue collection

Fiddler crabs were obtained from the Gulf Specimen Co., Panacea, Florida. They were acclimatized for a week in the laboratory under a constant temperature of 30°C and a 14:10 hour light:dark cycle. Experimental crabs were kept separately in plastic boxes containing artificial sea water (Instant Ocean, specific gravity: 1.02). Seven limbs including six walking legs and a large cheliped were autotomized from each crab by pinching the merus with a forceps. Data is expressed in number of days after autotomy (abbreviated: A + number of days).

Seven limb regenerates from each animal were harvested from three different stages of limb regeneration using four criteria: time after autotomy, concentration of

circulating ecdysteroids (measured via radioimmunoassay), size of limb regenerates (Regenerative index or R-value; Bliss, 1956), and growth rate of limb regenerates (Experimental growth rate or ER; Bliss and Hopkins, 1974). We collected limb regenerates from two biological replicates. In each treatment, we collected enough tissue to ensure isolation of at least 10 µg total RNA. Since blastema size is significantly smaller than both D_0 and D_1D_4 limb regenerates, this required harvesting blastemas from multiple crabs (e.g. N = 16 for A+4-1 library; Table 1). Table 1 depicts the sample size and characteristic features of limb buds collected to build the eight transcriptome libraries. The limb regeneration stages are:

1) Blastema (A+4): Limb regenerates were collected on day four following autotomy from crabs with low ecdysteroid titers.
2) Early proecdysial (D_0): Tissues were collected from crabs with low circulating ecdysteroid titers and limb regenerates with R-values ranging from 12 to 15 and ER values over 50.
3) Late proecdysial (D_1D_4): Tissues were collected from crabs with ecdysteroid titers above 60 pg/µl and limb regenerates with R-values above 19 and ER values less than 20.

Along with the three stages of limb regenerates, we also collected limb regenerates that were injected with dsRNA, either ds*EGFP* (control) and ds*EcR/*ds*RXR* (experimental) on days 1 (A+1) and 4 (A+4) following autotomy. The synthesis of dsRNA and the injection protocol are detailed in Chapter II (Das and Durica, 2013). The experimental limb buds were collected on A+6, when there is a significant lowering of circulating ecdysteroids ($p=<0.001$) and a lack of blastemal

proliferation in comparison to the control limb regenerates (Das and Durica, 2013). The number of animals required to generate A+6 cDNA libraries was increased to 42, as we only collected the injected limb regenerates (two per animal) for the 10 µg of total RNA. The experimental transcriptome library is designated as A+6-E and the control as A+6-C.

On the day of limb regenerate dissections, hemolymph was also collected from individual crabs to measure the ecdysteroid titers via radioimmunoassay (RIA; Hopkins, 1983). All limb regenerate samples were stored in RNAlater (Qiagen) at -20°C until further processing.

2.2. cDNA library production and Illumina sequencing

Total RNA was isolated using the RNeasy™ Mini Kit (Qiagen) following the company's protocol. The total RNA was sent to the Next Generation Sequencing (NGS) facility located at the Oklahoma Medical Research Foundation (OMRF) for further processing. Approximately 10 µg of total RNA from each library was used to isolate mRNA. The cDNA libraries were prepared from mRNA using the TruSeq™ RNA Sample Preparation Kit (Illumina) following the manufacturer's protocol. The 100 bp paired-end sequencing of the cDNA libraries was carried out using an Illumina HiSeq™ 2000 at OMRF.

2.3. Transcriptome assembly and analysis

Paired-end Illumina sequencing generated raw reads that were assembled using the Short Oligonucleotide Analysis Package *de novo*-Trans (SOAPdenovo-Trans) V1.01 assembly program (Xie et al., 2014). Prior to assembly of raw reads, the SOAP denovo-Trans program filtered out the low quality reads. We used 31 bp k-mers to assemble the 100 bp paired-end and clean short reads to build longer contiguous sequences (contigs).

The assembled contigs from each library were annotated via BLASTx alignment (searching the protein database using a translated nucleotide query) against four protein databases including the NCBI NR (non-redundant), NCBI refseq (Reference Sequence project), EMBL (European Molecular Biology Laboratory), and DDBJ (DNA Database for Japan) databases **(**Altschul et al., 1997; Nakamura et al., 2013). The e-value cut-off used for BLASTx was set at 10^{-3}. The BLASTx result of each contig listed the top three hits, and a script was written to generate an output summary file listing only the top hit for the contig. Following BLAST assignments, the contigs with length ≥200 bp were extracted from the BLASTx output files for downstream analysis. The resulting BLASTx data was sorted into two categories: contigs with hits and contigs with no hits. The contigs with BLASTx hits were used to generate Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways via MEtaGenome Analyzer (MEGAN 4) software (Kanehisa et al., 2006; Kanehisa et al., 2014; Huson et al., 2011; Mitra et al., 2011**)**. The read count or redundancy for each

contig was generated via the SOAPdenovo-Trans program and represents the number of short reads mapped to generate a particular contig. These read counts were used to generate normalized expression values, RPKM (read count per kilobase per million mapped reads, see below), for each contig. The protocol to generate KEGG summary files associated with RPKM values is provided in Appendix A. Eight separate KEGG summary files were generated. They were merged using R statistical software (R Development Core Team, 2012) to build a set of non-redundant BLASTx hits to our database. The pipeline for generating an annotated transcriptome is shown in Figure 1.

2.4. Identification of differentially expressed genes and statistical analysis

The expression levels of the assembled contigs are calculated by normalizing read count or redundancy per kilobase per million mapped reads (RPKM) (Mortazavi et al., 2008). The formula used for calculating RPKM is:

$$\frac{10^9 \times \mathrm{C}}{\mathrm{N} \times \mathrm{L}}$$

where,

C represents the number of reads that fall on the contig;

N represents total number of mapped reads in the experiment;

L represents the length of the contig in base pairs.

The total number of mapped reads for each library is represented in Table 2.

The RPKM values were calculated for each contig and were also associated with KEGG pathways to identify differentially expressed contigs/transcripts/open reading frames (ORF) and pathways. Differentially expressed transcripts were

discriminated through either RPKM values or as log_2fold changes in comparison to A+4 gene expression levels (for the development time course experiments) or A+6-C expression levels (for the RNAi experiment), unless otherwise noted.

We have used R software (R Development Core Team, 2012) to perform two separate statistical analyses. We applied Pearson Correlation tests to analyze correlation coefficients of RPKM values between each pair of biological replicates as well as between libraries from different developmental time-points. We also used R to run Pearson Chi squared tests with Yates' continuity correction to determine statistical significance for differentially expressed genes.

3. Results and discussion

3.1. Transcriptome profiling

3.1.1. Characteristic features of transcriptome libraries

The paired-end Illumina sequencing of eight libraries generated 848 million raw reads and was saved in FASTQ file format containing sequence and quality information. The raw reads were assembled using the SOAPdenovo-Trans assembly program (Xie et al., 2014). Following removal of low quality reads, 50% – 67% of raw reads, depending on the library, were assembled separately to generate a total of 3.04 million contigs. Table 2 represents the number of raw reads generated and assembled into contigs in each library.

Although a large number of contigs were generated in each library, contig annotation via BLASTx against the NR databases revealed that a majority (84% –

93%) of contigs had no hits. Since short contigs have a comparatively lower probability of being annotated than longer contigs, we set the contig length cut-off at ≥200 bp for further downstream analysis. Between 16% – 31% of the total contigs in each library were ≥200 bp in length. The average contig length for the different libraries ranged from 498 bp – 693 bp, while the longest contig length ranged from 10,631 bp – 21,071 bp. A total of 648,288 contigs (≥200 bp) were compiled (all libraries combined) of which 178,259 contigs were assigned BLASTx hits (Table 3). The BLASTx data from all libraries at this point in the pipeline analysis is also redundant. We have annotated 21% – 34 % of the contigs depending on the library (Figure 1; Table 3). This low percentage of annotation is similar to or better than other recently published crustacean transcriptome analyses, suggesting a limitation of current *de novo* assembly programs in generating assemblies, problems with database search algorithms in producing annotation hits and/or the presence of a large number of unknown and novel transcripts, including non-coding RNAs, that are entering the databases (Li et al., 2012; Harms et al., 2013; Jin et al., 2013).

In order to generate a set of contigs that are assigned to a non-redundant accession number (including NR, RefSeq, DDBJ and EMBL), we have used KEGG output data. The KEGG output data clusters annotated contigs with the same accession numbers even though that accession number may not be assigned to a KEGG pathway. Using this strategy we have compiled a unique set of BLASTx hits for each individual library (Figure 2A); these sum to 146,861 accession assignments when the individual library clusters are combined. By using the R software, 146,861 BLASTx hits in the combined libraries were further clustered to 61,373 unique accession numbers (Figure

2B). This high number of "unique" BLASTx hits implies that there is still redundancy in gene assignments. Since all eight libraries were assembled and annotated separately, we noticed variability in BLASTx assignments among libraries as well as between biological replicates (see Table 4 and Figure 3 for an example and explanation). This is one problem of *de novo* assembly due to the lack of a reference genome. Although this redundancy makes *de novo* quantification of steady state gene expression profiles difficult, the pipeline has been a valuable resource for identifying putative gene orthologs and estimating steady-state levels of gene expression for specific, characterized gene sequences.

3.1.2. Size distribution of assembled contigs and corresponding number of assigned BLASTx hits

The contigs (≥200 bp) in each library were sorted into three categories based on length. The three categories included contigs that ranged from: a) 200 bp – 999 bp, b) 1000 bp – 4999 bp and c) ≥5000 bp long. The number of assembled contigs decreases as the contig size increases (Table 5). Correlating the number of BLASTx hits among the three categories indicated that with increase in contig length, the percentage of gene assignment increases (Table 5; Figure 4). The highest percentage of BLASTx hits (over 90%) was found in the group comprised of contigs that are ≥5000 bp long, but there are much fewer contigs ≥5000 bp size than in the other two groups. In the mid-range of the size distribution, only 9% to 16% of contigs were assigned via BLASTx (Table 5). Taken together, these data suggest that optimization

of *de novo* assemblies is crucial for generating longer contigs, which in turn may increase the number of annotated contigs for non-model organisms without reference genomes.

3.2. Website Development

To make the crustacean sequence data easily accessible to the scientific community, we have developed a web-based approach to identify genes and sequences of interest (Figure 5). There are two ways in which the limb bud transcriptome assembly and BLAST data set can be searched:

a. Keyword search: This method utilizes text information, such as specific gene identifiers or names, to search through the annotated *Uca* database. The keyword search results provide both sequence information on the *Uca* putative ortholog of interest, as well as BLASTx information on best hits of that sequence to the protein databases (NR, RefSeq, DDBJ and EMBL). The website address to access this feature is: http://www.genome.ou.edu/crab_Illumina.html

b. BLAST search: This method utilizes sequence information (both nucleotide and protein) of genes that are annotated in other species and finds orthologous sequences in the *Uca* database. There are over 3.04 million sequences in the Illumina SOAP database that are searchable through BLASTn, tBLASTn or tBLASTx (Figure 5). The website address to access this feature is:

http://www.genome.ou.edu/blast/FiddlerCrab_Illumina.html

Both of the above-mentioned searches help in gathering information on sequences that have not previously been identified in crustaceans. In addition, database sequences with no BLASTx hits can be searched for novel crustacean genes. As proof of principle, this search method has led to the identification of novel, previously unidentified crustacean nuclear receptor isoforms for *EcR* and *E75*, putative orthologs of P450 genes that are involved in ecdysteroid biosynthesis, and a putative juvenile hormone receptor (*methoprene-tolerant*) ortholog. An example of using a keyword- based search strategy to retrieve hits to the transcriptome database is provided in Figure 5.

3.3. Correlation of Replicates

We have generated two biological replicates for each limb regeneration stage with the exception of the RNAi treated cDNA libraries. In order to analyze the equivalency of the replicates, we have used the expression levels (RPKM) of the BLASTx hits that are common to both replicates in each library to perform a statistical correlation study. Pearson Correlation Coefficients obtained from comparing RPKMs of BLASTx assignments between replicates of regeneration stages ranged from 0.52 – 0.56 (Table 6A). These low correlation values suggest high variability in transcript abundance between replicates. This variability can be attributed to several factors. First, as discussed in Table 4 and Figure 3, the lack of a defined annotated transcriptome produces multiple assignments for even the same gene in database string

searches. Contigs with exactly the same sequence were assigned to different unique identifiers present in protein databases and that in turn skewed gene expression levels (RPKM). Second, inter-animal variability might be a factor, due to both innate physiological differences and/or timing inaccuracies associated with staging. Variation attributable to these factors has previously been observed between individual animals while performing qPCR studies on individual genes. These results suggest that RPKM numbers based on a small sample size may not be representative; it suggests that pooled animal sampling may provide the best mean.

The above-mentioned variability might be avoided via clustering annotated contigs with a defined function. Assigning these contigs to KEGG pathways is one way to achieve assignments that are a part of the same molecular network. Using RPKM values of accession numbers assigned to 249 KEGG pathways, we calculated the Pearson Correlation Coefficients between two replicates of each library. The biological replicates show much higher correlation ($\geq$0.93) when comparing the KEGG pathway RPKMs rather than the RPKMs of unique accession numbers (Table 6A). We also analyzed correlation of transcript expression levels between different stages of limb regeneration by using the average RPKMs of KEGG pathways for biological replicates (Table 6B). As anticipated, the correlation values were lower between two different regeneration stages relative to their respective biological replicates. This indicates that clustering all contigs with a defined function is a more robust way to analyze differential expression. For this reason, analyses of reported differentially expressed genes in this paper were performed by manually identifying

all the contigs that represent transcripts of the same gene (i.e., that could be unambiguously identified on the basis of DNA sequence identity).

3.4. Functional Annotation via Kyoto Encyclopedia of Genes and Genomes (KEGG) database

The KEGG database is a series of manually annotated molecular interaction pathways that are related to metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases and drug development (Kinehisa et al., 2006; Kinehisa et al., 2014). The functional hierarchy of these global pathways can be viewed here: http://www.genome.jp/kegg/pathway.html

Using MEGAN 4 software, we have assigned contigs with BLASTx hits to KEGG pathways (Figure 6). Although the majority of contigs were not assigned (Table 7), we were able to assign 26,033 contigs to five global pathways in all libraries combined (Huson et al., 2011; Mitra et al., 2011). Approximately two to four times more contigs were assigned to the metabolism pathway than other pathways (Figure 6). It is interesting to note that regardless of the variation in percentage of BLASTx hits among limb regenerate libraries (21% – 34% of all contigs ≥200bp), the relative assignments of BLASTx hits to different pathways were similar across the eight libraries.

The KEGG pathways are created in a generic manner that is applicable across "all" species by assigning manually defined KEGG ortholog groups (KO) for proteins

obtained from various organisms. However, the organism list is highly limited (http://www.genome.jp/kegg/catalog/org_list.html). The only crustaceans listed in KO entries are *Daphnia pulex* (water flea) and *Petrolisthes cinctipes* (porcelain crab). This becomes a major limitation for KEGG assignments when analyzing *de novo* assemblies of crustaceans.

The KEGG assignments are also used to study the expression levels of various pathways by using their corresponding RPKM values. However, due to the limited number (~15%) of BLASTx hit assignments to KEGG, we were not certain whether the relative expression levels of genes within a pathway would be informative across limb regeneration stages. The Pearson Correlation Coefficients of KEGG pathway RPKMs suggest that the steady-state transcript expression levels between limb bud stages are more variable from each other than the expression profiles for biological replicates of each individual stage. Since we are interested in cellular proliferation (required during formation of blastema), we analyzed expression levels of two KEGG pathways: the Cell Growth/Death and the Replication/Repair pathways and their sub-pathways (Figure 7A and 7B). As per our expectation, we noticed a trend towards higher expression levels of these selected pathways in A+4 compared to D_1D_4 libraries. Both the Replication/Repair and Cell Growth/Death pathways, however, also appear up-regulated (4.7 and 4.6 fold respectively) in D_0 stages in comparison to D_1D_4 stages. This is contradictory to previously reported data stating that the rapid growth of limb regenerates during D_0 stages is due to an increase in cell size but not cell proliferation (Adiyodi, 1972; Hopkins et al., 1999; Hopkins, 2001). These results suggest that cell division might also occur during the early proecdysial stage along

with an increase in cell size. Direct assays of DNA synthesis and cell proliferation will be required to confirm this model.

3.5. Identification and quantification of an alternate EcR A/B domain splice variant

As mentioned in the introduction, the ecdysteroid receptor is a heterodimer protein complex of the nuclear receptors EcR and RXR. Both these receptors are expressed in all stages of limb regeneration. In crustaceans, LBD and hinge domain isoforms of EcR and RXR have been reported, but unlike insects, no EcR A/B domain isoforms were previously isolated via screening of cDNA libraries (Durica et al., 2002). Sequencing of genomic bacteriophage clones, however, identified an intron splice junction within the 5' A/B domain of *EcR*, suggesting the possibility of alternative amino terminal isoforms (Durica et al., submitted). In order to examine contig assembly and depth of sequencing, we have used the known sequence of the *EcR* and *RXR* genes to perform BLAST searches against the Illumina SOAP database. All previously identified hinge and LBD isoforms for these two nuclear receptors were present in the database. In addition, following BLASTn and tBLASTx, we have isolated an alternatively spliced isoform present in the A/B domain of the receptor, *EcR-A*, (Figure 8A; Durica et al., submitted). No A/B domain variants were identified for RXR.

Analyzing the relative transcript abundance of contigs containing the new EcR receptor variant suggests that *EcR-A* is the more abundant isoform compared to the

EcR-B isoform during the D_1D_4 stage (Figure 8B). Further, the transcript abundance of contigs containing either isoform is higher in blastema and D_0 stage relative to D_1D_4 stage. This variation in expression level was replicated using isoform-specific primers via qPCR (data not shown). It is interesting to note that in the limb regenerates, *EcR* expression is higher in blastema and D_0 stages, when the circulating ecdysteroid titers are low, and *EcR* transcript abundance appears down-regulated when the ecdysteroid titers are at maximum level. The evidence for higher receptor gene expression during stages when hormone titers are low, along with immunohistological evidence that the receptor is nuclear-localized (Hopkins et al., 1999; Wu et al., 2004), suggests that the receptor might function as a repressor of transcription during the A+4 and D_0 stages.

Alternative A/B domains have been reported in two other crustacean species, *Eriocheir sinensis* (gene: *EcR* variant L; GenBank: AHG30901.1) and *Macrobrachium nipponense* (gene: *EcR-L2*; GenBank: AHA33384.1 and gene: *EcR-S2*; GenBank: AHA33386.1). In *M. nipponenese*, measurement of relative expression levels of *EcR* splice variants revealed that *EcR-S2* is the dominant variant in both ovaries and testes (Shen et al., 2013).

3.6. Identification of genes involved in ecdysteroid biosynthesis – limb regenerates as a putative site for ecdysteroid synthesis?

Ecdysteroids, like vertebrate steroid hormones, are synthesized from cholesterol. In crustaceans, ecdysteroids are synthesized in a paired set of glands called the Y-organ (see Lachaise et al., 1993, for review). In *Drosophila*, the ecdysteroid biosynthetic pathway has been well characterized, and mutants in the

enzymatic conversion of cholesterol to active hormone have been identified (see Huang et al., 2008, for review). In crustaceans, there has been little success in the biochemical characterization of enzymes catalyzing the series of reactions leading to synthesis of ecdysone, 20E, and PonA (Rewitz and Gilbert, 2008). More progress has been made on the basis of structural homology to the insect p450 enzymes involved in this process. An ortholog of the enzyme responsible for the conversion of 5β[H]-diketol to 2,22-dideoxy-3-dehydroecdysone (*phantom*) was successfully cloned from the Y-organ of the kuruma prawn, *Marsupenaeus japonicus* (Asazuma et al., 2009). The crustacean sequences for genes encoding ecdysteroid biosynthesis pathway enzymes were also identified computationally following genome sequencing of *Daphnia pulex* and transcriptome analysis of Y-organs in the crayfish, *Pontastacus leptodactylus* (Rewitz and Gilbert, 2008; Tom et al., 2013).

Unexpectedly, we have recovered putative orthologs of genes encoding enzymes in the ecdysteroid biosynthetic pathway from the transcriptomes of limb regenerates. A keyword search of the transcriptome database first identified a strong hit to a putative *neverland* ortholog (encodes a Rieske oxygenase, the first enzyme in steroid biogenesis; BLASTx hit accession number: BAK39960.1; Score: 401 bits; Expect: E-109) and to a *disembodied* ortholog (encodes an ecdysteroid 22-hydroxylase enzyme; BLASTx hit accession number: EFX63066.1; Score: 412 bits; Expect: E-112). This prompted a more thorough search to identify potential transcripts that encode ecdysteroid biosynthesis enzyme orthologs. These genes are called Halloween genes as their mutations result in embryonic lethality before cuticle is synthesized (Gilbert, 2008).

We have recently generated transcriptome data from Y-organs of another brachyuryan crab, *Gecarcinus lateralis* (blackback land crab) (in collaboration with the Mykles laboratory, Colorado State University). We have used *Drosophila* protein sequences and conceptually translated nucleotide sequences from crayfish and land crab NGS data to search for orthologous sequences encoding ecdysteroid biosynthesis enzymes in the *Uca* limb bud transcriptome. This BLAST search produced putative orthologs for four 'Halloween genes' (*spook*, *phantom*, and *shadow*, in addition to previously identified *disembodied*), that encode cytochrome P450 enzymes involved in ecdysteroid biosynthesis (Figures 9 and 10). We have also recovered the *Uca* ortholog for the gene encoding a 3-oxo-Δ^4-steroid 5β-reductase (aldosereductase) enzyme that catalyzes the conversion of Δ^4-Diketol to 5β-Diketol (Figures 9 and 11). Following BLAST string searches, matches for potential orthologs were conducted using the OMA browser (Schneider et al., 2007; Altenhoff et al., 2011) or ORTHOdb (Waterhouse et al., 2013), and alignments were performed using MUSCLE (3.8) (Edgar, 2004; Figure 11A and 11B). The cladogram reflects the expected phylogenetic relationships; the *Uca* sequences are more closely related to the corresponding *Gecarcinus lateralis* sequences, and all the crustacean sequences cluster together (fiddler crab, land crab, kuruma prawn and crayfish). In addition, the different cytochrome P450 enzyme sequences from these different species cluster with their putative orthologs.

The finding that limb buds contain transcripts for these genes is rather unexpected, as the above mentioned genes are predicted to be active in Y-organs and have not been reported to be expressed in peripheral tissues. In addition, another

Halloween gene, *shade* (which encodes the last enzyme in the pathway that converts ecdysone to 20E and 25-deoxyecdysone to PonA), was not recovered from our database, although this gene is predicted to be active in peripheral tissues, such as the limb regenerates (see Mykles, 2011, for review).

Although the above tests strongly suggest that the individual *Uca* cytochrome P450s identified in these libraries are orthologous to ecdysteroidogenic enzymes, the functional significance of this finding is unclear. First, all the orthologous genes in the ecdysteroid biosynthesis pathway were not present at all stages of limb regeneration. Although *disembodied* and *spook* were expressed in all libraries, we recovered *shadow* only from D_1D_4 libraries, while *neverland* was absent in D_1D_4 libraries and present in blastema and D_0 libraries. Second, the expression levels of different transcripts were highly variable. The RPKM values for *phantom* and *shadow* were below five and one respectively, whereas, *spook* was highly expressed in A+4, at moderate levels at D_0 (419 – 1510) and at low levels in D_1D_4 (<5). Given the inconsistency and variability in Halloween gene expression across stages, and the lack of expression of the terminal hydroxylase, *shade*, we think it unlikely that complete ecdysteroid biosynthetic pathway is active in limb regenerates. What ecdysteroids or steroid metabolites are synthesized, and their potential role as an endocrine signal, will require biochemical characterization and validation of enzyme activity.

3.7. Identification of differentially expressed genes in RNAi treated blastemas

3.7.1 Expression profiles of *EcR* and *RXR*

As reported in chapter II, qPCR experiments indicated the ecdysteroid receptor complex was significantly down-regulated in ds*EcR*/ds*RXR* treated blastemas 48 hours post injection (A+6). To validate the transcriptome data we calculated the RPKM gene expression levels for both *EcR* and *RXR* (all isoforms combined) from A+6-C and A+6-E libraries. We saw a significant decrease in both receptors in A+6-E blastemas when compared to A+6-C ($p=0.005$ for *EcR* and $p=0.01$ for *RXR*, Pearson Chi Square; Figure 12). This data was obtained from 84 pooled blastemas (in one biological replicate for Illumina sequencing) in comparison to 16 from qPCR. The replication of receptor down-regulation validates the use of this technology as a means to analyze differential gene expression.

The two phenotypes associated with down-regulation of EcR and RXR that we observed were a significant decrease in cell proliferation and a 60% increase in cuticle deposition. We next analyzed some of the known genes and pathways associated with the above-mentioned phenotypes.

3.7.2. Expression profiles of cuticle proteins

Cuticle proteins (CP) are structural components of the arthropod exoskeleton and in crustaceans the cuticle is hardened via biomineralization (see Nagasawa, 2012,

for review). The exoskeleton in crustaceans provides protection from predators and infections but is a hindrance for body growth. It is shed periodically during each molt cycle via mineral resorption and apolysis of old cuticle, and deposition of new cuticle. The histological structure of cuticle shows the presence of four layers: epicuticle, exocuticle, endocuticle and membranous layer. The epicuticle and exocuticle are secreted by underlying epidermal cells during pre-molt, the endocuticle secretion and mineralization begins at the post-molt. The major chemical components of cuticle layers are cuticle proteins, along with chitin (a polysaccharide) and calcium carbonate (Roer and Dillaman, 1984; Nagasawa, 2012, for review).

As mentioned in Chapter II, histological studies of limb regenerates showed that there was a significant increase (60%) in cuticle deposition in A+6-E when compared to A+6-C. This led to the hypothesis that there is an up-regulation of CP and/or chitin synthesis. We have analyzed the expression profiles of transcripts of cuticle protein homologs identified in transcriptome libraries. Through BLASTx we have identified 59 unique accession numbers for CPs of which 22 were present in both A+6-E and A+6-C. Of these 22 CP homologs identified in limb regenerates, 12 were differentially expressed (10 up-regulated and 2 down-regulated; p-value <0.03; Pearson Chi Square test) in A+6-E when compared to A+6-C (Figure 13A) blastemas. The up-regulated CP transcripts in A+6-E had a $\log_2$ fold change ranging from 2 to 9.5, suggesting this up-regulation could be contributing to the thickened cuticle phenotype observed in histological sections of A+6-E compared to A+6-C blastemas.

Eight of the 12 differentially expressed cuticle proteins have been previously identified as differentially expressed across the molt cycle (Table 8, Anderson, 1999;

Faircloth and Shafer, 2007; Kuballa et al., 2007; Seear et al., 2010). It is interesting to note that several of the CPs that are normally expressed later in molt cycle (D_1D_4) were mis-expressed during blastemal development in the A+6-E library (Figure 13B). The up-regulation of several of these CPs following experimental ecdysteroid receptor knockdown via RNAi suggests that the ecdysteroid receptor signaling pathway might function as repressors for these CPs at the blastema stage.

3.7.3. Expression profiles of cell proliferation markers and cell cycle regulatory genes

In the fiddler crab, formation of the blastema during limb regeneration is dependent on epidermal cell proliferation. In chapter II, we have previously documented a decrease in blastemal cell proliferation following significant down-regulation of *EcR* and *RXR* transcripts in RNAi-treated animals (Chapter II, Figure 6). This result suggests that transciptome analysis might provide information on experimental differences in expression profiles for components of the cell cycle pathway. Since the BrdU incorporation assay detects cells in S phase of the cell cycle, we expect cell proliferation markers like proliferation cell nuclear antigen (*PCNA*) and minichromosome maintenance complex component 2 (*MCM2*) to be down-regulated (Linden et al., 1992; Köhler et al., 2005; Hanna-Morris et al., 2009). Upon assessing the gene expression levels of putative orthologs for *PCNA* (BLASTx hit accession number: ACK58408.1; Score: 221 bits; Expect: 2.1E-70) and *MCM2* (BLASTx hit accession number: XP_002407137.1; Score: 367 bits; Expect: 2.1E-98), we observed a

significant down-regulation of both transcripts in the A+6-E library compared to the control library (Figure 14A, p-value <0.0001 for both; Pearson Chi Square test). This data suggests that it is possible to use *PCNA* and *MCM2* as markers for cellular proliferation in *Uca*.

The regulatory protein complexes driving the cell cycle are comprised of a cyclin (Cyc) protein coupled with a cyclin dependent kinase (cdk) enzyme. These Cyc and Cdks complexes are also known to be modulated during embryonic development (see Budirahardja and Gönczy, 2009, for review). Diverse Cdks and their Cyc complement regulate initiation and transition through cell cycle phases. We have identified putative transcripts that encode four different Cyclins: *CycA* (BLASTx hit accession number: ADK13092.1; Score: 528 bits; Expect: 1E-147), *CycB* (BLASTx hit accession number: ACC77698.1; Score: 292 bits; Expect: 5.1E-76), *CycD* (BLASTx hit accession number: EFX84986.1; Score: 80 bits; Expect: 1E-16) and *CycE* (BLASTx hit accession number: EFX67141.1; Score: 253 bits; Expect: 8.1E-64). Although all four of these cyclin genes were down-regulated in A+6-E, only the change in *CycB* expression levels was statistically significant (Figure 14B, p-value=0.0001; Pearson Chi Square test).

The transition of G2 (growth) phase to M (mitosis) phase of the cell cycle is triggered by cdk1 and its associated protein CycB (see Ohi and Gould, 1999; Budirahardja and Gönczy, 2009, for review). Although we observed a significant down-regulation of *CycB*, we did not observe the same for *cdk1* (BLASTx hit accession number: ACI43009.1; Score: 188 bits; Expect: 1.1E-57) (Figure 14B). The down-regulation of *CycB* suggests G2/M arrest of the cell cycle, which in turn

obstructs cell proliferation in blastemas when ecdysteroid signaling has been disrupted. This suggests that *CycB* down-regulation might be a downstream effect of *EcR* and *RXR* knock-down in A+6-E blastemas.

Since growth of the limb bud ceases at terminal plateau, the cell cycle genes monitored in Figure 14 were not expressed in D_1D_4 libraries. However, the cyclins and replication core genes were expressed in both A+4 and D_0 stages (RPKM values ranged from 5 –140 depending on the transcript). This provides additional evidence, along with KEGG data, showing that cell division might be a component of early proecdysial growth.

3.7.4. Role of HR3 in G2/M arrest: Is *CycB* regulated via ecdysteroid signaling?

Recent studies on *Polidia interpunctella*, the Indian meal moth, have demonstrated that the 20E signaling pathway can induce G2/M arrest via nuclear receptor HR3 (Siaussat et al., 2004; Siaussat et al., 2008). In this moth rising titers of ecdysteroids induce transcription of HR3, which in turn inhibits CycB. This prompted us to analyze HR3 expression profiles in the RNAi treated A+6 libraries. A putative HR3 ortholog (BLASTx hit accession number: CAJ90621.1; Score: 365 bits; Expect: 3.1E-97) was identified and a $\log_2$ five-fold increase in transcript abundance was observed in the A+6-E library compared to A+6-C (Figure, 14B, p-value<0.0001; Pearson Chi Square test). This increase in HR3 is consistent with the observed down-regulation of CycB, and correlates with the block in cell proliferation observed following ecdysteroid receptor knockdown. In this instance, however, HR3 up-

regulation is occurring in a tissue at a time where circulating ecdysteroid titers are abnormally low.

The decrease in dividing blastemal cells is correlated with a significant lowering of circulating ecdysteroid titers. Monitoring optic neurogenesis in tissue culture using *Manduca sexta,* Champlin and Truman (1998) demonstrated that cellular proliferation occurs when ecdysteroid concentrations were maintained within a certain threshold. At sub-threshold levels of ecdysteroid, the cells were arrested in the G2/M checkpoint of the cell cycle. The mechanism of G2/M arrest is unknown. We propose that a critical level of ecdysteroid is required to maintain cellular proliferation in blastema and it is obstructed via knockdown of ecdysteroid receptors. The data further suggest that ecdysteroid receptors might repress *HR3* in limb regenerates under normal conditions and that when the former is down-regulated, HR3 expression increases, inhibiting CycB and resulting in G2/M arrest. This model is contrary to the notion that HR3 is an ecdysteroid response gene in early blastemal development. The model postulates limited or cell-type specific induction of this transcription factor.

3.8. Identification of putative *Up_MIH* (Molt inhibiting hormone) – a possible explanation for the systemic effect of RNAi injection in A+6-E

In Chapter II we postulated two hypotheses to explain the systemic effect of ds*EcR*/ds*RXR* injection (A+6-E):

a) Leakage of RNAi into the circulatory system, following injection, leading to down-regulation of ecdysteroid receptors in the Y-organ that in turn lowers the ecdysteroid titers below the critical level required for blastema proliferation.

b) Cross-talk between RNAi injected blastemas and the Y-organ via a secreted factor released from ds*EcR*/ds*RXR* injected blastemas that is competent in inhibiting ecdysteroid synthesis.

Several factors make the second hypothesis most plausible, particularly in light of new transcriptomic data in support of a secreted factor capable of inhibiting Y-organ ecdysteroidogenesis:

(i) Although we observed a significant lowering of circulating ecdysteroids 48 hours post RNAi injection, the titers in experimental crabs recovered to control levels later in the molt cycle (see Chapter II, Table 2D). This recovery happened as early as A+18 (data not shown).

(ii) In the experimental crabs, the percentage of emerged uninjected contralateral blastemas was higher (55%) than ds*EcR*/ds*RXR* injected claws (19%) and third walking legs (6%) (See Chapter II, Table 3A).

(iii) There were no significant differences in the growth rates of emerged uninjected contralateral limb buds between control and experimental crabs. The emerged blastemas injected with ds*EcR*/ds*RXR* plateaued at an R-value of 10.5 (±1.5), however, the control counterparts reached an R-value of 18.0 (±0.97) (See Chapter II, Figure 4A, 4B).

Taken together, we observe that the gradient of severity of RNAi phenotypes decreases from the site of injection. We are monitoring RNAi-mediated changes in the

transcriptome in cells from that site. Surprisingly we observed an increased expression of a putative ortholog that encodes a negative regulator of ecdysteroid synthesis. A *Uca pugilator* (*Up*) MIH ortholog (molt inhibiting hormone) (BLASTx hit accession number: ABF06632.1; Score: 184 bits; Expect: 2.1E-57) was recovered from the blastema stage (Figure 15A). As explained in Chapter I, MIH is a peptide hormone that inhibits production of ecdysteroids from the Y-organs. We observed a significant up-regulation of putative *Up_MIH* in the A+6-E library (Figure 15C, p-value<0.0001; Pearson Chi Square test). The MIH peptide belongs to the Crustacean CHH/MIH/GIH neurohormone family (pham01147) (Chan et al., 2003). As the name suggests, this group includes peptide hormones like crustacean hyperglycemic hormone (CHH) and gonad inhibiting hormone (GIH) along with MIH. In addition to *Up_MIH*, we have also recovered a putative *Up_CHH* ortholog (BLASTx hit accession number: ABA70560.1; Score: 129 bits; Expect: 8.1E-37); but putative *Up_CHH* was not differentially expressed in RNAi treated libraries (Figure 15C, p-value=0.62; Pearson Chi Square test). The cladogram of closely related crustacean protein sequences belonging to the CHH/MIH/GIH family shows that both *Up*_CHH and *Up*_MIH are grouped with previously identified, respective peptide hormones (Figure 15B). The presence of a pro-peptide *MIH* transcript in the blastema suggests that the mature peptide might be released into the circulation with the potential of inhibiting ecdysteroid synthesis in the Y-organs, thereby, lowering circulating titers. The rebound in ecdysteroid titers seen later in the molt cycle could then reflect loss of Y-organ sensitivity to MIH inhibition (see Mykles, 2001, for review).

This is the first report of *MIH* transcript expression in limb regenerates. The *MIH* transcript was also expressed in normally growing blastemas (A+4), but not in D_0 and D_1D_4 libraries. Previous work has shown that following secondary limb bud autotomy, the extracts from newly growing blastemas induced an inhibitory effect on circulating ecdysteroids (Yu et al., 2002). Through biochemical assay of the secondary limb bud extracts, a protein inhibitory factor was predicted to be an MIH-like peptide (Yu et al., 2002). The presence of this inhibitory factor was first proposed by D. M. Skinner and was termed LAF_{pro} (Limb Autotomy Factor proecdysis) (Skinner, 1985, for review). The lowering of ecdysteroid titers following secondary limb bud autotomy stopped the growth of the remaining limb buds and allowed the newly autotomized limb buds to recover and attain the size equivalent to the primary limb buds (see Mykles, 2001, for review). A similar situation is also observed during *Drosophila* imaginal disc regeneration, where 20E synthesis is impaired via release of the Insulin-like peptide 8 from the damaged tissue (Garelli et al., 2012; Colombani et al., 2012; see Chapter II for details). Hence, it is likely that lowering of ecdysteroid titers following down-regulation of *EcR* and *RXR* is re-capitulating the above phenomena and perhaps this is mediated via MIH.

Based on the information that the *MIH* expression might occur during early blastemal growth and not during early proecdysial growth, we predict that MIH is necessary to maintain a critical level of ecdysteroid titers for blastema formation. In addition, the RNAi experimental data suggests that the down-regulation of hormone receptors resulted in the up-regulation of *MIH* expression, leading to lowering of hormone titers below a sub-threshold level and subsequent systemic arrest of cell

proliferation in blastema. This information also leads to the hypothesis that following secondary limb bud autotomy, the newly developing blastemas might express *MIH*, which in turn inhibits the growth of the primary limb buds.

4. Conclusions

Illumina sequencing of limb regeneration stages has generated one of the largest transcriptome databases among crustaceans. This dataset enabled us to recover putative orthologs and novel isoforms of thousands of genes that have unique KEGG identifiers, many that are previously unidentified in the fiddler crab. Only a small subset has been described here; these genes are examined in the context of the phenotypic changes observed when ecdysteroid signaling is perturbed during early blastemal regeneration. This includes the identification of ecdysteroid biosynthesis genes, cell cycle pathway genes, the HR3 nuclear receptor, genes encoding cuticle proteins, and a peptide hormone inhibitory to ecdysteroid synthesis, *MIH*.

We hypothesize that blastemal development is dependent on a critical window of circulating ecdysteroids. Titers must normally be held low, but titers at sub-threshold levels inhibit cell proliferation in blastema, most likely via G2/M arrest. MIH synthesis in the blastema is down-stream of EcR/RXR signaling, as evidenced by increased levels of *Up_MIH* in blastemas from RNAi-treated animals. A drop in ecdysteroid titer below a required threshold might be triggered by MIH mis-expression in the blastema and subsequent down-regulation of ecdysteroid synthesis in the Y-organs.

The successful study of the role of ecdysteroid signaling during early regeneration is dependent on the tools available to manipulate and monitor genes of interest in a non-model organism. Our observation that ecdysteroid signaling mediates the early limb regeneration process, despite low circulating titers, was demonstrated by utilizing RNA interference. The model linking a drop in ecdysteroid titer to CycB-mediated G2/M arrest was built from a transcriptomic analysis made possible by advances in NGS sequencing and the accompanying software to handle 'big data'. The candidate genes and their postulated roles in this model are now open to further testing using these established technologies.

A major unknown in this model is how response element occupancy by the EcR/RXR heterodimer corresponds to the observed changes in gene expression for candidate genes, and how occupancy and levels of expression are affected by circulating hormone. To examine this issue, the genome sequence for this or a closely related brachyuran is critical.

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Zhang, W., Wan, H., Jiang, H., Zhao, Y., Zhang, X., Hu, S., Wang, Q., 2011. A transcriptome analysis of mitten crab testes (*Eriocheir sinensis*). Genet. Mol. Biol. 34, 136–141.

Table 1. Characteristic features of limb regenerates and sample size of transcriptome libraries.

Library name	Regeneration stage	Days past autotomy	R-value	ER	Ecdysteroid titers (pg/µl)	N
A+4-1	Early basal growth (blastema)	4	NA	NA	11.58 (±1.06)	**16**
A+4-2		4	NA	NA	09.01 (±0.91)	**17**
D_0-1	Early proecdysial growth	13	12.1	58	23.96	**1**
D_0-2		13	12.8	60	24.82	**1**
D_1D_4-1	Late proecdysial growth	18	22.3	0	70.29	**1**
D_1D_4-2		19	21.2	0	72.21	**1**
A+6-C	ds*EGFP* injected blastema	6	NA	NA	18.89 (±0.93)	**42**
A+6-E	ds*EcR*+ds*RXR* injected blastema	6	NA	NA	6.97 (±0.70)	**42**

Table 2. Representation of number of reads generated via Illumina sequencing and the total number of quality reads mapped to generate transcriptome for each library.

Library Name	Total number of reads generated from Illumina sequencing	Total number of mapped reads in SOAP *de novo* assembly	Total number of contigs
A+4-1	111,718,734	68,655,640	547,167
A+4-2	41,561,270	24,848,703	307,709
D_0-1	110,954,780	57,869,732	279,613
D_0-2	100,593,856	52,729,257	287,315
D_1D_4-1	87,396,092	58,831,590	220,274
D_1D_4-2	96,953,454	47,549,734	211,912
A+6-C	217,254,236	126,978,372	837,793
A+6-E	82,341,606	42,643,329	352,047

Table 3. Summary of transcriptome statistics following SOAP *de novo* assembly (contig length ≥200 bp).

Library name	Total contigs	Average length of contigs	Longest contig (bp)	Number of BLASTx hits	% of BLASTx hits
A+4-1	96,522	543.6	14,406	23,387	24
A+4-2	66,027	585.8	10,631	19,980	30
D_0-1	77,964	655.7	15,884	23,006	30
D_0-2	81,204	661.2	16,037	22,804	28
D_1D_4-1	65,563	660.8	21,071	20,258	34
D_1D_4-2	64,709	662.8	20,328	19,961	31
A+6-C	135,046	497.9	16,671	27,831	21
A+6-E	61,253	658.6	13,689	21,032	34

Table 4. Distribution and RPKM values of BLASTx hits for beta-actin in all eight libraries. We found seven different accession numbers assigned to transcripts that are homologs of the beta-actin gene. The protein sequence alignment of five BLASTx hit accession numbers (ABL63468.1, AFC88033.1, AAL40078.1, ACZ60616.1, AEI88062.1) and two conceptually translated D_1D_4 contigs (in yellow background) is shown in Figure 3. By manually combining all the RPKMs in each library we may avoid the problem of redundant gene assignments, but this technique cannot be applied for all 61,373 unique accession numbers. Circumventing this problem will require the assembly of a reference transcriptome by combining all raw reads from the eight libraries (120 gigabytes). Following annotation of the reference transcriptome, we can map the raw reads of individual libraries to the annotated reference transcriptome. In this manner we can avoid performing BLASTx searches for individual libraries, which in turn will reduce redundancies in assignments.

Accession number; Gene name; Species	**A+4-1**	**A+4-2**	**D_0-1**	**D_0-2**	**D_1D_4-1**	**D_1D_4-2**	**A+6-C**	**A+6-E**
gb|ABL63468.1| beta actin [*Scylla serrata*]	NA*	NA*	NA*	NA*	25647.3	NA*	181.4	NA*
gb|AEY68535.1| beta-actin [*Exopalaemon carinicauda*]	962.3	1.3	1498.6	1421.4	NA*	NA*	1059.3	NA*
gb|AFC88033.1| beta-actin [*Scylla paramamosain*]	NA*	3.1	31643.7	48684.1	NA*	21084.3	NA*	NA*
gb|AAG16253.1|AF300705_1 beta-actin [*Litopenaeus vannamei*]	NA*	1017.4	NA*	NA*	NA*	NA*	NA*	1091.7
gb|AAL40078.1|L76530_1 beta-actin, partial [Gecarcinus lateralis]	NA*	NA*	NA*	NA*	NA*	1366.8	NA*	0.46
gb|ACZ60616.1| beta-actin [*Panulirus argus*]	NA*	NA*	NA*	NA*	1593.78	1732.4	NA*	NA*
gb|AEI88062.1| beta-actin [*Scylla paramamosain*]	NA*	NA*	NA*	NA*	7.23	19.2	NA*	NA*

*NA: absence of corresponding BLASTx hit to the accession number.

Table 5: Size distribution of contigs and corresponding percentage of BLASTx hits.

Library Name	200 bp – 999 bp		1000 bp – 4999 bp		≥5000 bp	
	Total contigs	**# of BLASTx hits**	**Total contigs**	**# of BLASTx hits**	**Total contigs**	**# of BLASTx hits**
A+4-1	85,968	16,855	10,348	6,336	206	195
A+4-2	57,237	14,142	8,659	5,706	131	129
D_0-1	65,296	15,006	12,376	7,722	292	278
D_0-2	67,763	14,373	13,091	8,106	350	325
D_1D_4-1	54,693	13,180	10,641	6,693	229	220
D_1D_4-2	53,923	13,020	10,563	6,730	223	211
A+6-C	123,201	20,711	11,621	6,909	224	212
A+6-E	60,819	14,127	10,806	6,630	289	275

Table 6. Pearson Correlation Coefficients.

6A. Pearson Correlation Coefficients between replicates.

Library Name	Accession number RPKMs	KEGG pathway RPKMs
A+4	0.56	0.96
D_0	0.52	0.95
D_1D_4	0.53	0.93

6B. Pearson Correlation Coefficients between limb regeneration stages using average KEGG pathway RPKMs.

Libraries	A+4	D_0	D_1D_4	A6+C	A6+E
A+4	1	0.83	0.78	0.95	0.85
D_0		1	0.86	0.84	0.69
D_1D_4			1	0.79	0.67
A6+C				1	0.82
A6+E					1

Table 7. Percentage of annotated sequences not assigned to a KEGG pathway in each library.

Library Name	Not assigned (%)
A+4-1	85.10
A+4-2	86.02
D_0-1	86.16
D_0-2	86.21
D_1D_4-1	87.62
D_1D_4-2	87.78
A+6-C	88.61
A+6-E	74.75

Table 8. Cuticle proteins previously reported as differentially expressed across molt cycle in other arthropod species (order of proteins based on Figure 13A). The proteins in red had similar expression profiles in the D_1D_4 and A+6-E libraries (Figure 13B).

Accession number; name of protein; species	Abbreviation in Figure11	Paper	Site of expression	Time of transcript up-regulation or time of expression
sp|P81577.1|CUPA3_CANPG Cuticle protein AM1199 [*Cancer pagurus*]	AM1199	Anderson, 1999	Arthrodial membrane	Intermolt/down-regulated during premolt
gb|ABB91677.1| arthrodial cuticle protein AMP16.5 [*Callinectes sapidus*]	AMP16.5	Faircloth and Shafer 2007	Arthrodial membrane	Intermolt
gb|ADI59754.1| early cuticle protein 6 [*Callinectes sapidus*]	ECP6	Shafer et al., Unpubl.	Pre-exuvial cuticle	Premolt
ref|XP_002424768.1| structural constituent of cuticle, putative [*Pediculus humanus*]	PSCC	Kirkness et al., Unpubl	Unknown	Unknown
gb|ABB91676.1| calcified cuticle protein CP14.1 [*Callinectes sapidus*]	CP14.1	Faircloth and Shafer 2007	Calcifying hypodermis	Premolt
gb|ABM54466.1| cuticle protein BD1 [*Portunus pelagicus*]	BD1	Kuballa et al., 2007	Multiple tissues	Intermolt
gb|ABB91675.1| calcified cuticle protein CP19.0 isoform B [*Callinectes sapidus*]	CP19.0B	Faircloth and Shafer 2007	Calcifying hypodermis	Intermolt
gb|ADI59752.1| early cuticle protein 4 [*Callinectes sapidus*]	ECP4	Shafer et al., Unpubl.	Pre-exuvial cuticle	Premolt
sp|P81589.1|CUC11_CANPG Full=Cuticle protein CP575; [*Cancer pagurud*]	CP575	Anderson, 1999	Calcified exoskeleton	Intermolt
gb|ADI59750.1| early cuticle protein 2 [*Callinectes sapidus*]	ECP2	Shafer et al., Unpubl.	Pre-exuvial cuticle	Premolt
gb|ABR27687.1| cuticle proprotein proCP5.2 [*Callinectes sapidus*]	proCP5.2	Seear et al., 2010	Head	Premolt
gb|ABM54465.1| cuticle protein CB6 [*Portunus pelagicus*]	CB6	Kuballa et al., 2007	Multiple tissues	Not differentially expressed

Figure legends.

Figure 1. Transcriptome pipeline. Steps used in bioinformatics analyses for generating non-redundant set of BLASTx hits to accession numbers from Illumina raw reads. The values in parentheses indicate the number of hits/assemblies for the respective category.

Figure 2. Number of contigs (≥200 bp) with unique and redundant accession number in each library. Redundancy (in red) in accession numbers were reduced via KEGG (read text) and only unique (in green) contigs were used for further analyses (Figure 2A). Further redundancy was reduced by combining all libraries and isolating only the unique accession numbers (Figure 2B).

Figure 3. Multiple sequence alignment analysis of crustacean beta-actin protein sequences. Beta-actin is a highly conserved cytoskletal protein. We have used this gene as an example to show problems associated with annotation of *de novo* assembled transcriptome libraries. Table 4 indicates that there are BLASTx hits to seven different accession numbers, all beta-actin orthologs, which in turn skew the associated RPKM values for this mRNA. This also affects the correlation between the biological replicates. In order to test sequence relatedness between two contigs assigned to two distinct accession numbers (ABL63468.1 and AFC88033.1, yellow background in Table 4), we extracted and translated the corresponding contig nucleotide sequences (http://web.expasy.org/translate/). The alignment of protein sequences using MUSCLE (3.8) (Edgar, 2004) revealed the two contigs (#372806 in

D_1D_4 -1 and #363682 in D_1D_4 -2) are identical, the only difference being ORF length (Figure 3A). In addition, multiple alignment of the crustacean beta-actin protein sequences from Table 3 showed that due to extreme sequence similarity between these orthologs Figure 3B) the pipeline assembly can assign different contigs to different accession numbers. The protein identity in Figure 3B is represented by the first letters of genus and species names followed by its corresponding beta-actin accession number (Table 3), for example, gb|ABL63468.1| beta actin [*Scylla serrata*] is represented as S.s. ABL63468.1.

Figure 4. Distribution of BLASTx hits and no hits percentages among three ranges of contig length. The percentage of BLASTx hits (in green) increases as contig length increases, but the number of contigs decreases as its length increases (Table 5).

Figure 5. An example of a keyword search of the Illumina Transcriptome database to retrieve homologs/orthologs to NCBI-characterized genes present in the limb bud libraries. 5A. Web page interface for keyword search; the text term 'methoprene-tolerant' is used for search; alternatively, the built-in BLAST search utility could be used (using insect query sequences) to identify hits in library. 5B. Retrieval results to 'methoprene-tolerant' text search. By clicking on the appropriate topics, the nucleotide sequence of the identified contig can be retrieved (shown in 5C), or the results of the best BLAST hits to the library can be visualized). 5D. BLASTx results for library contig D_1D_4 439450 using NCBI database. The three contigs retrieved from the limb bud library by keyword search were identical; only largest contig is shown.

Figure 6. Representation of number of contigs among global KEGG pathways in all libraries. In all libraries, the most number (~2 – 4 fold) of contigs were assigned to metabolism network, followed by either cellular processes or genetic information processing.

Figure 7. Differential expression of KEGG pathways associated with cellular proliferation. We have chosen two major pathways (Figure 7A; Replication/Repair and Cell Growth/Death) and their sub-pathways (Figure 7B). The sub-pathway Cell cycle is one of the component of Cell Growth/Death, whereas, Replication/Repair pathway include DNA Replication and Base excision repair sub-pathways. In all the selected pathways we see a trend in down-regulation of cell proliferation pathway expression levels at the D_1D_4 stage. This is expected, as the limb buds stop growing at late proecdysial stage. Similar pathway expression profiles are observed in D_0 and A+4, however, suggesting that cell division might be part of early proecdysial growth (see text for details).

Figure 8. A/B domain isoforms of ecdysteroid receptor. Figure 8A represents sequence information on alternate A/B domain isoforms of EcR. The headers of both sequences denote - domain isoform type (library: contig identifier associated with the sequences). The different domain color matches the color of domain name present in the header. The different domains are colored separately – blue: A/B; brown: DBD; purple: hinge; black: LBD. The "*" represents stop codon. The yellow background represents the variable regions within the A/B domain. The underlined amino acids in

Figures 8A1 and 8A2 represent the variable exons within the LBD domains. Graphical representation of relative expression of contigs containing *EcR-A* and *EcR-B* isoform variants during three stages of limb regeneration is shown in Figure 8B**.** The $\log_2$ fold change was calculated relative to A+4 RPKM values. A decrease in *EcR-B* isoform to relative *EcR-A* isoform is observed in D_1D_4 library. The error bars indicate standard error.

Figure 9. Orthologs for ecdysteroid biosynthesis enzymes. We have recovered full length sequences for the P450 ecdysteroid biosynthesis pathway orthologs characterized in insects, with the exception of *phantom,* for which a partial sequence was obtained (Figure 10).

Figure 10. MUSCLE alignment of Phantom ortholog from two brachyuran crabs. We recovered partial sequence data for *Uca* pugilator *phantom* (Up_Phantom_partial) and it is highly similar to *Gecarcinus lateralis phantom* (Gl_Phantom) recently isolated from a Y-organ transcriptome library.

Figure 11. Cladogram of orthologs required for ecdysteroid biosynthesis. The cladogram was built via alignment of protein sequences using MUSCLE (3.8) (Edgar, 2004). Figure 11A shows the different P450 family protein orthologs in different colored boxes along with the outlier protein sequence for the enzyme aldosereductase. Figure 11B represents a cladogram of Neverland orthologs. [Up – *Uca pugilator*, Gl – *Gecarcinus lateralis*].

Figure 12. Comparison of down-regulation of ecdysteroid receptor complex in A+6-E when tested via two independent methods. We observed significant down-regulation of both EcR and RXR in data obtained via two different methodologies. The $\log_2$ fold change is relative to A+6-C RPKM levels.

Figure 13. Expression profiles of cuticle proteins. There was a significant up-regulation of 10 cuticle proteins (CPs) and down-regulation of two CPs in A+6-E compared to A+6-C (Figure 8A, p-value<0.03 for all genes; Pearson Chi Square test). Of the 12 CPs, eight show similar expression profiles in A+6-E and D_1D_4 (Figure 8B). The $\log_2$ fold change in both graphs is relative to A+6-C RPKM levels.

Figure 14. Expression profiles of putative orthologs involved in cellular proliferation. We observed significant down-regulation of *Uca* orthologs for proliferation marker genes: *PCNA* and *MCM2* genes (p-value <0.0001 for both; Pearson Chi Square test) (Figure 14A). We observed significant down-regulation of *CycB* (p-value=0.0001; Pearson Chi Square test), but not for *CycA, CycD, CycE, cdk1* (p-value=0.32, 0.11, 0.09 and 0.32 respectively; Pearson Chi Square test) in A+6-E library. The NR HR3 was significantly up-regulated in A+6-E (p-value <0.0001; Pearson Chi Square test) (Figure 14B). The $\log_2$ fold change is relative to A+6-C RPKM levels.

Figure 15. MIH sequence and expression levels in blastema phase. The BLASTp result of conceptually translated putative *Uca pugilator* MIH (Up_MIH) shows it is closely related (Expect: 3E-56) to *Gecarcinus latelaris* (Gl_MIH) (Lee et al., 2007).

The Up_MIH sequence contains regions corresponding to both signal and mature peptide. The following are the regions/amino acids that are characteristic of crustacean molt inhibiting hormones (Figure 15A).

Signal peptide in *Gecarcinus lateralis* sequence. Rest of the amino acids represent mature peptide.

Amino Acid – Six cysteines that form di-sulphide bridges in the mature peptide.

Amino Acid – conserved amino acids that are characteristic for the CHH/MIH/GIH family (2 Arginine, 1 aspartic acid, 1 phenylalanine and 1 asparagine).

Amino Acid – Glycine in the 12th position of mature protein is characteristic of this group of peptides.

Figure 15B represents a cladogram of closely related peptide hormones in crustaceans that include crustacean hyperglycemic hormone (CHH) and gonad inhibiting hormone (GIH). The Up_MIH clustered with the Gl_MIH and not with Up_CHH or other crustacean species CHH or GIH sequences. The accession numbers of peptide hormone sequences utilized in the cladogram are:

gb|AAQ81640.1| molt-inhibiting hormone 1 [*Eriocheir sinensis]*

emb|CAA53591.1| moult inhibiting hormone precursor [*Carcinus maenas*]

gb|AFH36334.1| molt-inhibiting hormone [*Scylla paramamosain]*

gb|AEJ54622.1| prepro-gonad-inhibiting hormone isoform A [*Macrobrachium nipponense*]

gb|AAL33882.1| gonad-inhibiting hormone [*Metapenaeus ensis*]

gb|AAR89517.1| molt-inhibiting hormone 2 [*Penaeus monodon*]

gb|ACX47134.1| gonad-inhibiting hormone [*Penaeus monodon*]

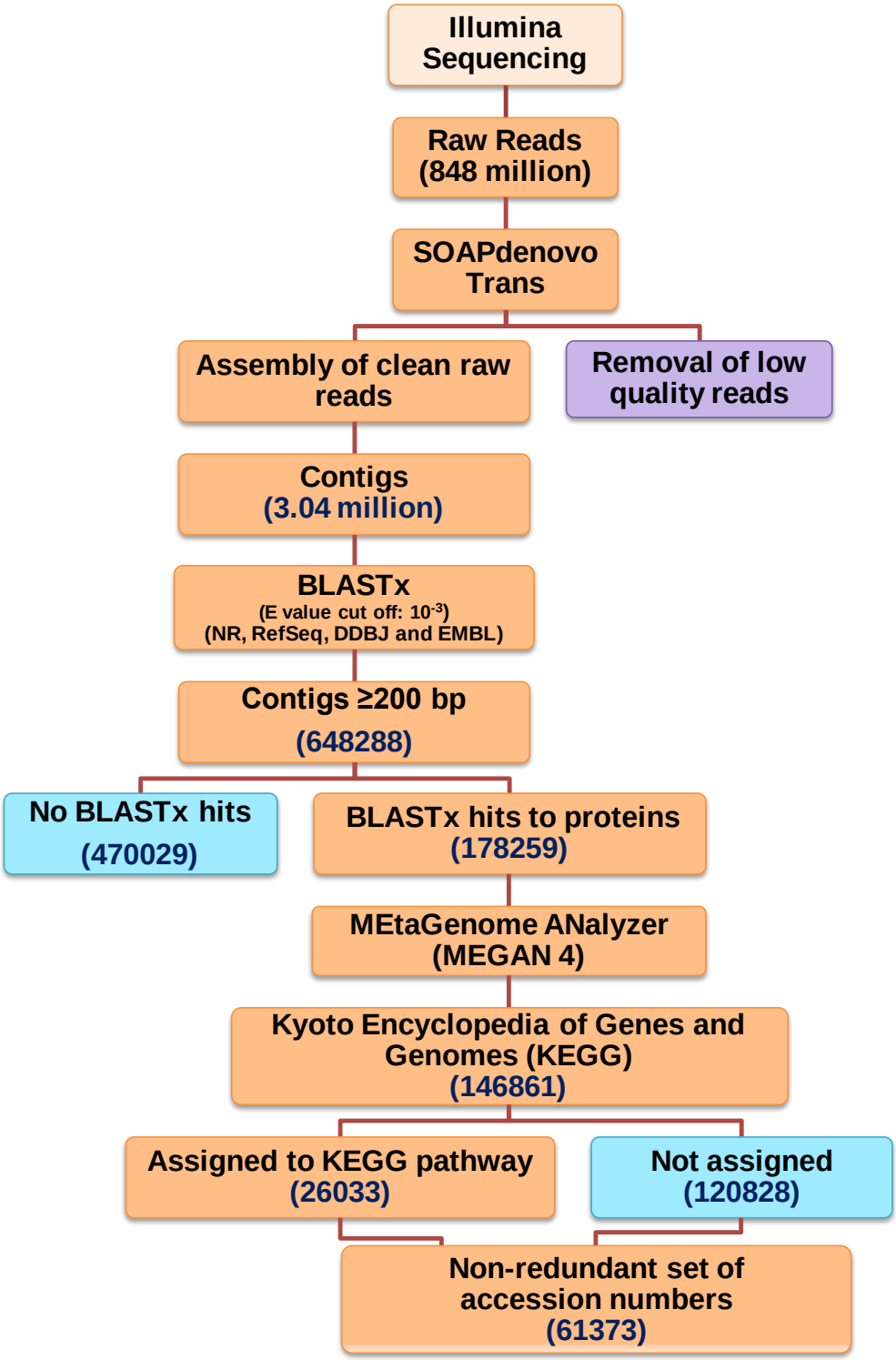
gb|AAQ24525.1| crustacean hyperglycemic hormone 1 precursor [*Penaeus monodon*]

gb|ABF58090.2| crustacean hyperglycemic hormone D [*Gecarcinus lateralis*]

gb|AAG32670.1|AF288680_1 CHH preprohormone [Carcinus maenas]

In addition, of putative *Up_MIH* transcript was significantly up-regulated in A+6-E (p-value <0.0001; Pearson Chi Square test), but no significant difference was observed for *Up_CHH* transcript expression relative to A+6-C. (Figure 15C).

Figure 1.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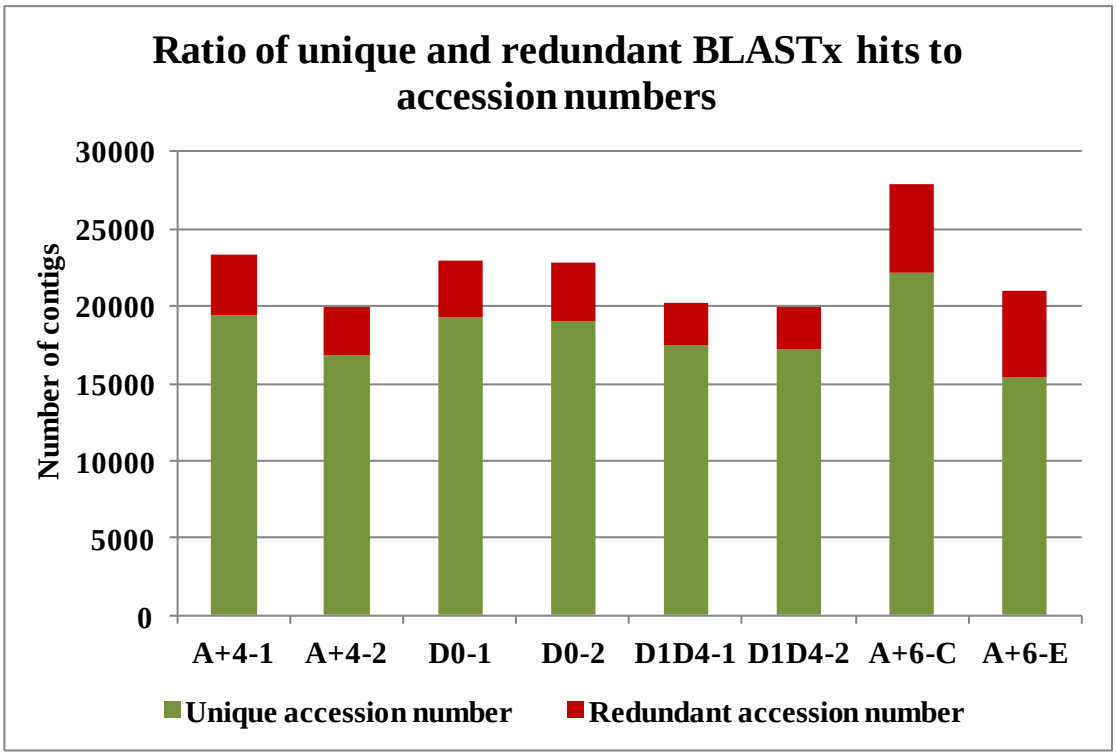
Raw Reads (848 million)
SOAPdenovo Trans
Assembly of clean raw reads
Removal of low quality reads
Contigs (3.04 million)
BLASTx (E value cut off: 10-3) (NR, RefSeq, DDBJ and EMBL)
Contigs ≥200 bp (648288)
No BLASTx hits (470029)
BLASTx hits to proteins (178259)
MEtaGenome ANalyzer (MEGAN 4)
Kyoto Encyclopedia of Genes and Genomes (KEGG) (146861)
Assigned to KEGG pathway (26033)
Not assigned (120828)
Non-redundant set of accession numbers (61373)

Figure 2.

2A.



2B.

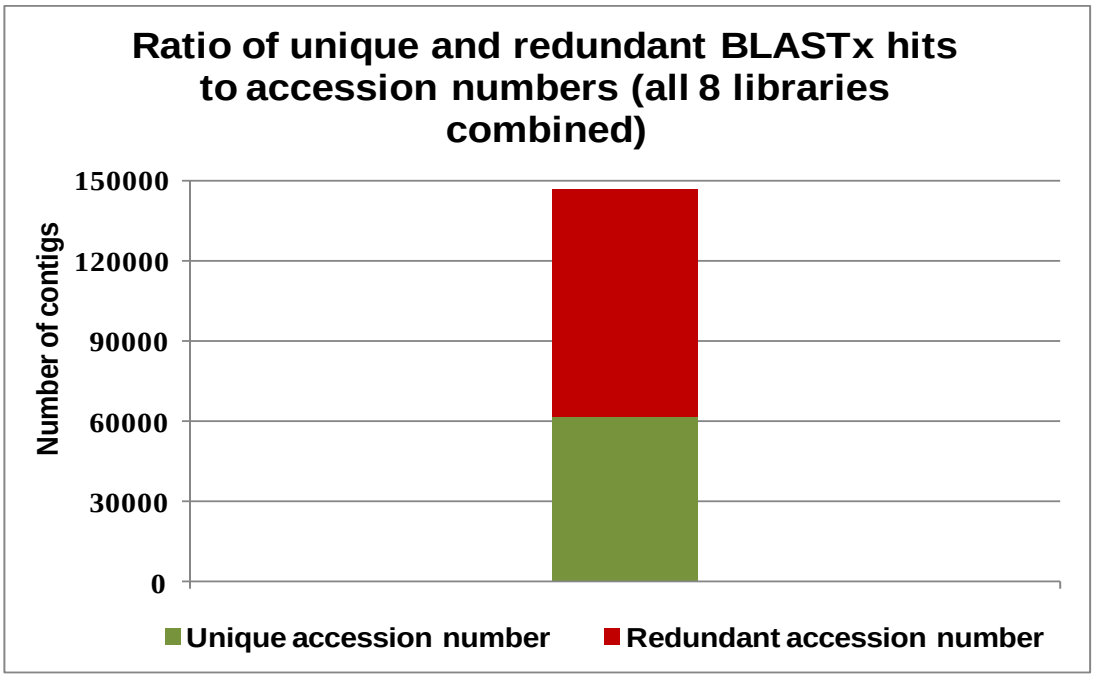
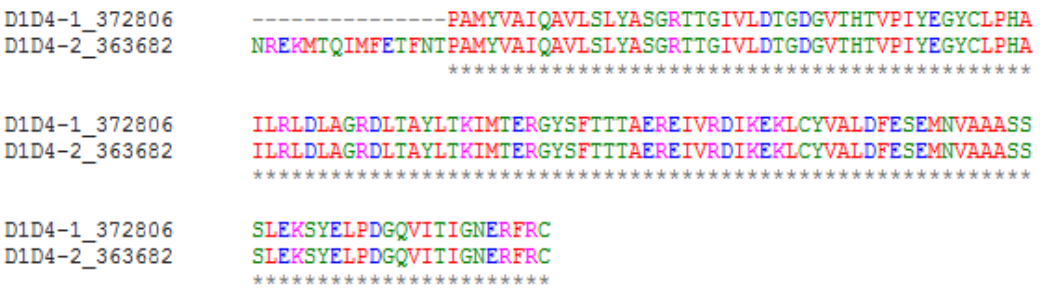


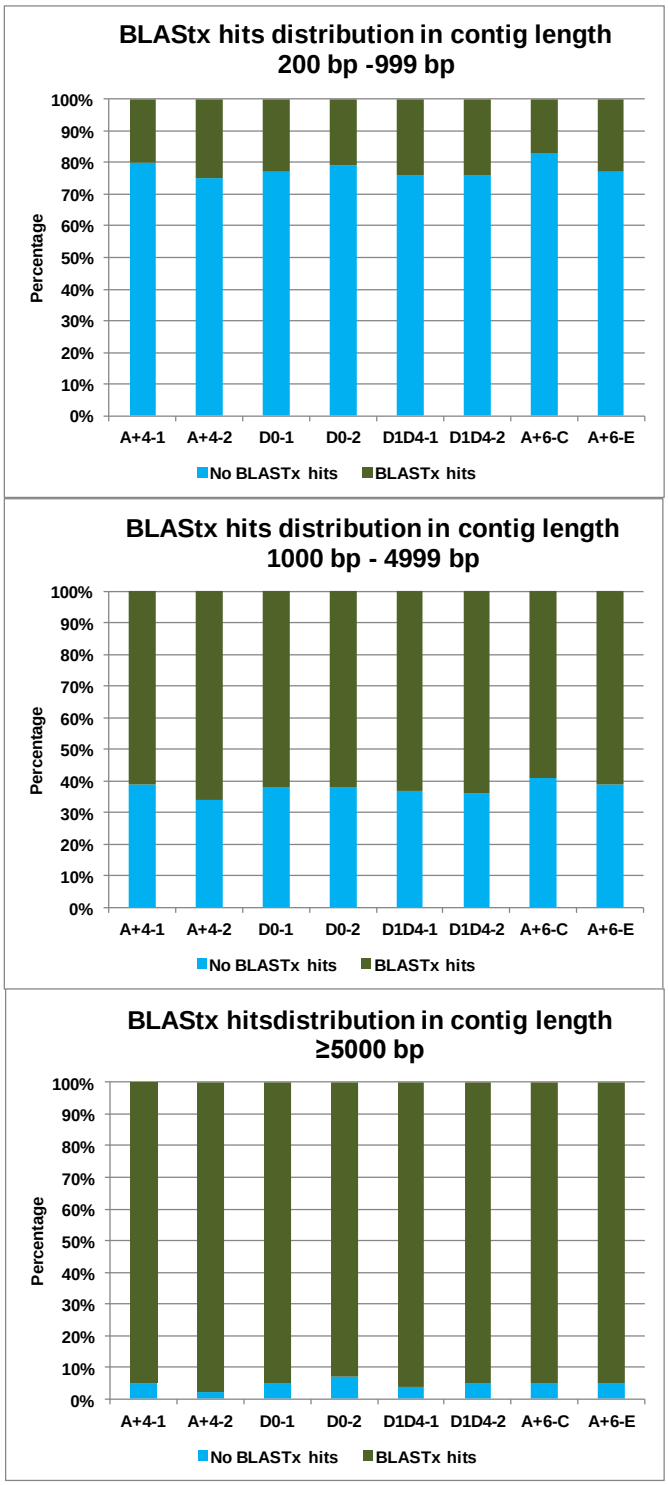
Figure 3.

3A.



3B.

Figure 4.


BLAStx hits distribution in contig length 200 bp -999 bp
Percentage
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A+4-1
A+4-2
D0-1
D0-2
D1D4-1
D1D4-2
A+6-C
A+6-E
No BLASTx hits
BLASTx hits
BLAStx hits distribution in contig length 1000 bp - 4999 bp
Percentage
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A+4-1
A+4-2
D0-1
D0-2
D1D4-1
D1D4-2
A+6-C
A+6-E
No BLASTx hits
BLASTx hits
BLAStx hitsdistribution in contig length ≥5000 bp
Percentage
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A+4-1
A+4-2
D0-1
D0-2
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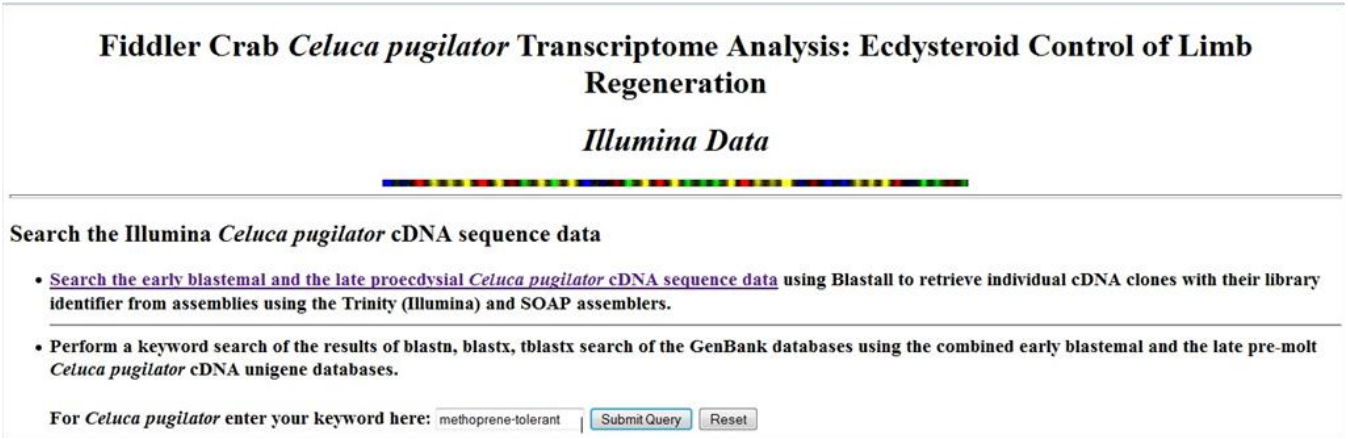
A+6-E
No BLASTx hits
BLASTx hits

5A.



5B.

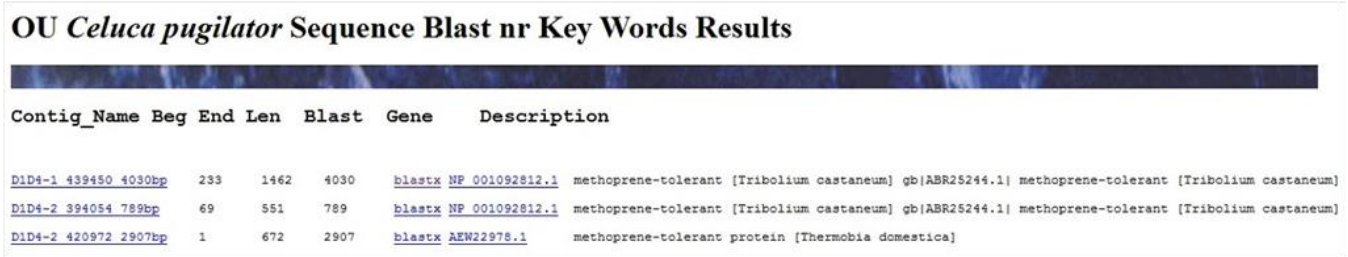


Figure 5.

Figure 5 (Contd.)

5C.

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AGTGCAGTGATGAGTGTGGTGAAGGGCCAGACGGGAGGTACTCATCACGCAAGTATAGACGAATGTAGTGGTGAAGACAGGAATTC
TATATCGACGCAGCTCAAGCCAGAGGCAAGCACTTCATCAGCCGACATGGGAGGCGAACAGAAGTTTCCCAGATCCTCGAGGGAAA
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TTGAGAGGGAACAATTTAAACAAACTCAAGCACACCCTGAACAGCATCTAAGTCCATCTTCTTCTCCTCAAGTGCAGATATGTCCG
GCTTCTCCACAGTATGGCAGTAAAAGCTGTTATCAGTCTCCATTAAATCAGGGTGAAGAACTCACAGACCAAAACTTTGCTCAAGG
CCTTGGTAGTAATAAATGTCATCCAAACAAGGACACTCAGTCTAATCCTTCAGACTACAGTCCTTTTGGAAATAGAAGTGAATTAA
GTCCAAGTACTGCCAATTATTTTATTGATGGCAGCATCACTTCTTCAAGAGACCAAAAGAAAGAAGACTGTTCACTTAAAACTCCT
GATCAGATAAATATCAATGTAGGTTTTCCTAAATCACAGTATAGTGCCGTGTCACTGAAGGAGAGCAAAGATAGCAGAATTTTATA
CGGACAAGGAGACTCTCAGATTATAGCTGCTGGCATGAGCTCTCCAGGGAGCACTTATGTCAGAAGTGAAGGAAGTGTTTCTCCCA
GTGGTTCATATCCTGTACATACTATCAGTGATGGGCCCATCACCAACAGCCCTCAAGATAGTGGAATAAGTGTAGGTTCTTATGCA
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CCATAGCCCTCAGCAAATGTCATTTGGTGGGTCTGTAAACCTCATAAACCCGTCCGAACCAGCTCATCAGATCAATATCCAACCAC
AGTTTTCTCCAAACTCCTTGTTGGGCACCAAGGATCACTATCACCAAGTGCAACAGCAGCAACACCATATATATAATCAACAGCAA
CTACTACAGCAACAACATCAGCAACAGCAACCACATCAACAGTTGCAGTTACAGCAGCAGTGCCATCAGCAACAGCACCAGCAACA
ACAACTACAACTAGGACAACAGAAACTTAAACATTTCCATCACCAGCCAACTATCACTGCAGCCAACATTAGACAGAACCTAAGTA
TCTCATCAACCCCAAAGTATGATGTAAAAGTGCAAGGGCACCTTAACCAAATAGTTCAGAGTTATGGTGCAGTAACAAACTGGCAA
TCAAGAACTGGTGATACTTCGTCTCATCCTTCAAATGCATCAGGAGAAAACCCAGATGTGTTCCACTCAACAAATATGTTACCTCA
CATGGAGAGTGAGGGTAGTCATGCCTTGCAATGTGACACCAGCTTCCACCTCAACAGTGCCAACCCAAGCTCAAAAAACAAAAGTG
TTCCGGTACAGCCTGAAGCCTGCTACAGTCATTGTTCTCCACCTCAGAGTATTGTTGAAAATCACCTGGGGACCACCTGTATTGCT
ACAAAAAAGGCTTACAACACACAATTTCAGTGTAGTCCATCACAAAGTGAGAGTAAAGGTAAATTCCCATGACAAGCAAAGCCCTG
TGAGTCAAATTACACATGATGCAAAAAATCACTGAAGATGAACACAACATGTTCTCCAAGAATTGCCTACTTTACCAGATTATGAG
GTAATGTCTATATGATTTACTAAAAGTTACACTTTGAGAAAGTTATTATAATTCACTTACAATAAAGGTAAAGGTAAACTTGGGGT
TATACACCACACTGTGCATGGTGAAGTGGTTAGTTGGTAATTTTGGATGGTTCTTAAACGTGTATCTGAGGAAAACTAATGAAGGT
AAACTATGGTAACACAGATGTCACACTGATTCTGTAATTCACCTCGGTCTTGACTGTGTGCTGGACCTATGTATGCCAGTTCCTTA
CCCAATAGGAAAGAGCTCAGAGCTCATAGAGTTCCAGTCTCTAGGTAATGCTGTGACCTAACACACCACACACCTCATCCTCCTTG
TTCAAGGAGGGGCAGTAGCCACCTGTGTTAATGGAAAAATCTGGAGATTTTCTTGAGCAAGGATCAAACTGCTGACCCTTGGGTGA
GAGATTCAGCCATTACCAACTGAACTATCAGTTTCCCAGGGTAGTGGGTTTTGGCATTCCTCAGAATGAAAGGCCAAGCAGTGGAG
ATGAACCACTCCATGTGAAGGTTTGTGTATGCCCCCAACTTTACAACACTTAGTAGGCAAACCTGATATTATAT

Figure 5 (Contd.)

5D.

```
methoprene-tolerant protein [Thermobia domestica]
Sequence ID: gb|AEW22978.1|Length: 516 Number of Matches: 1
Related Information

Score           Expect             Method          Identities     Positives     Gaps       Frame
291 bits(744)    1e-82   Compositional matrix adjust. 159/414(38%) 231/414(55%) 78/414(18%)   +2

Query  371   RDKLNNYINELAGIVPLGSGSSKRLDKTSTLRLAANYIRMHKIL-VKEDEAEKVPLVLG-  544
             RDKLN YINELA +VP+ + S+KR+D+TS LRL+A ++RMH+ L   E + + +P  L
Sbjct  1     RDKLNMYINELATLVPMVASSTKRMDQTSILRLSATFLRMHRTLNSTEFQMQLLPSYLEL  60

Query  545   GNIAHNLAEAVGGFLLVVTSTGRVVYVTEAVDQFFGHSQVDLLGHSIYNVIHPDDHEIFQ  724
              NI+  + E + GF+L+VTS+G++V+++E V++  GH+Q+DL+G SIY + HPDDH   +
Sbjct  61    ANISQQMLEDMEGFMLIVTSSGKIVFISEMVEKLLGHTQMDLMGQSIYGITHPDDHSEMR  120

Query  725   QQLIP-----------------------------------------------------KGNNR  754
               L                                                     + N R
Sbjct  121   MNLSANNDDSDSSSTDSQSGDDSSSEDASASPQSESSPRPSETSSSPAATTSDPCQTNQR  180

Query  755   VSFFCRMMEKALTRNDPGRYEIIHIVGQLKPIPASATIVPCSPSTSVISPGGRSNSDAEE  934
              SF+ R+ EKA++R DP RYE+IHIVG L+        VP  P  +   P  +S     E
Sbjct  181   RSFYVRLAEKAVSRGDPSRYELIHIVGHLR--------VPPRPEAT---PSTQSRRRQRE  229

Query  935   NYESDGDVDNQTLKAAINRIGTHILVSFVRVVKDRPITELSLVESTQDEYITRHGLGGNI  1114
                 S  D               ++LV   R+V+DR ITELSL+E+ +DEY+TRH L G I
Sbjct  230   PVTSMND---------------YVLVGVGRLVRDRRITELSLMEAVKDEYVTRHLLDGRI  274

Query  1115  LYTDHRISVVTGLMPGEVVGTSAFAYMYPDDRVWSLVAQKLMFTSTQGQGMVSYRLQCRD  1294
             +Y DHRISVV G +  EV G SAF +M+ +D  W+++A + M+ S  G G   YRL  ++
Sbjct  275   IYVDHRISVVAGYLAEEVSGLSAFNFMHAEDSRWTMIALRQMYGSADGCGNSCYRLLAKN  334

Query  1295  GSLVTLRSRGYLEVNKQTGQVESFVCINTVVSVKEAENEIKNQRRKLLPVISSQ  1456
             G  + LR+ GYLE +K T +VESF+C+NT+VS +E E  I++ R +  P I+ Q
Sbjct  335   GQFIYLRTHGYLEFDKNTQKVESFICVNTLVSDEEGEQGIQDMRDRYSPFIAFQ
```

Figure 6.

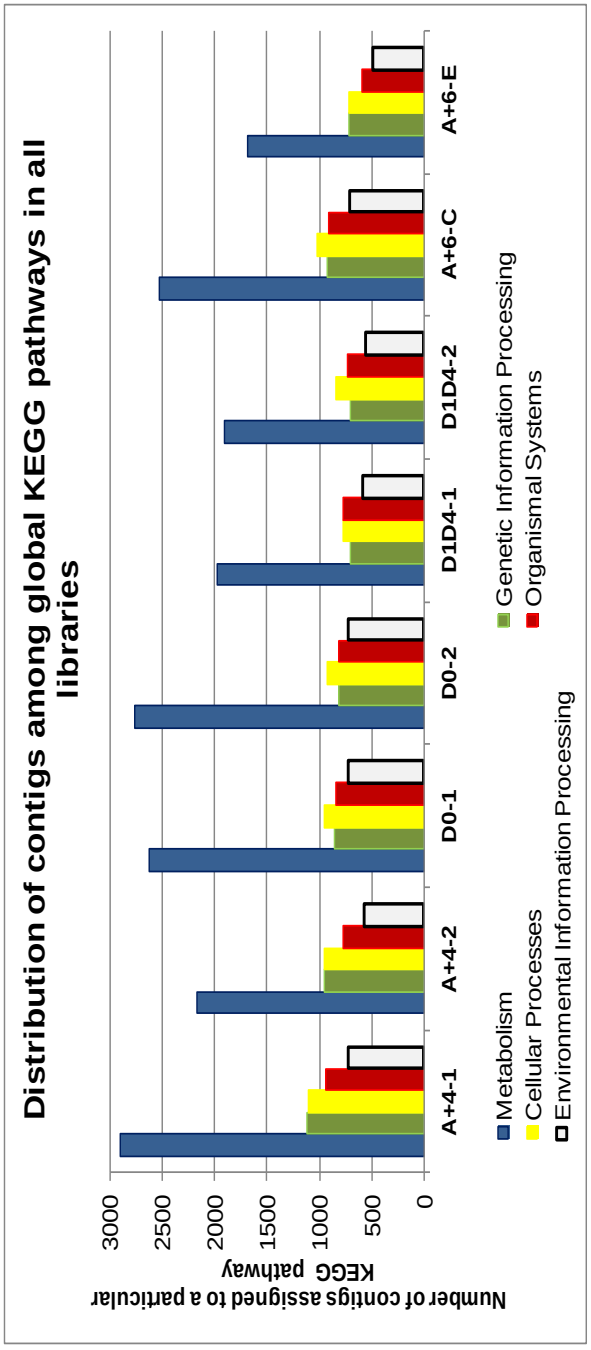
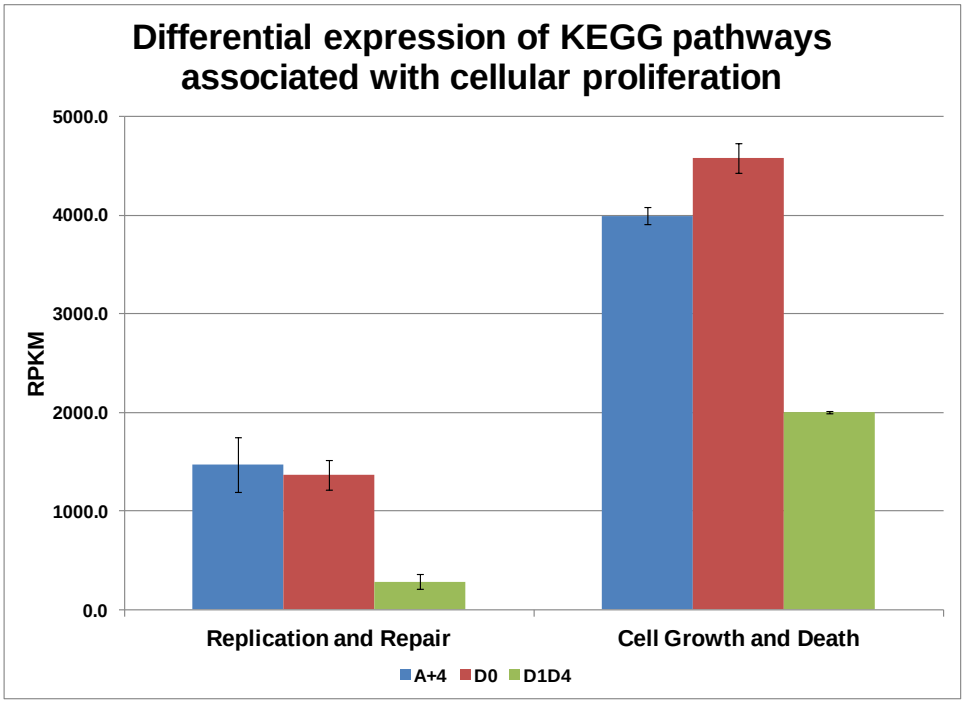

Distribution of contigs among global KEGG pathways in all libraries
Number of contigs assigned to a particular KEGG pathway
3000
2500
2000
1500
1000
500
0
A+4-1
A+4-2
D0-1
D0-2
D1D4-1
D1D4-2
A+6-C
A+6-E
Metabolism
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Organismal Systems

Figure 7.

7A.



7B.

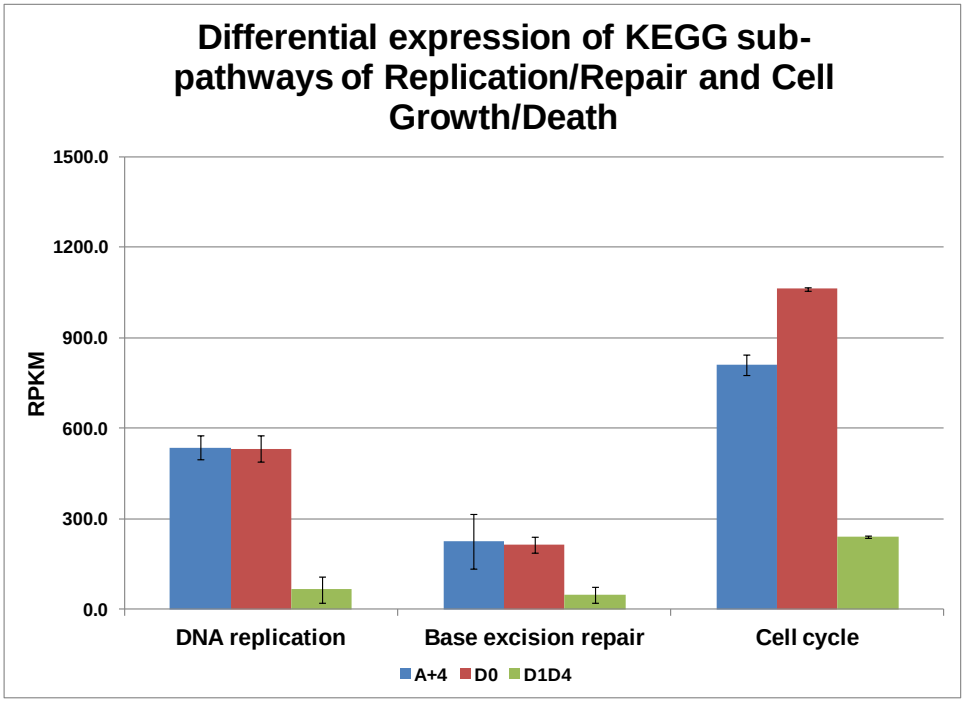


Figure 8.

8A1.

EcR-A isoform_Alternate-hinge-4a_LBD-exon_6a (D_1D_4-1: 440118)

MDLPRDSPFRGRGLMGGRSPTPHSSLIHTVVSPKMDHSPTSPPYVANSPLLGESPSGIPRSPVSPLGVL
VKSEPPVSPSGPSEYQVKPKKPRSDGERAEWVNSPTMSIDSLSPPPQNTNGVGGSLGHPSSGMSPMSSS
SYDPSSPYLSRSGRDDMSPPSSLSNFGADSYGDLKKKKGPIPRQQEELCLVCGDRASGYHYNALTCEGC
KGFFRRSITKNAVYQCKYGNNCEMDMYMRRKCQECRLKKCLNVGMRPECVVPESQCQVKREQKKARDKD
KTYPSLGSPIAEDKAAPISPCKSKGPSTACAMPFKNLVDSSSTVQSPMSAAPRLNVKPLTREQEELINT
LVYYQEEFEQPTEADVKKIRFNFDGEDTSDMRFRHITEMTILTVQLIVEFSKQLPGFATLQREDQITLL
KACSSEVMMLRAARRYDAKTDSIVFGNNYPYTQASYALAGLGESAEILFRFCRSLCKMKVDNAEYALLA
AIAIFSERPNLKELKKVEKLQEIYLEALKSYVENRRLPRSNMVFAKLLNILTELRTLGNINSEMCFSLT
LKNKRLPPFLAEIWDVSGY*

8A2.

EcR-B isoform_DBD_Alternate-hinge-4a_LBD-exon_6b (A+4-2: 609880, 489979, 614358)

MAKVLATARVDGMFVLGSGVATLNLSTMGDESCSEVSSSSPLTSPGALSPPALVSVGVSVGMSPPTSLA
SSDIGEVDLDFWDLDLNSPSPPHGMASVASTNALLLNPRAVASPSDTSSLSGRDDMSPPSSLSNFGADS
YGDLKKKKGPIPRQQEELCLVCGDRASGYHYNALTCEGCKGFFRRSITKNAVYQCKYGNNCEMDYMRRK
CQECRLKKCLNVGMRPECVVPESQCQVKREQKKARDKDKTYPSLGSPIAEDKAAPISPCKSKGPSTACA
MPFKNLVDSSSTVQSPMSAAPRLNVKPLTREQEELINTLVYYQEEFEQPTEADVKKIRDYEISDDDSDA
KFKHITDMTILTVQLIVEFSKRLPGFDTLLREDQITLLKACSSEVMMLRAARRYDAKTDSIVFGNNYPY
TQASYALAGLGESAEILFRFCRSLCKMKVDNAEYALLAAIAIFSERPNLKELKKVEKLQEIYLEALKSY
VENRRLPRSNMVFAKLLNILTELRTLGNINSEMCFSLTLKNKRLPPFLAEIWDVSGY*

8B.

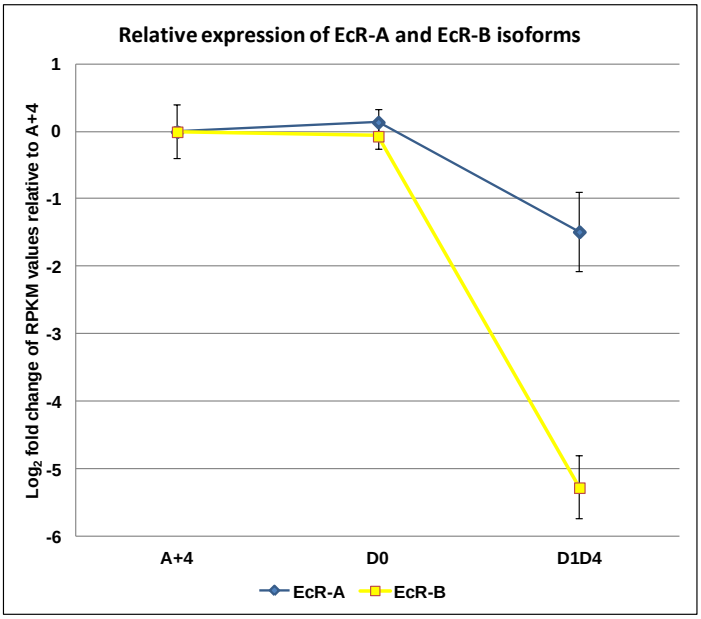


Figure 9.

>*Up*_Neverland
MLTDSLVRLVTECFHHNNVTTPRAASPPLGLGLGLLRAGDCWRWTEDGLALLTQEGAAWHLLYYL
AGTLLLLFVYRVAFSPVDMVKDLTDVGWGCLGGGRGSSLTERIREVQRLRKKGNLPPVYPNGWFT
VVESQELAVGQVKSVQVFGQTLAVFRGRGGEVHVTDAYCPHIGANMAVGGVVKGDCLECPFHGWL
FSGSDGKCVEIPYSSKVPRTASVKHWESRELNGFVFVWYDAEGRDPLWELPEVPEVADGSWAFRG
RTVHQILAHIQEMPENGADVAHLGHLHVPSIFKGSDLRDIFASSTLLDIAKHSWSGEWQARAAPE
SHVADLKVTHAFSLFGGRLKLFSMTVKVEQLGPGVVYLYFNTSVGSGVLIQTVTPLEPLRQKVVH
QFFSSRTFIAPFAKFVILSEARHFERDIMVWNNKQYLSQPLLVSEDRFIVKFRRWYSQFYSENSP
KFSFTKENSLDW

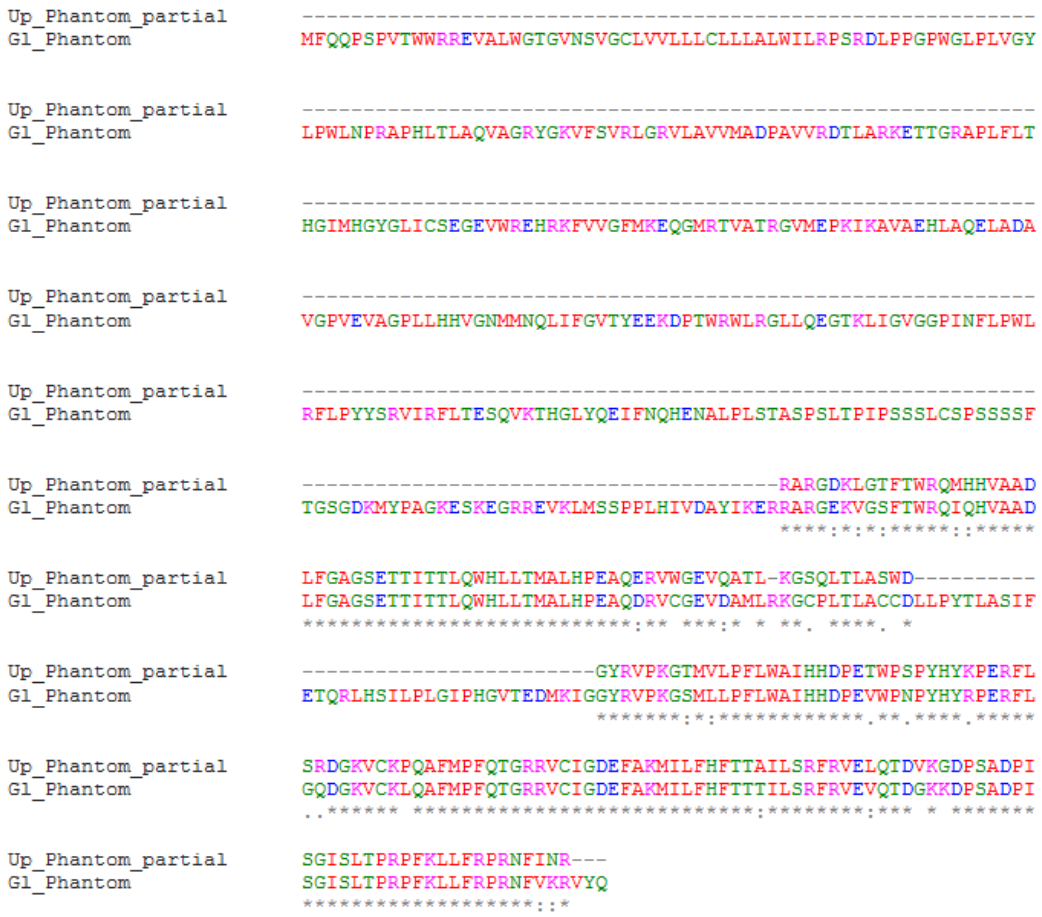
>*Up*_Spook
MVFVLAPATLIIMMMVLVAIAVQETARRRRKHQKQQYFQGTLTTPTASSDDLEIAKPTPPPGPTP
LPFIGNLLNLRNHSDCPYQGFSELKDKYGPVYSLKLGSSNAVIVNTYDTIKEVLINKANSFDARP
NLSRFNLYFGGERQHSLALCDWSDHQKRRMTLARAFLMFRGQQDHFSKFEANAVSEMPTLITEFD
KVLGRPVEAKEILSYCALNIFTGYMCSKKFQYEQDDFKTLTQNFDYIFRDINTGHIIDFLPGLEP
LFPSYINEIKKTATDIRQNILNNICLEKYEKLKQNPNDVEDLVDACFANLLTENEGEKWDWQTIL
YIVEDLLGGSMAVSNIVMRLLGHILQHPHVVQALRKEIDEKIGERPATLEDRHQMLYSQAVLYET
LRLTSSPIVPHVATEDATIGGYFVEKGSIVFPNNFEMNTSPSLWEEPTKFMPERFLKDGGLKKPE
YFIPFSTGKRACVGSKVVANVAFLVITTLLQRYDISLAEGKPEMPRGKISLDWNPFQMVFAMRQ

>Up_Aldosereductase
MAARIPLVTLNNGKKIPILGLGTWKSKPGEVTQAVKDAIACGYRHIDCALVYGNEPEVGAAIKAK
IEDGTIKREDLFITSKLWNTFRSKDLVIPSLKKSLTNLGLDYLDLYLIHWPMGYKEGGELFPADS
NGKTLYSDVDYVETWLELEKAQKEGLVQSIGVSNFNKEQIERILAAGEIVPATNQVECHPYLNQK
KLIDFCRSKNIYITAYSPLGSPDRPWAKPGDPLLMDDPKIVAIAEKYKKTPAQILIRYQIQRDVI
VIPKSVTKSRIESNFQVFDFTLSDEDMKTIDSFDCNGRLLHLNWVNDHKYYPFHAEF

>*Up*_Disembodied
MRGVVLGRLAAAVGDRLTCPRLLRHDAGAFTVSPTGYFLSIRAFSEGAGVTWEDAKPFDDIPGPA
ALPVVAGLHHYLPYVGKYSFSRLHQSGRLKLEQYGPIVRERLPGNVNLLLLFDPEDIETMYAKEG
RYPCRRSHLALQKYRLDRPHMFFTAGLLPTNGKHWWELRRRAQKSLSRVSAVASRLPHVDEVSRE
FADVVGRVRSGGSGRIPHFVDLGKRLFLELTMVSLLDTRLGDLTDHNEEADTLMAAADETNALTL
PTDNGMQLWRYLDTPKYRRLVRAQDTLYRIALKYVESKDEELRHARQERQAAGKEPEGKSSSSIL
ESFFESGLEDKDIVGLVSDTFIGGVDTGSYTLTYVLHSLACNPEKQDMLANEAKRLLGGSGGKVT
VGVLSEAKYLKAVLKETYRLHPISVGVGRIMQEDCVIRGYRIPKDTVVVTQNQVSSRMPEYFPDP
LHFLPERWLHKAPPAHPFLVLPFGHGPRSCIGRRMAEQNLQAVILHLMLRYRVGWLGGELDCVSK
LINEPVGHIEFTFTNR

>*Up*_Shadow
MSKAATNSILGRHLYRLGLQLRSSHRRHRATLANVTDDVQASHEARSITPQPAPTKSYKELPSPA
GYPVLGTLPKFVAAGGVQRHHKYVSQLHQELGSVFRDNLLGMELVFVSDQAAVREVFAAEGQYPQ
HFVPEPWLLYNKDRQVRRGIFFMDGEEWKRHRTVVLNRRLLRPGPLLAHLPAVNRIADALVDRWT
SRFPCRPIPDLERELYHWALESLGVMILGDKVGLLGDAPSNAEEQQRRREMLRFVEAIHGIPKLA
RALRLPAWKRMFGSLDEALASGHALVSAGLKTSRERRARGEDHHPPSLLDHLLHDEQMEEHEIIP
HLTDLFLAAADTTSYTAIWALYLLARHPEATQRLRQEVLEVTGGTGQVEGEHLAAMPYLKGVVKE
ALRMYPVAPFQSRVAQRNINLLGYKVPAGLMVILSVYTMGRDPTVFPNPDSFHPERWLRDTTAPS
ASAPCPFGSGPAAPRANSHAFFPFGIGSRSCIGRRLAENELYVLLAKLVARTDFRVLNQVDMAIR
MIGVTSEPLQLQVEPLTSTTTGRH

Figure 10.


Up_Phantom_partial
Gl_Phantom
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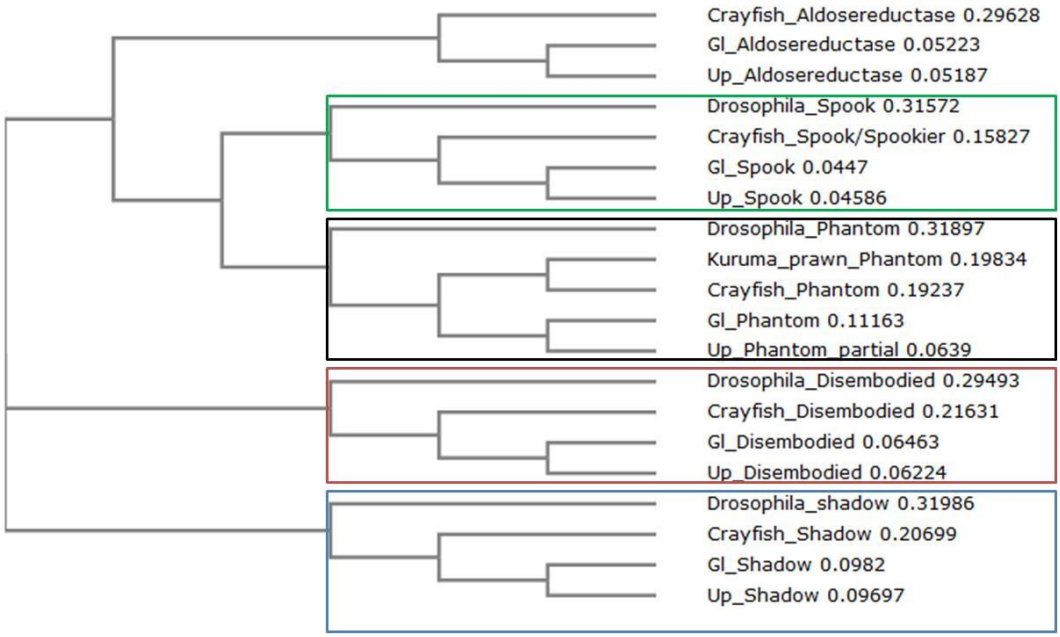
LPWLNPRAPHLTLAQVAGRYGKVFSVRLGRVLAVVMADPAVVRDTLARKETTGRAPLFLT
HGIMHGYGLICSEGEVWREHRKFVVGFMKEQGMRTVATRGVMEPKIKAVAEHLAQELADA
VGPVEVAGPLLHHVGNMMNQLIFGVTYEEKDPTWRWLRGLLQEGTKLIGVGGPINFLPWL
RFLPYYSRVIRFLTESQVKTHGLYQEIFNQHENALPLSTASPSLTPIPSSSLCSPSSSSF
TGSGDKMYPAGKESKEGRREVKLMSSPPLHIVDAYIKERRARGEKVGSFTWRQIQHVAAD
LFGAGSETTITTLQWHLLTMALHPEAQERVWGEVQATL-KGSQLTLASWD----------
LFGAGSETTITTLQWHLLTMALHPEAQDRVCGEVDAMLRKGCPLTLACCDLLPYTLASIF
ETQRLHSILPLGIPHGVTEDMKIGGYRVPKGSMLLPFLWAIHHDPEVWPNPYHYRPERFL
SRDGKVCKPQAFMPFQTGRRVCIGDEFAKMILFHFTTAILSRFRVELQTDVKGDPSADPI
GQDGKVCKLQAFMPFQTGRRVCIGDEFAKMILFHFTTTILSRFRVEVQTDGKKDPSADPI
SGISLTPRPFKLLFRPRNFINR---
SGISLTPRPFKLLFRPRNFVKRVYQ

Figure 11.

11B.



11B.

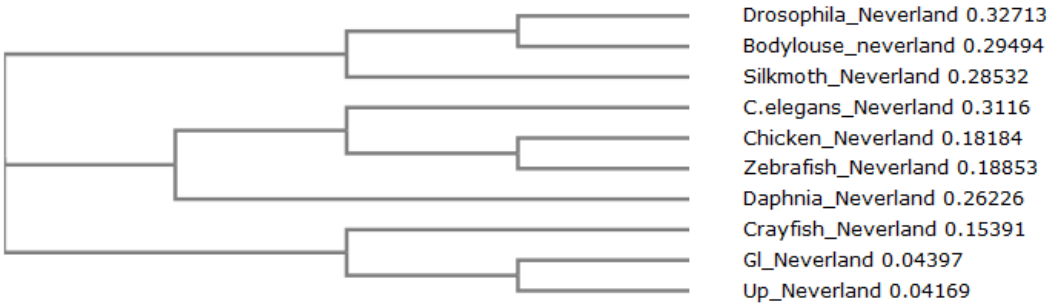


Figure 12.

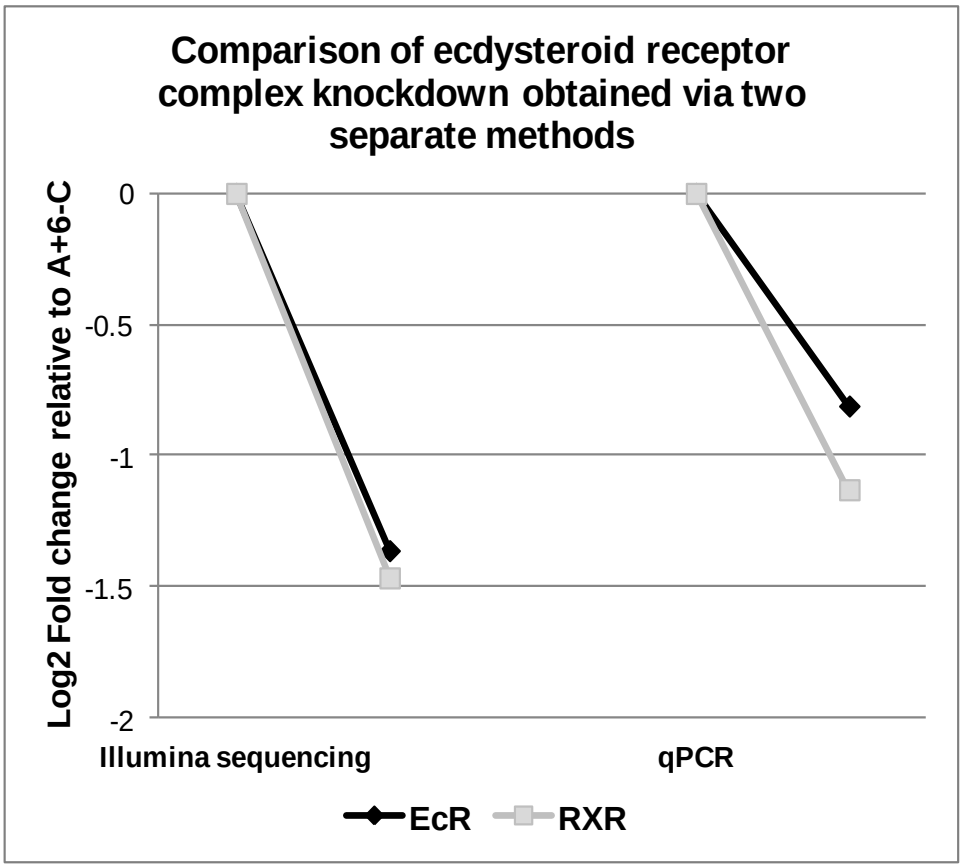
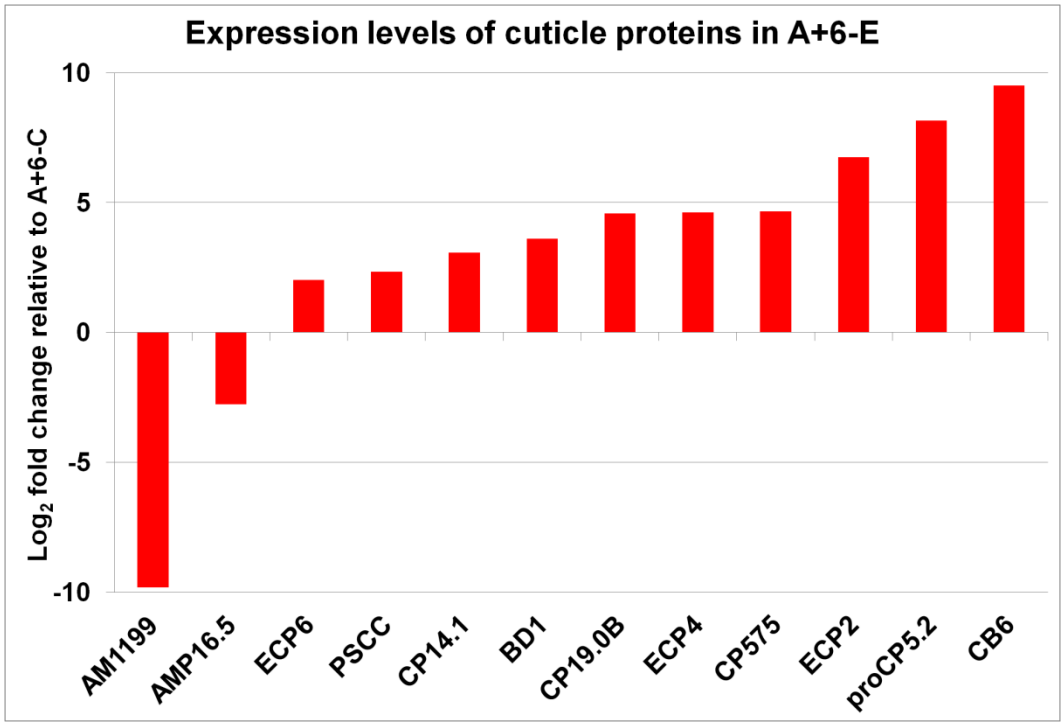

Comparison of ecdysteroid receptor complex knockdown obtained via two separate methods
Log2 Fold change relative to A+6-C
0
-0.5
-1
-1.5
-2
Illumina sequencing
qPCR
EcR
RXR

Figure 13.

13A.



13B.

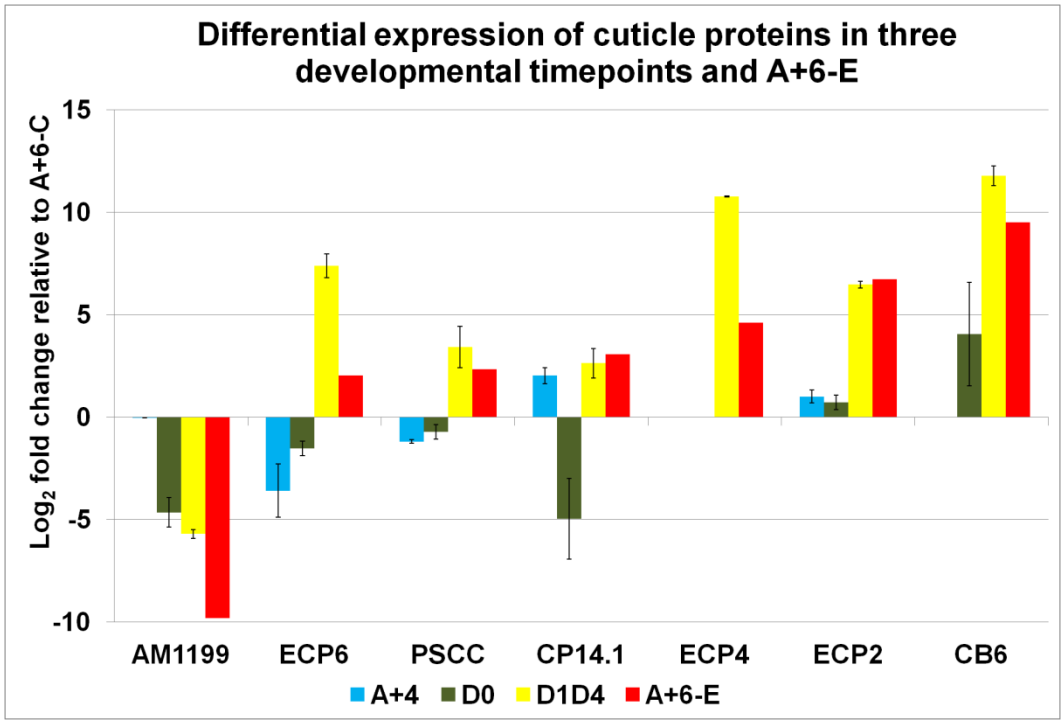
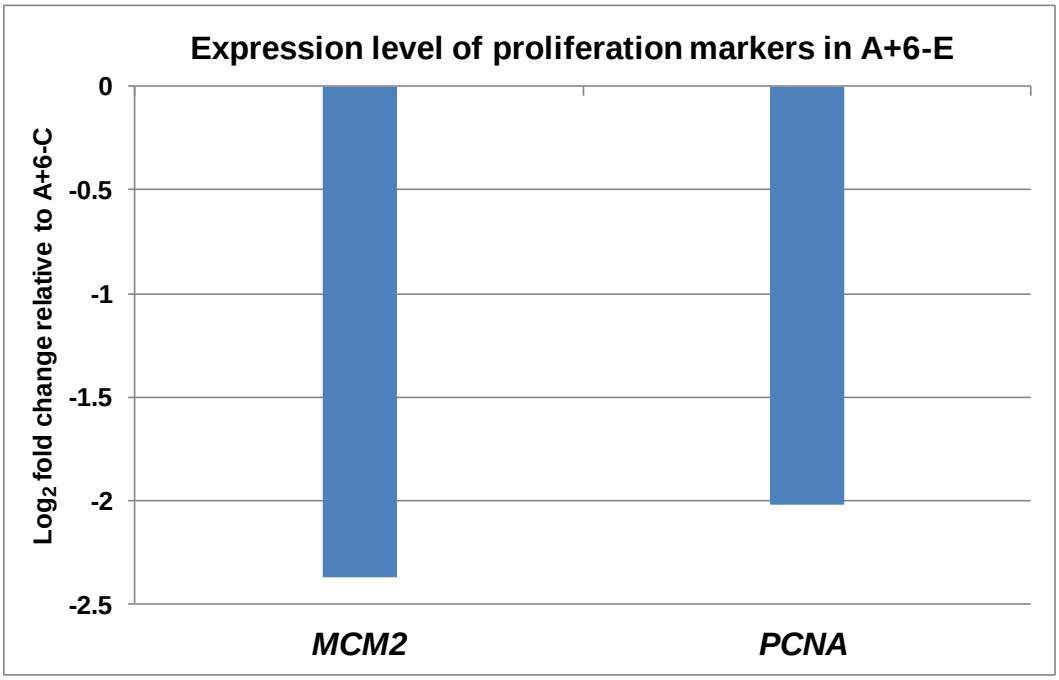


Figure 14.

14A.



14B.

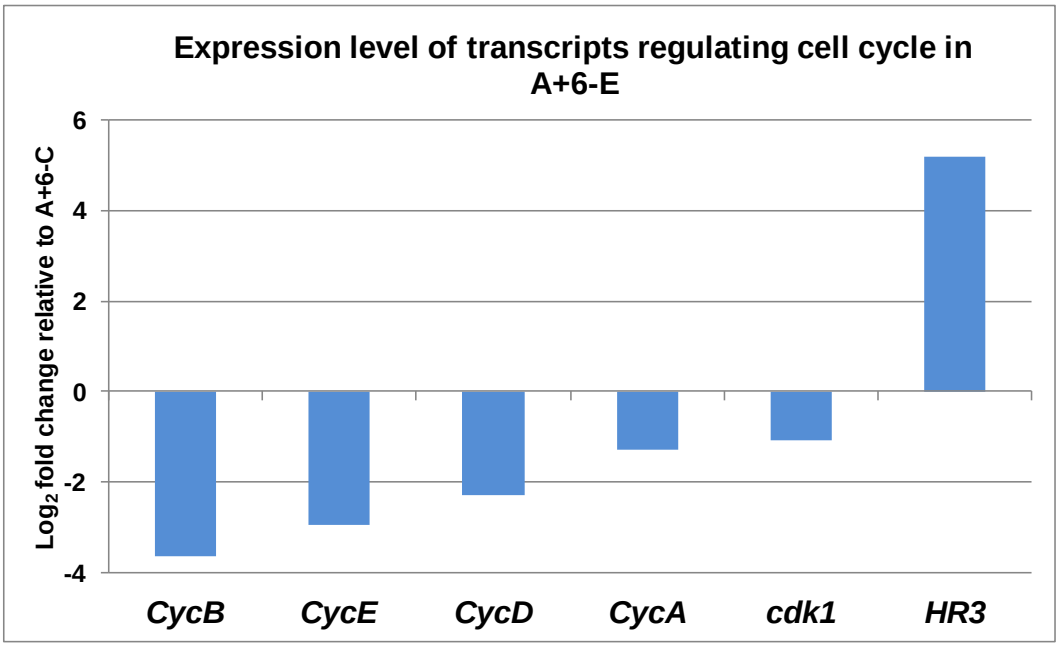


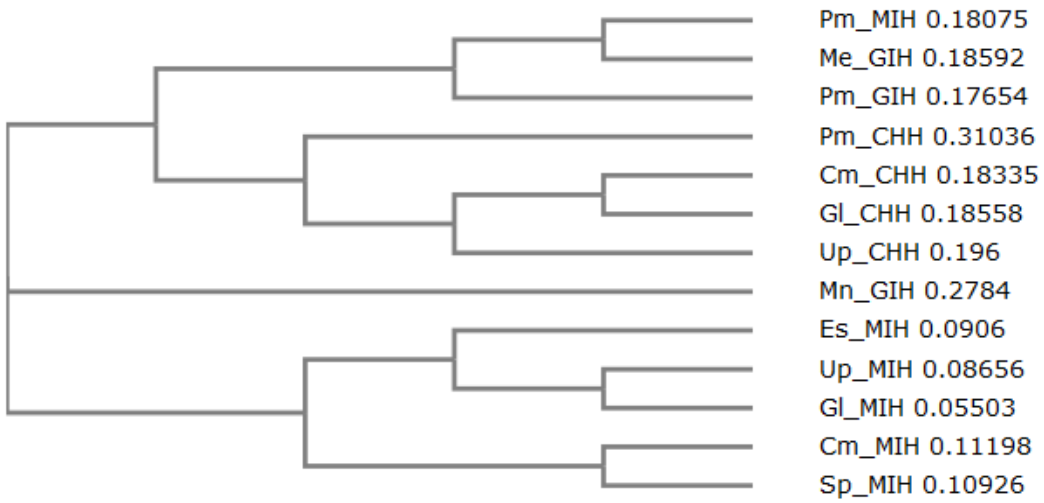
Figure 15.

15A.

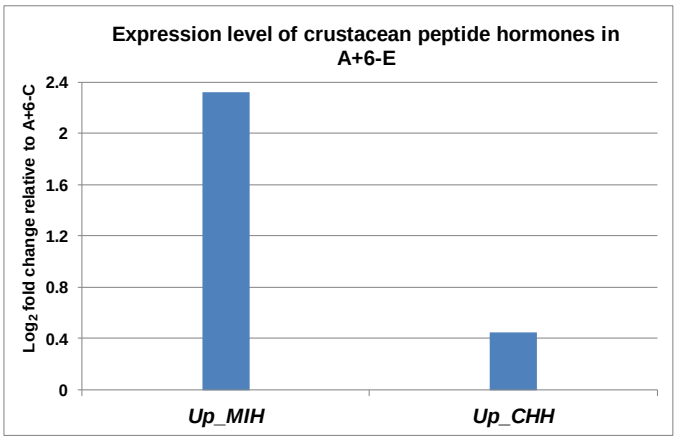
```
Up_MIH   1    MMSRNASRFTSQRTWLVAVAVLAVLWSCGVQRAAAGVIHDECSNRIGNRDIYKMVDWICE  60
              MMSR  SRF SQRTWLVAV VLAVLWS GVQRAAA VI+DEC N IGNRDI+K VDWICE
Gl_MIH   1    MMSRAESRFASQRTWLVAVVVLAVLWSIGVQRAAAAVINDECPNVIGNRDIFKKVDWICE  60

Up_MIH   61   DCANIFRIDGLSTLCRKNCFLNTDFLWCVYASERQAQKDELTRYVSILRAGS  112
              DCANIFRIDGL+TLCRKNCF N DFLWCVYASERQA+KDELTRYVSILRAGS
Gl_MIH   61   DCANIFRIDGLATLCRKNCFRNIDFLWCVYASERQAEKDELTRYVSILRAGS  112
```

15B.



15C.



APPENDIX A

Supplementary methods

Protocol to generate KEGG summary file for transcriptome libraries

This protocol is written by using data from D_1D_4-2 as an example. The Unix command lines for running perl scripts are written in red color.

1. Download and install MEGAN 4 from:

   http://ab.inf.uni-tuebingen.de/data/software/megan/download/welcome.html

2. Open the following website:

   ftp://ftp.genome.ou.edu/pub/for_Durica/Illumina_data_2011/D1D4-2_dir/soap_assembly/for_web_dir/

3. Download the following files:

   a. BLASTx file (D1D4-2_LargerThan200.X)

   b. Redundancy file (Redunduncy_SUB.xls)

   c. Perl scripts:

      i. summarize_blast_4_contigs5_X_pl2

      ii. summarize_MEGAN_KEGG_v3_pl

      iii. match_soap_redund_2_blast_summ_pl

4. Generate *.rma file by importing BLASTx file to MEGAN 4 following user manual (Huson, 2012):

   http://ab.inf.uni-tuebingen.de/data/software/megan4/download/manual.pdf

5. Run perl script to generate the BLASTx summary file as follows:

perl summarize_blast_4_contigs5_X_pl2 [D1D4-2_LargerThan200.X] NA NA [output.xls] 1

6. Run perl script to match redundancy data to BLASTx summary file as follows:

perl match_soap_redund_2_blast_summ_pl Redunduncy_SUB.xls [output from step #5]

This generates a file named “blastSummary_redundancy.xls”.

7. Use the output file from step 6 to calculate RPKM values for each BLASTx hits using the formula mentioned in methods and material section of chapter III. Make sure the file is saved as *.xls.
8. Open *.rma file in MEGAN 4.
9. Following MEGAN 4 user manual instructions open KEGG dialog box and create two separate files:
    a. i. Select all global KEGG networks including “Not Assigned”.

       ii. Uncollapse the KEGG tree to the maximum node by using “Tree”; “uncollapse Subtree”.

       iii. Select both Pathway nodes and KO numbers along with the “Not Assigned”.

       iv. Extract and save “KEGG names” and “read names” information by using "Export" under the "File". Choose "export CVS"; "Kegg-names, Read-names"; "tab format".

    b. i. Collapse the KEGG tree to the pathway node ONLY (i.e. hide KO numbers).

       ii. Select the pathway nodes along with the “Not Assigned” circle.

       iii. Extract and save KEGG "path" information by using "Export" under the "File". Choose "export CVS"; "Kegg-path, counts"; "tab format".

10. Run perl script to generate KEGG summary file by using on the files generated from steps 7 and 9.

perl summarize_MEGAN_KEGG_v2_pl [output from 9.a.iii.] [output from 9.b.iii.] [Output from step 7]

Following completion of step 10 the program generates a KEGG summary file named “FinalKEGG1.xls”.This output file will contain all assigned and not assigned BLASTx hits of a particular library associated with KEGG pathways along with their respective RPKM values.