Chapter 3
Crustacean Ecdysteroids and Their Receptors

Penny M. Hopkins

Abstract Ecdysteroids in crustaceans differ substantially from those of their fellow arthropods, the insects. Crustacean ecdysteroids and ecdysteroid nuclear receptors are similar to those of insects, but differ in the number of hormones and in the number and structure of the receptor isoforms. Moreover, the control(s) of ecdysteroid synthesis by crustacean Y-organs is primarily inhibitory - through molt-inhibiting hormone (MIH) - whereas in insects ecdysteroid synthesis is positively stimulated by a very different neurosecretory hormone. The *in vivo* effects of ecdysteroids are less understood in crustaceans than in insects but appear to have some concordance. Ecdysteroid-responsive genes in crustaceans are just beginning to be uncovered and may have some identities to insect genes. The differences in ecdysteroid control between insects and crustaceans are thought to have evolved to accommodate the differences in life-histories seen in these diverse arthropod groups.

Keywords Crustaceans • Y-organs • ecdysteroids • invertebrate nuclear receptors

3.1 Introduction

During the course of evolution the utilization of ecdysteroids by arthropods has undergone much divergence. Crustaceans and insects both draw on ecdysteroids to control a variety of important physiological events especially growth and differentiation. It is becoming increasingly obvious, however, that the overall control by ecdysteroids in crustaceans is very different from that of insects. Unlike most insects, crustaceans have a rather complex adulthood, where control of growth and reproduction must be alternated. The predominant hormones coordinating insect larval molts and metamorphosis differ from the number and variety in crustaceans. Differences are also seen in the number and structure of functional crustacean ecdysteroid receptor isoforms when compared to insect receptors.

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3.2 Ecdysteroids in Crustaceans

Levels of circulating crustacean ecdysteroids differ from animal to animal and from researcher to researcher because of natural variations but also because of variations in methods used. RIA remains the most sensitive method to measure total circulating ecdysteroids and RIA coupled with HPLC and/or LC-MS is used to identify individual circulating ecdysteroids. Results, however, disagree due to differences in the sensitivity and specificity of the antibodies. Levels of total ecdysteroid in the hemolymph of crustaceans remain very low during the intermolt portion of the molt cycle – a time when animals are feeding and mating. Lowest reported levels of circulating ecdysteroids during intermolt range from undetectable levels in many crustaceans to 3.3 ng/ml in the crab, *Callinectes sapidus* (Lee et al., 1998) and 16 ng/ml in the shrimp, *Panaeus vannamei* (Chan et al., 1988). Ecdysteroid levels rise rapidly as crustaceans approach molt (or ecdysis -E) and then abruptly fall just prior to E itself (Fig. 3.1a; Hopkins, 1992; Nakatsuji et al., 2000; Martin-Creuzburg et al., 2007). Highest reported levels of circulating ecdysteroids occur just prior to ecdysis and range from 600 ng/ml in the lobster, *Homarus americanus* (Chang, 1985) to 120 ng/ml in the crayfish, *Procambarus clarkii* (Nakatsuji et al., 2000).

Ecdysteroids in malacostracans are produced in hypertrophied strips of hypodermis called Y-organs. Y-organs are found in varying locations in the anterior body

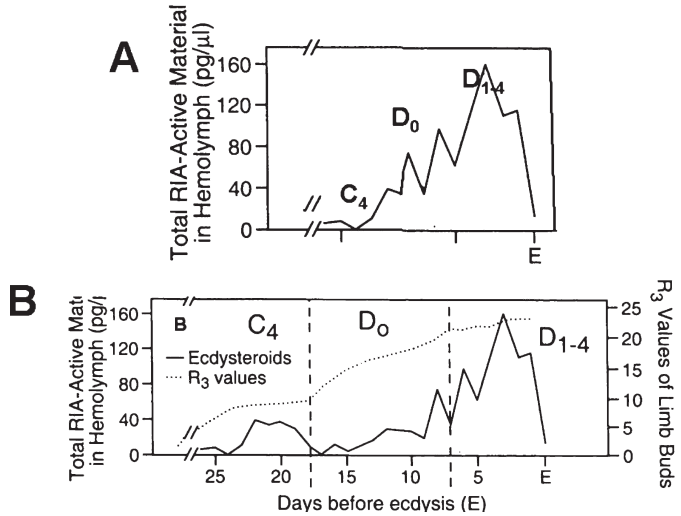
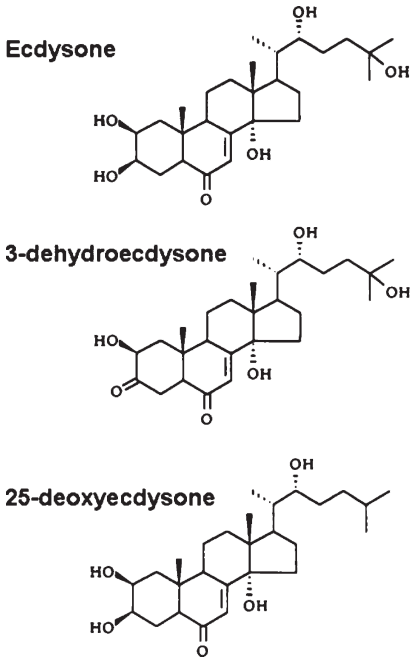


Fig. 3.1 Schematic representations of circulating levels of ecdysteroids in the hemolymph of the crab, *Uca pugilator* during a molt cycle. (**a**) Total RIA active ecdysteroids in the hemolymph (pg/ul) from an eyestalk-intact, non-regenerating crab. (**b**) Total RIA active ecdysteroids in hemolymph from a regenerating crab. Dotted line represents the R-value (= length of regenerating limb bud divided by the width of carapace times 100). C_4 is intermolt stage of molt cycle, D_0 is early proecdysis stage, D_{1-4} is late proecdysis stage, E is ecdysis (Redrawn from Chung et al.,1998a)

cavity of these crustaceans and Y-organ anatomy is extremely variable (see Lachaise et al., 1993). Y-organs are currently considered the major source of ecdysteroids but within the microrcrustaceans (copepoda, phyllopoda, and cladocera) Y-organs probably do not exist – the hypodermis may be the major source of ecdysteroids in these smallest crustaceans.

Most arthropods depend on dietary sterols and cholesterol for ecdysteroid synthesis. A major intermediate in ecdysteroid biosynthesis is 7-dehydrocholesterol which is converted to 2,22,25-trideoxyecdysone-ketodiol (Rees, 1985). In the insect prothoracic gland, there are sequential hydroxylations at the 2, 22 and 25 positions. The primary product in many insects is ecdysone (Meister et al., 1987; Smith and Sedlmeier, 1990) which is subsequently converted to 20-hydroxyecdysone in peripheral tissues (Gilbert et al., 2002). The Halloween enzymes that are responsible for the multiple hydroxylations of ketodiol in insects are discussed elsewhere in this volume.

Steroid metabolism in decapod Y-organs is different from that of insect prothoracic glands (Fig. 3.2): The 25-deoxy form of ecdysone (25dE), as well as ecdysone (E), are produced and released by Y-organs of the crab, *Carcinus maenas* maintained *in vitro* (Lachaise et al., 1986, 1989). A third compound (3-dehydroecdysone) has been shown to be produced by *in vitro* Y-organs of the crab *Cancer antennarius*



E only: *Pachygrapsus crassipes*, Chang and O'Connnor, 1977:

E + 3dE: *Cancer antennarius*, Spaziani et al., 1989; *Procambarus clarkii,* Sonobe et al., *1991*;*Orconectes limosus*, Bocking et al., 1993; *Macrobrachium rosenbergii* , Okumura et al., 2003.

E + 25dE: *Carcinus maenas*, Lachaise et al., 1989; *Uca pugilator*, Hopkins, 1992.

25 dE +3dE: *Menippe mercenaria*, Wang et al., 2000.

Fig. 3.2 The major ecdysteroid products of crustacean Y-organs. On the left are the structures of the three major secretion products from crustacean Y-organs. On the right is a selection of references reporting the secretion products either *in vivo* or *in vitro*

the freshwater prawn, *Macrobrackium rosenbergii* (Spaziani et al., 1989; Okumura et al., 1989) and the crayfish, *Procambarus clarkii* and *Orconectes limosus* (Sonobe et al., 1991; Okumura et al., 2003). 25dE and E released by the crab Y-organ are further hydroxylated at the 20-position by tissues other than the Y-organ. 25dE and its 20-hydroxylated metabolite ponasterone A (PA), as well as E and its metabolite 20 hydroxyecdysone (20E), have been found in the blood of three species of crabs (McCarthy, 1979; Lachaise and Lafont, 1984; Hopkins, 1992).

Hydroxylation at the 25 position of the side chain must be the first step in formation of ecdysone in *Drosophila* because the 2,22-dihydroxy form of the ecdysteroid cannot serve as a substrate for 25 hydroxylase enzyme (Warren et al., 2004). This P450 hydroxylase may be a control point for the selective production of ecdysteroids in the crustacean Y-organ. The 25 hydroxylase is not necessary for the production of 25dE but it is necessary for the synthesis of E and 3dE. Due to the lack of an ecdysone oxidase in Y-organs, 3dE cannot be a metabolic product of E and its production and release by some Y-organs argues for a separate pathway (Lachaise et al., 1993). 3dE, E, and 25dE are all found in the hemolymph of crustaceans but in varying ratios and amounts during the molt cycle. Since both E and 25dE are found simultaneously in the hemolymph of the crab *Uca pugilator* (Hopkins, 1992), the 25 hydroxylase activity must be closely regulated in this crab Y-organ. Indeed, down regulation of the 25 hydroxylase gene (*phantom- phm*) appears to be a mechanism used to control circulating levels of ecdysteroid in *Bombyx*. In *Bombyx* fourth larval instar prothoracic glands the expression of Bm*phm* decreases 90% from its peak value and does not rise to previous activity until late in the fifth larval instar (Warren et al., 2004). Down regulation of the 25-hydroxylase in *U. pugilator* during late PE (D_{1-4}) could account for the relatively higher levels of PA seen at that time (Hopkins, 1992).

The variety of ecdysteroids found in the hemolymph of some crustaceans is, therefore, more diverse than that found in most insects. This difference may be a function of the differences in insect and crustacean life histories. The insect life strategy is, in effect, compartmentalized into larval and adult stages. Larval insects do not reproduce and most adult insects do not molt. Many crustaceans, on the other hand, continue to molt and reproduce well into adult stages. Because insects partition growth and reproduction into different stages, fewer hormones may be able to serve more functions. Whereas the more complex adult stage of crustaceans may require a greater variety of ecdysteroids.

3.3 Control of Ecdysteroid Synthesis/Release in Crustaceans

The *in vivo* control of Y-organ ecdysteroid synthesis has long been thought to be via neurosecretory hormones secreted by ganglia located in the stalked eyes of crustaceans (Carlisle, 1954). There are three ganglia located in the eyestalk that are extensions of the brain. These eyestalk ganglia contain a cluster of large neurosecretory cell bodies (the X-organ) that send axons to a single neurohemal site on the surface of the eyestalk ganglia (the sinus gland –Bliss and Welch, 1952). The brain controls and

integrates environmental and physiological input that regulates the production/release of a variety of neuropeptides from the X-organ/sinus gland complex. At least one of these neuropeptides is thought to control ecdysteroid synthesis (Watson et al., 2001; Lago-Leston et al., 2007). The control of ecdysteroid synthesis *in vivo*, however, is more complex than a simple on/off control mechanism: During the course of the molt cycle, the blood of *U. pugilator* shows complicated patterns of various steroids and their metabolites. These patterns appear to be the result of a carefully controlled, complex program that seems to involve more than a single factor (Hopkins, 1983).

The *in vivo* control of ecdysteroid production in crustaceans differs substantially from that in insects. In insects a neurosecretory hormone from the brain (PTTH) stimulates the release of ecdysteroids (Gilbert et al., 2002) while in crustaceans control of ecdysteroid production/release appears in some aspects to be an inhibitory control. It has been known for over a century that eyestalk removal (ESX) leads to molting (Zeleny, 1905). ESX also leads to elevated levels of circulating ecdysteroids in some crustaceans and re-implantation of eyestalk ganglia can reverse these preparations for molt (see Skinner, 1985).

3.3.1 Molt-Inhibiting Hormone (MIH)

Evidence that eyestalk extracts contain a putative molt-inhibiting hormone (MIH) capable of inhibiting the onset of molt was first presented for the crab *Ocypode macrocera* in 1965 (Rao, 1965). Since then a series of workers have shown, using *in vitro* techniques, that eyestalk or sinus gland extracts may affect molt by controlling production of steroids by the Y-organ (Soumoff and O'Connor, 1982; Watson and Spaziani, 1985; Webster and Keller, 1986; Naya et al., 1988; Lachaise et al., 1988; Sonobe et al., 1991). ESX crabs that proceed into a successful ecdysis, produce about 1.5X more total RIA-active ecdysteroids than do eyestalk-intact crabs demonstrating the inhibitory effect the eyestalks have on total ecdysteroid production (Hopkins, 1992). Eyestalk extracts also inhibit *in vitro* conversion of ketodiol to 25dE and E in Y-organs from the crab *C. maenas* (Lachaise et al., 1988).

The pattern of circulating ecdysteroids during stage D is of low levels followed by a large peak four to five days prior to E. This pattern is exactly the same in eyestalk intact and ESX crabs. The large increase in ecdysteroids days after eyestalk removal cannot be due to further reductions in MIH titers because the half life of MIH in the hemolymph is only 10–20 min (Chung and Webster, 2005). It would seem that the patterns of circulating ecdysteroids must be controlled by more than eyestalk-derived inhibiting factors.

Moreover, ESX in crustaceans does not always result in molting (Carlisle, 1954; Flint, 1972; Lachaise, 1992). In the crab, *U. pugilator*, half of the crabs undergoing ESX fail to molt (Hopkins, 1992). The total RIA-active ecdysteroids in the hemolymph of these non-molting crabs fails to reach the very high late PE levels observed in molting crabs (Hopkins, 1989a). Apparently these crabs never prepare

for E since the Y-organs are not activated, even though the eyestalks are removed. Both the large PE peak of ecdysteroids in ESX crabs that molt and the failure of a large number of other ESX crab to enter PE argues very strongly for extra-eyestalk source(s) of Y-organ control. One such extra-eyestalk control could be feedback effects of ecdysteroids on the Y-organ itself. The Y-organs of the shrimp *Marsupenaeus japonicus* contain the highest levels of ecdysteroid receptors of any tissue examined (Asazuma et al., 2007). A mixture of ecdysteroids extracted from the hemolymph have been shown to have a stimulatory effect on *in vitro* release of ecdysteroids from Y-organs in *U. pugilator* (Hopkins, 1986) while in the crayfish, *Orconectes limosus*, exogenous 20E and RH-5849 had inhibitory effects on both *in vivo* circulating levels and *in vitro* Y-organ release of ecdysteroids (Dell et al., 1999). It is hard to determine what holds the Y-organs in abeyance for such a long time after ESX or why Y-organs in some ESX crabs fail to produce ecdysteroids in preparation for molt but it is clearly more complicated than first envisioned.

Purified MIHs have been isolated and sequenced from the crabs, *Carcinus maenas* and *Charybdis teriatus*, (Webster, 1991; Chan et al, 1998), the shrimps, *Penaeus japonicus*, *Penaeus vannamei*, *Penaeus monodon and Metapenaeus ensis* (Yang et al., 1996; Wang et al., 2000; Krungkasem et al., 2002; Gu et al., 2002), and the lobster, *Jasus lalandii* (Marco et al., 2000). Endogenous MIH levels *in vivo* are quite low ranging from 4–6 fmoles/ul of hemolymph (Nakatsuji and Sonobe, 2004; Chung and Webster, 2005). A number of different MIHs have been identified with 74–78 amino acids in length and six highly conserved cysteine residues (Webster, 1991; Yang et al., 1996; Marco et al., 2000). Based on similarities in sequences, MIH is considered to be part of a larger peptide hormone family that includes the crustacean hyperglycemic hormone (CHH), vitellogenesis-inhibiting hormone (VIH), mandibular organ-inhibiting hormone (MOIH), and other peptides from other organisms (Wainwright et al., 1996; Soyez, 1997; Chen et al., 2005). Peptides from the CHH family have overlapping pleiotropic activities. Some of the members of this family are produced in the X-organs of the eyestalk but many of them are produced and released from multiple sites throughout the *Cancer* nervous system where they may be released into hemolymph in response to a variety of clues and thus may serve as extra-eyestalk sources of Y-organ controlling hormones (Hsu et al., 2006).

In the crab, *Charybdis feriatus*, levels of MIH mRNA in the eyestalk do not change during the molt cycle suggesting that secretion of MIH may be post-transcriptionally regulated Chan et al., 1998. This is supported by the fact that immunopositive MIH in the sinus gland correlated negatively with circulating levels of ecdysteroids (Lee et al., 1998; Nakatsuji et al., 2000). These observations suggest that there is a constant MIH production in and release from the X-organ/sinus gland during intermolt but intermittent release with concomitant storage in the sinus gland during PE. This could account for the constant level of inhibition of the Y-organs during the molt cycle and the episodic release of ecdysteroids during PE.

Crustacean MIH cDNAs have been cloned, sequenced and expressed from the shrimps, *Panaeus vannamei*, *Penaeus japonicus*, *Metapenaeus ensis*, *Marsupenaeus japonicus* (Sun, 1994; Ohira et al., 1997; Gu and Chan, 1998; Okumura et al., 2005),

the prawn, *Macrobrachium rosenbergii* (Yang and Rao, 2001), and the crabs, *Cancer magister*, *Callinectes sapidus*, *Gecarcinus lateralis* (Klein et al., 1993; Umphrey et al., 1998; Watson et al., 2001; Chen et al., 2007; Lee et al., 2007a). The presence of MIH in the circulation *in vivo* and its ability to lower circulating ecdysteroids *in vitro* have been determined (Lee et al., 1998; Nakatsuji et al., 2000; Chung and Webster, 2005). Circulating levels of MIH *in vivo*, however, are lowest in the hemolymph during intermolt in the crab, *C. maenas* (Chung and Webster, 2005) when levels of circulating ecdysteroids are lowest. MIH levels in hemolymph of *P. clarkii* and *C. maenas* are highest during late proecdysis, when circulating levels of ecdysteroid are also highest (Nakatsuji and Sonobe, 2004; Chung and Webster, 2005). Levels of MIH in the hemolymph of these crustaceans are, therefore, inconsistent with MIH as the sole inhibitor of Y-organ activity. These data also argue for a more complex control system of the crustacean Y-organ than a simple on/off system.

An MIH receptor protein has been isolated from Y-organs in the crab *C. sapidus* (Lee et al., 1995). This receptor is a membrane-bound receptor that regulates intracellular cGMP (and possibly cAMP) levels in the Y-organ tissue (Nakatsuji et al., 2006). A recombinant MIH was able to maximally suppress ecdysteroid production by only 65% (Nakatsuji et al., 2006).

MIH may function to suppress Y-organ activity during specific molt stages and not exert any effect during other stages. There is some evidence that the receptor for MIH may be down-regulated during most of proecdysis in the crayfish, *P. clarkii* (Nakatsuji and Sonobe, 2004). The exact function of MIH in controlling the production of circulating ecdysteroids awaits further clarification.

3.3.2 Methyl Farnesoate

Methyl farnesoate (MF) is a sesquiterpenoid – an unepoxidated form of insect juvenile hormone (JHIII). It has been implicated in a number of hormonal activities in crustaceans (see Laufer and Biggers, 2001; Borst et al., 2001). MF is secreted by the mandibular organs (MOs) of crustaceans (Borst et al., 1987; Tobe et al., 1989; Nagaraju et al., 2005, 2006). The MOs – which are analogous to the insect copora allata – are located in the anterior body cavity of crustaceans in very close proximity of the mandibles (Byard et al., 1975) and at one time were often confused with the Y-organs (Sochasky et al., 1972). In the crab, *Scylla serrata*, the MOs secrete farnesoic acid (FA) in much greater amounts than MF (Tobe et al., 1989). Moreover, the enzyme that converts FA to MF (O-methyl transferase) is found in many peripheral crustacean tissues (Kuballa et al., 2007). MF has been implicated in crustacean reproduction control and may enhance ovarian maturation (Laufer et al., 1998; Jo et al., 1999).

MF has also been shown to stimulate ecdysteroid synthesis in crustacean Y-organs (Borst et al., 1987; Tamone and Chang, 1993). Thus, MF may serve as another extra-eyestalk source of Y-organ control. There is also some evidence that

methyl-farnesoate (MF) may have anti-ecdysteroid actions (Yu et al., 2002; Mu and Leblanc, 2004; Tuberty and McKenney, 2005). Exogenous MF administration results in the formation of larval intermediates and retards larval development in freshwater prawns (Abdu et al., 1998). Due to its chemical similarities to molecules known to function as ligands for nuclear receptors, it is possible that MF could interact with a crustacean nuclear receptor in the same way as it does with an insect nuclear receptor (Jones et al., 2006). It has been suggested that the anti-ecdysteroid actions of MF may be effected through its binding to a nuclear receptor and that by doing so in some way inactivates the functional ecdysteroid receptor (Mu and LeBlanc, 2004). MF binds with low affinity to UpRXR, the diner partner of the ecdysteroid receptor in the crab, *U. Pugilator*, and may modulate the action of the functional ecdysteroid receptor in this crab (Hopkins et al., 2008).

3.4 *In Vivo* Effects of Ecdysteroids in Crustaceans

Ecdysteroids in crustaceans have been reported to control many physiological processes. Most of the processes under the sway of ecdysteroids are related to the molt cycle. There is some evidence that ecdysteroids may play a role in reproduction (Subramoniam, 2000) but the effects of ecdysteroids on reproduction in male and female crustaceans remains contradictory and poorly documented (Lafont and Mathieu, 2007).

3.4.1 Growth in Crustaceans

To grow, crustaceans must first shed (molt) the old exoskeleton, then synthesize, expand and harden a new one. This is followed by growth in muscle and organ mass (Skinner, 1985). The crustacean molting cycle is divided into five stages (Drach, 1939) on the basis of changes in cuticle morphology. Subsequently other parameters (such as rate of limb regeneration and levels of circulating ecdysteroids) have been added to further refine Drach's stages (Hopkins, 1989a, 1992).

The old exoskeleton is shed at Drach's stage E, ecdysis (Fig. 3.1a). During stages A and B, metecdysis or postmolt, the new exoskeleton expands and hardens. During stages A/B, levels of total circulating ecdysteroids in the crab *U. pugilator* are low (<20 ng/ml). Feeding, increases in organ mass, and reproduction occur during stage C_4 (anecdysis or intermolt) which can last for an extended period depending on physiological conditions and is characterized by low levels of circulating ecdysteroids (<20 ng/ml). Intermolt cycles can vary substantially but once a crab commits to enter proecdysis (stage D) the length of proecdysis is usually about the same (in *Uca* about 24–27 days; Hopkins, 1982) unless the crab is regenerating limbs. Stage D, involves preparation for molt. The early part of stage D (D_0) is variable in length and is characterized by low but increasing levels of circulating

ecdysteroids, while later stage D ($D_{1–4}$) is characterized by apolysis, the separation of the old exoskeleton from the underlying epidermis, as well as the synthesis of new exoskeleton and very high levels of ecdysteroids (>75 ng/ml).

ESX does not affect the length of D. There is no statistical difference in the length of D between control and ESX animals (Hopkins, 1982). The levels of ecdysteroid are higher in the ESX animals and the actual increase in size is affected. Control animals increase an average of 2.4% (+0.2) in carapace width after a molt and ESX animals increase an average of 11.1% (+0.8). This effect on final body size supports a role for ecdysteroids in crustacean growth control.

3.4.2 Ecdysteroids and Limb Regeneration

Many crustaceans can regenerate lost limbs after reaching full adult maturity (see Hopkins, 2001). When a limb becomes damaged, some crustaceans can jettison it in a reflex termed autotomy (McVean, 1984). In the crab *U. pugilator*, regeneration of the new limb occurs in two phases: The first is a period of basal growth where a miniature intact limb organizes *de novo* from a blastema at the wound site. Limb loss can occur at any time during the molt cycle and basal growth will proceed but generally basal growth occurs during an ecdysial stage C_4 when circulating levels of total ecdysteroids are lowest (and 20E is the predominant circulating ecdysteroid). The second phase is characterized by limb bud hypertrophy and occurs only during the prolonged early proecdysial stage (D_0) of growth that is induced when regeneration occurs (Hopkins, 1993; Hopkins et al., 1999). A fully regenerated limb is often indistinguishable from the other non-regenerated limbs on the same animal (Fig. 3.3).

Normally, without regeneration, circulating levels of ecdysteroid begin to rise during stage D_0 and levels of PA rise relative to 20E. In an animal that has lost two (or more) limbs during C_4 (called multiple autotomy), ecdysteroids start to rise as the animal enters D_0, but then they plummet to intermolt levels and composition (<20 ng/ml total ecdysteroids – with 20E as the predominant ecdysteroid) within a day or two. Circulating ecdysteroid levels stay low until regeneration is well under way. When regeneration is imposed upon the molting cycle during stage $D_{0,}$ stage D_0 is prolonged (Fig. 3.1b). The regenerating limb bud grows very rapidly during this extended D_0. As the limb reaches a greater size and development, ecdysteroids levels once again start to rise (levels of 20E and PA become almost equal at this time ~16–17 ng/ml respectively). When the late PE (stages $D_{1–4}$) very high levels of ecdysteroids build, the limb bud stops growing. The titers of total circulating ecdysteroids peak during this time reaching levels of 75–135 ng/ml with PA as the predominant circulating ecdysteroid (Fig. 3.3; Hopkins, 2001).

When limb regeneration occurs it can have a feed-back effect on the circulating levels of ecdysteroids and consequently on the entire molt cycle. If limb buds are removed during later D_0, circulating levels of ecdysteroids will drop again, allowing for new blastemas to form and limb buds to grow to mature size (McCarthy and

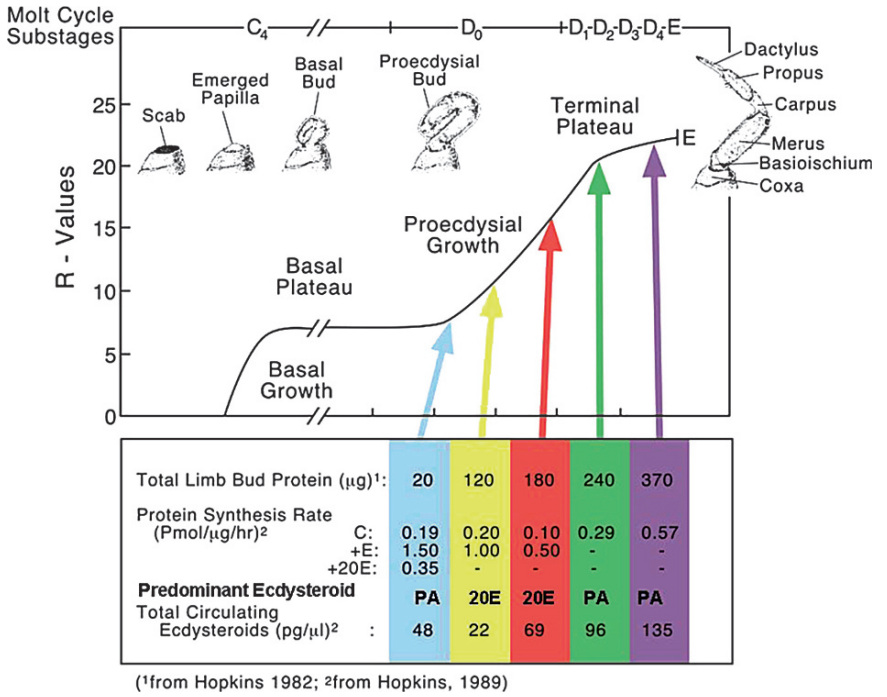


Fig. 3.3 Comparison of the growth of a regenerating limb bud from *Uca pugilator* to amounts of protein in the limb bud and total circulating ecdysteroids, as well as predominant circulating ecdysteroid. At top are molt cycle stages (see legend for Fig. 3.1). Solid line represent growth curve of limb buds as R-values (see legend for Fig. 3.1 for description). PA = ponasterone A and 20E = 20-hydroxyecdysone (Redrawn from Hopkins, 1993) (*See Color Plates*)

Skinner, 1977). The regenerating limb bud itself may be responsible for controlling circulating levels of ecdysteroids (Skinner, 1985). Limb bud extract was able to slow limb bud growth *in vivo*, but there is no evidence that the extract did so by lowering circulating levels of ecdysteroid (Yu et al., 2002).

The changes that occur in the composition of circulating ecdysteroids to accommodate basal and D_0 growth suggest that low levels of E/20E are supportive of regenerative growth while 25dE/PA may not be. Changes in ecdysteroid titers and ratios during the crustacean molt cycle are temporally correlated with major physiological events presumably under hormonal control (for review see Chang, 1989; Hopkins, 1992). *In vitro* rates of protein synthesis during early proecdysial limb bud growth (early stage D_0) are responsive to E and 20E (Fig. 3.3; Hopkins, 1993). *In vivo* injection of high levels of 20E during basal growth, however, significantly delays limb bud papillae emergence in the crab, *G. lateralis* (Hopkins et al., 1979). 20E can inhibit the expression of the *UpEcR* gene *in vitro* (Durica et al., 2006).

What is not known is the effect of other endogenous ecdysteroids, such as PA, on blastema growth and papillae emergence. Although a role for ecdysteroids in claw muscle atrophy accompanying molt has been observed (Kim et al., 2005) ecdysteroid regulation of many molt-cycle related physiological events (e.g. proliferation of epidermal cells, secretion of new cuticle, withdrawal and storage of calcium salt from old cuticle, construction of new exoskeleton) has not been examined at the molecular level.

3.5 Ecdysteroid Receptors in Crustaceans

Comparisons of nuclear receptors (NRs) across phyla, show that they are similar in their structure. They all contain characteristic and similar regions (regions A through F) from N-terminal to carboxyl terminal (see Renaud and Moras, 2000). The amino terminal contains the A/B domains. It is the least conserved of the domains – even among closely related orthologs – and is associated with transcriptional activation. The C domain is conserved across groups and primarily serves as the DNA binding domain (DBD). The D domain is more variable and represents a flexible hinge region, mediating nuclear localization and subunit pairing. The E domain, or ligand binding domain, LBD, contains a hydrophobic pocket for ligands and is involved in receptor dimerization. The LBD also mediates interactions with other proteins that can serve as co-activators or co-repressors of transcription. The LBDs of NRs are responsible for protein-protein interaction, ligand-binding specificity and ligand-dependent transactivation.

Ecdysteroids in all arthropods exert their effects through nuclear receptors (NRs), that function as ligand-dependent transcription factors (Koelle et al., 1991). In insects, the functional ecdysteroid receptor consists of a heterodimer between two members of the steroid hormone/NR superfamily: the ecdysteroid receptor (EcR) protein and the ultraspiracle (USP) protein. While the EcR protein is quite distinct relative to vertebrate steroid receptors, the insect USP protein is a homolog of the vertebrate retinoid-X receptor (RXR) family (Oro et al., 1992; Thomas et al., 1993; Swevers et al., 1996). In several different insects, A/B domain isoforms of both the EcR and USP proteins have been identified, which arise through alternate promoter usage and differential splicing of variant N terminal A/B domains with "common" DBD and LBD-containing exons (see Riddiford et al., 2001).

3.5.1 Crustacean EcRs

Crustacean homologs of the insect EcR have been cloned from the fiddler crab, *Uca pugilator* (Durica and Hopkins, 1996; Chung et al., 1998b), the tropical land crab, *Gecarcinus lateralis* (Kim et al., 2005), the kuruma prawn, *Marsupenaeus japonicus* (Asazuma et al., 2007) and the water flea, *Daphnia magna* (Kato et al., 2007).

In general, crustacean receptors are rather conserved with the four EcRs sharing around 90% identity with each other and a high degree of similarity to insect EcRs. Only a single invariant N-terminal A/B domain for UpEcR was isolated from the crab, *U. pugilator*, but hinge region variants were identified. The partial consensus sequence of GlEcR was 93% identical to the homologous regions of the deduced amino acid sequence of the UpEcR (Kim et al., 2005). In the kuruma prawn two MjEcR isoforms were uncovered differing by an exon in the LBD coding sequence occurring at the helix 2–3 boundary through helix 4 (Asazuma et al., 2007). Three isoforms of the *Daphnia* EcR were found. These isoforms, like those of insects, differed in the A/B domain and not in the LBD region as in the other crustacean EcRs. Thus, there is consistency in the general form of malacostracan EcRs with a variance seen in the microcrustacean *Daphnia* EcR.

3.5.2 Crustacean RXRs

Crustacean USP/RXR homologs have been cloned from the same crustaceans as the EcR homologues listed above. *U. pugilator* cDNA clones of *UpRXR* are structural homologs of the retinoid-X classes of nuclear receptor (Durica and Hopkins, 1996; Chung et al., 1998b; Durica et al., 2002; Wu et al., 2004). cDNA variants of the RXRs have been isolated, representing different transcripts that apparently arise through alternative splicing. Similar LBD isoforms have been isolated from, *G. lateralis* (Maestro et al., 2005; Kim et al., 2005). In addition to the UpRXR LBD isoforms, cDNA variants were also observed in the hinge region of UpRXR. The UpRXR variants differ by five amino acids (aa) within the "T" box, the conserved domain concerned with hormone response element (HRE) recognition (Beckett and Petkovich, 1999). Isoform variants in this region were also observed in GlRXR (Kim et al., 2005). The MjRXR DNA binding domain showed high homology to insect USP but the LBD was most similar to UpRXR. The UpRXR and MjRXR LBDs are overall more similar to vertebrate RXRs than to dipteran USPs (Chung et al., 1998b; Asazuma et al., 2007) whereas the *Daphnia* USP LBD is more similar to insect USPs that to crustacean RXRs.

When the crystal structures of the LBDs from two insect USPs were determined, it was shown that the LBDs adopt an "antagonist" conformation, rather than the "agonist" conformation associated with transactivation/coactivator binding (see Egea et al., 2000; Billas et al., 2003). A structural element important in the antagonist conformation is the connecting loop (L1–3 region) between helices H1 and H3. Both UpRXR and GlRXR have LBD isoforms containing inserts within this region. These isoforms differ by the inclusion or exclusion of an exon encoding approximately 33 amino acid residues. In *G. lateralis*, another exon variant in the LBD helix 7–8 region was detected, although a similar variant is not found in either *U. pugilator* or two related *Uca* species (unpublished observation).

3.5.3 Receptor Isoform Functions

RXR isoform variation in the T box and LBD region could influence *in vivo* transactivation properties, ligand affinities, or the ability to interact with EcR, i.e. contribute to LBD isoform specificity (see Talbot et al., 1993). The two UpRXR variants are found in regenerating blastemas as well as in growing PE limb buds. Moreover, UpRXR expression in the limb bud stays fairly constant during blastema differentiation, D_0, and PE growth (Chung et al., 1998a). The isoform variants may have different physiological roles *in vivo*, because different combinatorial arrays of receptor isoforms differ in their ability to mediate distinct responses via their DNA- and ligand-binding properties.

3.5.3.1 DNA-Binding Properties

The two UpRXR variants (lacking the five amino acid inserts) either having or lacking the 33 amino acid insert in the LBD can interact with UpEcR, but only the larger variant heterodimer is able to bind to the IRper-1 HRE. This heterodimer pair is not able, however, to bind to a DR-1G HRE. The small variant (lacking the 33 amino acid insert in the LBD) heterodimerizes much more readily with EcR (Fig. 3.4) and will bind to a DR-1G HRE (Wu et al., 2004).

3.5.3.2 Ligand-Binding Properties

UpEcR binds PA with differing affinities depending on the UpRXR isoform with which it is paired (Fig. 3.4). *In vitro* binding studies using Sf9 expressed receptors have revealed that the UpEcR will not bind to PA as a monomer (or as a homodimer). When the UpEcR gene is co-expressed in Sf9 cells with one or the other of the UpRXR isoforms, it readily binds to PA. When UpEcR is paired with the smaller of the two UpRXR isoforms (the one that most closely resembles insect USP), the binding affinity of EcR for PA is greater (Hopkins et al., 2008). This is the dimer pair that binds to the DR-1G HRE (and not to the IRper-1 HRE). When UpEcR is paired with the larger UpRXR (containing a 33 amino acid insert in the loop between helix 1 and 3 of the ligand binding domain) the binding affinity is less and this pair will not bind to DR-1G but will bind to the IRper-1 HRE quite readily (Hopkins et al., 2008; Wu et al., 2004). The IC_{50} of the two pairs of heterodimers show that PA is most effective in inhibiting PA binding. For the larger of the isoforms: IC_{50} for PA (2×10^{-8}M) > 20E (9×10^{-7}M) > E (1.6×10^{-6}M). These data suggest that the binding kinetics of UpEcR to ligand and to DNA differ significantly depending upon the UpRXR isoform with which it is paired.

Whereas the insect EcR/USP heterodimer has been shown to bind ecdysteroids (see Riddiford et al., 2003), it is presently unclear whether the insect EcR is a

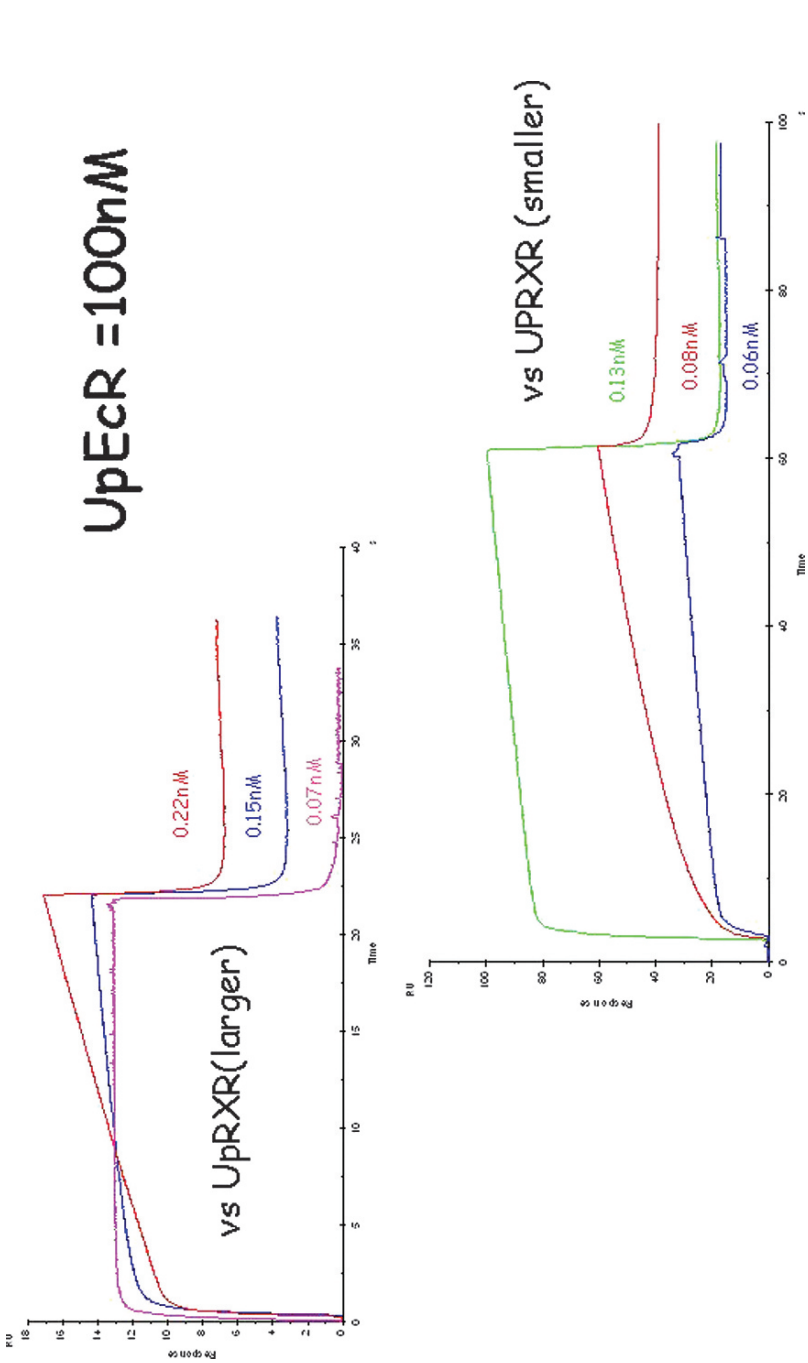


Fig. 3.4 Comparative binding affinities of *Uca pugilator* ecdysteroid receptor (UpEcR) to two isoforms of the retinoid-X-receptor (UpRXR). Surface Plasmon binding assay used. Sf9 expressed UpEcR (100nM) interacted with verious concentrations of UpRXR isoforms – larger and smaller. Response is relative amount of binding compared to control flow cells (*See Color Plates*)

permissive or non-permissive binding partner for insect USP ligand binding. The LBD of the lepidopteran and dipteran USP has diverged significantly from vertebrate RXRs (which bind 9-cis retinoic acid and fatty acids). This together with the crystal structure studies has led to the speculation that USP has no cognate ligand or a different ligand-binding specificity (Kapitskaya et al., 1996; Guo et al., 1998; Chung et al., 1998b; Hayward et al., 1999). Ligand-binding and transcriptional activation studies have tested a variety of ecdysteroid, retinoid and juvenile hormone (JH) analogues for USP binding activity with negative results (Oro et al., 1990; Yao et al., 1993; Harmon et al., 1995). Low affinity binding of *Drosophila* USP to JH III, however, and USP-mediated transactivation in response to JHIII, has been reported (Jones and Sharp, 1997; Jones et al., 2001; Xu et al., 2002), as has JHIII mediated repression in transactivation studies (Maki et al., 2004). Methyl farnesoate (a product of the *Drosophila* ring gland – Richards et al., 1989) may also be a ligand for dmUSP (Jones et al., 2006).

There is no question that JH activity is involved in the modulation of the ecdysteroid response (see Dubrovsky, 2005; Riddiford et al., 2003), but the mechanism of how crosstalk is modulated is still unclear. The anti-ecdysteroid effects of juvenoids in *Daphnia magna* (which has an EcR partner that is more like insect USP than crustacean RXR) were strongly synergistic indicating that they modulate ecdysteroid signaling by a mechanism that may sequester the EcR binding partner (RXR/USP) into other partnerships that prevents heterodimerization with EcR and thus leads to reduced EcR-mediated activity (Mu and LeBlanc, 2004).

One of the best-studied ligands involved in the control of vertebrate limb morphogenesis is retinoic acid (RA; Oro et al., 1992; Means and Gudas, 1995; Brockes, 1997). RA derivatives function in many vertebrate tissues to induce differentiation by controlling the production of morphogenic signals and exogenous RA has profound, disruptive effects on the regeneration of vertebrate limbs (see Brockes, 1997; Maden, 2000 for reviews). All-trans retinoic acid (atRA) disrupts the normal regeneration of limbs in *U. pugilator* (Hopkins and Durica, 1995). There are two episodes of cuticle secretion observed during early limb regeneration. The first episode is unaffected by the presence of RA but the second episode is disturbed, leading to excessive cell proliferation but no subsequent differentiation (Hopkins and Durica, 1995). The effects of retinoids on limb bud differentiation may be similar to the anti-ecdysteroid effects of juvenoids in *Daphnia* (Mu and LeBlanc, 2004) in that retinoids may promote UpRXR homodimer (or other heterodimer) formation that reduces or eliminates UpEcR/UpRXR formation and thus normal limb bud differentiation (Hopkins and Durica, 1995). Retinoids may be endogenous components of the regenerating limb blastema. There is evidence of endogenous retinoid production in blastema tissue and that retinoids can synergistically affect PA binding by UpEcR (Hopkins et al., 2008). An aldehyde-hydrogenase enzyme necessary for endogenous retinoid synthesis is present during early blastema development in *U. pugilator* (Hopkins, 2001) and the *U. pugilator* EST library indicates the presence of cellular retinoic acid binding protein and retinaldehyde-dehydrogenase – both of which have active roles in retinoid synthesis and signaling.

3.5.4 Tissue Distribution of Receptors

Probes derived from these clones have identified putative ecdysteroid target tissues throughout the molt cycle in both *U. pugilator* and *M. japonicus* (Chung et al., 1998b; Hopkins, 2001; Asazuma et al., 2007). Virtually all tissues examined express EcR and RXR receptors but in variable patterns that sometimes do and sometimes do not correlate with circulating levels of ecdysteroids. Expression of the *UpEcR* and *UpRXR* genes monitored with Northern blots, ribonuclease protection assays (RPA), quantitative reverse transcription PCR (Q-RTPCR) and immunocytochemistry show that UpEcR and UpRXR are widely expressed in a large number of non-regenerating crab somatic tissues, in ovarian tissue, and in regenerating limb buds (Durica and Hopkins, 1996; Chung et al., 1998a, b; Durica et al., 2002; Wu et al., 2004).

The pattern of expression of UpEcR mRNA is greatest in regenerating limb buds when 20E is present in low titers and is the predominant ecdysteroid in the hemolymph. UpEcR mRNA in the large cheliped is upregulated during late PE when total ecdysteroids in the hemolymph are reaching their maximum peak and PA is the predominant ecdysteroid (Chung et al., 1998b). These differences may, in turn, lead to implementation of different genetic programs in crustacean tissues throughout the molt cycle. UpRXR variants and UpEcR are present throughout the two phases of limb regeneration but clearly each variant is subserving different transactional activities.

3.6 Ecdysteroid Responsive Genes in Crustaceans

Arthropod models were one of the first biological systems used to investigate the molecular mechanisms of steroid hormone action. Although experiments demonstrating ecdysteroid regulation of gene transcription were historically at the frontiers of classical endocrinology, we still know comparatively little about how circulating ecdysteroids are capable of programming differential gene expression and ultimately distinct physiological responses. The function of ecdysteroids in the regulation of gene transcription during insect metamorphosis has been the object of intense study. Variations in 20-hydroxyecdysone (20E) titers have long been associated with molting and metamorphosis in insects (see Karlson, 1996), and a hierarchy of transcription factor gene expression mediated by ecdysteroid exposure has been characterized in *Drosophila* and *Manduca* (Talbot et al., 1993 see Riddiford et al., 2003).

The *Drosophila* E75 gene was one of the first loci suspected to be under ecdysteroid control (Segraves and Hogness, 1990). Studies in several insect systems indicate that both *EcR and E75* are primary ecdysteroid-responsive genes (Karim and Thummel, 1992; Wang et al., 2002; Riddiford et al., 2003; Siaussat et al., 2004). During early blastema development (8 days after autotomy – A + 8), short intervals of 20E *in vitro* exposure was ineffective in increasing *EcR* gene transcript

levels in limb bud explants (Durica et al., 2006). Exposure of more developed limb buds (A + 12) to 0.5–1 μM 20E for periods greater than 2 h led to decreases in *EcR* transcript levels. Bud explants taken from animals in late proecdysial stages (when circulating levels of PA are highest) appear refractory to 20E exposure; neither Q-RTPCR nor RPA indicate significant changes in *EcR* transcript abundance. The limb buds used in these induction studies were removed from animals with very low levels of total circulating ecdysteroids. During this time 20E is present in the circulation at about 20 pM concentration and injection of high levels of 20E in vivo inhibited blastema development (Hopkins et al., 1979).

In the crab *G. lateralis* there are strong correlations between elevated circulating ecdysteroids and *GIRXR, E75* and intracellular cGMP expression suggesting that these loci may be ecdysteroid-responsive genes (Kim et al., 2005; Lee et al., 2007b). The appearance of a growth arrest-specific protein (GAS7) from the X-organs/Sinus gland complex of the white shrimp *Fenneropenaeus indicus* and phosphorylation events for the signaling protein ERK2 shows molting stage specific correlations (Devaraj and Natarajan, 2006) that suggest that these gene/proteins may be controlled by ecdysteroids.

Crustacean tissue-specific EST data bases are currently available on line. Data from *U. pugilator*, *C. sapidus*, *C. maenas*, and *H americanus* can be found in EST libraries that are BLAST and keyword searchable; the data set is also available through GenBank (Durica et al., 2006). The first characterized library from *U. pugilator* was made from mRNA isolated 4 days post-autotomy (A + 4), when the first sign of morphological differentiation, cuticle secretion, is observed. Analysis of 2,030 individual cDNA clones led to assignment of 473 contigs and 417 singlets, for a total of 890 different transcripts. Of these, approximately 63% showed no BLAST homology on database searching, while approximately 11% could be assigned to a known ortholog in the COG (Clusters of Orthologous Groups) database. The *U. pugilator* EST database screened against the Gene Ontology (GO) database. tBLASTx searches (Blast2Go, Conesa et al., 2005) produced a significantly greater number of biological functional assignments than for the COG database at the same expect criterion (10^{-5}); approximately 40% of the domains identified among the assembled sequences could be assigned a GO annotation (see website for assignments). Nevertheless, a majority of ESTs from this library are as yet unassignable as to molecular function, cellular process or cellular component.

Sequence similarity searches between other crustacean EST databases produced hits between 13–30% of the *U. pugilator* query sequences (Pymood program; see Durica et al., 2006 for example of program output). Comparisons indicate that approximately 65% of the *U. pugilator* sequences are not found in any of the other three crustacean databases. There are also clear differences between the libraries in terms of BLAST hits to the *U. pugilator* database. The *H. americanus* database, which represents an olfactory-organ-specific library, contains hits to only 14% of *U. pugilator* ESTs, while the *C. sapidus* database, derived from hypodermal tissue, contains hits to 30% of *U. pugilator* ESTs. Although this comparison is influenced

by the number of ESTs present in the target databases, it also undoubtedly reflects similarities in gene-expression profiles for subsets of genes not related to "house-keeping" functions.

The *U. pugilator* early blastema library represented a tissue that is undergoing both proliferation and remodeling. An EST screening from a library constructed from a later proecdysial stage of limb bud regeneration, where the individual limb segments are already formed, and are undergoing an increase in mass due to muscle tissue synthesis (Hopkins, 1989b). From this library, 1,337 sequence reads resulted in 239 contigs and 399 singlets for a total of 638 different transcripts. Pymood comparisons indicate that the early and late *U. pugilator* regenerating limb bud databases show a greater degree of similarity (33% BLAST hits) than to the other crustacean databases, although tBlastX analysis indicates that many of the shared sequences from the *U. pugilator* regenerating limb databases are still undefined as to biological function.

The most abundant sequences (i.e. greatest number of times a specific sequence is detected among the ESTs) in the two different *U. pugilator* regenerating limb bud mRNA populations show profound differences. Constitutive 'house-keeping' genes were represented in both populations, the 25 most abundant sequences in the two libraries are distinctly different. In early blastemal library, there is a group of proteins (TNFRSF1a modulator protein; macropain; cyclophorin; TGFβ inducible nuclear protein) that has been linked to proliferative or apoptotic decisions tied to TNF signaling pathways (Gu et al., 1998; Li et al., 2004; Bouwmeester et al., 2004). For the proecdysial library, proteins involved in muscle biogenesis and arthrodial cuticle deposition (i.e. differentiation, not determination) are among the most abundant (muscle LIM protein; muscle actin; myosin three light chain; cuticle protein LCP18; arthrodial cuticle protein AMP9.3). This suggests that, even with limited sample size screening, information on gene networks and genes involved in important biological processes relevant to a particular physiological state can be obtained from these studies.

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