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

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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 h 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 pro-ecdysial 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 *U. pugilator*.

1. Introduction

Regeneration in invertebrates has been widely studied (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 (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, and *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 *U. pugilator*. Limb regeneration in these crabs occurs in two phases, basal and pro-ecdysial 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

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(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 (Chang and Mykles, 2011, for review). In *U. 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_{1-4} 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_{1-4}). 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 (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 have 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 (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 its 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 h 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 (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. Three walking legs and large cheliped were autotomized from each crab by pinching the merus with a forcep. Data is 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 are 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) 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). Control treatments consisted of RNase free water or RNAi complementary to a full-length green fluorescent protein encoding transcript (ds*EGFP*; 720 bp; Life Technologies, transcribed and quantified as described above) 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. To allow animals to feed, the contralateral second walking limb was autotomized in lieu of the contralateral claw. Changes in morphology and growth rate of the third walking leg in *Uca* have been conventionally used as a standard for monitoring limb bud development (Hopkins, 1982, 1989, 1992, 2001). 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 (*E*75 and *GAPDH*) are given in Table 1. In Figs. 1 and 2, statistically different values are indicated by asterisks ($^{*} = p < 0.05$, $^{**} = p < 0.005$).

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 40× magnification.

Limb bud growth was monitored throughout the molt cycle for statistical differences between the control and experimental

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

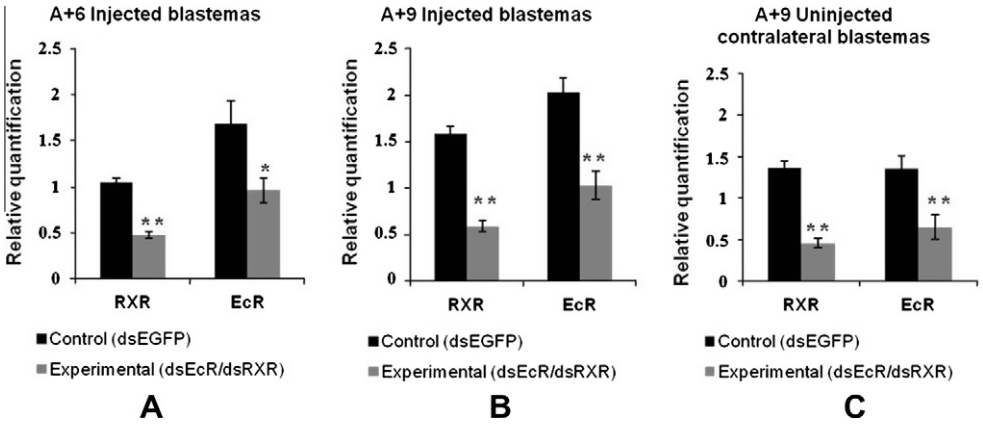

Fig. 1. Relative transcript abundance of *RXR* and *EcR* in blastema, 48 h post second injection with both ds*EcR*/ds*RXR* (420 ng). A. In the A1/4 injection protocol we observed that at A+6, both *RXR* ($p < 0.001$) and *EcR* ($p = 0.02$) in injected blastemas (pooled claw and third walking limb) were significantly down-regulated when compared to the controls ($N = 8$). B. In the 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. C. 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.

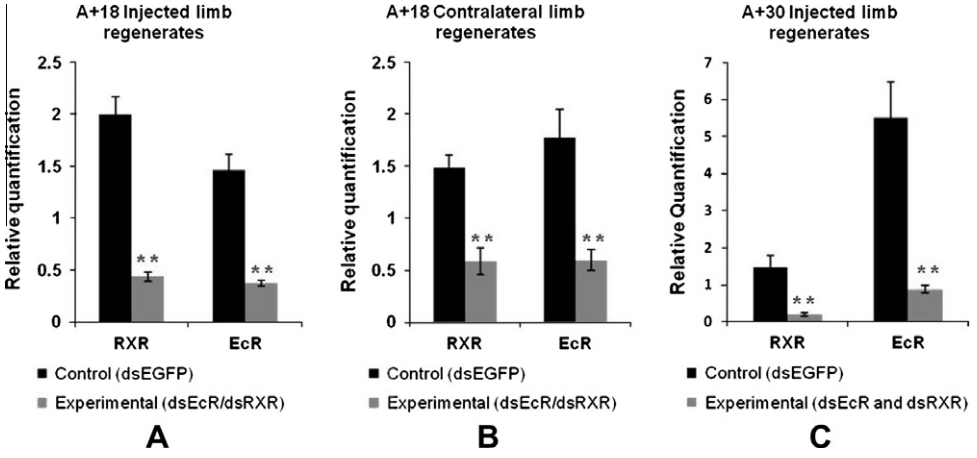

Fig. 2. Sustained down-regulation of *RXR* and *EcR* transcript abundance following RNAi treatment. At A+18 (A and B) following A1/4 injection, receptor transcript abundance in both injected and contralateral uninjected limb blastemas ($N = 8$) of experimental animals was 22–40% of their respective control blastemas ($p < 0.001$). C. 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.

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 h later and fixed in Lillie's fixative for 24 h. 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 1 l of water, pH 7.4) for an hour. The DNA was then denatured for 30 min using 2 N HCl at 37 °C followed by neutralizing the sections in 0.1 M 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 °C. 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 2 h at 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 h post final injec-

tion 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 observed in control injected blastemas (Fig. 1A and B). Upon examining receptor transcript abundance in the uninjected contralateral limb regenerates in experimental animals, we also observed statistically significant knockdown in both the A1/4 (47–51% reduction; data not shown) and A4/7 protocols (48–50% reduction; Fig. 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 for experimental animals showed that in both injected and uninjected contralateral limb regenerates the receptor steady-state mRNA abundance was 22–44% of control values, indicating a prolonged effect of RNAi (Fig. 2A and 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 receptor transcripts 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, Fig. 2C). To analyze whether ds*EcR*/ds*RXR* RNAi may have a non-specific inhibitory effect on mRNA accumulation, we examined transcript abundance of the housekeeping gene, *GAPDH* (A+9, A+18, A+30; Fig. 3A) and another related nuclear receptor that in insects is an early response gene in the ecdysteroid cascade pathway, *E*75 (Fig. 3B). Following injection of RNAi, we did not observe any significant differences in *GAPDH* and *E*75 transcript abundance in injected limb regenerates relative to controls.

3.2. Fluctuating ecdysteroid titers during molt cycle

We examined circulating hormone levels 48 h 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 bud as a papilla is associated with mitotic division of epidermal cells in the blastema and differentiation of the limb primordium into limb segments. Tables 3A and 3B document 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 (Figs. 1B and 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

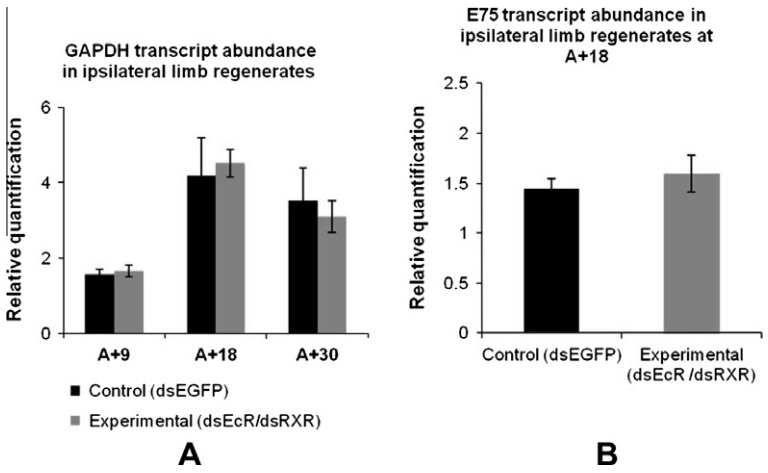

Fig. 3. Transcript abundance of *GAPDH* and *E*75 in ipsilateral limb regenerates. A. At A+9 ($N = 7$, $p = 0.67$), A+18 ($N = 8$, $p = 0.99$) and A+30 ($N = 6$, $p = 0.256$) no significant difference in *GAPDH* mRNA levels were observed between arrested ds*EcR*/ds*RXR* injected and ds*EGFP* injected crabs. B. Relative *E*75 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.

Table 2
Ecdysteroid titers at 48 h post second injection.

	Ecdysteroid titers (A+6)	*N*	*p*-Value (*t*-test)
A. Injection protocol – A1/4			
Control (ds*EGFP*)	11.4 (±1.3) pg/μl	62	<0.001
Experimental (ds*EcR*/ds*RXR*)	3.0 (±0.6) pg/μl	59	
	Ecdysteroid titers (A+9)		
B. Injection protocol – A4/7			
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	
C. Injection protocol – A4/7			
Control (ds*EGFP*)	20.13 (±1.03) pg/μl	35	<0.001
Experimental (ds*EcR*/ds*RXR*)	8.91 (±0.83) pg/μl	35	
	Ecdysteroid titers (A+28)		
D. 24 days post second injection – A1/4 injection protocol			
Control (ds*EGFP*)	52.08 (±5.64) pg/μl	9	0.72
Experimental (ds*EcR*/*RXR*)	49.02 (±7.44) pg/μl	13	

two-tailed, unpaired *t*-test, Fig. 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 (Fig. 4B). These results suggest that higher penetrance of RNAi occurs when it is injected earlier during limb regeneration and that higher penetrance in growth inhibition is 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 7 days following autotomy in the control limb regenerates (Fig. 5A) (Hopkins et al., 1999). Cellular proliferation of the control limb regenerates led to formation of papilla by A+11 (Fig. 5B). Histological analysis revealed that there was a lack of cuticular invaginations in the arrested ds*EcR*/ds*RXR* injected limb regenerates (Fig. 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 Fig. 5A). A significantly thicker cuticle deposition was observed in arrested experimental limb regenerates indicated by a double-headed arrow in Fig. 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, Fig. 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 (Fig. 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 (Fig. 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 pro-

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.

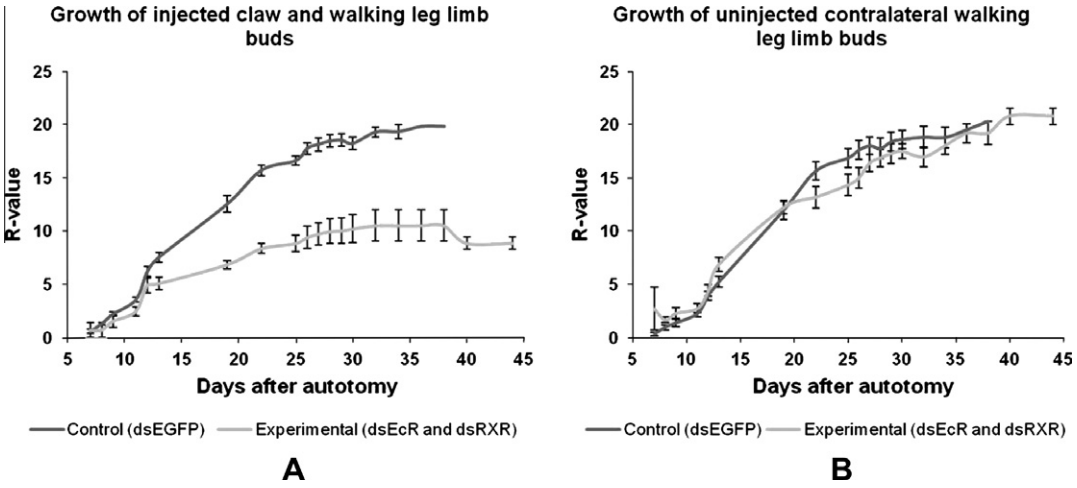

Fig. 4. Regenerating limb bud growth (*R*-values) in experimental and control crabs that escaped basal growth arrest following A1/4 injection protocol. A. Growth pattern of injected ipsilateral limb buds following ds*EcR*/ds*RXR* ($N = 6$) and ds*EGFP* ($N = 19$) injection ($p < 0.001$ at A+32, two-tailed,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

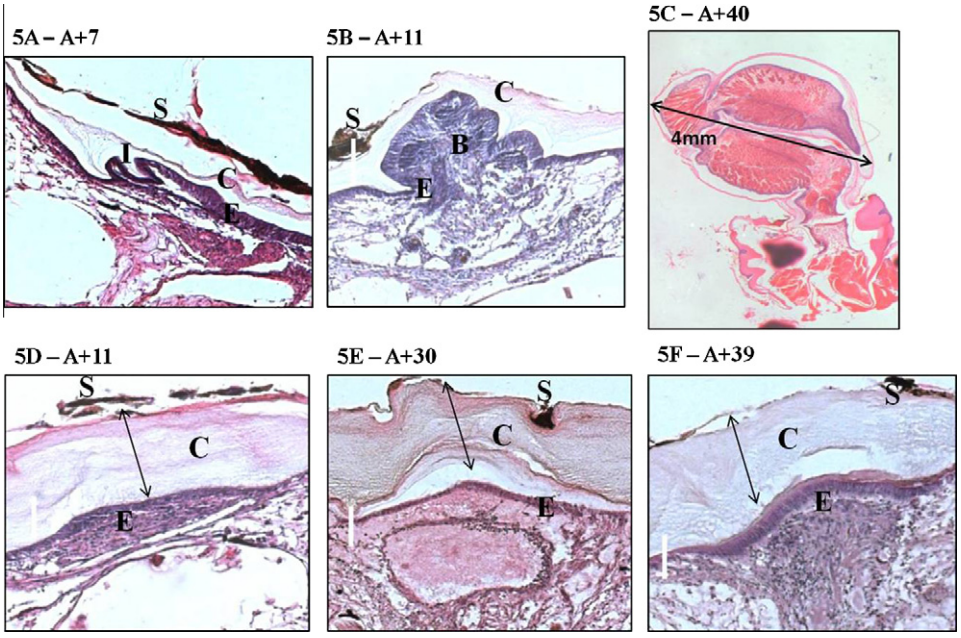

Fig. 5. Histological paraffin sections of blastema stained with Hematoxylin and Eosin from A4/7 injection protocol. A and B are RNase-free water injected control blastema sections (10 μm thick). D, E and F are experimental blastema sections injected with ds*EcR*/ds*RXR* (10 μm thick). The epidermal cells (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: 100 μm. Figure C represents a section of a late proecdysial fully grown limb bud (4 mm in width) that will give rise to a functional limb.

liferation assay, however, suggests that, due to the down-regulation of the ecdysteroid receptor transcripts in the experimental blastemas, a block in cell division is 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 (Tables 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

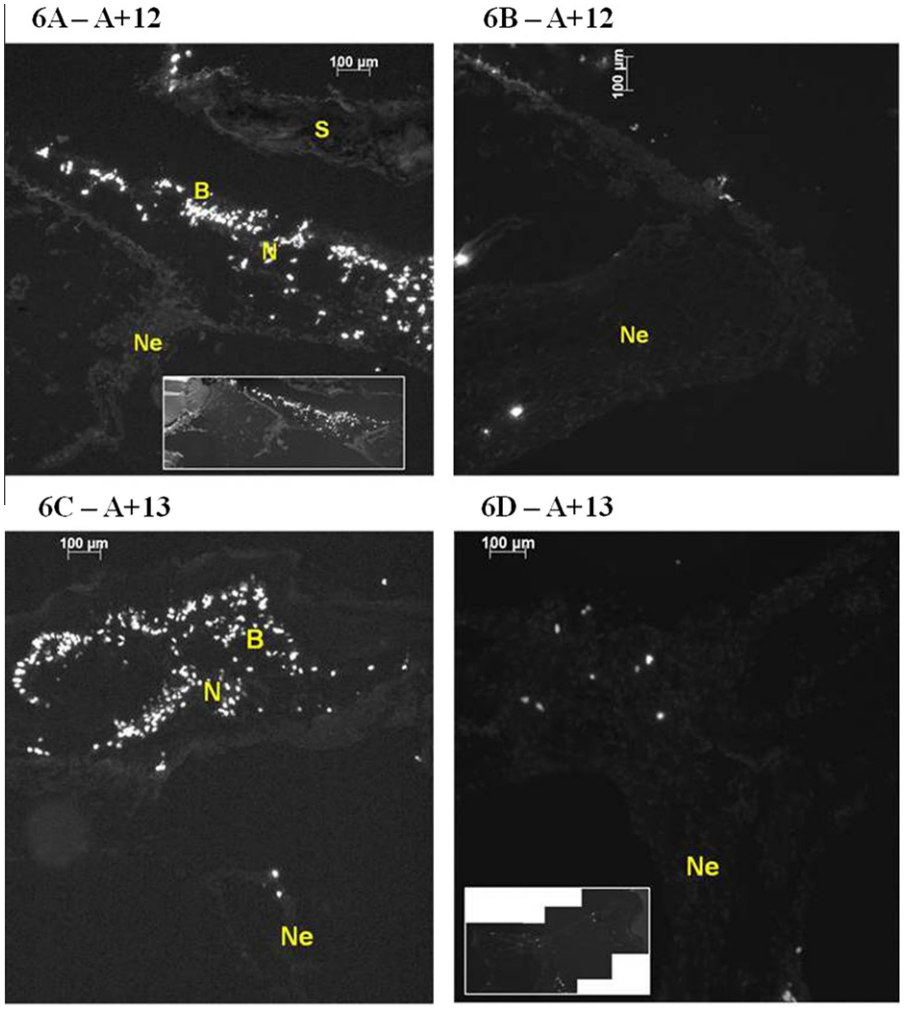

Fig. 6. Cell proliferation assay of limb regenerates from A1/4 injection protocol. BrdU staining shows that in the ds*EGFP* injected blastemas (A and C), the cells divide (N) along the nerve (Ne) and underneath the scab (S) in the area where the blastema (B) organizes. The ds*EcR*/ds*RXR* (B and D) injected blastemas however, fail to proliferate. A and D insets: panoramic view of entire section of ds*EGFP* and ds*EcR*/ds*RXR* injected blastema sections reduced to 20% and 12.5% respectively in comparison to A and D. Sections were examined using a Zeiss AxioImager fluorescent microscope.

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	<0.001
Failed to molt	0	31	
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	<0.001
Failed to molt	2	1	39	
Total	28	19	41	

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, 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 increases 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 *U. 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 h post treatment and the genes remained silenced as late as A+30 in post-treatment blastemas. In *Caenorhabditis 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 *E*75 transcripts were not observed in experimental crabs, supporting sequence specificity of RNAi to down-regulate *EcR* and *RXR*. Although *E*75 is an ecdysteroid inducible gene in *Uca* (Durica et al., unpublished) 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 h 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 h 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 ds*EcR*/ds*RXR* injected crabs, at 48 h 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 *G. 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; 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 (*dilp*8), 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). *Dilp*8 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 (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 (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 (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. While 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 regenerate via proliferation and regenerating imaginal discs express *wingless* (*wg*) and *d-myc* (Repiso et al., 2011, for review). An ecdysone pulse drives cell cycle division during larval development in *Drosophila* (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 *bcl*2 (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 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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