ECDYSONE REGULATION OF GENE EXPRESSION DURING *DROSOPHILA MELANOGASTER* DEVELOPMENT

by

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(Under the Direction of Michael Bender)

ABSTRACT

Pulses of the steroid hormone ecdysone punctuate each stage of the *Drosophila melanogaster* life cycle. The ecdysone signal is transduced to the genomic level via the Ecdysone Receptor Complex, made up of one of three *EcR* isoforms and a heterodimeric partner, *USP*.

In this work, we show that the *EcR* isoforms have distinct developmental functions, based on the unique mutant phenotype of the *EcR-A* mutants in comparison to the *EcR-B1* mutants (Chapter 2). While *EcR-B1* is required for pupariation, the *EcR-A* isoform is required for completion of pupal development. The nature of the *EcR-A* mutant deletions also revealed some variation in the phenotypes. This observation, in conjunction with a putative novel *EcR-A* isoform further substantiates the hypothesis of the *EcR* isoforms having unique functions.

We employed a genomics approach to ascertain a novel set of ecdysone targets. We focused on a specific pulse of ecdysone which occurs in the middle of the third instar, which we refer to as the mid-third instar commitment pulse (Chapter 3). We show there are many ecdysone regulated genes which have a diverse expression pattern during this pulse, substantiating the presence of an ecdysone pulse at this timepoint. We identified several genes that are considered ecdysone target candidates based on their expression patterns.

This work contributes more detail to the elucidation of the ecdysone signaling pathway, and also lends a template for the identification of gene targets in vertebrate steroid pathways.

INDEX WORDS: Ecdysone, EcR, isoforms, steroid, hormone, Drosophila, genomics, nuclear receptor, mutant, microarray analysis

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DEDICATION

To Yahweh… A million thank you's is not enough… I give you my eternal praise!

To Salome'… "not yet, but soon"…

To my mama, aunt Emma and Fern, uncle Preacher, Granddaddy, aunt Lois and uncle J, "snoopy", daddy and Linda… and everyone else who ever TRULY loved me…

What I hope for you (and me):

Truest love… with an unconditional flare… sustained and ignited by a spiritual connection that pierces through time [and distance]`

`Sweetest peace… enlightened by wisdom… imparted with grace by those newly introduced to their selfishness, evoking the transformation… to selflessness`

`Purest health… complimented with a resilience of youth despite the ripeness of the vessel, completed by attaining the eternalization of the internal essence [my soul]`

ALL ORDAINED BY GOD!

-Melissa Boneta '03

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TABLE OF CONTENTS

Chapter 1

Introduction

(Review of Ecdysone Signaling and Ecdysone Receptor Function

During Insect Development)

I. General Mechanisms of Steroid Hormone Signaling

Production and release of steroid hormones are processes that are essential to the proper development and physiological changes that occur throughout the life cycle of most organisms. Specifically, hormones regulate homeostasis and activate morphological changes, such as those that occur during metazoan embryogenesis and reproductive development (Beato, 1989; Burns and Matzuk, 2002; De Luca, 1991; Riad et al., 2002; Rocha and Funder, 2002), and cue specific adaptations in response to environmental changes. Hormones travel throughout the vascular system, released in specific concentrations which are regulated on both a temporal and spatial scale. These pulses of hormone prepare the organism for the impending alterations programmed into developmental progression. The various changes that occur are specific to each type and function of the hormone studied. Examples of such hormones include estrogen, testosterone, and glucocorticoids (Beato, 1989; Melcangi et al., 2002).

The receptor proteins for these hormones, and for other small lipophilic hormones such as thyroid hormone and retinoic acid, have been localized to the cellular nuclei of target tissues, which suggest their role in transcriptional regulation. A superfamily of nuclear receptors with over 300 members (Whitfield et al., 1999) was established based on DNA and protein homology across vertebrate and non-vertebrate organisms in which all the members share highly conserved sequences conferring their conserved DNA and ligand binding functions (Di Croce et al., 1999; Whitfield et al., 1999). These receptors have a C_2C_2 zinc finger DNA binding domain (Fig. 1.1), which further indicates their function in transcription regulation, and a ligand binding domain at their

carboxyl termini which is necessary for binding the hormone and proper transduction of the hormone signal. The amino termini of these receptors are not as highly conserved as the other functional domains, but are necessary for the proper transactivation of steroid target genes (Beato, 1989; Di Croce et al., 1999; Evans, 1988; Green and Chambon, 1988; Ham and Parker, 1989; Whitfield et al., 1999) and therefore necessary for proper function of the receptors. The receptors regulate target gene expression by binding promoter regions on specific sequences called response elements. The overall consensus for these response elements is a 15 nucleotide palindromic sequence (Beato, 1989). The precise nucleotides of each receptor's response element are specific for each receptor and vary for certain target genes. These nucleotide variations directly correlate with the level of regulation of target gene response (Grad et al., 2002; Lan et al., 1999; Renaud and Moras, 2000).

The biochemical mechanism of nuclear receptor action is well studied in vertebrate systems (Fig. 1.2). Studies that reveal the structure and the function of DNA and ligand binding domains indicate the hormone-specific nature of the regulation (reviewed in Renaud and Moras, 2000). Binding of the ligand creates a conformational change which transforms the receptors into their active forms (Fig. 1.2) thereby causing the receptor to activate transcription of the target genes via ligand induction. Also, studies have revealed there is an interaction between nuclear receptors and huge chromatin-remodeling complexes (Robinson-Rechavi et al., 2003). Several hormone receptor cofactors are components of the chromatin-remodeling complexes and have been shown to harbor histone deacetylase activity (HDAC), which compacts chromatin (Fig. 1.2). This

tightening effect prevents other transcription factors, such as the general transcription machinery, from interacting with the gene promoter regions and therefore prevents transcription. Other receptor cofactor complexes have histone acetyl-transferase (HAT) activity, which relaxes or loosens chromatin, permitting transcription to occur by allowing the general transcriptional machinery to interact with the promoter region (Fig. 1.2). Whether or not the particular hormone ligand is present and bound to the nuclear receptor determines the type of cofactor complex (repressor or activator) to be recruited to the target gene promoter. These cofactor interactions permits the receptor to act as a repressor in the absence of the ligand and an activator in the presence of the ligand (reviewed in Renaud and Moras, 2000).

Defects in either hormone synthesis, hormone release or hormone receptor expression and function are associated with such medical disorders as cancers, growth defects, and reproductive disorders (Burns and Matzuk, 2002; Hodgin and Maeda, 2002). An example of one hormone related disorder is testicular feminization syndrome, which is caused by a hormone receptor defect. In this syndrome, male patients have functional testes, which release proper levels of testosterone; however, the testosterone receptors are defective, inhibiting the transduction of the hormone signal and causing sexual traits to become feminized (Brinkmann et al., 1996).

Hormone receptors are essential in transduction of the proper signal to the hormone target genes of specific tissue types. Oftentimes, it is observed that multiple receptor isoforms or variants are produced for specific hormone receptors (Whitfield et al., 1999). These isoforms have been shown to require an

interaction with other nuclear receptors as heterodimers to fully achieve their functional diversity. Because of these findings, a central question in nuclear receptor studies is whether the different hormone receptor isoforms have unique functions or if they act redundantly. One of the goals of our research is to address isoform specificity through mutational analysis of the specific isoforms, using the steroid hormone ecdysone signaling pathway in *Drosophila melanogaster* as a model system (see Nuclear Receptor Functions section of this chapter and Chapter 2).

II. Hormone Signaling During Insect Development
Historical review of hormone research

Insect hormone signaling has been studied for nearly a half century and is probably the most extensively defined hormone system in invertebrates. Specifically, research on the steroid hormone ecdysone has been a cornerstone in developmental biology and has aided in the development of tools to unravel the intricate processes that are under hormonal control. The extent of research done in this area increases the efficacy of utilizing this system for discovery of novel ecdysone hormone target genes and new components of the ecdysone pathway. The ultimate goal of our research is to define completely the entire ecdysone signaling pathway, including the comprehension of each component of the receptor complex and its downstream targets. The final step would be characterization of the products and functions of these target genes throughout the entirety of Drosophila development. The following two sections are a brief

review of almost ten decades of historical literature that is the foundation of today's ecdysone research.

Elucidation of the Insect Endocrine Organs and Regulation of Hormone Secretion

Classical studies in zoology and entomology revealed that the morphological changes seen within metamorphic events are under hormonal control. These mechanisms were initially observed during amphibian development and later in insect development (reviewed in Henrich et al., 1999). Because the developmental stages during molting and metamorphosis are so well synchronized and controlled, it was speculated early on that there must be a signal to trigger the appropriate changes at the appropriate times.

Some of the earliest work in the insect was done by Kopec at the turn of the 19th century and on into the 1920's. In his studies, he defined a developmental signal that was localized to the anterior region of the moth Lymantria (Kopec, 1922). Based on studies done previously in tadpole metamorphosis, he applied the theories modeled after amphibian metamorphic controls to the metamorphosis of moths. Amphibian investigators found that removal of either the thyroid gland, or the brain (specifically, the hypophysis) resulted in the inhibition of metamorphosis. They eventually deduced that the brain controls secretion of thyroid material, and it is the active component of metamorphosis. This was observed from sets of experiments where metamorphosis was initiated by supplementing animals with the thyroid material.

First, Kopec investigated the effect of removing the brain from insect

larvae that were about to undergo metamorphosis (Kopec, 1922). Publications of

earlier studies reported that there was no need for the brain. Kopec found this not

to be true. He discovered that pupation was blocked by extracting either the

whole brain or the subesophogeal ganglion region of the brain if done so in larvae

aged to seven days after the last molt. However, if he waited to remove the organ

on the tenth day following the last molt, these animals still underwent

metamorphosis. He concluded that the brain is necessary for metamorphosis

and releases a signal at a very specific time, after which the brain is no longer

necessary to complete the process. Other work that substantiates Kopec's finding

in this regard include ligature experiments, using larvae of various insects,

including the silkworm and the blowfly (Kopec, 1922). Next, Kopec wanted to

investigate the medium by which the signal was sent by the brain (Kopec, 1922).

To determine if the signal was mediated through the nervous system, particularly

through the nerves, he severed the connections of the nerves to the posterior

region of the brain and found the animals still pupated. Then to determine if the

signal was sufficient, he transplanted organs (gonads) of younger animals into

older animals just before they were to pupate, and found the younger organs did

not change. However, when older organs, such as wing germ tissues staged just

before pupation, were transplanted into younger animals, these tissues still

underwent their scheduled metamorphosis. Amazingly, he ended up with larval

animals that "grew wings"! In conclusion, Kopec reported that the subesophogal

ganglion of the brain is necessary for metamorphosis, the signal is not transduced

by nervous system circuitry, the signal is not sufficient to induce metamorphosis

on tissues that are not physiologically prepared, and once the signal is sent to the tissues, they will continue their metamorphosis without the brain. He also hypothesized that the brain itself is the secretory organ releasing the metamorphic factor because insects lack the thyroid gland found in the amphibian model (Kopec, 1922).

Discovery of ecdysone and JH activity

During the thirties, Soichi Fukuda (Fukuda, 1976; Fukuda and Takeuchi, 1967) investigated the "molting activity" seen at work in the silkworm, *Bombyx mori*. This animal normally undergoes five larval stages before a complete metamorphosis into the adult animal. Because of an altered morphological state of the corpus allatum (c.a.) at the onset of these stages, Fukuda and others speculated that this organ is responsible for release of the molting signal. Indeed, they found that if the organ is removed, the animal will undergo precocious metamorphosis. Fukuda did detailed studies of the secreted factor in these animals during its development and found that at each molt the amount of substance (activity of the c.a.) reduced. He determined that by the end of the last larval stage the activity of the organ was "nil". Other studies revealed the concentrations of the substance took a sharp increase during pupation, which lead Fukuda to do the following investigations. In animals where the c.a. is removed prior to the last molt (allatectomized by extraction operations), Fukuda tested the activity of the pupating c.a. by transplanting to earlier staged animals (Fukuda, 1976; Fukuda and Takeuchi, 1967). Fukuda found that during the early stages of pupation, although slightly active, the activity of the c.a. is not sufficient to promote molting in these animals, and they metamorphose early. However, in

8

the second half of the pupation stage, the activity of the c.a. is able to sustain the molting of allatectomized larvae, as they entered their fifth molt and then pupated. He found this activity was independent of the brain being present in the donor, or the host. This confirmed that the signal necessary for the molt is secreted by the c.a. This signal was eventually found to be the Juvenile hormone (Fukuda, 1962).

In later experiments Fukuda also performed transplantations of the prothoracic gland into the posterior region of ligatured larvae (Fukuda, 1976). He found that in animals where the posterior region is sequestered from the anterior region, so that their posterior would not undergo metamorphosis with the anterior region, implantation of the prothoracic gland into the ligatured posterior will induce its pupation (as measured by cuticle darkening and hardening). These data indicate that the metamorphosis factor is secreted by the prothoracic gland in the silkworm. Hence, the prothoracic gland is most likely Kopec's "missing" thyroid gland.

In addition to Kopec's and Fukuda's work, Fraenkel investigated the physical and biochemical nature of the signal, using the blowfly Calliphora (Fraenkel and Bhaskaran, 1973; Hsiao and Fraenkel, 1966; Zdarek and Fraenkel, 1971). His interest was mainly in determining how the signal was transduced through the organism. While he agreed with Kopec's findings, that the brain is necessary, he doubted that the brain itself was the secretory organ. Fraenkel began his work with ligature experiments where he noted the placement of the ligatures greatly affects the resulting partial metamorphoses. He found that in order for the posterior region NOT to pupate, it must be completely ligated from

the region containing the ganglion organs. The anatomy of the blowfly is slightly different from that of the moth and silk worms used in the above studies. In fact, in our insect of interest, Drosophila, these organs (c.a., prothoracic gland, ganglion, and the corpus cardiacum, which transduces neurosecretory signals to the c.a.) are all "fused" into a single structure known as the ring gland. Fraenkel's initial work supported the previous findings of the necessity of the brain and the secretion potential of the prothoracic gland and c.a. To investigate the medium of transduction he first made temporary ligations which caused the nervous system to be permanently severed but allowed blood to flow freely, and found these animals pupate normally. He then decided to further prove the blood was the medium of the signal by making blood transfusions. He ligatured and removed the anterior portions of larvae and injected them with the blood of pupating animals. This transfusion of blood initiated pupariation in the ligatured animals. This confirmed that the signal is transduced through the blood.

The metamorphic hormone had already been shown to be ecdysone, upon purification of the hormone by P. Karlson in the late fifties (Karlson, 1956). Karlson also used ecdysone injections to rescue the non-pupating phenotype of animals that are defective in ring gland formation (Hoffmann et al., 1974; Karlson, 1967; Karlson, 1968). He was also able to rescue the non-pupating posterior region of ligatured larvae with a posterior injection of the hormone.

The inhibitory interaction of JH, produced by the c.a., on ecdysone produced by the prothoracic gland, was shown by V. Wigglesworth. He utilized a body fusion technique between Rhodnius (ticks) animals at different developmental stages (reviewed in Henrich et al., 1999). To conduct the

experiments, he decapitated the ticks, which removed the brain and retained the c.a., and he found that the c.a. factory inhibits metamorphosis. He fused larvae which only underwent a single larval molt and fused them with animals that were in their last molt. The older animals underwent an extra molt and produced a novel larval stage in which the larvae was much larger than normal.

In summary (Fig. 1.3), throughout the course of the above historical works, we now understand that in most lepidopterans, the c.a. is the site of release of the Prothoracicotropic Hormone (PTTH) which then activates the prothoracic gland to release ecdysone. Juvenile hormone, which is also released by the c.a., negatively regulates the ecdysone signal, causing animals to molt in the presence of both ecdysone and JH, but undergo metamorphosis in the presence of ecdysone alone (Henrich et al., 1999).

Advantages of the Drosophila melanogaster model system in hormone research

Utilization of the Drosophila melanogaster system allows not only for the genetic manipulation (which can produce results within a week vs. a month in mice), but also for the physical manipulation of organs and tissues as seen in the studies described above. The maintenance of Drosophila is fairly economical in comparison to vertebrates and even to some other invertebrates.

The experimental capacity of Drosophila provides advantages not present in many other model organisms (St Johnston, 2002). There are hundreds of mutant lines available for study which were isolated through various mutagenesis screens, such as the Nusselin-Volhard screens (Nusslein-Volhard et al., 1985; Nusslein-Volhard and Wieschaus, 1980), mass EMS screens (Bentley et al.,

2000) and P-element insertion projects (Bourbon et al., 2002; Roch et al., 1998; Salzberg et al., 1997). Each of these mutational screens seeks to eventually knock out each gene of the Drosophila genome individually, in an effort to determine specific gene function. Additionally, with the recent sequencing and annotation of the Drosophila genome (Adams et al., 2000), the ability to immediately determine the locus of a specific gene and its putative function makes these phenotypic screens even more powerful (St Johnston, 2002). The Drosophila genome project has created a new field in the world of functional genomics and has added an array of genetic tools for elucidation of gene function to a genetic model system that already has versatile genetic capability (See Functional Genomics section later in this chapter).

As an added bonus, the complex events that occur during Drosophila development are comparable to the developmental changes in response to steroid hormones that occur in other organisms. Because these various steroid signals mediate similar functions in development, reproduction, and tissue morphology during changes such as metamorphosis, wound healing and injury response, the molecular components of the hormone pathway tend to have conserved gene sets (Burns and Matzuk, 2002; Di Croce et al., 1999). Therefore, the elucidation and definition of specific gene targets in Drosophila can be readily applied to the hormone signaling pathways of other organisms. Mutant screens of Drosophila, such as those referred to above, allow the mechanistic dissection of the signaling components and their effects which readily yield global candidate genes in the steroid hormone pathway. Studies in Drosophila are the key to unlock elucidation of steroid hormone signaling in vertebrates.

III. Signaling by the Steroid Hormone Ecdysone in Drosophila
melanogaster

Ecdysone Regulation of Puffing in Polytene Chromosomes

Peter Karlson first observed the effect of the ecdysone hormone on polytene chromosomes in the salivary gland (Karlson, 1996). Karlson's work was based on *in vivo* studies, which included investigating the effects of exposure to ecdysone to fixed salivary glands. Karlson found that ecdysone triggers a puffing pattern within the chromosomes, and this pattern is very precise and reproducible. These puffs were physical evidence of the remodeling of the chromatin in these areas, which indicated areas of transcriptional activity. The size of a puff is indicative of the amount of activity; hence a large puff denotes heavy transcriptional activity. Karlson's colleague, Clever, suggested a regulatory pathway induced by ecdysone, in which the later puffs were induced by the earlier puffs (Karlson, 1996). Their investigations were limited by the constraints of the *in vivo* system. Subsequently, Michael Ashburner and colleagues developed a new technique of in vitro culture of salivary glands which allowed a more controlled environment of ecdysone manipulation and application. Ashburner's work consists of four major projects (Ashburner, 1972; Ashburner, 1974; Ashburner et al., 1974; Ashburner and Richards, 1976; Richards, 1976a; Richards, 1976b; Richards, 1978):

1- Elucidation of the sequence of puffing due to ecdysone (Ashburner, 1972)

2- Effect of varying concentrations of ecdysone (Ashburner et al., 1974)

3- Effect of removal of ecdysone, also termed washouts (Ashburner and Richards, 1976)

4- Effect of protein synthesis inhibition (Ashburner, 1974).

They defined specific subsets of chromosomal "puffs", indicative of transcriptional activity, that were responsive to ecdysone hormone. They were able to define a patterning of these puffs by mimicking the physiological signaling of ecdysone during the Drosophila life cycle by adjusting concentration and timing of the ecdysone application. They were able to discern two broad groups of puffs, early and late, relative to the timepoint of initial ecdysone signal. The early puffs were directly responsive to the ecdysone signal, and were sensitive to the concentration levels of the hormones. These early puffs peaked within 4 hours of the hormone signal and then regressed. The late puffs, lagging behind the early puffs for 3 hours, were sensitive to protein synthesis inhibition, and their peaks varied from 6-8 hours following ecdysone signaling. In all, Ashburner observed 125 puffs and found that the puffing patterns were very reproducible in each experiment. Additionally, premature removal of the hormone signal caused the early puffs to regress earlier than normal, and caused precocious induction of the late puffs. The extent of the late puff activity, as measured by puff size, correlated with the amount of the early puff induction. These experiments led to the formulation of a explanatory model for ecdysone signaling. This model states that ecdysone, in complex with its receptor, directly induces the transcription of the early genes, and directly represses the transcription of the late genes. The protein products of the early genes then repress their own transcription while simultaneously inducing the expression of the late genes (Fig. 1.4). These target

genes (both late and early) have since been under investigation for function and mechanism of their specific response to ecdysone.

The data that helped solidify the signaling model is as follows. The desensitization of the early puffs to ecdysone (after their regression) was inhibited by lack of protein synthesis. Also, the induction of the late genes did not occur without protein synthesis. This indicated that the induction seen by the early genes on the late genes was due to the production of the protein products of the early genes. These protein products are also the factors that cause the early genes to become repressed following their peak. Today, this long standing theory is known as the Ashburner Model (Fig. 1.4) and is the backbone of ecdysone hormone signaling research in *Drosophila*.

Ecdysone signaling during Drosophila development

The *Drosophila melanogaster* life cycle consists of embryogenesis, three larval stages, and the pupal stage, during which the larvae metamorphose into the adult animal. Ecdysone release punctuates each stage of the life cycle, initiating the onset of the subsequent stage (Fig. 1.5). During metamorphosis, most of the larval tissues degenerate while the adult structures emerge from the imaginal tissues. The imaginal discs and histoblasts (nests of imaginal cells) are present throughout the larval life, but remain undifferentiated until onset of pupation. Imaginal discs evert and elongate to form the rudimentary appendages, while histoblasts form the external adult abdominal epithelium and gut.

We now know that ecdysone physiologically triggers molting and metamorphosis during the *Drosophila* life cycle (Delattre et al., 2000; Richards,

1981a; Richards, 1981b). Release of ecdysone initiates a complex pathway of gene regulation that results in the spectrum of physiological and behavioral changes that characterize the morphological changes specific to each life cycle stage. These changes do not occur in the absence of the hormone, and it is therefore called the molting hormone and or metamorphosis hormone. If ecdysone is removed prematurely at any stage, the animal will not develop any further (Fristrom and Fristrom, 1993). This single molecule affects specific tissue types in a widely variant manner. At the point of metamorphosis, some tissues may be triggered to grow or develop; others may be triggered to undergo cell death or histolysis while some tissues may not respond at all.

It is believed that a small pulse of ecdysone, which occurs during the middle of the 3rd instar stage, is necessary for the tissues to undergo the metamorphic transition. There is a very distinct change in gene expression patterns at the point of the mid third instar event. This event makes the tissues competent to receive the signal for metamorphosis, rather than simply undergoing another molt. The initiation of larval tissue apoptosis and the eversion of the imaginal structures during metamorphosis depends on the proper signaling of ecdysone (Fristrom and Fristrom, 1993). Several genes identified from ecdysone puffing experiments were investigated in detail and they show a sharp increase in expression, while others that were on throughout previous stages of the life cycle are completely turned off.

Of the 100+ genes known to be ecdysone responsive and presently under investigation, most have been identified through the puffing experiments described earlier. Because salivary glands were the only tissues used in the

16

puffing studies, gene expression patterns identified in these experiments is specific to the salivary gland tissue type. These tissues do not undergo a morphological metamorphosis, but degenerate during this stage. The tissues cued for metamorphosis are where we expect to find upregulation of genes required for tissue competency and morphological transitioning. Therefore, because the different tissues types respond differently to the ecdysone signal, it is plausible that the 100+ set of genes identified in the salivary glands are not the same targets that are cued in other tissue types, or at least the may not be regulated in the same manner in other tissues.

The small mid-third instar pulse of ecdysone, also known as the commitment pulse, is necessary for onset of metamorphosis though it is not sufficient to induce metamorphosis. The subsequent pulse of ecdysone, just before pupariation, is required to induce pupariation and initiate pupal development. However, ligations and transplantation experiments suggest that the pupariation pulse, without the mid-third instar commitment pulse would only evoke an additional larval stage, rather than induce metamorphosis (Kopec, 1922). Studies suggest that this pulse of ecdysone affects yet another set of genes separate from those affected during the molts, and separate from those affected during the onset of metamorphosis. Mutations in genes identified as ecdysone sensitive at these timepoints either cause defects in the metamorphic processes or prevent metamorphosis altogether. We investigate the relevance of this commitment pulse, as well as define novel candidate ecdysone target genes in Chapter 3 of this dissertation.

IV. Nuclear Receptor Functions; Isoforms and Specificity

The proper transduction of hormone signals to the genomic level is dependent on the receptors of the hormone ligand. As described in the first section of this chapter, all hormone ligands have specific receptors that recognize specific gene targets. The amazing implication in this mechanism of gene regulation is that although all cell and tissue types are exposed to the same signal, different tissue types respond in distinct manners. To achieve this tissue specific perception of the signal, many receptors have alternate isoforms that are believed to interact with a plethora of different tissue and or receptor specific cofactors.

Steroid Receptor Isoforms

In steroid receptor studies, there are two underlying themes that occur across phyla of organisms studied (Robinson-Rechavi et al., 2003; Whitfield et al., 1999). The first common characteristic, which was described earlier, is that all the receptors have the same gene structure, with the C-terminus harboring the ligand and DNA binding domains, while the N-terminus is the least conserved yet is necessary for proper function (Beato, 1989; Renaud and Moras, 2000; Whitfield et al., 1999). Secondly, many receptors have multiple isoforms or variants (Bommer et al., 2002; Cheng et al., 2001; Conneely and Lydon, 2000; Giangrande et al., 2000; Mollard et al., 2000; Richer et al., 2002; Vienonen et al., 2002; Whitfield et al., 1999; Yudt and Cidlowski, 2001; Yudt and Cidlowski, 2002). The isoforms can arise through gene duplications, homologs or can be produced from the same gene as a result of utilizing alternate promoters or alternative RNA splicing.

The observation that most receptor isoforms have distinct temporal and spatial expression patterns is intriguing. The patterns of their expression often times mimic the resulting physiological effect of the hormone signal. Specifically, tissue types that have similar expression patterns, or harbor the same isoform, tend to have the same or similar developmental fates or responses to the signal. In the case of Drosophila ecdysone signaling, this phenomenon is well documented and still under current investigation (Bender et al., 1997; Cherbas, 2002; Dela Cruz et al., 2000; Kim et al., 1999; Li and Bender, 2000; Mouillet et al., 2001; Schubiger et al., 1998; Talbot et al., 1993).

Structure and Mechanism of the Ecdysone Receptor

The ecdysone signal is transduced to the genomic level via the Ecdysone Receptor Complex. This hormone/protein complex is a combination of the only protein known to bind ecdysone, the ecdysone receptor protein (EcR), Ultraspiracle protein (USP) and the steroid hormone ecdysone. Both protein components are members of the nuclear receptor superfamily.

The Drosophila EcR protein has three isoforms (EcR-A, EcR-B1 and EcR-B2) (Fig. 1. 5). The EcR isoforms are derived from a single gene locus and are products of the use of two promoters as well as alternative splicing (Talbot, 1993). The EcR-B isoforms are transcribed from one promoter while the EcR-A isoform is transcribed from an upstream promoter. Each isoform shares the common C-terminal DNA and ligand binding domains, with unique N-terminal domains that function in transcriptional regulation. These isoforms have been shown to have different spatial and temporal expression patterns throughout the Drosophila life cycle. The EcR-B1 isoform is expressed throughout the embryonic, larval and

pupal stages. EcR-B1 is expressed at high levels in strictly larval tissues and lower

levels in imaginal discs. The EcR-A isoform is expressed for shorter periods

within the embryonic and pupal stage. EcR-A is expressed at high levels in

imaginal discs and at lower levels in strictly larval tissues (Talbot et al., 1993).

This distinction between EcR isoform expression patterns is seen in other insects

as well. The temporal and spatial expression of EcR isoforms has also been

defined in Manduca (Jindra et al., 1996) as well as mosquito (Wang et al., 2002).

The patterns and regulation of expression in these insects mimic those seen in

the Drosophila studies. These data suggest that the different EcR isoforms may

control different developmental fates in these tissues. Recently, it has been

shown through transfection experiments that each isoform regulates the

activation of target gene expression differently (Mouillet, 2001).

The DNA binding sites for the ecdysone receptor complex are specific

sequences called ecdysone response elements (EcRE's) located in the promoter

regions of ecdysone-responsive genes (Fig. 1.6) (Cherbas, 1993). The repertoire of

these elements is still not fully characterized, as the total number of target genes

under regulation of the ecdysone receptor is still unknown. The ecdysone

receptor binding affinities of these sites seem to vary with not only the structure

of the element itself, but also the flanking sequences within the enhancer regions

of the target genes (Antoniewski et al., 1996). These binding affinity differences

confer a type of competition between element sites. Therefore, the genes with the

EcRE of highest binding affinity will be more responsive to the ecdysone signal.

It is hypothesized that this degeneracy along with the affinity differences of the

EcRE's are one mechanism of conferring tissue and developmental stage-specific

coordination of the hormone signal (Olson, 1998). To further complicate this signaling model, the EcR complex is also believed to interact with several co-activators and co-repressors, which are necessary for the proper regulation of the target genes (Di Croce et al., 1999). These co-factors include factors that are specific to steroid receptors and others that are general transcription factors. This level of co-factor regulation is largely based on chromatin structure remodeling which occurs within the promoter regions of the target genes, and may also facilitate the tissue-specific effects of ecdysone.

All of this evidence provides the background to support the relevance of an investigation to determine differential gene targets between the isoforms. In fact, one would expect to find differential target genes based not only on the isoform expression patterns, but also on the fact that the tissues that have complementary expression of the isoforms also have different developmental fates (Talbot et al., 1993). Additional evidence in other insects shows that the two isoforms of the EcR partner (USP-1 and USP-2) also differentially regulate target genes (Lan et al., 1999) which is also the case for its vertebrate homologue, the Retinoid X Receptor (Chambon, 1994).

Investigations of Isoform-specific Function of EcR

Recent investigations of the isoform-specific regulation of EcR have given promising results. In one study (Li and Bender, 2000), a transgene driving a specific isoform was expressed in an EcR null background. Li et al. found that only the EcR-B2 isoform was sufficient to rescue embryonic lethal mutants past larval stages into pupariation. They found that any of the three isoforms could rescue mutants through the molts between instars, though less efficiently than

EcR-B2. In Schubiger et al. (1998), EcR-B isoform function was removed via imprecise P-element excision of the EcR-B promoter region. In this study, both EcR-B1 and EcR-B2 were removed and they show that the EcR-B isoforms are specifically required for larval molting as well as neuronal remodeling and that the EcR-A isoform is not sufficient. Bender et al. (1997) show that polytene chromosomes from EcR-B1 mutants lose their characteristic puffing in response to ecdysone, indicating ecdysone-sensitive genes are no longer properly regulated. This puffing was totally rescued by expressing an EcR-B1 transgene, and partially rescued with an EcR-B2 transgene; however, the EcR-A transgene did not rescue the puffing at all (Bender et al., 1997). Additionally, Lee et al. (2000) have shown that EcR-B1 is specifically required in neuronal remodeling for mushroom body dendrite pruning (Lee et al., 2000).

In more traditional mutagenesis studies (Bender et al., 1997; Carney et al., 2003; Davis et al., 2003) a mutagenesis screen is used to isolate mutations that only remove single isoforms and the resulting phenotypes are compared. Compelling data from these studies shows without question that the isoforms have distinct developmental functions and or requirements. (The work on EcR-A mutants is described in Chapter 2 of this thesis (Carney et al., 2003; Davis et al., 2003). Each of the isoform specific mutants reported previously have distinct lethal time points and phenotypes, which supports the claim that the isoforms have distinct developmental functions (Bender et al., 1997). Because the isoforms are distinctly spatially expressed, the isoform-specific mutant phenotypes could reflect an absence of the ecdysone signal due to certain tissues no longer having an ecdysone receptor, as opposed to some unique function of the receptor

isoforms. To address this caveat, isoform-specific and tissue-specific rescue must be done in a tissue specific null background, which was the premise behind the work done by (Cherbas et al., 2003).

In (Cherbas et al., 2003), a dominant negative isoform of EcR was constructed and utilized to uncover the separate functions of the EcR isoforms. This technique has been found to be quite useful in this endeavor (Chandler and Werr, 2003), as it has been utilized in other works as well. The construct used in Cherbas et al., 2003 was driven by a Gal4 promoter-driver system which inhibited all EcR function in the specific tissues targeted. Subsequently, specific EcR isoforms were driven simultaneously in the EcR-blocked tissues in such a manner as to overcome the repression of the dominant negative construct. This tissue-specific rescue with specific isoforms was used to determine if tissue types that normally unambiguously express distinct isoforms can be rescued with any other isoform. The investigators found that only certain tissues or mechanisms require specific isoforms while the majority of other tissues and mechanisms can be regulated by any of the isoforms. This means that the isoform specific requirement only exists in certain tissues or for certain ecdysone-regulated mechanisms. This lays a foundation to establish which genes in particular will be regulated by distinct isoforms.

V. Functional Genomics capabilities in Drosophila and Hormone Research

Emerging field of functional genomics

Functional Genomics has been a rapidly emerging field of science in the last five years. With new technological advancements that allow for the

sequencing of entire genomes of organisms over a period of only months (as opposed to decades), genome wide studies have become increasingly popular. Several model organisms have benefited from the annotation of the genome, such as yeast, several microorganisms, C. elegans and Drosophila (Reinke and White, 2002). Functional genomics studies address very specific questions but on a broad scale. For instance, many studies focus on particular biological behaviors, or developmental events, but investigate the entirety of the gene expression pattern at the particular event in question (Reinke and White, 2002). This provides an important perspective because little if any biological function or behavior occurs in a vacuum, or is unaffected by other events occurring simultaneously in the organism. To fully understand all the components that mediate development and behavior, we must elucidate all interactive components, which requires the investigation of the entire genome and all genes that are active during these events. Microarray experiments are the technique utilized for these endeavors (Chandler and Werr, 2003; DeRisi et al., 1996; Eisen et al., 1998; Schena et al., 1995).

Microarrays in Hormone research

More specifically, microarrays have proven to be useful in the identification of hormone target genes (Bubendorf, 2001; Cheon et al., 2002; DePrimo et al., 2002; Igaz et al., 2002; Leo et al., 2002; Mousses et al., 2002; Pinette et al., 2003; Power et al., 2002; Reinke and White, 2002; Schapira, 2002; Smit and Romijn, 2001; Soulez and Parker, 2001; Strohman, 2002; Willson and Moore, 2002; Zhu et al., 2002) in metazoan systems. In every case microarrays are used, numbers of genes averaging in the high hundreds are identified. This

puts a new perspective on the scope of molecular interactions involving hormonal target gene expression. While one cannot delve beyond sequence homology into the possible functions of the gene products uncovered in microarray techniques, this technology gives a blue print to start with, and can quickly address whether a hypothesis is even worth pursuing, as is the case with hormone drug target analyses (Pinette et al., 2003).

In our case, microarray analysis will be a phenomenal tool to identify the genes that are under the direct regulation of ecdysone, and subsequently if these genes are regulated differently by distinct ecdysone receptor (EcR) isoforms. Given that 100+ genes were found to be targets in a single tissue type (salivary glands), there are probably hundreds of distinct target gene sets in other tissue types with distinct developmental fates. Therefore, it is conceivable that there are thousands of genes under the regulation of ecdysone. To identify such a vast number of genes, a genomic approach is absolutely necessary. Whereas classical approaches to identify gene targets, such as Westerns and Northerns, would take years and/or heavy man power, with microarrays a single scientist could perform these experiments over a period of several months.

Functional genomics in Drosophila

Functional genomics has been a priceless tool when coupled with the power of the Drosophila system (Gorski and Marra, 2002; Heckel, 2003; Reinke and White, 2002; Schlotterer, 2003; Tickoo and Russell, 2002; White, 2001). Upon sequencing and annotation of the Drosophila genome (Adams et al., 2000), development and utilization of genomics tools was significantly simplified. Elucidation of transcriptional profiles of genes during specific events in

Drosophila development and even within specific tissue types has become easily

achievable (Furlong et al., 2001; Jin et al., 2001). In particular, (Li and White,

2003) have shown that the metamorphic events in specific tissue types with

different terminal fates have very distinct expression patterns of particular gene

sets. Most findings in these studies support the already implied functions (such

as cell adhesion during tissue morphogenesis), but occasionally, surprises of

unexpected gene sets from a particular biochemical pathway emerge, and it is

these surprises that make the experiments worthwhile. Additionally, these

experiments may reveal components of gene sets that were not previously

identified through conventional methods such as mutagenesis (Biesecker, 2002;

Gorski and Marra, 2002; Reinke and White, 2002; Tickoo and Russell, 2002).

Such gene identities would not have been identified in any other way, as mutant

phenotypes do not necessarily tell the entire story of a gene's complete

functionality, as is the case with early lethal mutations.

A transcriptional profile of the entire Drosophila melanogaster life cycle

has recently been established (Arbeitman et al., 2002), which now allows us to

study the profile of a gene's expression pattern throughout development. In this

instance we will be able to determine if specific genes, which may be required for

survival through embryonic development (and therefore also makes amorphic

mutants in these genes embryonic lethals) are also expressed, and therefore

required during other stages of the life cycle. In this instance, we will be able to

attribute more functions to gene products that we otherwise would not have been

aware of. This developmental profile resource also allows us to confirm findings

in other developmental studies when investigating the expression of specific

genes at specific timepoints during development. In my work, I will utilize this data set to confirm expression of novel ecdysone regulated genes identified in my work. The novel ecdysone targets should have expression transitions that coincide with the pulses of ecdysone. The functional genomics studies are described in chapter 3 of this thesis.

More recently, coupling mutant analyses and transgenics with functional genomics has opened a new door in the world of genomics studies (Chandler and Werr, 2003; Dow and Davies, 2003; Horn et al., 2003). With techniques such as RNAi, dominant negative allele constructions as well as cell culture and tissue culture studies we are able to determine more specifically the effects of changing certain variables and conditions on the entire genome that normally would not have been possible to manipulate. The integration of these traditional genetics techniques with bioinformatics allows a more efficacious investigation of the genetic pathways that make up the phenotypic and physiological events during development as well as in mutant analysis and disease research. The fine tuning of these functional genomic techniques in Drosophila (Ballatori and Villalobos, 2002; Bubendorf, 2001; Cooper, 2002; Katze et al., 2002; Rasmuson, 2002; Reinke and White, 2002; Rose et al., 2002; Schlotterer, 2003; Sreekumar et al., 2001; Tickoo and Russell, 2002; White, 2001) will be priceless in their application to human genetic and disease research, which more than validates its biomedical value.

The work presented in the following chapters is a culmination of classical genetics and functional genomics studies. This work was done for the completion

of the PhD degree requirements of Melissa B. Davis and contributes to the field of steroid hormone signaling as well as developmental genetics and genomics.

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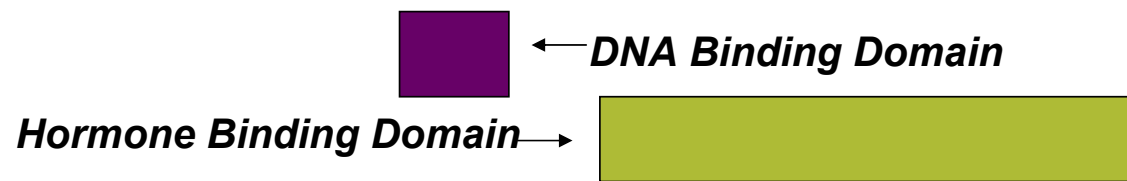
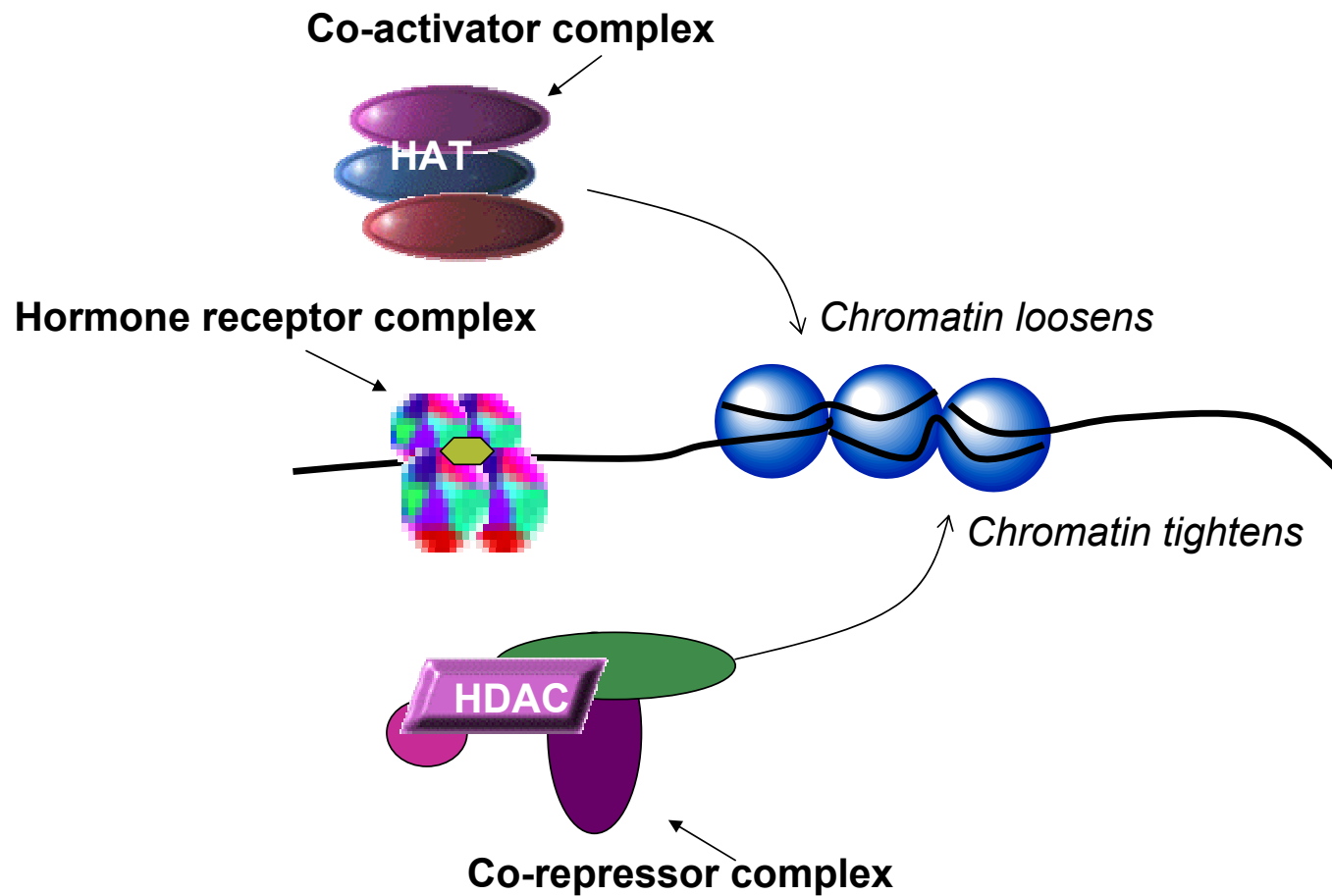**Figure 1.1 The conserved domains of the Nuclear Receptor Superfamily**

Figure 1.2 Interacting co-factor protein complexes interact with the nuclear receptors. Co-activators have HAT and co- repressors have HDAC activity which causes chromatin remodeling, under the regulation of the hormone ligand.
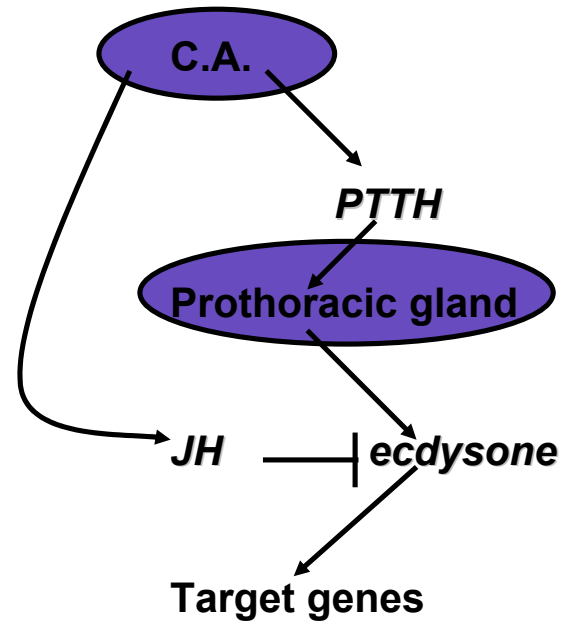
Figure 1.3 The ecdysone signaling pathway. PTTH is released from the c.a. which then activates the prothoracic gland and stimulates release of ecdysone. JH is released from the c.a. and has a retardant influence on ecdysone action until metamorphosis, when JH is no longer present.
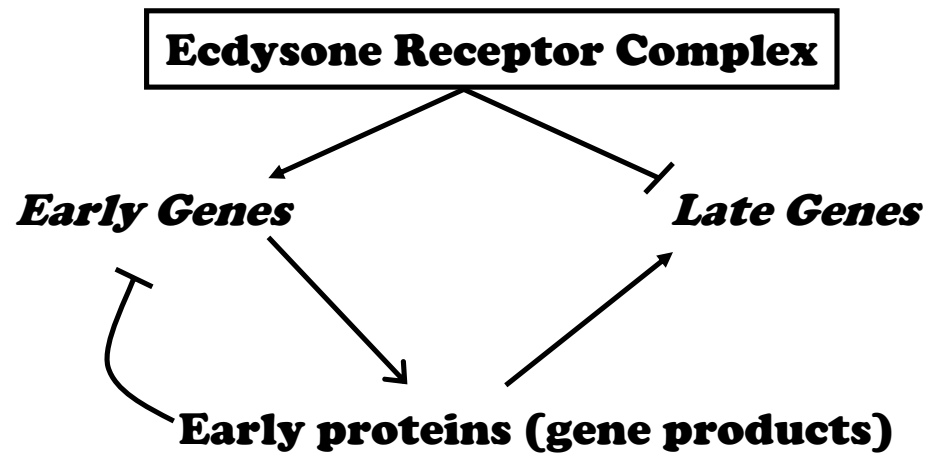
Figure 1.4 Schematic of ecdysone regulation as described by the Ashburner Model. Early genes are activated by the EcR complex, simultaneously repressing late gene activation. The products of early genes then autonomously repress themselves and activate late gene expression.
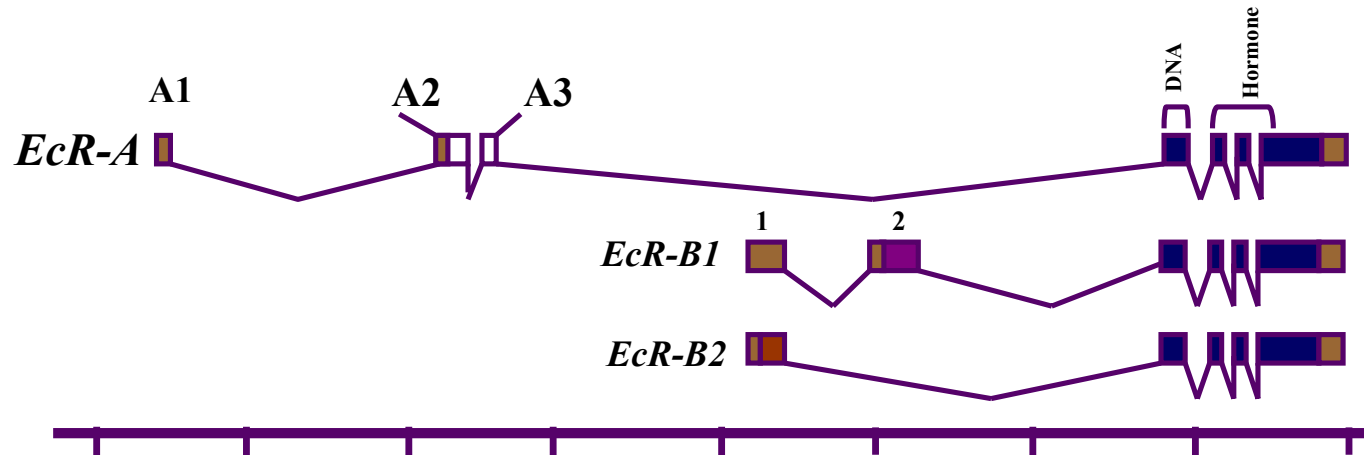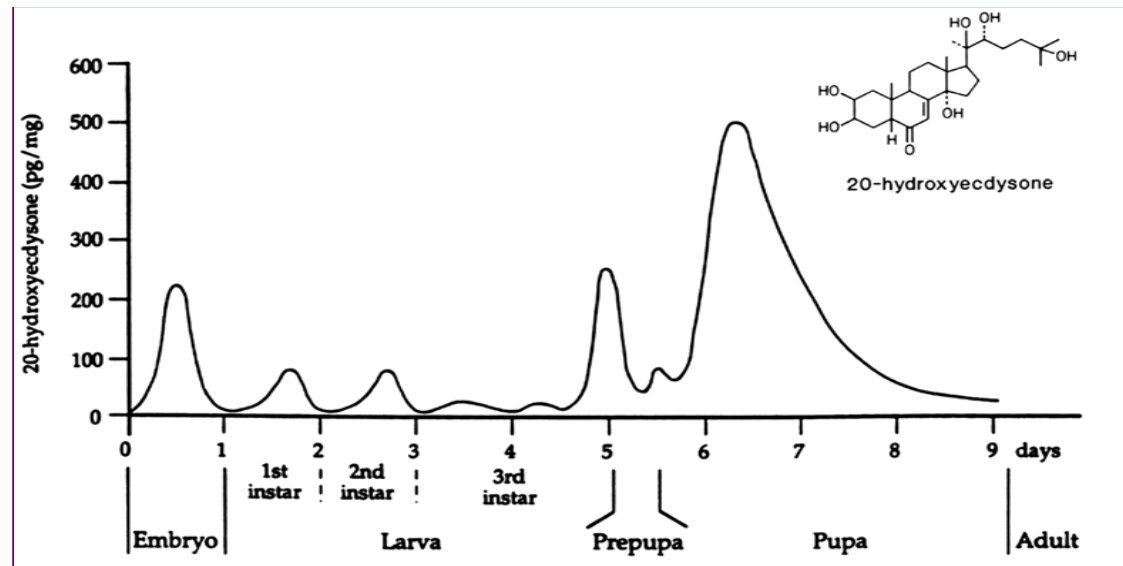
Figure 1.5 Pulses of ecdysone are released at each stage of the *Drosophila* life cycle (top panel). Also shown is a structure of 20H ecdysone and the Ecdysone Receptor gene and it's isoforms. The Ecdysone Receptor has three distinct isoforms, which are products of use of two promoters and alternative splicing. They share the same ligand and DNA binding domains, with unique N-terminal domains (bottom panel).
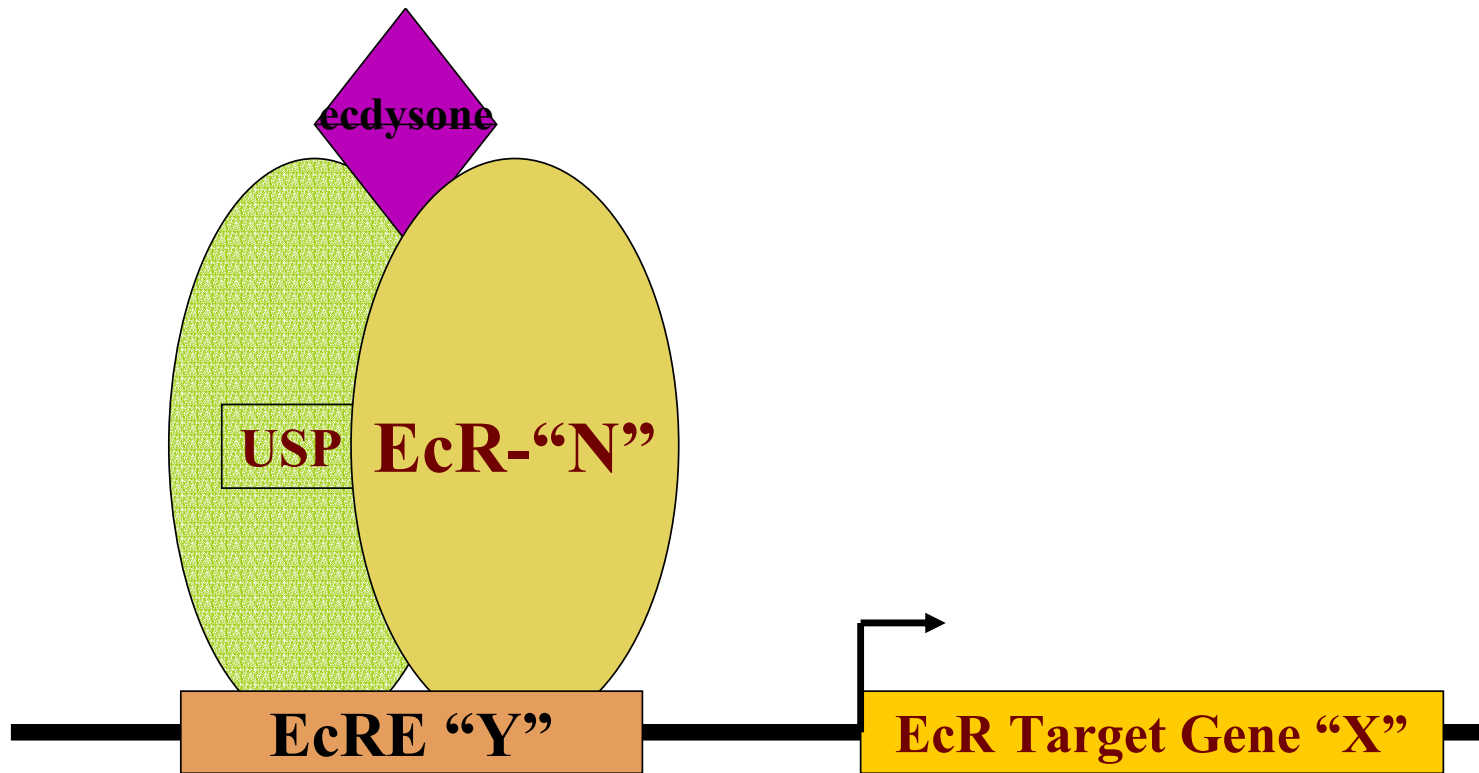
47

Figure 1.6 The Ecdysone Receptor Complex. EcR coupled with the Ultraspiracle protein USP in a heterodimeric complex binds ecdysone and DNA thereby regulating the transcription of ecdysone target genes. The binding sites of the ecdysone receptor complex are called ecdysone response elements (EcRE's) located in the promoter regions of ecdysone responsive genes

Chapter 2

***Phenotypic Analysis of EcR-A Mutants Suggests; EcR Isoforms Have
Unique Functions During Drosophila Development[1]***

[1] Davis, M.B., Carney, G.E., Robertson, A.E. and Bender, M. 2003. To be submitted to Development.

Summary

The steroid hormone ecdysone acts through a heterodimer consisting of the EcR and USP nuclear receptors to trigger transitions between developmental stages in Drosophila. The *EcR* gene encodes three protein isoforms (EcR-A, EcR-B1, and EcR-B2) that contain a common carboxyl-terminal region including DNA-binding and ligand-binding domains but have unique amino termini. EcR-A and EcR-B1 are expressed in a spatially complementary pattern at the onset of metamorphosis, suggesting that specific responses to ecdysone require distinct EcR isoforms. Here we describe phenotypes of three *EcR-A* specific deletion mutants isolated using transposon mutagenesis. Westerns show that each mutant lacks EcR-A protein, while EcR-B1 protein is still present. The EcR^{112} strain has a deletion of *EcR-A* specific non-coding and regulatory sequences but retains the coding exons, while the EcR^{139} strain has a deletion of *EcR-A* specific protein coding exons but retains the regulatory region. In these mutants, developmental progression of most internal tissues that normally express EcR-B1, with the exception of the salivary glands, are unaffected by the lack of EcR-A. The EcR^{94} deletion removes the *EcR-A* specific protein coding exons as well as the introns between the *EcR-A* and *EcR-B* transcription start sites. This deletion places the *EcR-A* regulatory region adjacent to the *EcR-B* transcription start site. EcR^{112} and EcR^{139} mutant animals die during mid and late pupal development, respectively, while EcR^{94} mutants arrest prior to pupariation. *EcR-A* mutant phenotypes and lethal phases differ from those of *EcR-B* mutants, suggesting that the EcR isoforms have separate and unique functions.

Introduction

During the *Drosophila melanogaster* life cycle the steroid hormone ecdysone is released at specific times and converted to a more active form, 20 hydroxyecdysone, via several intermediate forms which also harbor some reduced biological activity. All ecdysteroids will be referred to here as ecdysone. Pulses of ecdysone punctuate each stage of the life cycle (Richards, 1981) triggering the transition into the subsequent stage. Without proper regulation of this hormone, the animal will not develop properly, or in extreme cases where loss of the hormone has taken place, animals will not transition to the subsequent stage of development and will die prematurely (Berreu et al., 1984; Henrich et al., 1993). Ecdysone has been shown to be required for proper molting between larval instars as well as onset and completion of metamorphosis for pupation and adult development (Borst et al., 1974; Henrich et al., 1993; Sliter and Gilbert, 1992).

Ecdysone tightly coordinates the array of physiological changes that characterize each stage of the life cycle. Interestingly, while all tissues are exposed to the hormone, different tissue types have unique responses to the signal. Perhaps the best example of these differences is at the onset of metamorphosis when the majority of the larval cells and tissues degenerate by undergoing apoptosis, autophagy, and histolysis (Baehrecke, 2000; Baehrecke, 2002; Lee et al., 2002; Thummel, 2001), while the progenitor imaginal cells and discs proliferate and differentiate into pupal structures and ultimately into their respective structures of the adult fly (Riddiford, 1993; Robertson, 1936; Roseland and Schneiderman, 1979).

The ecdysone signal is transduced to the genomic level via the ecdysone receptor complex. This complex is made up of a heterodimer of the Ultraspiracle

51

protein (USP) and the Ecdysone Receptor (EcR) proteins (Yao et al., 1993). Once

EcR is bound to ecdysone it is more readily able to bind USP, and they both then

bind to the promoter and enhancer regions of ecdysone target genes (Elke et al.,

2001; Koelle, 1992; Yao et al., 1993). The receptor complex recognizes its target

genes by specific DNA sequence motifs called ecdysone response elements. This

molecular interaction is the means by which ecdysone regulates the genes that are

responsible for the plethora of physiological changes that are characteristic of the

developmental progression through the life cycle.

Three protein isoforms (EcR-A, EcR-B1, and EcR-B2) are produced from the

EcR gene via the use of two promoters and alternative splicing (Talbot et al., 1993).

These isoforms share the same carboxyl terminus, which includes the hormone

binding and DNA binding domains, while the amino termini are unique to each

isoform. The question that drives this and similar work in the arena of hormone

receptor research is whether the different isoforms of a given receptor have unique

functions, either by regulating different sets of genes, regulating the same set of

genes but in a differential manner, or if they are functionally redundant and simply

regulate the same genes in the same manner (Conneely and Lydon, 2000).

The three EcR isoforms are hypothesized to have unique functions based

upon studies that define their distinct temporal and spatial expression patterns

(Kim et al., 1999; Robinow et al., 1993; Sung and Robinow, 2000; Talbot et al.,

1993; Truman et al., 1994) and the distinct biochemical properties of their unique

amino terminal domains (Dela Cruz et al., 2000; Mouillet et al., 2001). Tissues with

analogous responses to ecdysone at the onset of metamorphosis express EcR

protein isoforms in similar patterns. The *EcR-B1* isoform is expressed at high levels

in cells with strictly larval functions that do not contribute to the adult structures and in gut imaginal cells that give rise to pupal abdominal epithelium and adult midgut. In contrast, the *EcR-A* isoform is expressed at high levels in the imaginal discs, which ultimately differentiate into the appendages of the adult fly during metamorphosis, and also at low levels in larval tissues and imaginal histoblasts (Talbot et al., 1993). Similarly, expression of EcR-A is correlated with neuronal maturation while EcR-B1 expression correlates with neuronal regression (Truman et al., 1994).

Mutational analysis of the *EcR* gene has produced three types of *EcR* amorphic mutants: *EcR* nulls, in which all isoforms are disrupted; *EcR-B* mutants, where both *EcR-B1* and *EcR-B2* are removed; and *EcR-B1* mutants, in which only the *EcR-B1* isoform is removed (Bender et al., 1997; Schubiger et al., 1998). Phenotypic analysis of these mutant alleles revealed that different lethal phases and mutant morphologies are associated with each type of mutant. *EcR* null mutants arrest late in embryogenesis. *EcR-B1* mutants fail to pupariate and their ecdysone responses are inhibited in larval and imaginal tissues that normally express high levels of EcR-B1, while initiation of ecdysone responses in tissues that normally express high levels of EcR-A is permitted (Bender et al., 1997). *EcR-B* deletions reveal a requirement for the EcR-B isoforms in larval molting and neuronal remodeling (Schubiger et al., 1998).

Here we describe the phenotypes of three *EcR-A* mutant alleles that have been identified from a three-step local transposon mutagenesis screen (Carney et al., 2003). Each of these alleles lacks EcR-A, but retains EcR-B1 expression. Two alleles, *EcR^{112}* and *EcR^{139}*, exhibit a mid-pupal lethality. *EcR^{112}* carries a deletion

that removes the EcR-A transcription start site and dies during early pupal development. *EcR^{139}* carries a deletion that removes *EcR-A* coding exons A2 and A3 and dies later in pupal development. These mutant alleles reveal a requirement for *EcR-A* during the pupal-pharate adult transition during metamorphosis. The remaining allele, *EcR^{94}*, exhibits a phenotype very similar to the *EcR-B1* non-pupariating phenotype. The *EcR^{94}* allele carries a deletion that removes exons A2 and A3 as well as most of the intervening sequences between the *EcR-A* and *EcR-B* transcription start sites. Our results show that EcR-A is required during metamorphic development and suggest that EcR isoforms have distinct developmental functions.

Materials and Methods

Lethal Phase Scoring

EcR mutant strains were maintained as heterozygotes to a second chromosome balancer. Heterozygous *EcR* mutant strains were maintained at 25° under a regulated 12 hour light-dark cycle. Twenty five males (*yw; EcR-A/CyO,y+)* from each mutant strain were crossed to 25 virgin females of an *EcR* null allele (yw; *EcR^{M554fs}/CyO,y+*) and allowed to mate for approximately three days. Subsequently, 200 eggs were collected from each cross and mutants were separated from wild-type siblings using the yellow gene (*y+*) marker to identify yellow mouth hooks. The mutants were scored at six timepoints (first instar, second instar, third instar, white pre-pupa, pupa, and adult) throughout the developmental life cycle. For each scoring, the surviving mutants were counted and moved to fresh food and the survival percentages were calculated. The percentage is based on the ratio of

animals still alive at the time of scoring to the total number of mutants collected at hatching. At least two rounds of crosses were scored for each genotype and the data from each round were combined and are presented in the text as a single data set.

Broad lethal phases were established by determining when percentages of surviving mutant animals dropped to 0% at specific life cycle stage scorings. Once a broad lethal phase (within a specific life cycle stage) was determined, a more precise staging of the actual lethal timepoint (point of development during the specific stage) was defined. The precise timing was done by scoring the animals at smaller time intervals within the life cycle stages (e.g. during pupal development observations were made every 3-4 hours).

As controls, siblings (*EcR-A/CyO,y+* and *EcR^{M554fs}/CyO,y+*) for each mutant cross were also collected and scored, to ensure the treatment of the animals did not affect viability and that wild-type animals of a similar genetic background did not generate any lethality.

Western analysis

Whole animal protein extracts were isolated from late third instar (clear-gut) larvae (see (Andres and Thummel, 1994) for larval staging criteria) using standard extraction protocols (Talbot et al., 1993) and run on a denaturing 6% polyacrylamide gel. After proteins were transferred to a nitrocellulose membrane, blots were incubated in monoclonal antibody directed against EcR-A (15G1A) at a 1:3000 dilution or EcR-B1 (AD4. 4) (Talbot et al., 1993) at a 1:30 dilution for 2 to 4 hours. After washing, blots were incubated with HRP labeled goat anti-mouse secondary antibodies at a 1:5000 dilution for 3 hours. Lumi-light chemiluminescent detection sytem (Roche) was used to visualize immunoreactivity.

Immunohistochemical and DAPI Stains

The EcR-A (15G1A) and EcR-B1 (AD4.4) specific monoclonal antibody ascites fluids were used to perform the immunohistochemical experiments. *EcR-A* mutants and wild-type siblings were collected at stages indicated in the text, dissected and stained simultaneously in separate tubes. A standard protocol for immunostaining was used (Talbot et al., 1993). DAPI stains were done simultaneously on each antibody stain to ensure visualization of the nuclei. DAPI was done using a standard protocol.

Microscopy and Photography

EcR mutants were collected and dissected at stages indicated in text. The internal tissue images were produced using a Zeiss Axiophot compound microscope and photographed using an attached 35 mm camera. The images were transformed into digital images and minor adjustments made with Adobe Photoshop to correct for discolorations and background clearing. Whole animal images were taken with a digital camera attached to a Leica MZ6 dissecting microscope.

Results

Gene Structure and Protein Expression of EcR-A Mutants.

The three *EcR-A* mutants analyzed here (*EcR^{112}*, *EcR^{139}* and *EcR^{94}*) are the result of a local transposon mutagenesis strategy to isolate *EcR-A* specific deletions caused by transposase-induced imprecise excision or male recombination (Carney et al., 2003). Figure 2.1 shows the deletion endpoints of these mutants, summarizing the DNA structure of each mutant relative to the wild-type gene structure. *EcR^{112}* was isolated in a male recombination screen and retains the P element insertion located

just downstream of the A1 exon and a 10 kb deletion within the first intron, which is also seen in the parental strain, *EcR^{O11}* (Carney et al., 2003). Additionally, *EcR^{112}* has an approximate 4 kb deletion of the *EcR-A* regulatory region, including the transcription start site, exon A1, and at least 3 kb of sequence upstream of the start site. This upstream deletion additionally removes several unessential tRNA genes. Thus, *EcR^{112}* lacks the *EcR-A* transcription start site but retains *EcR-A* coding exons A2 and A3. *EcR^{112}* is therefore predicted to lack the *EcR-A* transcript originating from the *EcR-A* transcription start site.

The *EcR^{139}* deletion was obtained in an imprecise excision screen (Carney et al., 2003) and is structurally complementary to *EcR^{112}* in that the *EcR-A* upstream sequences and the transcription start site are retained while EcR protein coding exons A2 and A3 are deleted. *EcR^{139}* also retains the P element insertion and a 10 kb deletion of intron 1 seen in the parental strain, *EcR^{O11}* (Carney et al., 2003). However, *EcR^{139}* has an internal deletion of about 10 kb within the P element and an 11.5 kb deletion that removes exons A2 and A3 (Fig. 2.1).

EcR^{94}, also obtained in an imprecise excision screen, lacks the parental P element insertion and carries a deletion of approximately 36.5 kb which removes exons A2 and A3. The upstream endpoint is the same as the *EcR^{139}* upstream endpoint, which is at the point where the P element was inserted, 160bp downstream of the A1 exon, and the downstream endpoint is 266bp upstream of the *EcR-B* transcription start site. This deletion effectively juxtaposes *EcR-A* regulatory sequences and the *EcR-B* transcription start site.

Western analysis of *EcR-A* mutants heterozygous to an *EcR* deficiency or an *EcR* null mutant show that the EcR-A protein is no longer detectable in these animals (Fig. 2.2). The EcR-B1 protein is, however, still present. In comparison, a wild-type control at a comparable stage shows expression of both EcR-A and EcR-B1. There are breakdown products that are recognized by the EcR-B1 antibody which are elevated in the *EcR-A* mutants. Thus, the seemingly reduced levels of EcR-B1 may be due to degradation of the full length EcR-B1 protein product within the protein preps of the mutants. We do not believe this contributes to the phenotype of the mutants (see discussion). These western data substantiate the isoform specific nature of these *EcR-A* mutants.

EcR-A is required for pupal development.

Lethal phase analysis revealed varying lethality among the *EcR-A* deletion mutants. Each allele was tested in trans to either an *EcR* null allele *(EcR^{M554fs})* or a deficiency chromosome that removes *EcR (Df(2R)nap^{12}*; data not shown). Table 2.1 shows that each of the *EcR-A* mutant strains has a distinct lethal phase. Both *EcR^{112}* and *EcR^{139}* have a pupal lethal phase while the *EcR^{94}* mutants fail to pupariate. Interestingly, there seems to be some degree of lethality for each genotype at each life cycle stage, possibly in conjunction with the pulses of ecdysone that occur at each of these stages.

For *EcR^{112}* and *EcR^{139}* we made a more specific determination of the lethality time points occurring during pupal development (between stages P5 and P12; see Bainbridge and Bownes, 1981) (Fig. 2.3). While the *EcR^{112}* and *EcR^{139}* mutants generally both die during pupal development they die at distinct stages

during this development. There is a significant drop in viability for EcR^{139} at the molt to third instar, whereas the EcR^{112} strain has a higher rate of survival to pupariation (Table 2.1). While more EcR^{112} mutants survive to pupariate relative to EcR^{139} animals, the EcR^{139} mutants that pupariate survive longer than the EcR^{112} mutants that pass the pupal stage. The P8 stage of lethality for EcR^{112} refers to the pupal period of development past head eversion, when the eyes of the pupa have a slight yellow tint (Bainbridge and Bownes, 1981). Only a few EcR^{112} mutants (< 5%) survive to the P8 stage. The P15 stage of lethality for EcR^{139} refers to a completely developed pharate adult. At this stage the animals are ready to eclose. While a significant number of EcR^{139} mutants reach this stage, even displaying some movement in the pupal case, they rarely eclose (<1%).

Phenotypic analysis of EcR-A specific mutants

In order to define the phenotype of the *EcR-A* specific mutants, we examined external and internal phenotypes. The overall phenotype we attribute to the loss of *EcR-A* is lethality during the early pupal period. Between the EcR^{112} and EcR^{139} mutants, there were several different phenotypes observed with one predominant phenotype displayed for each genotype (Figs. 4 and 6A-C). The EcR^{94} mutants had the most consistent phenotype of non-pupariation. The severity of the phenotypes and lethal phases correlate with the amount of the *EcR-A* region deleted (compare Fig. 2.1 with Table 2.1 and Fig. 2.4). We also show that there are several alternative phenotypes for each of the mutants with earlier and later lethalities than that of the predominant phenotype (Fig. 2.5).

The *EcR139* strain has the longest survival, dying within the mid-pharate adult period. These animals have significant progression of development, including head eversion, eye development, and leg and wing morphogenesis (Fig. 2.4B). Usually, just following the pigmentation of the eyes, these animals appear to degenerate in their pupal cases (Fig. 2.4G) which we believe may be a type of desiccation event. Approximately 15% of the *EcR139* mutant animals survive to later pharate adult stages (P15) having fully developed wings, legs and bristles (Fig. 2.5A and 5B). These animals rarely eclose however, and after several days also degenerate in their pupal cases (Fig. 2.5A). Additionally, about 5% of these mutants do not make it to the mid pupal stage, but desiccate during the pre-pupal/early pupal stage, just after pupariation (Fig. 2.5C). We believe that the desiccation phenotype is a symptom of pupal cuticle imperfections, specifically dealing with its integrity. As compared to wild-type animals, these mutant cuticles clearly do not tan or shorten properly (Fig. 2.4H) and the rigidity of the pupal case is also reduced (data not shown).

The *EcR112* strain has an earlier predominant lethal phase relative to *EcR139* (Fig. 2.4C and D). These animals die during early pupal development, just after head eversion (P8). A common characteristic of the *EcR112* mutants is a misshapen cuticle which develops during pupariation. The larval cuticle case is somewhat misshapen due to a behavioral defect (see Fig. 2.5D) seen in both the *EcR112* and the *EcR139* strains. In preparation for pupariation, wild-type animals stop feeding, wander to the sides of the vial (wandering stage) and remain still for the onset of pupariation (still stage). Subsequently, the cuticle shortens, tans and solidifies into

its characteristic puparial state. The *EcR-A* mutants, however, do not always behave in this way. Several of the mutants that survive to pupariation continue to feed and seem to physically resist the changes of pupariation until the larval cuticle hardens, forming the pupal case. As they struggle to free themselves of the glue attaching them to the wall, the subsequent hardening of the cuticle during formation of the pupal case freezes the animals in awkward (misshapen) positions which create a curved pupal case (see Fig. 2.5D).

A large number of these mutants never leave the food, suggesting they either do not receive or do not respond to the signal that stimulates the wandering behavior normally occurring before pupariation. This behavioral defect may also allow for longer survival in that the animals that do not leave the food, or are not removed from the food for observation, do not show the signs of desiccation we described earlier. This observation suggests the integrity of the cuticle may be lacking in some way, which may allow the animals to dry out if not surrounded by moisture, such as the case when they are left submerged in the food.

Investigations of internal tissues reveal that the EcR-A mutants have salivary glands persisting past the stage they should be present. Following pupariation, the salivary glands normally undergo autophagy, but in the EcR-A mutants, this cell death often does not take place, and later animals retain swollen salivary glands. (Fig. 2.6). This degeneration is thought to be under the direction of EcR-B1, as it is the predominant isoform expressed in salivary glands. This suggests the EcR-A mutation may impede EcR-B1 functions. However, other internal structures that predominately express EcR-B1, such as gastric cecae and larval midgut cells, are

unaffected, still undergoing the cell death and autophagy seen in wild-type animals (data not shown).

In contrast, the *EcR^112* strain has a few (2%) escapers which survive to the pharate adult stage (Fig. 2.5E-G). These animals rarely eclose, and seem to lack a fully defined operculum (Fig. 2.5F). The heads of these animals also seem to be compacted into the anterior portion of the puparium, where there is normally a space or gap at this point of development (Fig. 2.5A vs. 2.5E). Also, the pharate adults that are dissected from the puparium have not ejected the mouthooks, which would normally reside in the anterior gap of the puparium and the appendages have not properly separated from the interior pupal case (Fig. 2.5G). The escapers which make it to the late pharate adult stage, usually have leg morphological deformities (Fig. 2.7). Figure 2. 7 shows the typical crooked and twisted deformities observed in these mutants. There are kinks in the coxa and tibia segments while the tarsal segments of the legs are swollen and arched when compared to the wildtype leg (Fig. 2.7A).

The *EcR94* strain has the earliest lethal phase, dying just before pupariation. These animals have the anterior and posterior gapping that is seen in the *EcR-B1* mutants (Fig. 2.4E, compare with 8A white arrows). Unlike the *EcR-B1* mutants, the internal tissues of the *EcR94* animals seem to become unstable undergoing apoptosis or autophagy at a significantly earlier time prior to necrosis. It is difficult to define any internal structures (either larval or imaginal) within the *EcR94* mutants with this phenotype once they reach the late 3rd instar larval stage. Although the majority of these animals die at the pre-pupal stage, a few escapers

make it to later stages of development. Approximately 2% of the animals pupariate, but do so improperly. Eversion of the anterior spiracles occurs more anteriorly (Fig. 2.8D) and at a 90° angle as opposed to the wild-type 45° angle (see Fig. 2.5). These escapers also undergo desiccation within 24 hours of pupariation (Fig. 2.8D).

Antibody stains of internal structures show that the *EcR94* mutants lack expression of EcR-A, as expected (data not shown). Interestingly, these mutants have an alteration in the expression pattern of EcR-B1. Whereas EcR-B1 is normally expressed predominantly in the larval cells of the proventriculous (pv), Fig. 2.9C shows EcR-B1 is no longer expressed in these tissues. Fig. 2. 9C also shows that the imaginal ring, which normally predominantly expresses EcR-A, is now expressing EcR-B1 in *EcR94* mutants. Accordingly, the larval cells of the salivary gland, which normally express high levels of EcR-B1 (Fig. 2.9F) no longer express EcR-B1 in these cells in the *EcR94* mutant (Fig. 2.9H). Western analyses show that the *EcR94* mutants still produce wild-type levels of EcR-B1, which may indicate then that the expression pattern of the EcR-B1 isoform has changed, and is presumably following the EcR-A expression pattern; however, not all tissues that highly express EcR-A, such as imaginal discs, show high expression of EcR-B1 in these mutants. This may indicate that the sequences of the regulatory region of the *EcR-A* promoter (Sung and Robinow, 2000) are not the only set of regulatory sequences that delineate the expression pattern of EcR-A.

Discussion

Here we describe the phenotypes of three EcR-A deletion mutants. Western analysis shows that the EcR-A protein isoform is no longer produced in these mutants while the EcR-B1 isoform is still present. We recognize there appears to be a reduction in the amount of full length EcR-B1 protein in these mutants. However, we do not believe this observation is indicative of an affect on the EcR-B1 protein production. In mutants where both EcR-A and EcR-B are removed, the phenotype is embryonic lethality. Therefore, we would expect there to be a phenotype that is at least more severe than the phenotype of the EcR-B1 specific mutants if both EcR-A and EcR-B1 were mutated in these lines. Because the increase of breakdown product that appears in the mutant westerns inversely correlates with the reduction of full length protein, we believe these are artifacts of the unstable mutant protein preps. Alternatively, this observation may indicate that EcR-A is required for the stability of the EcR-B1 protein, suggesting an auto regulatory effect between the isoforms. Other experimental observations support this autonomous regulation hypothesis, such as an increase in the EcR-A protein when an EcR-B1 transgene is induced (personal communication, Li and Bender). Therefore, we are confident that the mutants presented here (*EcR^{112}* and *EcR^{139}*) are EcR-A specific.

We find have shown that the removal of *EcR-A* leads to an overall pupal lethality. This finding indicates that the *EcR-A* isoform is required for the completion of pupal development. Although the phenotypes for each of these mutant strains are somewhat divergent, there are predominant phenotypes associated with each mutant.

The *EcR^112* mutant, which has a deletion of the *EcR-A* specific regulatory region and A1 exon, has an early pupal lethal phenotype. The *EcR^139* mutant, which has a deletion of the *EcR-A* specific protein coding exons (A2 and A3), has a late pupal lethal phenotype. The *EcR^94* mutant, with a deletion of nearly the entire genomic region between the A1 exon and the *EcR-B* transcription start site, has a non-pupariating phenotype. The *EcR^94* mutants seem to be a unique class of *EcR-A* specific mutants in that there is likely to be mis-expression of the *EcR-B* isoforms caused by the regulatory region of *EcR-A* driving the expression of the *EcR-B* isoforms (see below). All of the *EcR-A* mutant phenotypes share the commonality of incomplete metamorphosis and improper morphology of the puparium.

Isoform specific requirements

We anticipated that the phenotype of the *EcR-A* specific mutants would reflect the time of the earliest required and detectable endogenous EcR-A protein expression. The EcR-A protein is first detected during embryogenesis; however, this initial expression just after egg laying has been attributed to maternal contribution of the transcript (Talbot et al., 1993). The next transcript increase in *EcR-A*, during mid-embryogenesis, is believed to be endogenous to the embryo. Surprisingly, the data shown here indicate that the first occurrence of endogenous *EcR-A* is not when it is specifically required for survival.

Subsets of the *EcR-A* mutants die off at various intervals during early development and molting, as indicated in the lethal phase analysis in this paper. While we did not focus on these earlier lethals, there is a significant occurrence of lethality prior to the predominant lethal phase timepoint. Generally, the mutant

animals seem to show a pattern of lethal periods that coincide with the developmental intervals where ecdysone pulses occur. Because most *EcR-A* specific mutants live to the end of third instar and beyond early pharate adult development, the *EcR-A* isoform is not required for proper embryogenesis, hatching, or molting. The fact that some mutants die during these earlier phases of development indicates that it is difficult, yet possible, for the animal to survive these stages without *EcR-A*. These observations likely signify that the lack of *EcR-A* interrupts the ecdysone signal at the transition of each stage. This interruption may frequently be overcome, either by some other mechanism of transducing the signal or because of a dispensable role for *EcR-A* during larval molts, which allows the majority of the mutants to molt into the subsequent instar stages, and in the cases of *EcR[112]* and *EcR[139]*, to pupariate and develop to the mid-pupal stages.

The *EcR-B1* mutants live beyond the molting stages as well, surviving up to the pupariation timepoint (Bender et al., 1997), which indicates one of two possibilities. The first is that neither *EcR-A* nor *EcR-B1* are specifically required for embryogenesis, hatching, nor molting, which suggests that *EcR-B2* is the isoform responsible for these functions; or second, during these processes **any** of the three isoforms are sufficient to transduce the ecdysone signal. We favor the latter hypothesis, since *EcR* null mutants, which are normally embryonic lethal, can be rescued through the molting stages by ectopically expressing any of the isoforms. However none of them alone will faithfully rescue past pupal development (Li and Bender, 2000). This suggests that the ecdysone signal's role during the pulses detected and required for these earliest transitions into subsequent life cycle stages is more generalized. Explicitly, the genes that respond to ecdysone during the

processes of hatching and molting are most likely not the same set of target genes needed for specific tissue physiological changes, alleviating the necessity of specific isoforms to do different jobs. Rather, the ecdysone targets responsive during these phases of development would be more akin to cell growth regulators, metabolism and other homeostatic genes. At the point in development when the ecdysone signal has a more complicated role, such as the differentiation and tissue remodeling seen during metamorphosis, the specific isoforms take on their unique roles, probably with the aid of specific cofactors that are only expressed at the onset of metamorphosis.

EcR-A and EcR-B1 have unique functions

In comparing these *EcR-A* mutant phenotypes with those of the *EcR-B1* mutant, which removes the *EcR-B1* isoform specifically and the *EcR-B* mutants (Bender et al., 1997), which remove both the *EcR-B1* and *EcR-B2* isoforms, we find the *EcR-A* mutants have a novel phenotype. Despite the variability of the *EcR-A* mutant phenotypes, it is clear that these mutants reveal a difference in developmental functionality, at least between the *EcR-A* and *EcR-B1* isoforms, as these *EcR-A* mutant phenotypes are all different from the *EcR-B1* mutant phenotype. What is left to be determined is the specifics of this functional difference. To address this, a distinction must be made as to whether the functionality or requirement differences are a manifestation of a secondary cofactor interaction specific to each isoform, perhaps due to temporally regulated tissue specific cofactors, or if the unique activity is due to differences in the biochemical activity of the actual receptor isoforms.

Given that the *EcR-B* mutant phenotypes are different from the *EcR-B1* mutant phenotypes (Bender et al., 1997) and rescue experiments using *EcR-B1* vs. *EcR-B*2 have markedly different results (Cherbas, 2002; Li and Bender, 2000), one can extrapolate that *EcR-B1* and *EcR-B*2 isoforms also have unique functions, which suggests that all three isoforms have separate and unique functions. Biochemical assays of differential EcRE binding affinities between the EcR isoforms also support them each having distinct functions (Dela Cruz et al., 2000). Such findings are also in agreement with conclusions in vertebrate nuclear receptor research (Conneely and Lydon, 2000) which indicates distinct functions between the specific isoforms of other nuclear receptors. One possible explanation given for the mechanism of distinguishable isoform-specific functions is differential binding affinities to various response element architectures found in the promoter regions of target genes (Antoniewski et al., 1996; Crispi et al., 2001; Lehmann et al., 1997; Tsai and O'Malley, 1994); Niedziela Majka et al., 2000). However we note that the developmental uniqueness of isoform requirements does not rule out the possibility of biochemical redundancy. The definitive expression patterns of each isoform could in some way limit the full potential of the biochemical activity of each isoform via physical barriers. In the case of biochemical redundancy, specific tissue types that harbor certain isoforms may also harbor other tissue specific co-factors that could interact differently with specific isoforms, and thereby may regulate each isoform's action in respective tissue types (Arbeitman and Hogness, 2000; Beckstead et al., 2001; Lehmann and Korge, 1995; Pearce and Yamamoto, 1993; Thormeyer et al., 1999; Tran et al., 2001; Tsai et al., 1999). Alternatively, each of the isoforms may potentially interact with all of the cofactors in the same biochemical manner, but the

seclusion of the cofactors, and therefore the cofactors' regulatory capacity, into the specific tissue types could be what directs the observations we interpret as *EcR* isoform specific due to overlapping expression patterns of tissue-specific cofactors with specific *EcR* isoforms.

Different deletions have different phenotypes... a new EcR isoform?

Between the different *EcR-A* mutations, there is some diversity in the lethal periods and mutant phenotypes manifested from their respective deletions. This diversity may indicate some difference in the ability of the respective deletions to remove all *EcR-A* function beyond the point of detection by Western analysis, or more intriguingly, this may indicate a second *EcR-A* transcript which is removed or altered in the *EcR^{139}* allele and not in the *EcR^{112}* allele.

Data on cDNA's from the Berkley Drosophila Genome Project (BDGP) indicates there are two additional exons, which are present in a novel transcript of *EcR-A*. This new transcript apparently is produced from a unique promoter (Fig. 2.10), which implies the presence of new regulatory sequences, and lies downstream from the previously described *EcR-A* transcription startsite (Talbot et al., 1993). This new isoform could have been masked from discovery by the fact that it shares the same protein coding region as the previously described EcR-A isoform, and that the sizes of the additional two exons together are almost the exact same size as exon A1 (Fig. 2.10), which gives the transcripts similar mobility during gel electrophoresis and therefore would be indistinguishable in Northern analyses.

Upon comparison of the loci of this new transcription startsite and non-coding exons, we find that the *EcR^{139}* allele fully removes the functionality of the

EcR-A isoform, because the coding exons shared by both EcR-A isoforms are removed. However, the EcR^{112} mutant only removes the regulatory region of the EcR-A isoform produced from the upstream transcription start site, which suggests there may possibly be some EcR-A produced off of the downstream promoter. The lack of detectable EcR-A protein in the EcR^{112} mutants, by Westerns, indicates the residual nature of the "new" isoform, and also may explain why there isn't a greater difference in mutant phenotypes between EcR^{139} and EcR^{112}. Additionally, this new transcript may explain the drop in viability seen in the EcR^{139} line during 2nd instar that is not seen in the EcR^{112} line.

Additionally, the $EcR94$ strain has a unique gene structure feature that may be the cause of its divergent phenotype in comparison to the other *EcR-A* mutants. Analysis of the genomic structure reveals that the *EcR-A* regulatory region is placed adjacent to the *EcR-B* transcription start site (266 bp upstream). The *EcR-B* promoter region is largely deleted in this mutant strain, with the full regulatory region of the *EcR-A* isoform left intact. This suggests that the EcR-B isoforms may be expressed in an EcR-A pattern. This extraordinary event has given the truest opportunity to determine if one isoform can replace another and therefore answer the question of isoform function distinction.

Unfortunately, this deletion may also have an adverse affect on the expression of the *EcR-B* isoforms. It has previously been shown that a region of 500bp of the promoter region from *EcR-B1* is insufficient to produce proper levels of EcR-B1 in a transgenic (Li and Bender, unpublished), which means the portion of the *EcR-B* regulatory region remaining in the *EcR94* allele may be insufficient to

70

regulate the proper expression of the *EcR-B1* and *EcR-B2* isoforms. However, Western analysis clearly shows the *EcR94* allele produces wild-type levels of the EcR-B1 protein isoform (Fig. 2.2). This suggests that the production of EcR-B1 seen in Western analyses is under the regulation of the *EcR-A* promoter region. This implies that the EcR-B1 and EcR-B2 isoforms are expressed in an EcR-A pattern in this mutant strain. The fact that some tissues that normally express high levels of EcR-A are not expressing the EcR-B1 protein, under the regulation of the upstream *EcR-A* promoter in *EcR94* mutants, may signify the existence of the new promoter annotated in the genome database. This suggests that the downstream promoter is responsible for the EcR-A expression in tissues, such as in the imaginal discs and ring gland, since unusual over-expression of EcR-B1 is not seen in antibody stains of these tissues from *EcR94* mutants.

Alternatively, the difference in mutant phenotypes between the different *EcR-A* mutant genotypes could be due to the differences in genomic structures. Deletions for each of the mutants may displace regulatory elements within this genomic region and thereby may affect the expression of surrounding genes, or more pointedly the expression of the *EcR-B* isoforms, as well as the novel *EcR-A* isoform. Additionally, gene finder programs have detected a putative gene in the deletion region which is removed in *EcR139* and not *EcR112*. However, there is currently no transcript evidence and this putative gene doesn't show homology to any known gene of required function. Also, the variations of phenotypes between the deletion mutants could possibly have to do with the presence of several tRNA gene clusters within this region which are deleted in these mutants. This is unlikely

however; as these tRNA clusters have been extensively studied and previous mutational analyses show that they are not vital for development or survival (Gergen et al., 1981; Hovemann et al., 1980; Leung et al., 1991; Yen and Davidson, 1980). Obviously, we would like to determine the specific molecular interactions or aberrations that result in the variation of phenotypes between these distinct EcR-A mutants, and look forward to these studies in the near future.

Acknowledgements

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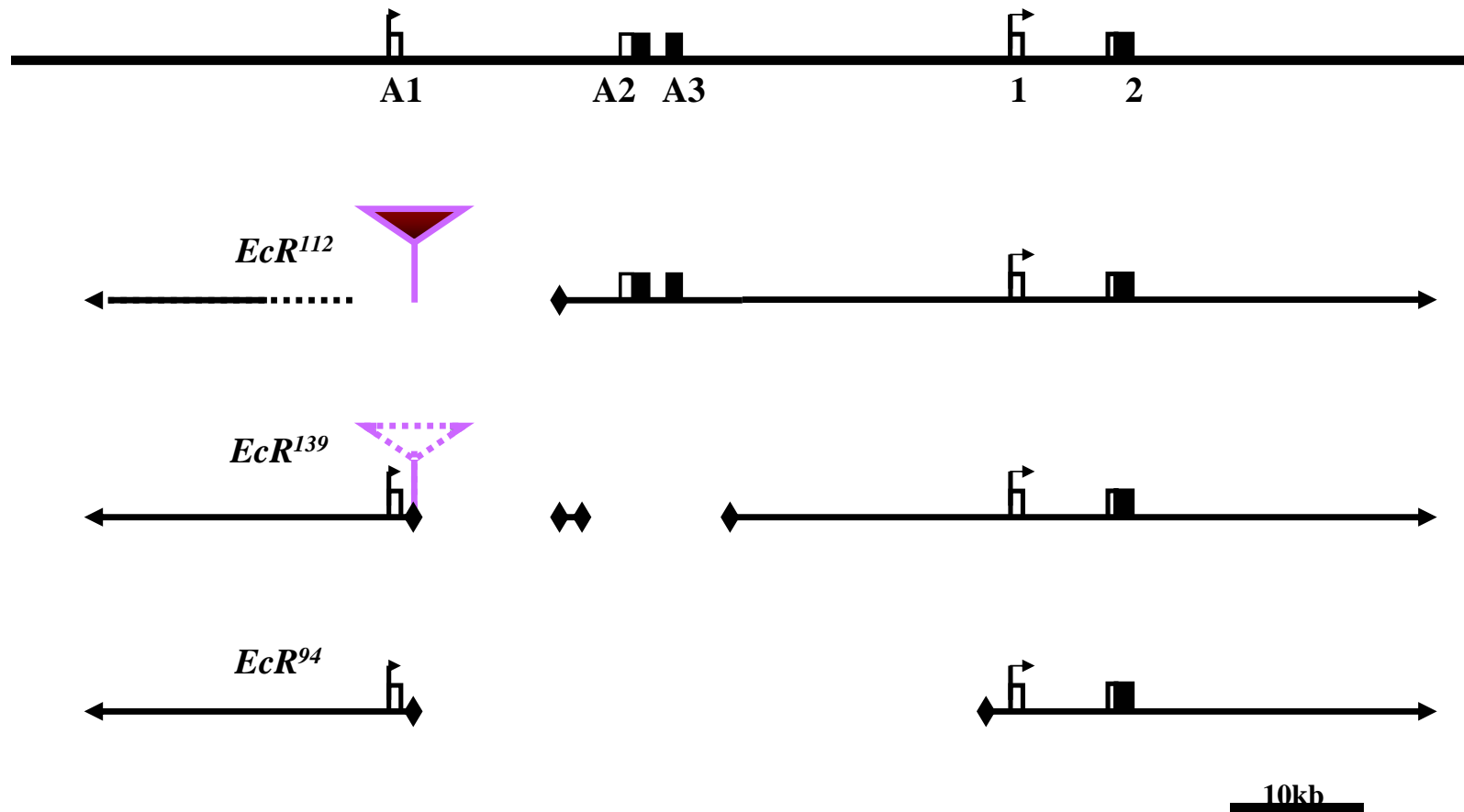
Figure 2.1. Deletions that remove *EcR-A* sequences. The top line represents genomic sequences including the *EcR-A* transcription start site (upstream arrow), *EcR-A* specific exons A1-A3, the *EcR-B* transcription start site (downstream arrow), and exons 1 and 2. Protein coding regions are indicated by filled boxes. The lower three lines indicate the structure of the *EcR112*, *EcR139* and *EcR94* deletions. The inverted triangle indicates a P element insertion (not to scale) completely or partially retained in *EcR112* and *EcR139*, respectively. Diamonds indicate endpoints of deleted sequences in these strains. The dashed line in the *EcR112* strain indicates the current imprecision in mapping of the upstream endpoint.
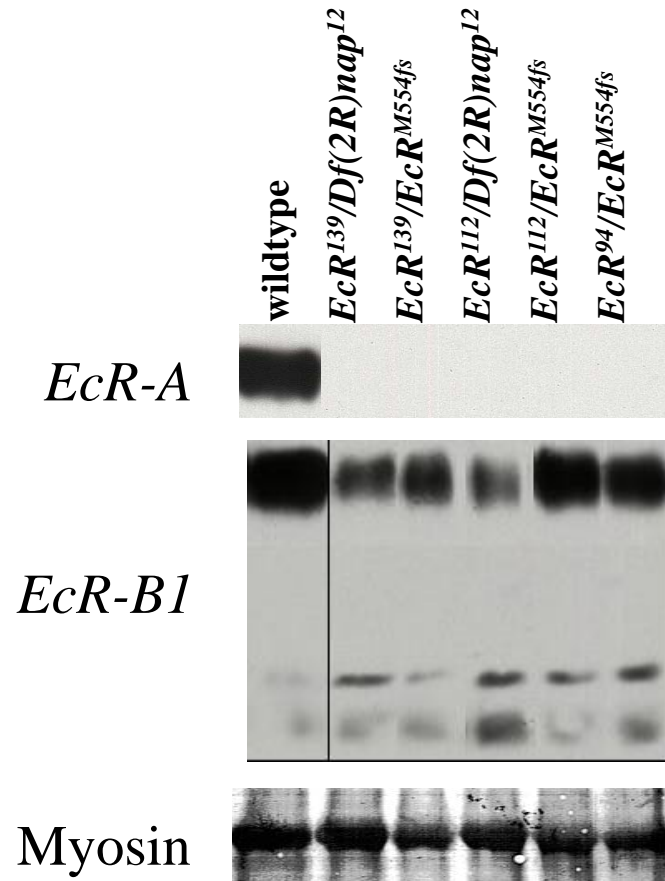
Figure 2.2. *EcR-A* **deletion mutants lack EcR-A expression but retain EcR-B1 expression.** Extracts from wildtype (*Canton-S*) and *EcR-A* deletion mutants heterozygous to *EcRM554fs* or hemizygous to *Df(2R)nap12* at white pre-pupal stages were assayed by Western blot for the presence of EcR-A and EcR-B1 protein. *EcR94/Df(2R)nap12* animals did not survive to this stage and were not tested. Western filters were first probed with the EcR-A specific antibody 15G1A (top panel) and then stripped and re-probed with the EcR-B1 specific antibody AD4. 4 (center panel). A separate filter comparably loaded with protein extracts was probed with an anti-myosin antibody as a loading control (bottom panel).

Table 1. *EcR-A* mutants have varying lethal timepoints

Paternal allele	Percentage of survival				
	1st instar	2nd instar	3rd instar	pupal	adult
EcR^{011} n=8	100%	100%	100%	88%	88%
EcR^{94} n=16	100%	75%	75%	0%	0%
EcR^{139} n=20	100%	60%	40%	40%	0%
EcR^{112} n=38	97%	88%	77%	74%	0%

Table 2.1. Lethal Phase of EcR-A mutants. *EcR-A* mutants heterozygous for the *EcR^{M554fs}* allele were observed six times during development (hatching is not shown). Percent survival is relative to the total number of mutants (n) collected at hatching from a collection of 200 eggs. *EcR^{112}* has a less than 100% score at 1st instar because mutant animals were found dead at the time scored for 1st instar. *EcR^{011M}* is the parental strain, serving as the positive control.
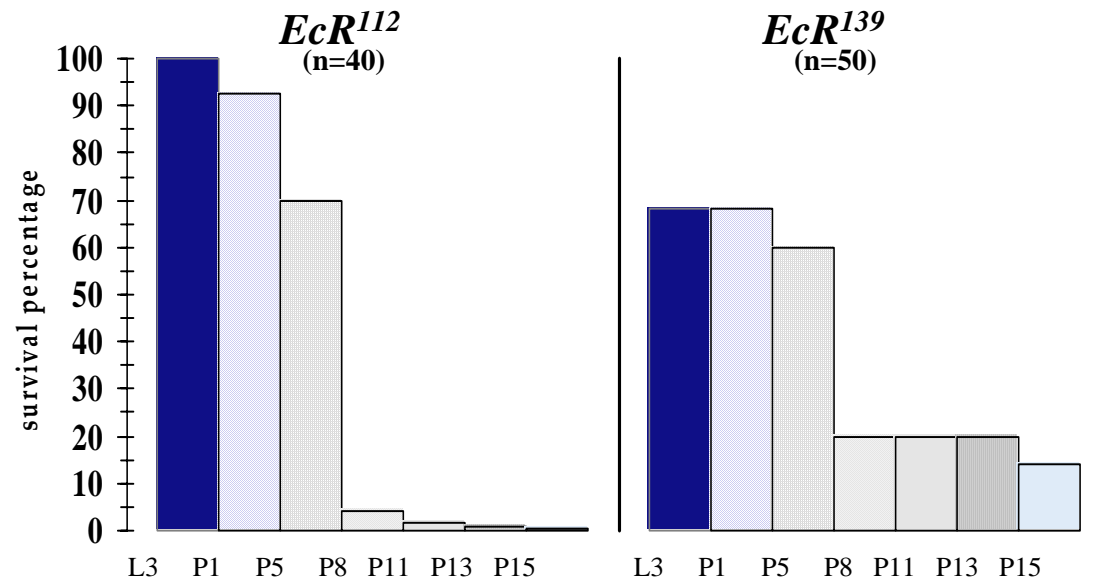
Figure 2.3. Prepupal and Pupal Lethality of *EcR112* and *EcR139*. Percent survival is shown for *EcR112 /EcRM544fs* and *EcR139/EcRM544fs* mutants. Developmental staging during the prepupal and pupal staging is according to Bainbridge and Bownes (1981). L3= late third instar stage.
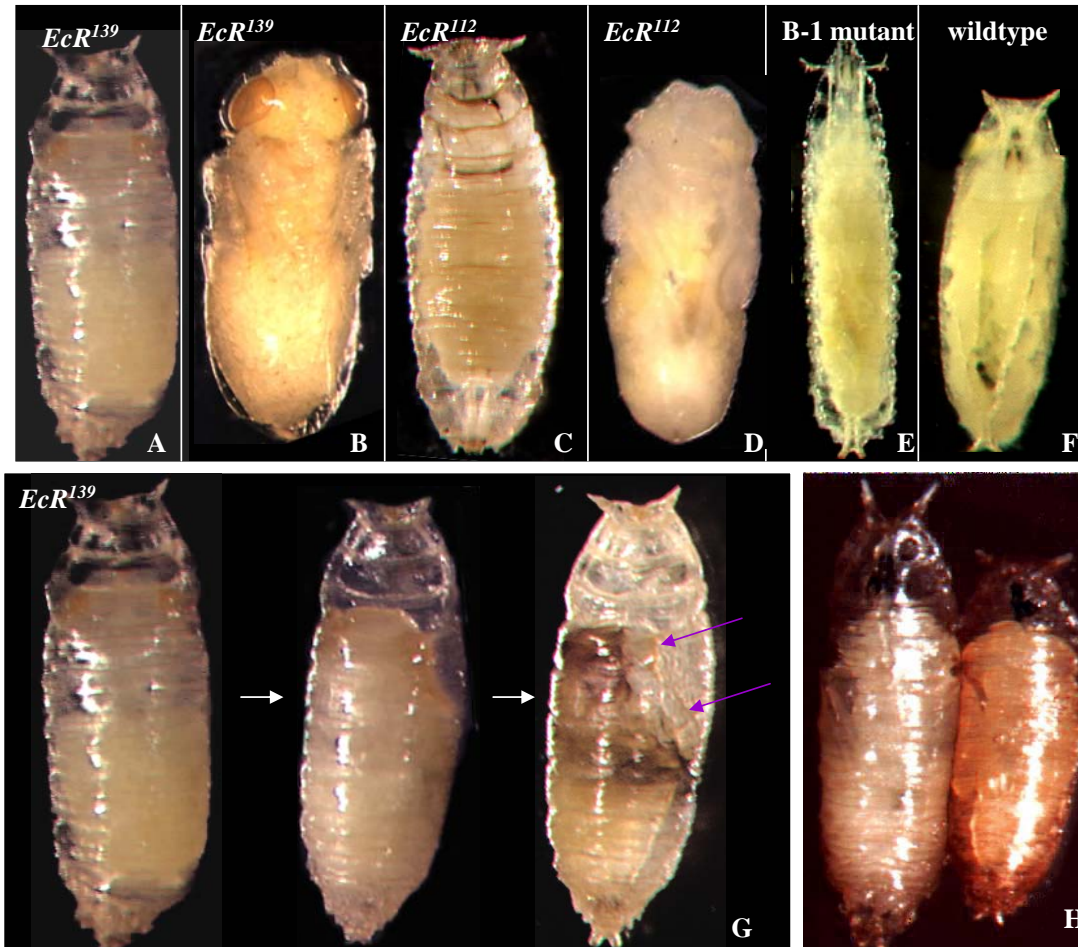
Figure 2.4. Predominant *EcR-A* mutant lethal phenotypes differ from *EcR-B1* mutant phenotype. The predominant phenotypes of *EcR139* and *EcR112* (heterozygous with *EcRM554fs*) are shown here. The animals are shown both in the puparium (A and C) and removed (B and D). The *EcR-B1* mutant phenotype is non-puppariating (E) at the time when the animal should be white pre-pupae (wpp) as shown in wildtype (F). Panel G shows a progression of the *EcR139* predominant phenotype in 24 hour intervals. Purple arrows indicate the outline of necrotic tissue in the puparium case. Panel H shows *EcR139* and *CS* to illustrate the lack of puparium tanning, and larval shrinkage seen in both *EcR139* and *EcR112*.

83
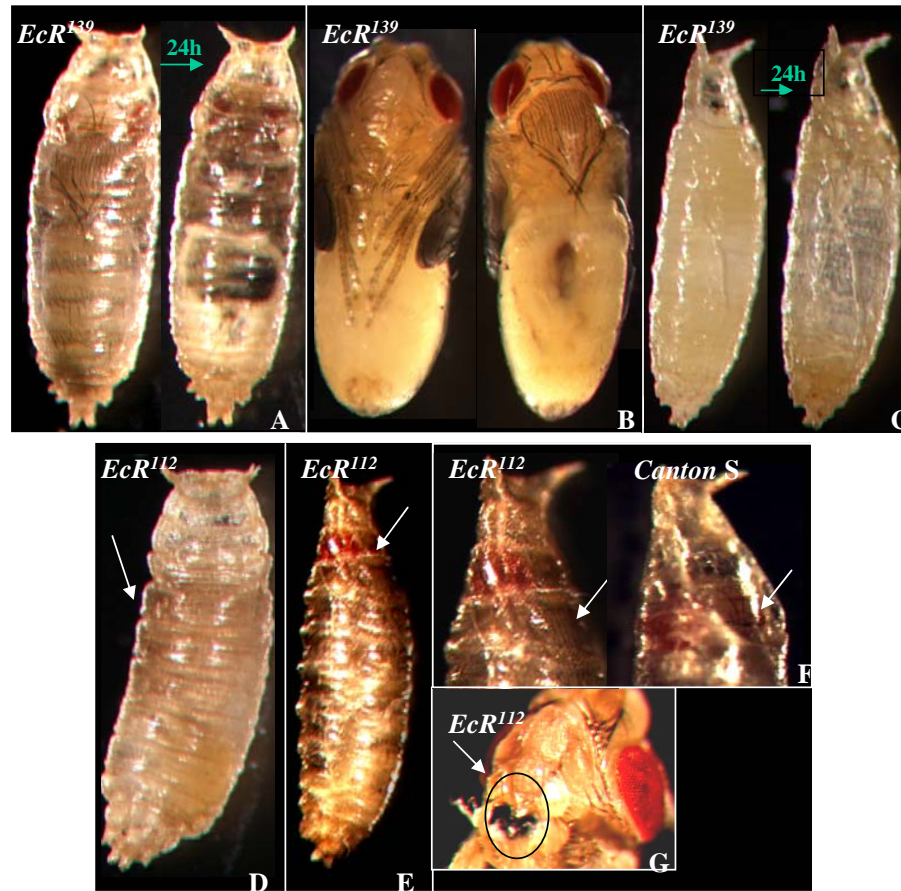
Figure 2.5. Alternative *EcR-A* mutant lethal phenotypes. *EcR139* and *EcR112* mutants have alternative phenotypes. The coupled animals separated by green arrows denote the same animal 24 hours apart. Panel A shows an early pharate adult of *EcR139*. A dissected pharate adult is shown in panel B. Panel C is a representation of *EcR139* mutants that arrest at the wpp stage and undergo desiccation within 24 hours. Some *EcR112* mutants arrest as wpp stage as well (panel D) with misshapen pupal cases (arrow). *EcR112* mutants surviving to pharate adults (panels E-G) have not completely formed an operculum (panels E and F arrows). Panel F shows a normal operculum from a *Canton S* strain. A pharate adult extracted from the pupal case (panel G) shows the mouthparts were still attached to the cuticle and the mouth hooks were not properly ejected during pupation (black circle).
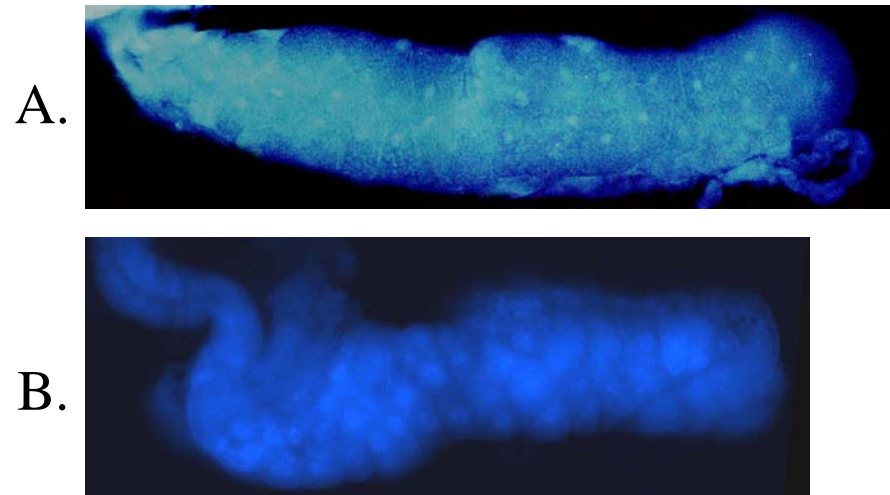
84

Figure 2.6. Persisting Salivary Glands. Panel A shows a representation of a persisting salivary gland commonly observed in the *EcR112* and *EcR139* mutants at stage P8. Panel B shows a degenerating salivary gland from a wildtype animal at stage P5.
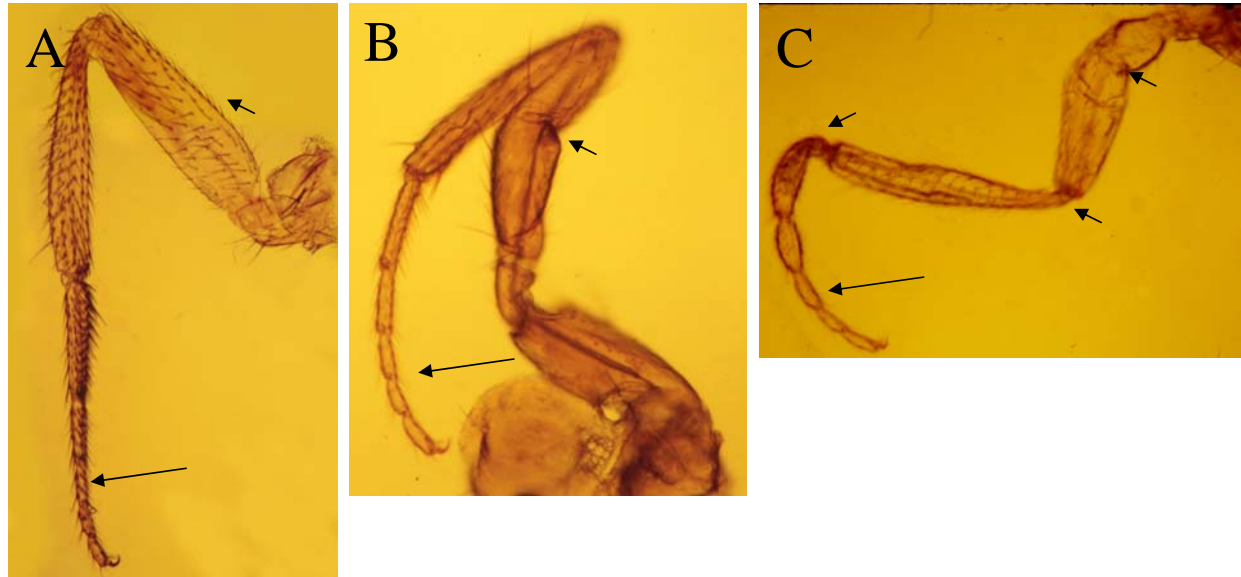
Figure 2.7. *EcR-A* **mutant leg deformities.** The *EcR112* and *EcR139* mutants which live to pharate adults and/or eclosion have leg deformities (Panels B and C). There are kinks present in the tibia/femur and coxa segments (short arrows), as well as swelling in the tarsal segments (long arrows) when compared to wild-type leg morphology (Panel A).`
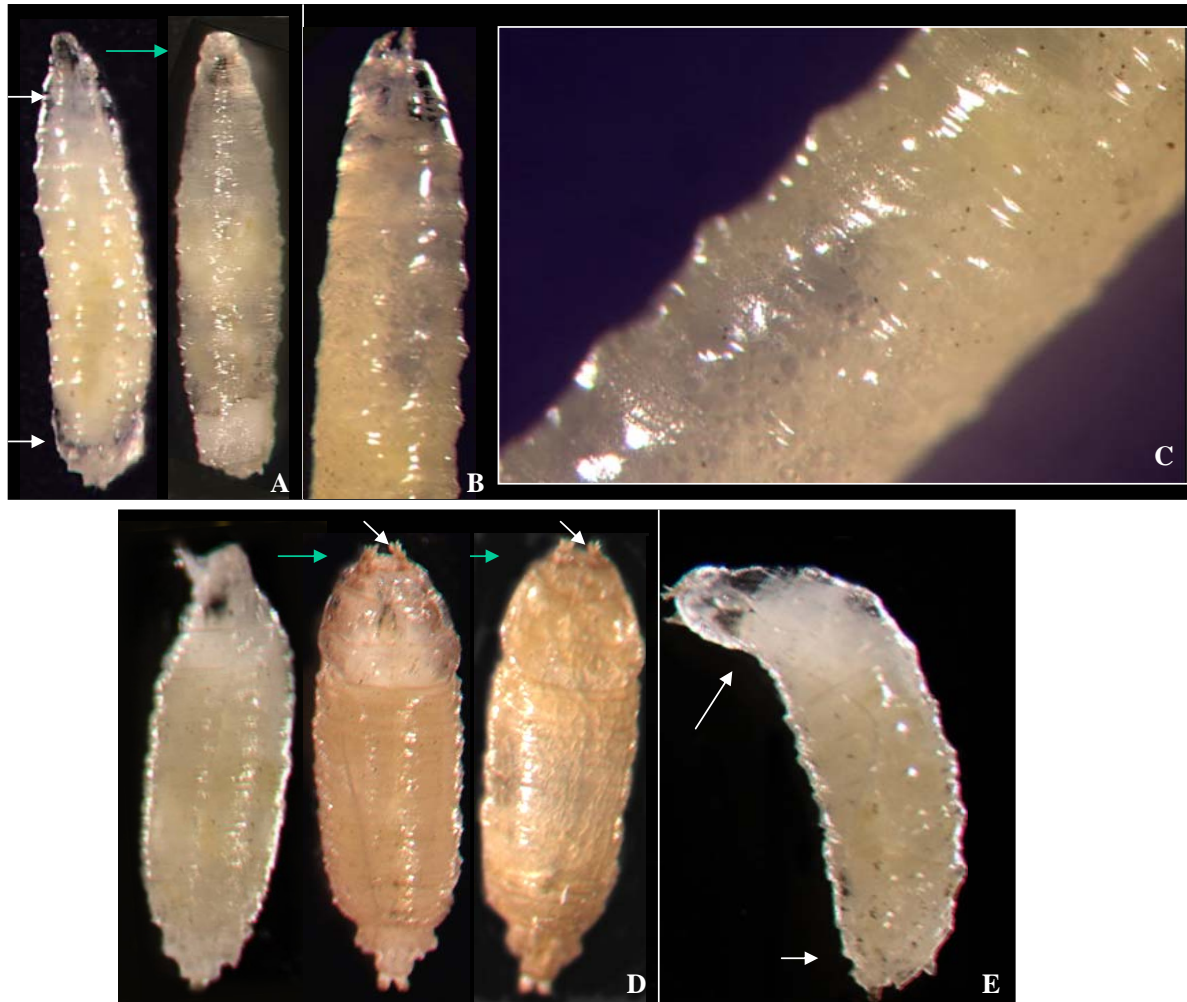
Figure 2.8. *EcR94* **mutant phenotype.** The *EcR94 (*heterozygous with *EcRM554fs)* allele has a predominant phenotype similar to the *EcR-B1* mutants, dying at late third instar with a non-pupariating gap stage (panel A). Panel B is higher magnification of the anterior gap. Panel C is an enlargement of the animal in panel B showing the degradation of internal tissues. The very few animals that pupariate (panel D) have misplaced anterior spiracles (short arrows) and a misshapen pupal case (panel E long arrow). The short arrow in panel E shows where cuticle stiffening has begun from onset of pupariation. Green arrows show the same animal at a later time.
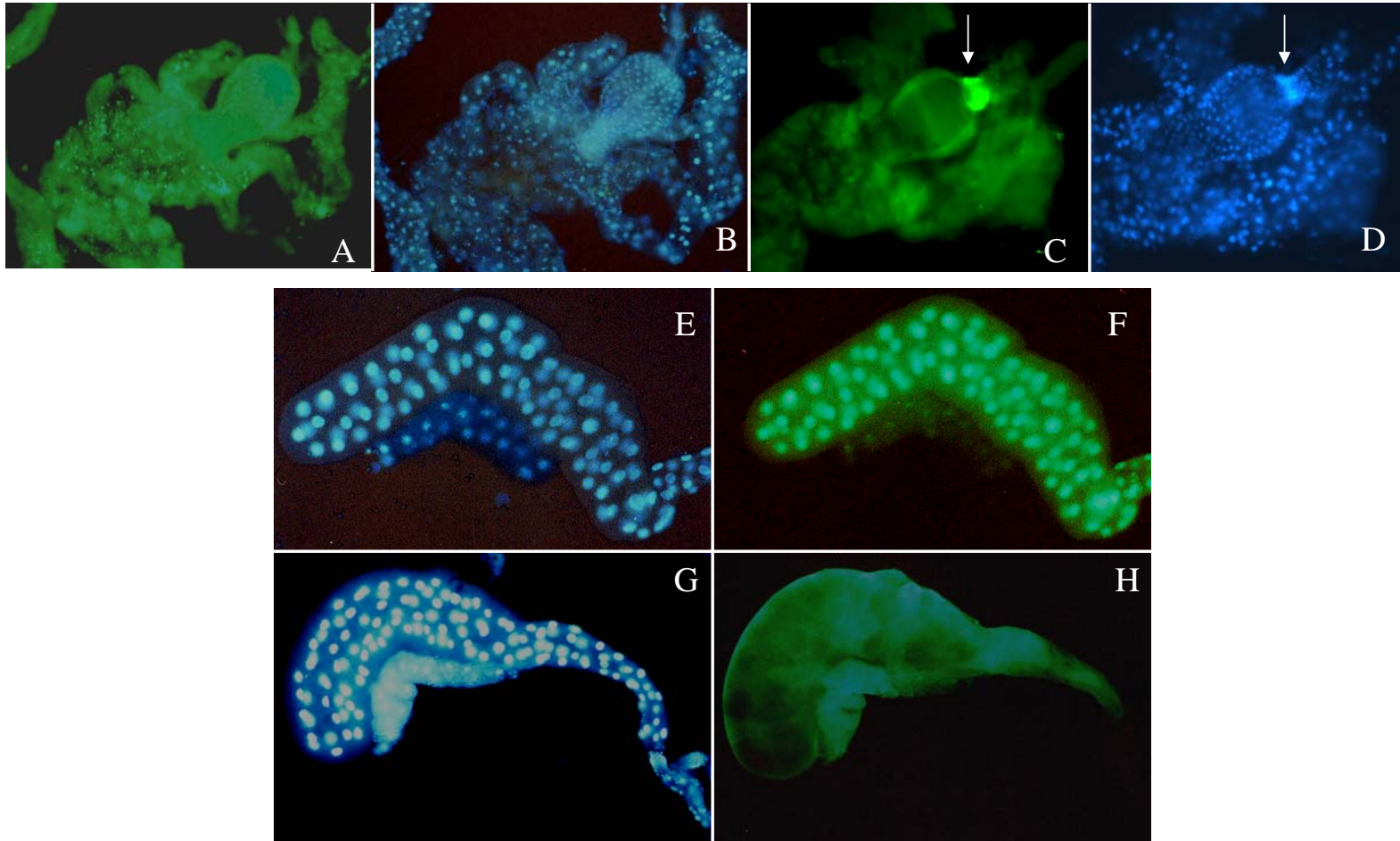
87

Figure 2.9. Antibody and DAPI stains of internal structures. Panels A-D show the proventriculous (pv) stains and panels E-H show salivary gland stains. Panels A, B, E and F are *Canton S* and panels C, D, G and H are *EcR94* mutants heterozygous with *EcRM554fs*. The antibodies used in panels A, C, F and H are EcR-B1 specific. The panels B, D, E and G are DAPI stains. Arrows highlight the imaginal ring of the pv.
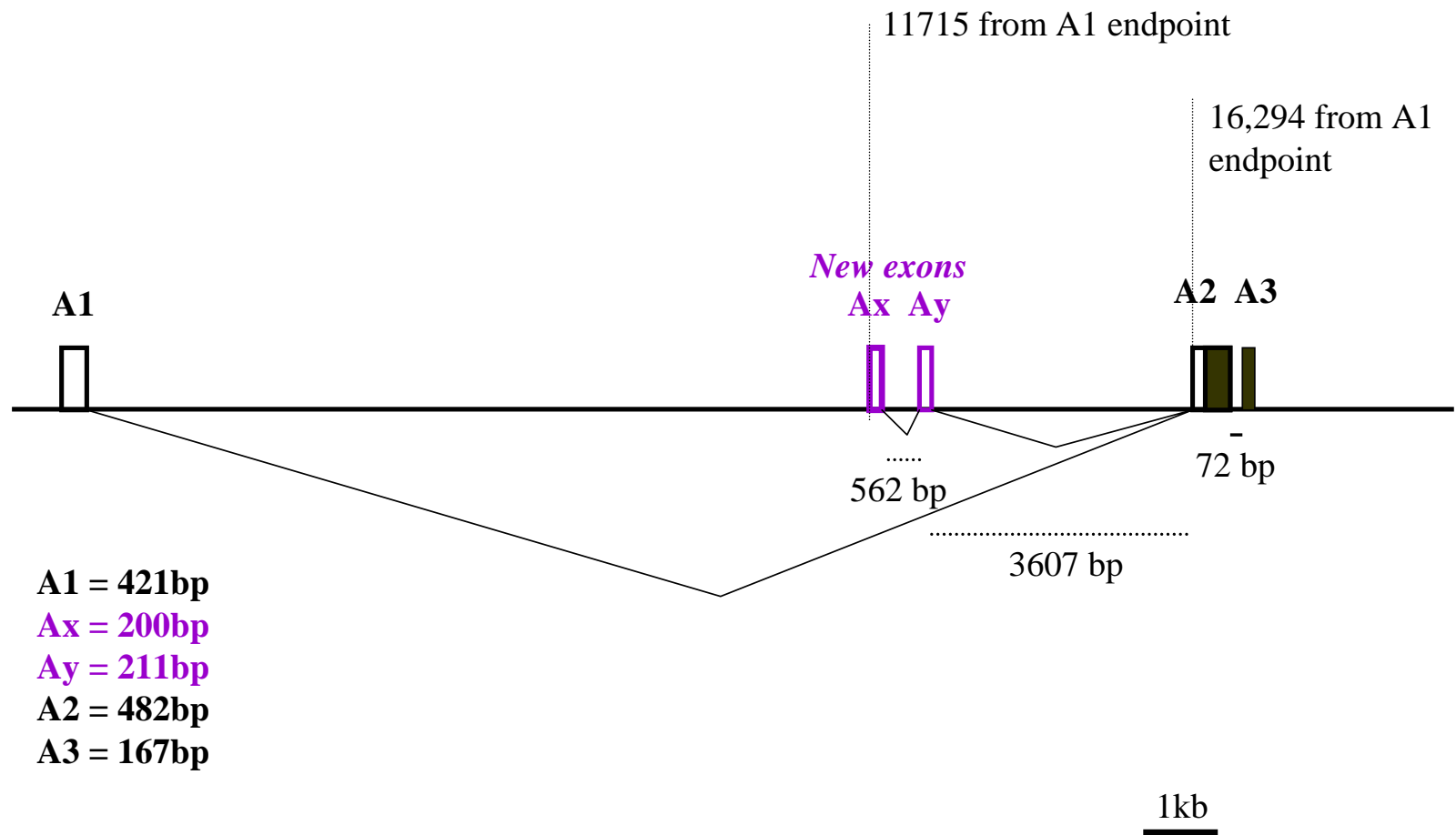
Figure 2.10. The new *EcR* transcripts. BDGP (Berkely Drosophila Genome Project) data shows there are two new *EcR* exons annotated within the *EcR* gene (Ax and Ay) which reside upstream of the A2 exon and downstream of the A3 exon. The sizes of the exons and intervening introns are indicated.

Chapter 3

Analysis of the Genomic Response to the Mid-Third Instar

Ecdysone Pulse

Introduction

During Drosophila development, large pulses of ecdysone initiate the transitions between life cycle stages. The largest pulse occurs at the end of the third instar, triggering puparium formation and the subsequent larval to adult metamorphosis. A small pulse of ecdysone occurs during the middle of the 3rd instar stage and is believed to be necessary for the animal to properly undergo metamorphosis. This mid-third instar pulse is responsible for the behavioral response known as wandering, when the animals leave the food source to find an adequate location for pupation just prior to the pupariation pulse of ecdysone. Because of this distinct behavioral change, as well as distinct changes in gene expression, this period of the life cycle is termed the "mid-third instar event" in some literature (Richards, 1981a; Richards, 1981b; Richards et al., 1999; Riddiford, 1995; Truman and Riddiford, 2002).

Previous studies in other insects have shown that the ecdysone signal that occurs in the middle of the third instar is necessary for subsequent metamorphosis (Richards et al., 1999; Riddiford, 1995). This event has been shown to make the tissues competent to receive the signal for metamorphosis in lepidopterans, rather than alternatively undergoing another molt and is therefore referred to as the competency pulse in some literature. In Drosophila, this mid-third instar pulse commits the larval tissues to undergo their specified metamorphic changes when exposed to the next ecdysone signal, the pupariation pulse, at the end of third instar (Truman and Riddiford, 2002). This relatively small ecdysone pulse is therefore oftentimes referred to as the "commitment pulse". Here we refer to the mid-third instar

pulse of ecdysone as the commitment pulse. This commitment pulse primes the ecdysone gene targets in a manner that results in a distinct response to the pupariation pulse of ecdysone in comparison to the other larval molt pulses. There is a very discrete change in Drosophila gene expression patterns and behavior at the time when the commitment pulse is released, referred to as the mid-third instar transition (Andres et al., 1993). This suggests that the commitment pulse commonly observed in Drosophila may also be necessary for metamorphosis in these insects.

There are most likely a unique set of genes targeted by the commitment pulse as well as by the pupariation pulse, in comparison to sets of gene targets affected by the larval pulses. Functional genomics studies have shown that the gene networks that are initiated during embryogenesis are re-activated during metamorphosis (Arbeitman et al., 2002). As ecdysone is required to maintain proper transition between the stages of the life cycle, these findings suggest that the genes controlled by ecdysone are differentially regulated at different points in the life cycle. Therefore, because the mid-third instar event is a unique ecdysone-triggered event during the life cycle, there are probably unique expression patterns for ecdysone regulated genes at this timepoint. To date, there are no reports of the novel genes associated solely with this stage of development or this specific pulse of ecdysone.

To determine in more detail the effect of the mid-third instar ecdysone commitment pulse on gene regulation, we utilized a functional genomics approach. To date several labs have defined approximately 100+ genes that

are ecdysone-sensitive, using experimental approaches such as in vitro assays of chromosomal responses to ecdysone (Huet et al., 1993; Thummel, 2002). Most of the genes defined in these studies were identified using salivary gland polytene chromosomes. To distinguish ecdysone-regulated genes in these studies, investigators identified regions of puffing on these chromosomes that indicated transcriptional activation that was caused by the ecdysone signal (Ashburner, 1972; Cherbas, 1993; Hurban and Thummel, 1993). Because salivary gland studies only reflect the ecdysone response in one type of tissue, a tissue that undergoes cell death at the onset of metamorphosis, it is reasonable to assume that there are hundreds of other ecdysone targets that are differentially regulated in tissue types that undergo alternative cell fates in response to the pupariation pulse of ecdysone. To define novel ecdysone genes expressed throughout the animal, we are using a whole animal transcriptional profile approach.

Recently, a whole animal developmental transcriptional profile was published (Arbeitman et al., 2002) showing the trends of gene expression for almost one third of the Drosophila genome. The staging of this experiment spans the entire life cycle, with the majority of the sampling done during embryogenesis. The resolution of sampling done during larval development was approximately 10 hours between sampling. This would not be precise enough to observe genomic changes from the commitment pulse. Based on these data, we cannot determine whether there are transcriptional pattern changes that could be attributed to the ecdysone commitment pulse. Therefore, we have developed a finely staged developmental synchronization

93

scheme and analyzed ~25% of the genome to uncover genes that may be specifically regulated by the mid-third instar commitment pulse of ecdysone.

In all, a total of ~600 genes were determined to be differentially regulated during or around the time of the commitment pulse. We chose to focus on characterizing the functions of genes that showed a regulation change specifically at the mid-third instar event, between 93 and 99 hours AEL. We found that nearly 150 genes are activated during this pulse, while around 100 are repressed. Additionally, more than 120 genes show differential regulation, transitioning between activation and repression at several points during the period analyzed. Only ~80 of the all genes categorized in these groups are known to be ecdysone targets. Therefore, the remaining genes that were not previously defined as ecdysone-regulated, but mimic the expression patterns of known ecdysone targets, may now be considered candidates for ecdysone regulation.

Materials and Methods

Staging of Animals

Canton S wild-type animals were synchronized at three points during the sampling process, starting at egg lay. Two egg collections were made for approximately 1.5 hours during peak egg lay times {as determined by circadian rhythm data (Gruwez et al., 1971)}. Synchronization steps were taken again at hatching by discarding any early hatchers (at 20 hours after egg lay) and taking collections of newly hatched animals hourly and transferring them to new collection plates. The animals were aged and

screened at the 2nd instar larval molt. Any animals lagging or prematurely molting were discarded at that time. To ensure simultaneous development, the animals were confined to constant temperature (25 degrees C) and regulated light cycles (12h light, 12h dark).

During the initial staging experiments, the animals were allowed to age until the time of pupariation in order to measure the time required to reach this stage from the onset of the third instar. The animals pupariated at ~116-118 hours after egg lay (AEL). This normalization was done to ensure that the animals collected for sampling were staged precisely during the middle of third instar, at the time of the commitment pulse of ecdysone. Behavioral changes known to occur subsequent to the commitment pulse, such as wandering of the 99-102 hour animals, allowed us to be certain the mid-third instar event had occurred, and our sampling was accurately timed.

The mid-third instar event was determined to occur between 93-99 hours AEL based on the relative timing of the molt to third instar, the time wandering initiated, and the time of pupariation. Experimental collections of synchronized animals were taken in three hour intervals starting at 87h AEL, (approximately six hours prior to the midpoint of the mid-third instar event) through 102h AEL (approximately six hours subsequent to the midpoint). This temporal range of sampling was done to ensure the detection of both early and late genes that are differentially expressed in response to the ecdysone pulse, and permitted us to observe the full range of the expression pattern in response to the commitment pulse.

The collection procedure was repeated until an adequate number of larvae were collected. Approximately 3500 animals were collected for each timepoint sampled. The sample collection procedure was repeated over the course of 10 weeks (~60 samplings).

RNA extraction.

Total RNA was extracted from larvae in groups of 200-300 using a TRIzol method protocol adapted from (Khodarev et al., 2002). mRNA was then isolated and purified using a Qiagen mRNA isolation kit following the manufacturers instructions. mRNA was then precipitated to increase sample concentration and quantified by spectrophotometry.

Microarray printing

The microarrays utilized have a full representation of the entire Drosophila genome. Targets spotted on the arrays were PCR fragments of the coding regions of each gene documented in the annotated genome. The primers used to develop the PCR fragments were developed and optimized by Kevin White as described in Rifkin et al. (2003). Printing was done as described by White (2001). Slides from three independent print runs were used during this project.

Microarray hybridization

Double dye labeling (Cy3 and Cy5) was used for microarray hybridization as described in Li and White (2003). A cumulative developmental reference sample (Li and White, 2003; White et al., 1999) was used to determine the relative expression levels in the experimental samples and labeled with the Cy3 dye. The experimental samples of the mid-third

96

instar timepoints were labeled with the Cy5 dye. mRNA was labeled using an

RT-PCR labeling method (Li and White, 2003; White et al., 1999). Prior to

each hybridization, equal amounts of mRNA (up to 5ug, measured to the 0.01

of a microgram) between samples and references were aliquoted for labeling

protocols to ensure proper correlation between the sample and the reference

signals. Slide hybridization was done in a heated (68 degrees Celsius) water

bath with hybridization chambers manufactured by Gene Machine. For each

timepoint, between 6-8 hybridizations were done. Three to five

hybridizations per timepoint were determined to be suitable for analysis after

initial visual screening for non-specific hybridization or printing defects.

Hybridizations were incubated overnight (~16 hours).

Microarray Scanning

Arrays were scanned subsequent to hybridization using Axon scanning

equipment and Gene Pix 3.0 software was used for visualization of the

hybridization signals as well as initial background normalization of readings

from each slide. This normalization step calculated the amount of

hybridization background noise in the vicinity of the spots, representing

genes, and normalized the gene data accordingly. Scanning protocol was

done as instructed by the manufacturer (Axon laboratories).

Data Mining

Secondary data filtering was done on raw slide data based on

background noise and faint spot calculations from algorithms developed by

the White lab (Li and White, 2003). This formula determines whether the

data for each gene (spot) is reliable based on the amount of background over

the entire slide. Individual gene ratio data that does not surpass the background cutoff (as calculated by an algorithm that measures the ratio of hybridization in blank spot regions of the slides) is removed from the data set. Therefore, the gene data remaining are only those data points (ratios) that pass background noise quality filtration. The resulting data set was then statistically tested using Analysis of Variance (ANOVA) to determine a true occurrence of gene expression changes, and also T-tested for the reliability/reproducibility of the repeats performed in each time point where applicable. Also, control gene correlations of all data repeats (from each reliable hybridization of all print runs) for each timepoint were analyzed to determine the reliability of the data.

The fold change method was employed in determining candidate genes, (Draghici, 2002). We used 3-fold as the cutoff for initial analysis. The program Cluster (developed by Michael Eisen) was used to isolate the genes exhibiting appropriate fold change from the data set as well as to analyze expression patterns and group sets of genes that were expressed in similar patterns. The program Treeview (developed by Michael Eisen) was used to visualize cluster data and isolate nodes of expression patterns of interest.

Because the entire data set collected was compiled from hybridizations done on arrays printed from different print runs, the genes were listed in different orders for certain prints. This ordinal disarray prohibited the straightforward cumulative analysis of all the hybridization repeats for each gene. Therefore, the data for each time point was sub-divided based on specific print run identification and clustered according to the print run with

the most repeats (at least two for each time point). Cluster nodes of interest

were obtained from these subsets of prints. Therefore, the data for timepoints

87 hours, 90 hours, 93 hours and 96 hours were the result of duplicate

repeats, whereas the 99 hour and 102 hour timepoint data are the result of

triplicate repeats. Ultimately, the complete compiled data will be used to re-

create the expression pattern graphs shown in this chapter once more repeats

are done for the specific timepoints. The data presented here represents the

filtered mean of all data repeats available for each time point. Data presented

for the control gene set includes a minimum of three independent

hybridization repeats, and therefore has a higher confidence and correlation

value than that of the experimental data sets.

Results

To ensure the accuracy of the timecourse during sampling of the mid-

third instar event, we undertook the task of calculating and documenting the

precise midpoint of the third instar. In previous work, pupariation has been

determined to occur at around 118-125 hours after egg lay (AEL) (Andres et

al., 1993; Baehrecke, 1996). In our hands pupariation occurs at ~116-118

hours AEL. We found that temperature variation as well as the light dark

cycle timing, greatly affected the tempo of development. At cooler

temperatures, development progresses at a much slower rate than at elevated

temperatures. Upon detailed observations, we determined the third instar

midpoint to be at ~96h AEL, when the animals were kept at 25 degrees C and

a consistent 12h light and 12h dark cycle.

Of the 54 hybridizations done for the entire experiment, 20 were utilized for data analysis, including at least 3 repeats for control genes of each timepoint and two repeats for experimental genes (see Material and Methods). After background noise filtration and statistical testing of the initial data set, ~3500 genes passed preliminary standards of having a value above background hybridization and having a complete set of data (ratios for each timepoint) and were analyzed by clustering. This clustered data set represents approximately 25% of the known genome.

Correlations for the repeats of each timepoint were calculated to determine the reproducibility and slide quality of each experiment. Control gene correlations are presented in Figure 3.1 and full data set correlations of the experimental genes are presented in Figures 3.2 and 3.3. For the control gene correlations, a subset of 96 control genes was chosen to determine the overall correlation for all hybridizations performed for each timepoint. Correlations for the repeats of each timepoint are shown on each relative graph (Fig. 3.1). Generally, the R^2 value should be within the range of 1 to 0.5 to be considered acceptable, with a value of 1 denoting perfect correlation (Smith, 2000). Most of the correlations of the control gene data falls within this cutoff, meaning the data for these gene expression values are reproducible and therefore are considered reliable. The experimental data correlations (Figs. 3.2 and 3.3) only represent the data obtained from hybridizations of a particular print run (see Materials and Methods). For this reason, the correlations for 87h, 90h, 93h, and 96h show only a single correlation between the two hybridization repeats utilized for data analysis of

the experimental genes (Fig. 3.2). Correlations for 99h and 102h show three

correlations among the three hybridization repeats (Fig. 3.3). The

correlations for the 87h timepoint are relatively low. This low correlation

lends some doubt to the reliability of the data for this timepoint, as they

indicate the resulting values are not highly reproducible values. In the

subsequent analyses shown, we have included the 87h timepoint values but

realize that they may change once additional repetitions are completed.

Expression patterns for genes such as actin isoforms are expected not

to show drastic changes in expression, and accordingly we observed little or

no change in expression levels at each time point for these genes (Fig. 3.4).

This finding indicates that the observed variations in candidate gene

expressions are authentic changes in expression level and not the result of

experimental error caused by sampling, labeling, or hybridization

abnormalities for specific timepoints, as these biases would have been

observed in the actin control genes' expression pattern. The entire set of

genes that passed normalization and background noise filtration (see

Material and Methods) was clustered (Fig. 3.5) using the gene cluster

program developed by the Eisen Lab of Stanford University.

 Of the ~3500 genes input into the cluster program, nearly 1200 genes

passed the initial cluster filtering which determines if each gene has a

complete set of data and shows at least one data point among the timepoints

is of a value +/- 1 above or below the reference sample over the timecourse.

In other words, 1200 genes had data points for each timepoint and at some

point were up regulated or downregulated, relative to the reference sample.

Of these 1200 genes, approximately 600 showed a change in expression of at least 3-fold between the lowest level and highest level of expression throughout the timecourse, which was also determined with the cluster filtration protocol. These ~600 genes were then grouped within nodes of similar expression patterns (Fig. 3.5). Expression patterns that reflect a distinct change in expression at the mid-third instar event are considered to be suggestive, indicating possible regulation by the ecdysone pulse that occurs at this timepoint. Nodes of genes with suggestive expression patterns were isolated and classified based on their specific expression trends. For the purposes of this body of work, we only chose nodes that had dramatic changes in expression specifically at the mid-third timepoint, concurrent with the commitment pulse of ecdysone, for more detailed characterization.

The following data includes representative patterns of the candidate genes we isolated in this study. For each pattern, there are only a small set of genes shown and from this set the standard deviations of several genes are plotted as an example of data reliability. The data shown are all in log2 transformation (e.g. 2 = 4 fold, 3=8 fold, etc.) as an effort to properly depict the extent of upregulation and downregulation with equity.

Control Gene Analysis Results

Approximately 96 genes analyzed were extensively researched genes of known function and expression. We consider these genes to be internal controls as the expression patterns of many of these genes are already known to some extent, and a few are specifically known for this timepoint (Andres et al., 1993). Of these 96, approximately 40 were known to be ecdysone-

regulated. These genes were of special interest to determine whether expected patterns of expression were occurring in response to the ecdysone pulse. If these ecdysone-regulated genes show a change in expression, this gives further supporting evidence to the claim of a commitment pulse of ecdysone occurring and the mid-third instar event.

Expression patterns for known ecdysone targets show the trends expected for their genes. Figure 3.6 shows the positive control genes which are activated by this pulse of ecdysone. Some of these genes are already known to be ecdysone target genes and show an expected trend of activation at the point of ecdysone release, such as Dhr38 and E74A, while some of these genes may not have previously been shown to respond specifically to this ecdysone pulse. Figure 3.7 shows the control genes which are repressed by this pulse of ecdysone. Some of these genes are ecdysone-regulated genes that are known to be repressed by pulses of ecdysone, such as Late (L) genes and Salivary Gland Secretion (SGS) genes (Baehrecke, 1996; Lehmann and Korge, 1995; Thummel, 2002).

To determine the function of certain genes, we searched the Flybase database for gene function information. There were several genes within the internal control group that while significantly studied but had not been previously associated with ecdysone. It is interesting to note that most of the novel ecdysone target genes that group with known ecdysone-regulated genes are also related in function. In Panel B of Figure 3.6, we see that the *Ecdysone receptor (EcR)* gene is a part of this cluster. Within this group is also another hormone receptor gene *Dhr38* (Fisk and Thummel, 1995), which

functions in epidermal differentiation. Additionally, we found that *unkempt*, also a transcription factor responsible for ommatidium and nervous system development (Mohler et al., 1992) not previously associated with ecdysone, also clusters with this group of genes. *EcR* (Bender et al., 1997; Carney and Bender, 2000; Li and Bender, 2000; Schubiger et al., 1998) has also been shown to be necessary for each of these functions (Lam et al., 1999; Matsuoka and Fujiwara, 2000). Similarly, *IMPE2* is also an ecdysone target (Hurban and Thummel, 1993) that clusters with this group, and functions in imaginal disc eversion. We know from previous studies that *EcR-A*, the isoform of *EcR* that is present in this group, is the predominant isoform in the imaginal discs. Therefore, the functional similarity as well as common spatial expression patterns with EcR further the support identification of the new ecdysone target candidates.

In a similar fashion, in panel A of Figure 3.7 and Panel A of Figure 3.8 we see a conglomerate of cell death genes (*reaper, drice, decay*) (Dorstyn et al., 1999; Jiang et al., 2000; Yu et al., 2002) clustering together as repressed genes. These genes have long been associated with cell death, as well as the cell death functions being associated with ecdysone signaling; however, the genes themselves have not previously been linked directly to ecdysone in a manner to describe them as ecdysone-regulated. Because these genes mimic known ecdysone targets, it is probable they are under the same regulation. Specific examples include *decay*, a cell death caspase (Dorstyn et al., 1999), which clusters with known ecdysone targets such as *E63-2* (Andres and Thummel, 1995; Stowers et al., 2000; Thummel, 2002) and *SGS-8*. Also

Cyclin D, expressed within imaginal discs, was not previously associated with ecdysone signaling, but clusters within the same group as *decay.* Additionally, in Panel B of Figure 3.7 the *LSP gamma* gene, associated with nutrient reservoir activity, is found clustered with two ecdysone targets, *L71-7 (late gene)* (Wright et al., 1996) and *Eip28/29 (Ecdysone induced protein)* (Hurban and Thummel, 1993; Jiang et al., 2000).

Experimental Genes Analysis: Activated Genes - Repressed Genes - Variant Genes

All of the experimental genes shown in the following figures and tables have a fold-change that is considered to be above the background noise threshold (Draghici, 2002). Each of the genes have at least a three fold change over the timecourse, which initiates or transitions at the mid third instar event. We have isolated these genes in groups called nodes as they are created in the Cluster program.

The nodes of interest chosen from the cluster can each be classified within three broad groups of variant gene expression. The first group, which we refer to as the activation group, includes genes that have a general trend of up-regulation and consists of 148 genes. The second group of candidate ecdysone targets, which we refer to as the repression group, has a general trend of repression and consists of 158 genes. The third and last group, which we refer to as the variant group, consists of 123 genes that display variant expression patterns. This variant group consists of genes with expression levels that move transiently between induction and repression throughout the period of the mid-third instar pulse.

In Figure 3.8 we show that certain unknown genes show similar trends of expression and are accordingly clustered with subsets of control ecdysone regulated genes. These examples of clusters containing both unknown and ecdysone regulated genes support the hypothesis that some of the genes uncovered in this study are ecdysone-sensitive. Genes that follow the same or similar expression may be under the same type of regulation, or within the same gene/biochemical network. It is important to keep in mind, however, that because the known ecdysone targets were isolated from a single tissue type, we expect to find novel ecdysone target genes that have a unique expression pattern which may not mimic the pattern of the known ecdysone regulated control genes, as they may function in different tissue types and therefore may have unique expression patterns.

Activated Genes of Interest

Within the group of 148 activation genes, there are four subgroups for which two examples of these expression patterns are represented in Figures 3.9-3.10 and Tables 3.1-3.2. The first group of genes, which we refer to as "up-level", has an activation in expression that levels off to a steady state of expression. There are a total of 49 genes in this group. Expression patterns for 6 of these are shown in Figure 3.9 and Table 3.1. These genes are activated starting at the 93h timepoint and then have a steady state of expression. (also see control genes in Figure 3.6 panel A). In other subgroups of the up-level group, the steady state of expression starts at 96h or 99h (data not shown). The second sub-group, of 41 genes, has a drastic spike of upregulation and then returns to normal levels (Fig. 3.10, Table 3.2). This

group is probably the most interesting, as it represents a group of genes tightly regulated and very sensitive to a signal at the specific time of the mid-third instar event, when we correlate the mid-third instar pulse of ecdysone. Most of the genes within this group are of unknown functions, which make future analysis of these genes very intriguing as it may introduce new functions under ecdysone regulation.

Repressed Genes of Interest

Of the down-regulated group, there are three sub-groups, similar to the sub-groups of the upregulated genes, and only one is represented here. This group of representative repressed genes has a drastic down regulation at the midpoint that is immediately reversed after the midpoint (Fig. 3.11 and Table 3.3). This group includes several genes of high interest, including several transcriptional co-factors, such as *snail* (Ashraf et al., 1999; Ashraf and Ip, 2001), *goosecoid* (Hahn and Jackle, 1996), and *broad*, which is a known ecdysone regulated transcription factor (Gonzy et al., 2002; Karim et al., 1993; von Kalm et al., 1994) (Crossgrove et al., 1996) (data not shown). We refer to the 71 genes in this group as "down-spiked" genes, which are similar to the up-spike genes, in that they are tightly regulated and most likely responding to a specific signal at the time of the ecdysone pulse. Because one of these genes is a known ecdysone target, it is reasonable to hypothesize that the signal these genes are responding to is the mid-third instar ecdysone pulse.

Variant Genes of Interest

The final group of 123 genes are those that show varying types of expression throughout the timecourse (data not shown). These genes have several spikes of activation and/or repression. An example of these expression patterns are shown in Figure 3.12. Because the expression pattern of these genes are so variant, they are probably not exclusively associated with the mid-third ecdysone pulse. These genes are likely some component of the ecdysone pathway, as they have significant expression changes at the mid-point, correlating with the pulse of ecdysone. However, the variation of expression suggests they are also some other regulatory cue, and therefore, we did not focus on characterizing the functions of these genes. The variant nature of their expression pattern does not negate the possibility of ecdysone regulation, but the constraints of this study will not allow for significant proof of ecdysone regulation.

Conclusions and Discussion

Data supports presence of a mid-third instar ecdysone pulse and a novel set of ecdysone regulated genes.

We have developed a synchronizing scheme to sample the mid-third instar event and determine the genomic response to a probable pulse of ecdysone at this timepoint, referred to here as the commitment pulse. Upon definition of the midpoint of the mid-third instar event, we then sampled animals at a 3 hour resolution to observe changes in gene expression at the time of the commitment pulse. Correlation data shows that our sampling and

hybridization data are statistically sound, in that most of the values of gene expression are reproducible. In some cases the range of up and down regulation of certain genes is more drastic than others, which skews the correlation coefficient; however, this does not negate the fact these gene expressions are significantly changing at the midpoint. Additionally, the analysis of internal control genes (both positive and negative controls) suggests that the data we have obtained is reliable in determining the actual transitioning expression patterns of novel genes.

We have shown that there are significant expression transitions during the mid-third instar event for hundreds of Drosophila genes. Additionally, we show that there are markedly significant changes in the expression patterns of known ecdysone target genes that were not previously associated with the commitment pulse of ecdysone. The distinct regulation of such a large number of at this timepoint suggests there is some regulatory/developmental signal at this stage which results in a genomic response, in addition to the previously described behavioral response. As some of these genes are directly ecdysone-regulated it is reasonable to hypothesize that the signal occurring at the mid-point is in fact a pulse of ecdysone.

We have described three broad sets of genes, characterized by the type of expression change they display in this study. There is a set of activated genes (148), repressed genes (158), and a variant regulation set of genes (~120). We believe these genes include subsets of ecdysone-regulated targets that are either repressed or activated in the presence of the hormone. Because of the presence of cofactors that may be temporally regulated or

tissue-specific, some of these target genes display a complex expression pattern (such as the variant group of genes). Further dissection of these genes may indicate they are not only under regulation of ecdysone, but other ligands and or cofactors associated with the Ecdysone Receptor.

Early vs Late genes… the Ashburner Model.

The Ashburner Model (Thummel, 2002) defines the molecular mechanism of the ecdysone signal, as depicted in salivary gland polytene chromosomes. This model states that ecdysone, in complex with its receptor, directly induces the transcription of the early genes and directly represses the transcription of the late genes. The protein products of the early genes then repress their own transcription while simultaneously inducing the expression of the late genes. Therefore, we can attribute placement or classification, as defined by the Ashburner model, of the ecdysone-sensitive genes identified here by the pattern of expression they display.

Genes that have spikes of upregulation or downregulation at specific points that correspond to the pulse of ecdysone within the mid-third period (93h-96h) may be grouped with the Ashburner early genes or late genes respectively. The genes with later (99h-102h) spikes or upregulation and/or repression are likely to be genes that are regulated by the transcription factors that are encoded by the early genes.

In Figure 3.12, Panel A, we show the expression patterns for the EcR isoforms. Panel B of Figure 3.12 shows a subset of genes that may possibly be under the direct regulation of EcR-B1 as they mimic the expression pattern of EcR-B1. They show an initial repression prior to the ecdysone pulse (87h and

90h), initiation of expression (93h) and subsequent repression (96h), correlating with the mid-third pulse. This type of expression pattern makes these genes possible early gene candidates. As this activation and repression mimic the expression pattern of the EcR-B1 expression (Fig. 3.12, Panel A) it also stands to reason that these genes are regulated by this isoform. The upregulation of these genes at 102h follows the upregulation of the EcR-A isoform, which could be due to a phenomenon known as isoform switching.

The known late genes follow expected patterns of expression and are also shown in this figure (Fig. 3.12, panel C). Interestingly, some of these genes show a later upregulation after the ecdysone pulse (102h). This may be due to relaxation of the repression, or perhaps the beginning of the subsequent pulse of ecdysone (Pre-pupal pulse).

New ecdysone targets... reaffirming ecdysone biological function

We show that some internal control genes that have not previously been associated with ecdysone regulation also cluster with the known ecdysone targets (Fig. 3.6 and Fig. 3.7). These data provides an additional link, by mimicking expression patterns of known ecdysone targets, to suggest they are also regulated by ecdysone. In Figure 3.6, these activated genes are associated with cell proliferation and biosynthesis (*cyclin C, cyclin J, DDC*) (Chen et al., 2002; Yu et al., 2000), ligand specific gene regulation (*Dhr38, Dhr96, Dhr3, IMPE2*) (Crispi et al., 1998; Fisk and Thummel, 1995; Lam et al., 1999; Matsuoka and Fujiwara, 2000; Sutherland et al., 1995), and tissue specific development or cell fate (*IMPE2, EcR-A, Cyclin J*). All of these

functions have been associated with ecdysone signaling biologically but not shown to be directly regulated by the hormone.

In Figure 3.7 and 3.8 several of the repressed genes here function in cell death. The fact that these cell death genes are within a repressed group indicates the need to delay the effect of these genes. We know that upon onset of metamorphosis, these cell death pathways are initiated in the very tissues they are found expressed in. *Decay* is known to be expressed in the salivary gland and midgut, while mutant analysis of this gene shows disturbance in nurse cell, oocyte, and egg chamber function, all parallel findings of the *EcR* gene (Bender et al., 1997; Buszczak et al., 1999; Carney, 1998; Carney and Bender, 2000; Li and Bender, 2000; Schubiger et al., 1998). Additionally, a gene that increases resistance to the Juvenile Hormone (Ashok et al., 1998; Shemshedini and Wilson, 1990) is drastically repressed during the midpoint (data not shown). This reduction in the resistance to JH suggests the animal is at this point more sensitive to JH. As we know JH counteracts the effect of ecdysone. Certain ecdysone targets genes are repressed at the mid-third instar event. These same genes are then subsequently activated at the next pulse of ecdysone (such as the cell death genes). This reverse response to ecdysone may be explained by the low level of JH at the midpoint. By the time of the pupariation pulse, there is no detectible JH. However, the low level of JH and the mid-third instar event may become more powerful in its inhibitory effect on ecdysone activation via JH sensitization. By lowering the animal's resistance to JH in implicitly

increases its sensitivity, therefore the lower levels of JH at the midpoint are capable of inhibiting the activation function of ecdysone.

Additionally, the *LSP gamma* gene, associated with food/ nutrient storage (Deutsch et al., 1989), shows a sharp decrease in expression and remains repressed (Fig. 3.7). This is probably a result of the onset of wandering. As the animal is no longer ingesting food, the food storage function is no longer necessary. *LSP gamma* clusters with two known ecdysone targets, which suggests it is also under the regulation of ecdysone.

Some previously studied genes, which have defined function and shown to interact with EcR have not been shown to be ecdysone-regulated. Two such genes are *crol* and *bonus* (Andres and Cherbas, 1994; Antoniewski et al., 1996; Cherbas, 1993). Both of these genes are believed to interact with EcR in a manner to confer specificity of gene regulation by the EcR isoforms (Beckstead et al., 2001; D'Avino and Thummel, 2000). In our data set, we observe a significant fluctuation in expression for both of these genes directly at the midpoint (data not shown). This suggests that these genes are also regulated by ecdysone.

Of all the experimental genes (~600) revealed to be differentially expressed ~500 were not previously defined as ecdysone-sensitive. Of these ecdysone target candidates, ~200 have unknown function. Genetic interaction databases may assist in attributing putative function to most of these unknown genes, and thereby help to reveal the mechanisms associated with the mid-third commitment pulse. Additionally, sequence similarity or

protein motifs may also provide a clue to the molecular function of the unknown genes.

Confirmation of ecdysone regulation… and other future directions.

In attributing gene regulatory mechanisms, several lines of independent data will be necessary in order to prove definitively the source of regulation. In the case of attributing ecdysone regulation, we must primarily show these genes have a change in expression at a time coincident with a pulse of ecdysone. Secondly, we must show that there are response elements that reflect possible EcR binding, necessary to be directly regulated by ecdysone. Finally, we must show that a lack of ecdysone and or ecdysone receptor disrupts the expression change initially observed and considered to be the result of the pulse of ecdysone. We have completed the first step of initial observation of gene expression transition during an ecdysone pulse. We look forward to confirming these candidates with the additional means discussed below.

Initial candidate status was attributed to genes that show an expression transition at the midpoint of third instar, which correlates with the pulse of ecdysone that occurs at this time. To further corroborate these candidates, we compared the expression patterns of candidates with the expression patterns of known ecdysone target genes. Certain subsets of genes show a pattern that may suggest their regulation by ecdysone, based on the positive correlation of their expression pattern with, or negative correlation against, their expression when compared to the ecdysone

receptors. As the EcR protein is the direct link of the target genes to the ecdysone hormones, genes that exhibit correlative expression behavior are likely candidates for ecdysone regulation. Figure 3.12 shows a subset of genes that are candidates with these criteria. To prove this definitively, we could undertake another genomic approach with the EcR isoform mutants to reveal the direct targets of the receptors. This would confirm the correlative expression patterns of candidates uncovered here.

The DNA binding sites of the ecdysone receptor complex are specific sequences called ecdysone receptor response elements (EcRE's) located in the promoter regions of ecdysone-responsive genes (Andres and Cherbas, 1994; Antoniewski et al., 1996; Cherbas, 1993; Crispi et al., 1998; Petersen et al., 2003). To definitively confirm the candidate genes found in this study are ecdysone-sensitive, we must confirm the expression patterns with additional biological repeats of the genomics analysis, as well as define the promoter regions to determine the presence of Ecdysone Response Elements. Subsequently, biochemical and wet bench experiments will be utilized to validate the differential pattern of expressions observed in this work. This future work will in turn prove the presence of the ecdysone pulse at the mid-third instar event and will also show the genes affected during this pulse of ecdysone are in fact directly regulated by the hormone.

The data timepoints that only reflect two repeats are not as statistically sound as desirable. Ideally, the correlation coefficients would be in the range of 0.8-1. In order to statistically confirm the reproducibility of gene expression transition values, repeats must be completed for these timepoints.

We are confident that the candidate genes shown here are legitimate as separately these genes tightly correlate over repeats (as indicated by standard deviation values) and cluster with the control genes. Additionally, most of the genes listed here have functions that are associated with the biochemical mechanisms that are regulated by ecdysone pathway. Keeping in mind that the purpose of this project was to screen the genome for ecdysone target candidates for further analysis, we have succeeded in showing dozens of potential candidates worthy of additional investigation.

Acknowledgements

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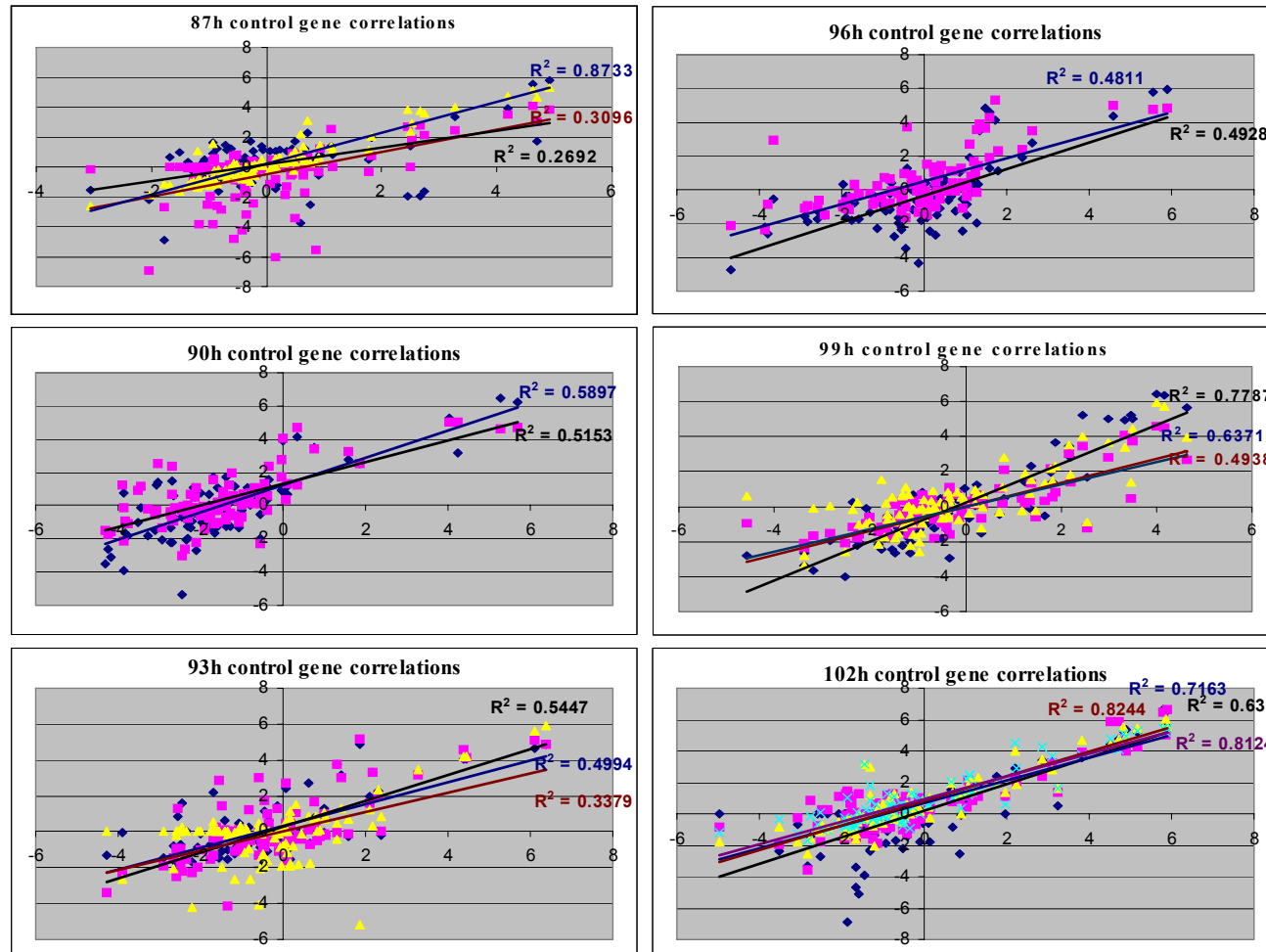
Figure 3.1. Scatter plots showing the correlation coefficients of the control gene repeats for each timepoint. The R^2 values are listed above each graph, and graphs are labeled for specific timepoints. The data presented is log2 transformed, e.g. 2 = 4 fold, 4 = 8 fold, etc. Different colors represent the comparison plots of different hybridization pairs.
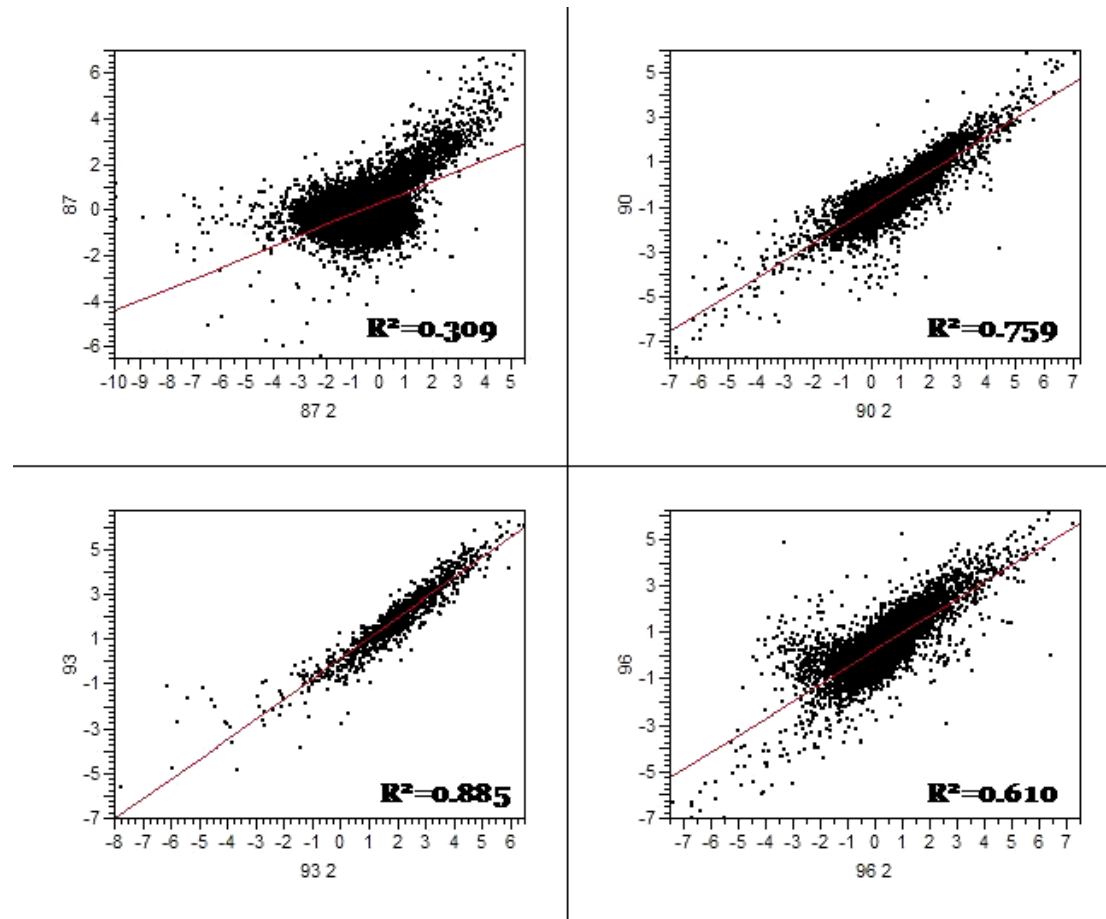
Figure 3.2. Experimental gene correlations for 87h-96h data points. These scatter plots show single correlations between the two repeats used for analysis. The R^2 values are shown on the specific graphs. The X and Y axes represent the gene expression value log2 transformed (2=4fold).
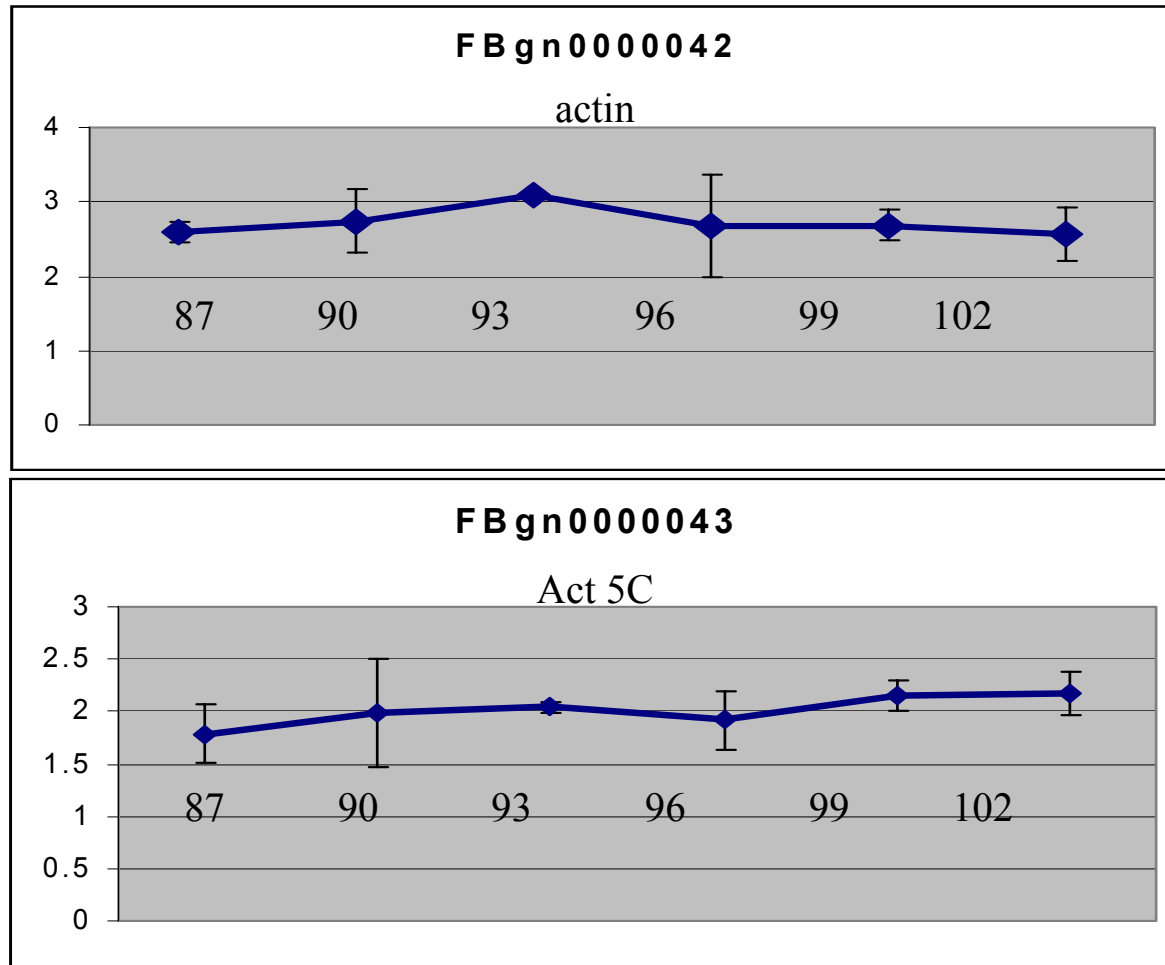
126
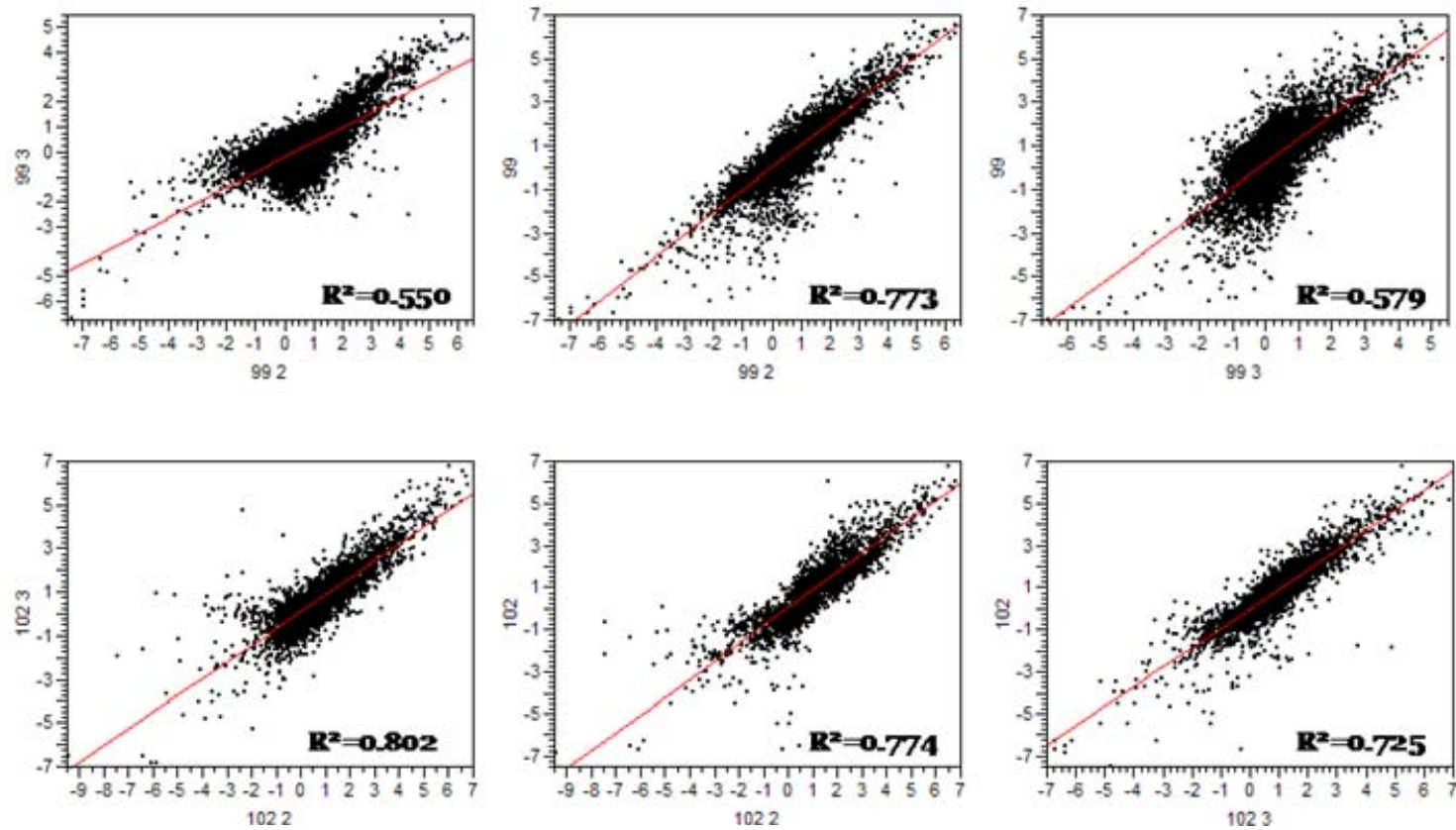
Figure 3.3. Experimental gene correlations for 99h-102h data points. These scatter plots show the single pair wise correlations between the three repeats used for analysis. The R² values are shown on the specific graphs. The X and Y axes represent the gene expression value log2 transformed.

127

Figure 3.4. Negative control genes. Actin isoforms shown here have no significant variability in expression across the mid third instar pulse of ecdysone. Panel A is actin and Panel B is another isoform of actin, Act5C. The X axis is the six time course timepoints (87h-102h), the Y axis is the level of expression (log2) relative to the reference sample.
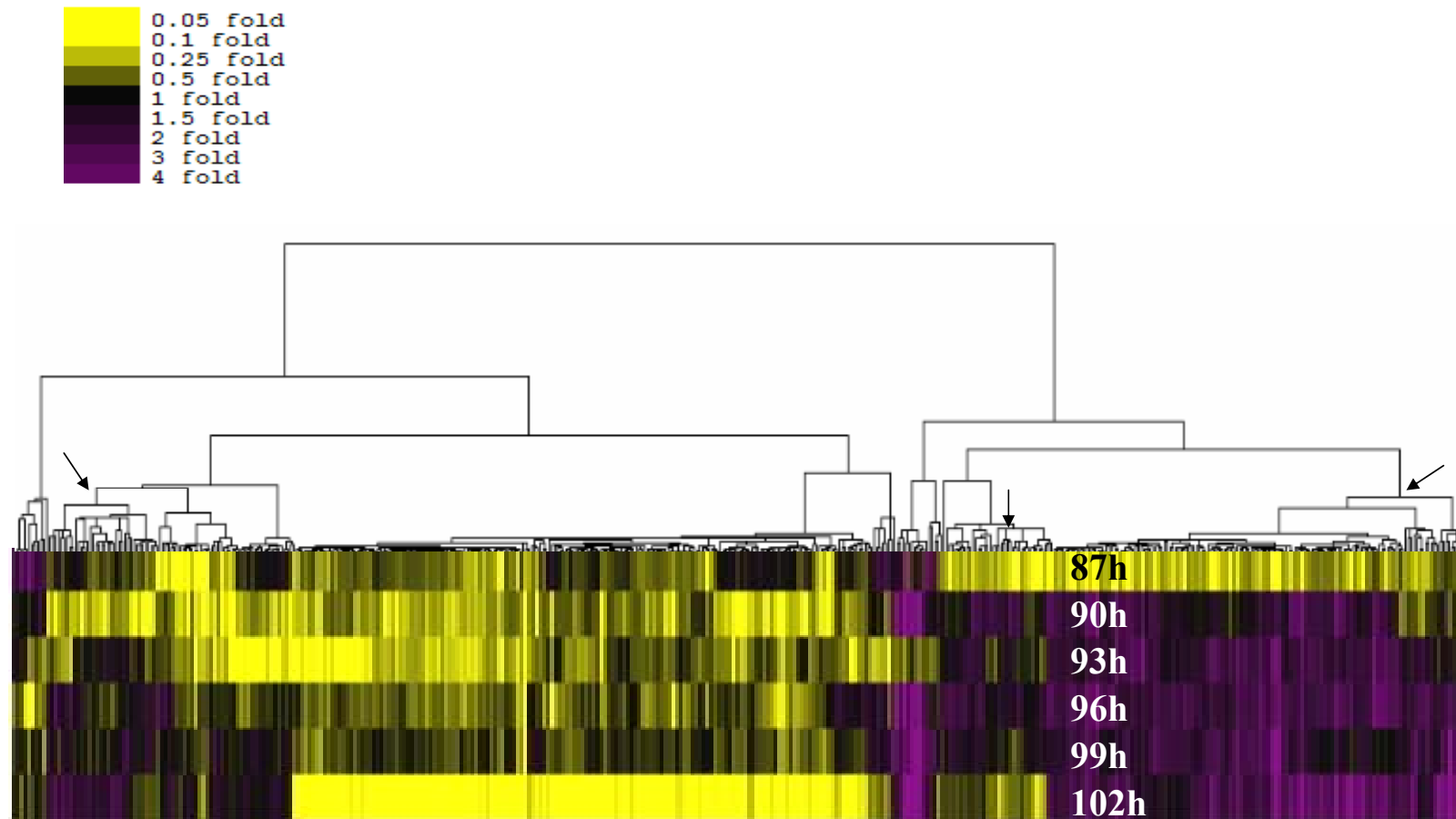
Figure 3.5. Clustergram of the complete filtered data set, including internal controls, of genes that show expression changes during the mid-third instar event. Arrows denote examples of specific nodes of interest that show a transition at the midpoint which were chosen for detailed analysis. Yellow indicates a negative ratio (decrease in gene expression relative to the reference sample), purple indicates a positive ratio (increase in gene expression relative to the reference sample) and black indicates no change in expression relative to the reference sample. A color key showing fold induction or repression is shown in upper left corner.
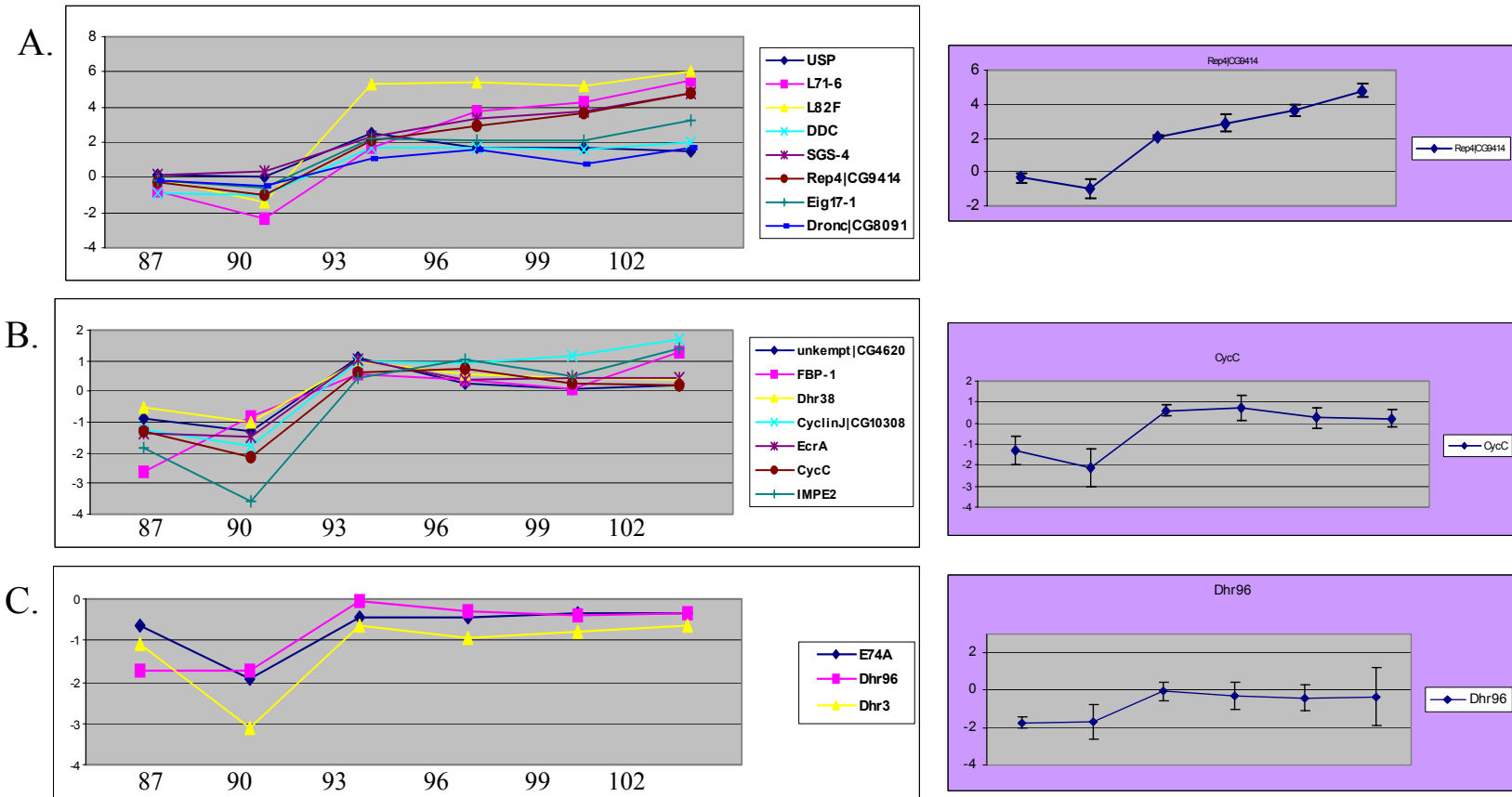
129

Figure 3.6. Positive control activation genes. Panel A shows genes that are activated at the 93h timepoint and retain the same level of expression with a slight increase towards 102h. Panel B shows genes that are slightly repressed at the 90h timepoint and then are activated at 93h and retain the same level of expression. Panel C shows genes that have a transient repression and then the repression is relieved at the 93h timepoint. Genes are labeled as specified in the key legend. The X axis is the six time course timepoints (87h-102h), the Y axis is the level of expression (log2) relative to the reference (2 = 4fold). Graphs to the right depict the standard deviation of a random set of example genes.
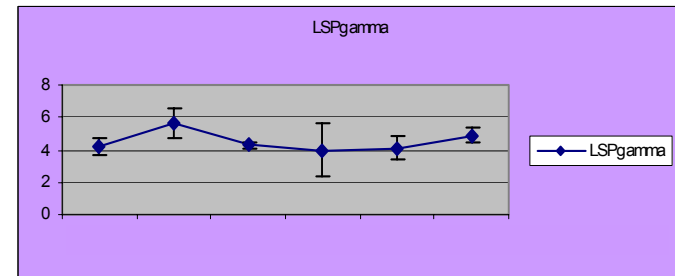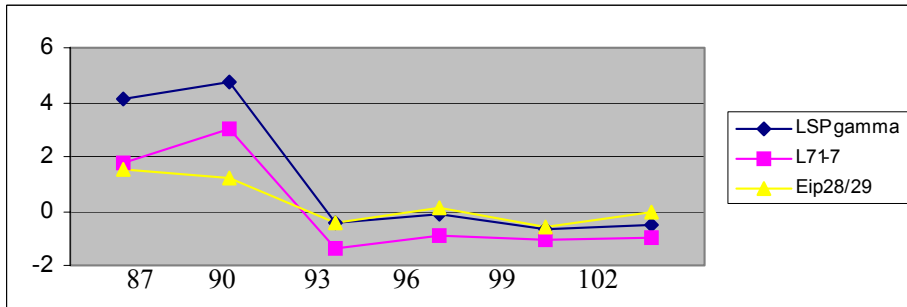
130

A.
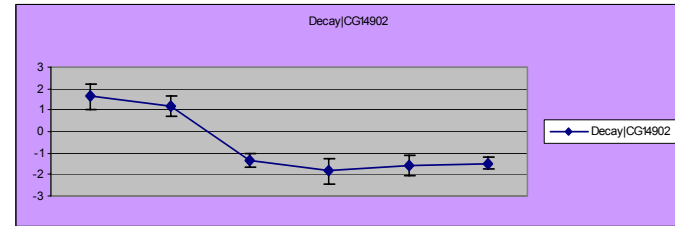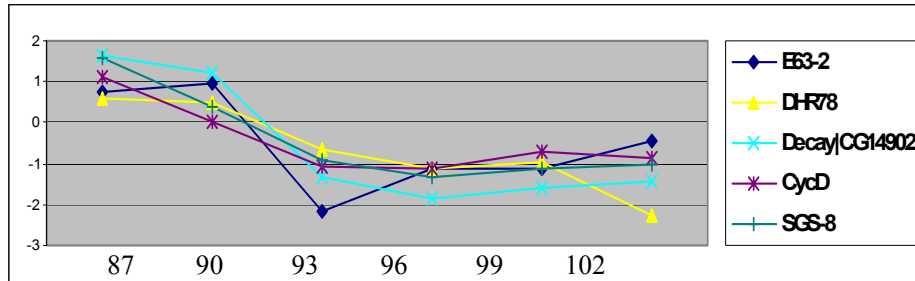
B.

Figure 3.7. Positive control repression genes. Panel A shows genes that have an overall gradual repression (with a sharp decrease at the midpoint). Panel B shows genes that have a sharp repression at the midpoint and remain repressed for the duration of the timecourse. The X axis is the six time course timepoints (87h-102h), the Y axis is the level of expression (log2) relative to the reference. Graphs to the right depict the standard deviation of a random set of example genes.
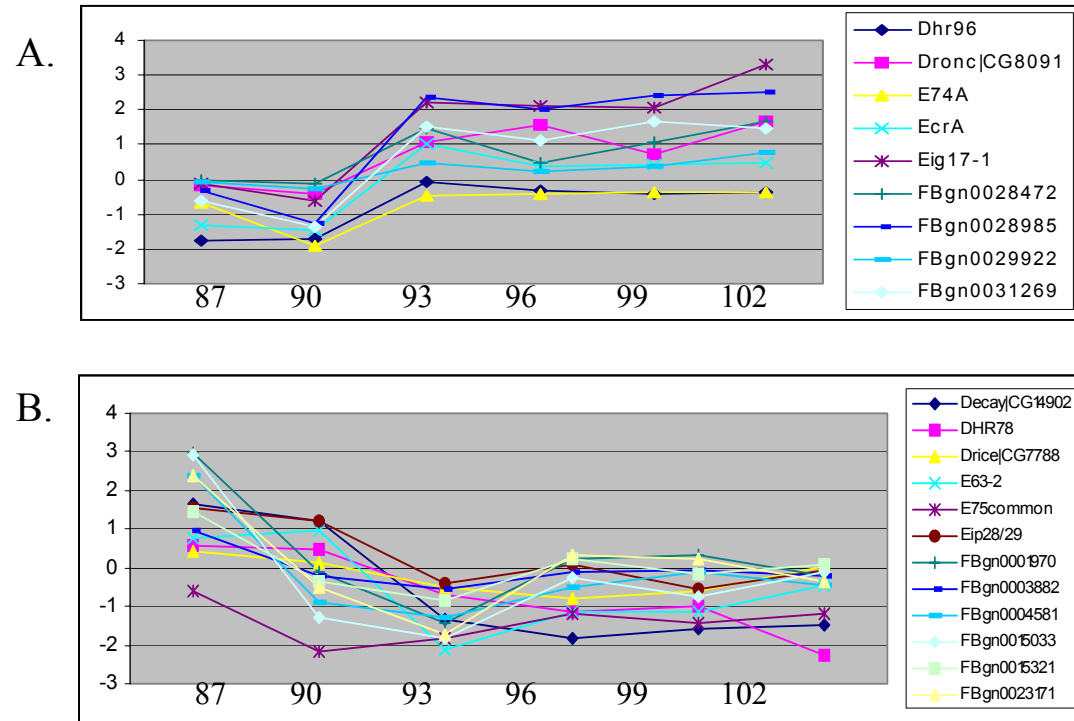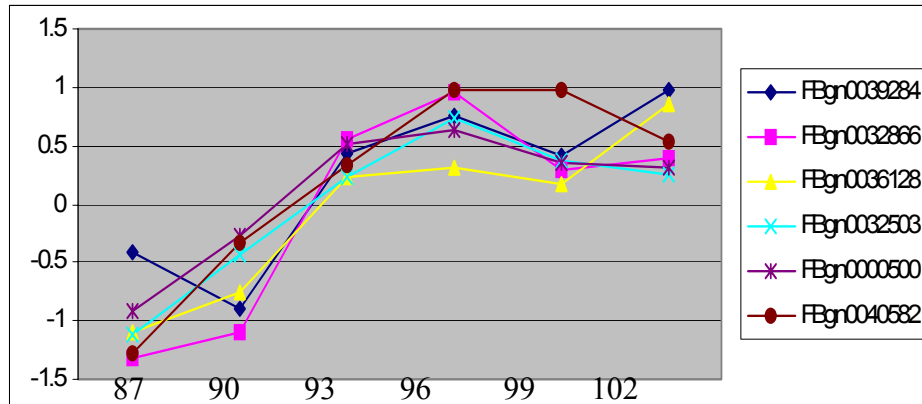
131

Figure 3.8. Comparison graphs. These graphs show the known ecdysone sensitive activation genes (panel A) and repression genes (panel B) that also cluster with unknown genes. The X axis is the six time course timepoints (87h-102h), the Y axis is the level of expression (log2) relative to the reference.
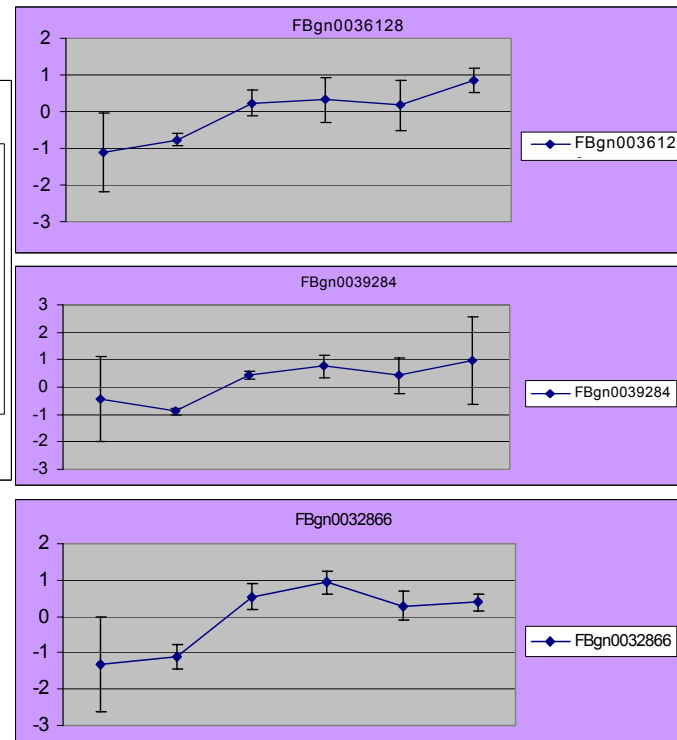
132

Figure 3.9. Set of unknown activation genes. This group shows an upregulation that peaks at the mid-third instar event and remains level. Panel B shows the standard deviations of each timepoint for a subset of genes from panel A. The X axis is the six time course timepoints (87h-102h), the Y axis is the level of expression (log2) relative to the reference.

Table 1. "Uplevel" activation candidates

Full name	Molecular function	Biological process	Protein domains	FlyBase ID
	ARF small monomeric GTPase		ADP-ribosylation factors family; Ras GTPase superfamily; SAR1 GTP-binding protein family	FBgn0039284
	high affinity inorganic phosphate:sodium symporter		Gastrin/cholecystokinin family	FBgn0032866
	neuropeptide hormone		Zinc finger, C2H2 type	FBgn0036128
	nucleic acid binding			FBgn0032503
Drosulfakinin		neuropeptide signaling pathway		FBgn0000500
				FBgn0040582

Table 3.1. List of genes for the subset of activated genes represented in Figure 3.9. Shown here are the proper names of the genes that have been previously studied, the molecular function, biological function and biochemically functional protein domains present in these gene products.

134
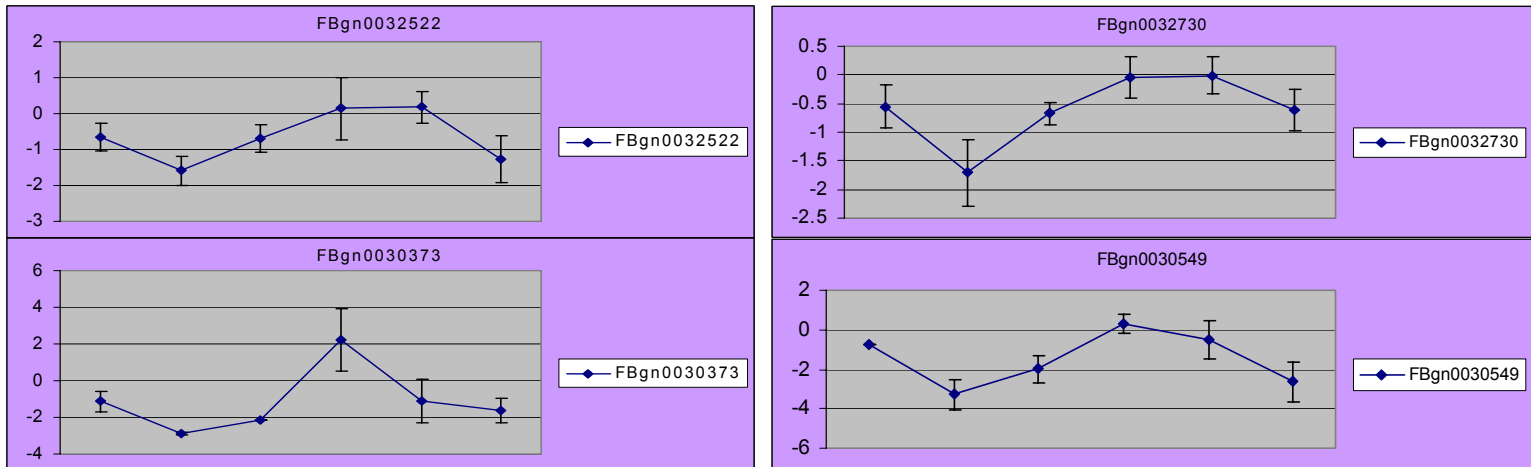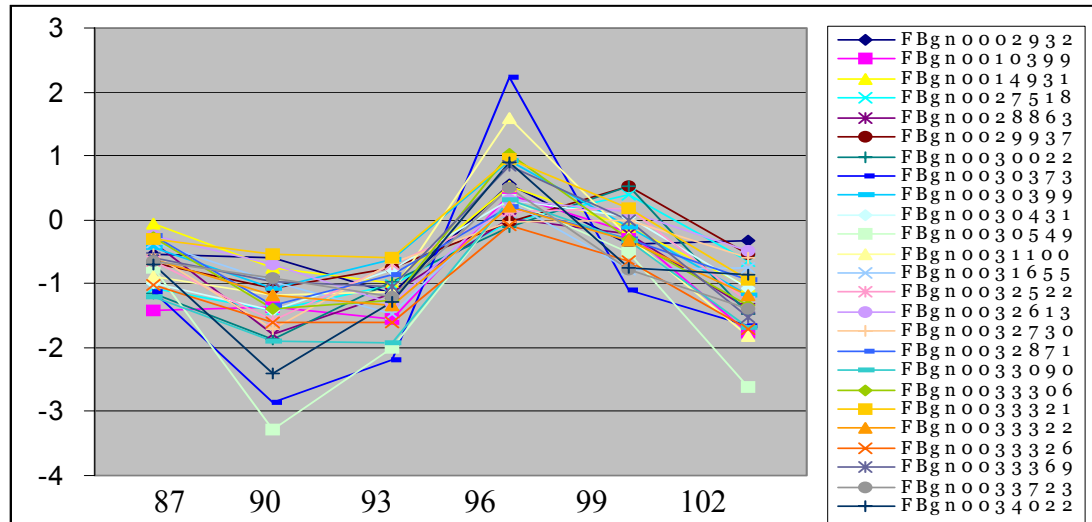
Figure 3.10. Upspike genes. These genes show a drastic upregulation at the midpoint followed by immediate downregulation (panel A). Panel B shows the standard deviations of each timepoint for a subset of genes from panel A. The X axis is the time course timepoints (87h – 102h), the Y axis is the level of expression (log2) relative to the reference.

135

Table 2. "Upspike" activation candidates

Full name	Molecular function	Biological process	Protein domains	FlyBase ID
			Concanavalin A-like lectins/glucanases; EGF/Laminin	FBgn0034070
	cytochrome P450	hormone metabolism; insecticide catabolism	Cytochrome P450 enzyme; E-class P450 group I; E-class P450 group IV; Cytochrome P450	FBgn0034053
	pre-mRNA splicing factor	mRNA splicing	D111/G-patch domain	FBgn0040024
			Hairpin loop containing domain of hepatocyte growth factor	FBgn0033306
	voltage-gated calcium channel		Integrin A (or I) domain	FBgn0028863
	FMN adenylyltransferase		Phosphoadenosine phosphosulfate reductase; Adenine nucleotide alpha hydrolases	FBgn0032522
	FMN adenylyltransferase		Phosphoadenosine phosphosulfate reductase; Adenine nucleotide alpha hydrolases	FBgn0030431
neuralized	DNA binding	determination of sensory organ precursor cell fate; eye morphogenesis (sensu Drosophila); neurogenesis; peripheral nervous system development	RING finger domain, C3HC4	FBgn0002932
			RNI-like	FBgn0031100
			RNI-like	FBgn0033369
	serine-type endopeptidase		Serine proteases, trypsin family; Chymotrypsin serine protease family (S1); Trypsin-like serine proteases	FBgn0033321
	helicase		SNF2 related domain; DEAD/DEAH box helicase; Helicase C-terminal domain; P-loop containing nucleotide triphosphate hydrolases	FBgn0031655
			Sodium/calcium exchanger protein	FBgn0033326
NMDA receptor 1	N-methyl-D-aspartate selective glutamate receptor		Solute binding protein/glutamate receptor; Ionotropic glutamate receptor; NMDA receptor; Potassium channel; Periplasmic binding protein-like I	FBgn0010399
	peroxisome targeting signal-2 receptor	peroxisome organization and biogenesis	Trp-Asp repeat (WD-repeat)	FBgn0027518
			Trp-Asp repeat (WD-repeat)	FBgn0035724
			Zinc finger, C2H2 type; C2H2 and C2HC zinc fingers	FBgn0032730
	transcription factor	regulation of transcription, DNA-dependent	Zinc finger, C2H2 type; C2H2 and C2HC zinc fingers	FBgn0014931

Table 3.2. List of genes for the subset of activated genes from Figure 3.10. Shown here are the proper names of the genes that have been previously studied, the molecular function, biological function and biochemically functional protein domains present in these gene products.

136

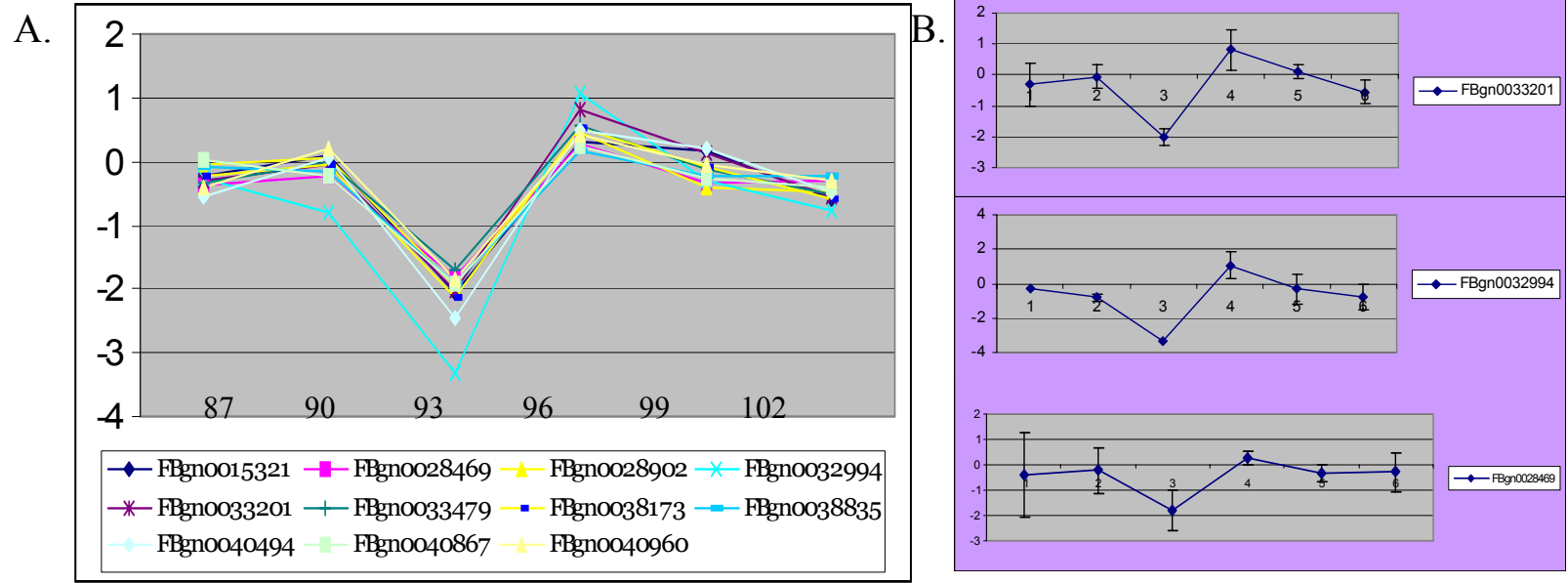**Figure 3.11.** Downspike genes. A subset of genes that have a drastic period of repression at the midthird instar event, coincident with the pulse of ecdysone at this timepoint. Panel B shows the standard deviations of each timepoint for a subset of genes from panel A. The X axis is the six time course timepoints (87h-102h), the Y axis is the level of expression (log2) relative to the reference.

137

Table 4. "Down spike" repression candidates

FlyBase_ID	Symbol	Full_name	Molecular_function	Biological_process	Protein_domains
FBgn0015321	UbcD4	Ubiquitin conjugating enzyme 4	ubiquitin conjugating enzyme activity;	ubiquitin cycle;	Ubiquitin-conjugating enzymes;
FBgn0028469	BcDNA:LD28120		monocarboxylic acid transporter activity;		Monocarboxylate transporter
FBgn0028902	Tektin-A	Tektin A	microtubule binding activity;	microtubule-based process;	Tektin
FBgn0032994	CG17482				
FBgn0033479	CG2292				Phosphatase/sulfatase, details
FBgn0038173	Adgf-C	Adenosine deaminase-related growth factor C	growth factor activity;		Adenosine and AMP deaminase; Metallo-dependent hydrolases, details;
FBgn0038835	CG17274		glutamate-gated ion channel activity;		Ionotropic glutamate receptor; Periplasmic binding protein-like II, details;
FBgn0040494	BcDNA:LD37196				

Table 3.3. List of genes for the subset of activated genes from Figure 3.11. Shown here are the proper names of the genes that have been previously studied, the molecular function, biological function and functional protein domains present in these gene products.
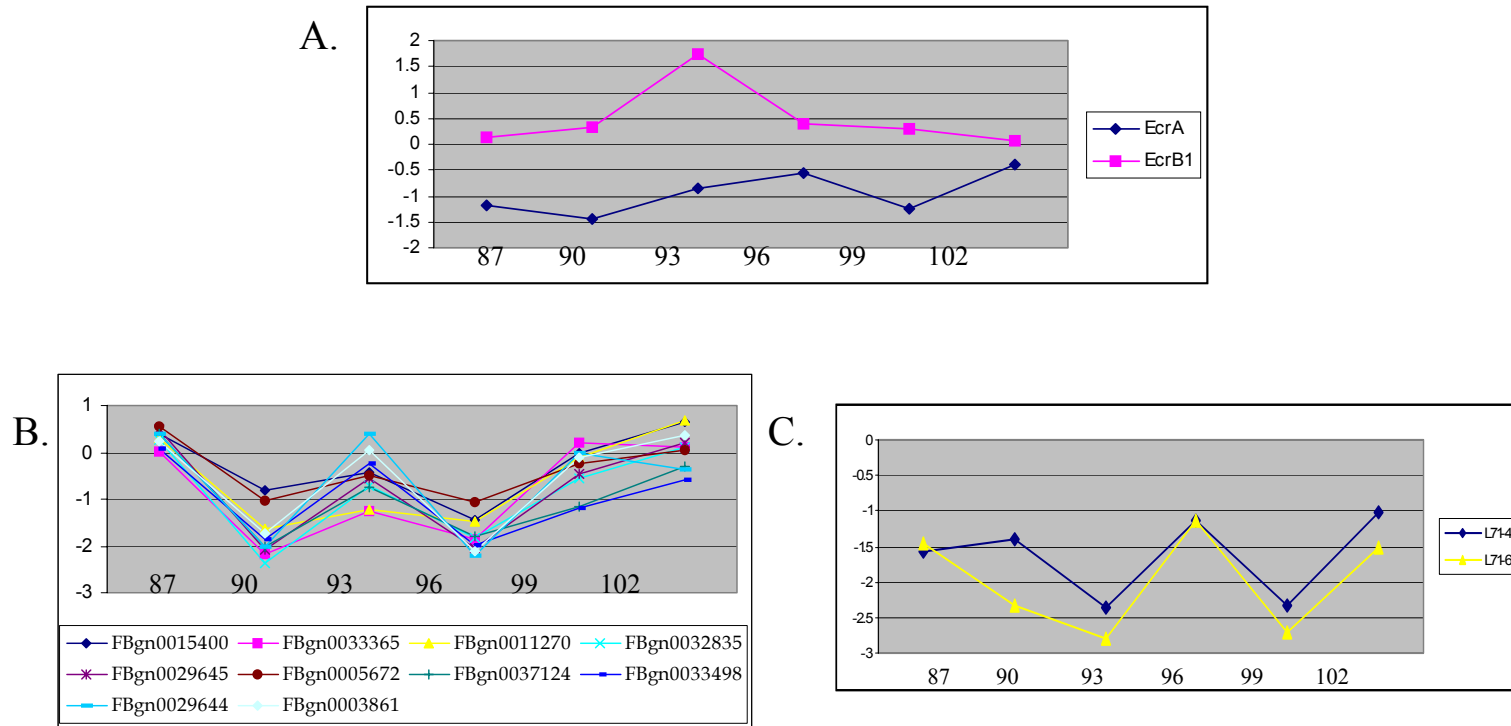
138

Figure 3.12. Genes regulated by ecdysone responsive transcription factors. These genes mimic the behavior of Ashburner models early (Panel A) and late genes (panel C). Panel A shows the pattern of expression for the EcR isoforms. Panel B shows genes that have a pattern that may suggest they are regulated by EcR-B1 based on the correlation of expression to EcR-B1 (panel A). The last two timepoints correlate with EcR-A, which may indicate an isoform switch in regulation. Panel C shows expression patterns of late genes that are under regulation of the early gene products.

139

Chapter 4

Summary and Conclusions

Steroid hormone signaling is essential to the proper development of all animals (Arnosti, 2003; Beato, 1989; Henrich et al., 1999). While we know much about steroid synthesis and the general mechanism of hormone signaling, relatively little is known about the specific molecular interactions of steroids and their receptors. As described in the introduction, we generally know that the hormone is released from an endocrine gland and is then bound by the hormone receptor. The receptor then transduces the signal to the gene targets via transcriptional regulation. However, there are a plethora of unknown co-factors that can and do interact with these receptors, altering their activity. In addition, we only have a minimal estimation of the entire set of target genes for any given hormone.

Many hormone receptors come in variant forms known as isoforms and these isoforms have distinct spatial and temporal expression patterns (Barsony and Prufer, 2002; Chambon, 1996; Conneely and Lydon, 2000; Di Croce et al., 1999; Evans, 1988; Glass et al., 1997; Green and Chambon, 1988). There are only a few instances where the distinct functionality of the isoforms has been proven, although it is probable that all isoforms have some type of functional difference. These differences in function could be as slight as a difference in the intensity of regulation on target genes, or as drastic as regulating a totally different set of targets.

As the hormone signal is mediated through the vascular system, all tissues receive the same hormone signal. Intriguingly, different tissue types respond in widely variant manners. In the case of Drosophila metamorphosis, in response to a hormone signal, most larval tissues under go cell death, while imaginal tissues

proliferate and differentiate into new structures (Baehrecke, 2000; Cherbas, 1993; Gorski and Marra, 2002; Karlson, 1996; Riddiford et al., 2000; Thummel, 2002). In order to fully understand the entirety of the hormone signaling pathway, these possibilities must be investigated.

To contribute to the elucidation of steroid hormone signaling, we have used the *Drosophila melanogaster* model system, with a specific focus on the steroid hormone ecdysone. Ecdysone punctuates each stage of the life cycle, facilitating the transitions into each stage of development. We have investigated the nature of specific isoform function, as well as conducted functional genomic studies to determine a novel set of ecdysone target genes. In the process of isolating novel target genes, we also address the presence and effect of the mid-third instar commitment pulse of ecdysone, the existence of which has often been in question.

Data Review

EcR isoforms have distinct functions

The ecdysone receptor (*EcR*) has three isoforms, and each form a heterodimer complex with *USP* to create a functional Ecdysone Receptor Complex (Hall and Thummel, 1998; Hodin and Riddiford, 1998; Hu et al., 2003; Koelle, 1992; Koelle et al., 1991). In Chapter 2, we have shown that the EcR isoforms have unique developmental requirements, which suggests they have unique molecular functions. Previous work already indicated that the various tissues that specifically express the different isoforms have different cell fates during metamorphosis. Our phenotypic analysis of the *EcR-A* mutants, in

comparison with the *EcR-B1* mutant, proves these isoforms have distinct developmental roles. The *EcR-B1* mutants are non-pupariating, arresting at the end of the third instar. These animals show minimal signs of pupal development. In constrast, the *EcR-A* mutants survive to late pupal stages and even up to pharate adult. This indicates that EcR-B1 is required for the onset of pupariation while EcR-A is required for the completion of pupal development. This suggests that the EcR-B1 isoform responds to the pupariation pulse, while EcR-A responds to the mid-pupal pulse. The functional implication could be that there are a different set of genes that are required for pupariation than for pupal development. This further implies that the different isoforms specifically and differentially regulate these different sets of gene targets.

In other studies of investigating distinct isoform function, we have learned that indeed the isoforms regulate certain targets uniquely, but also regulate some targets the in an identical manner. In tissue specific studies, we see that different isoforms have variant affects when expressed in certain tissues. In studies of testing the affinity of EcRE's, we see that different isoforms have different affinities to specific variations of EcRE sequences. In both cases, these differences could be explained by a unique set of gene targets between the different isoforms. To address this possibility, we sought to define a novel set of ecdysone target genes. Because most known target genes were isolated as such from a single tissue type of a specific developmental, we hypothesize that there are other target genes (responsible for other developmental fates) that would not be identified in this tissue. To address this, we undertook a functional genomics investigation of the mid-third instar ecdysone commitment pulse.

The mid-third instar ecdysone commitment pulse

In some literature, the mid-third pulse of ecdysone has not been observed in Drosophila. Several other studies in various insects have shown that this pulse is required for the animal to undergo metamorphosis in response to the subsequent puparation pulse of ecdysone. This event, termed the mid-third instar event, is very unique in the life cycle. At this event, a behavioral transition occurs, inducing the animals to cease feeding and "wander". Additionally, molecular data shows that a distinct set of ecdysone sensitive genes show expression level transitions at this mid-third instar event. Because of the precise timing of this behavior and gene expression transition, it is clear that there is a developmental cue that creates this effect. Animals that no longer receive the ecdysone pulse at this stage do not undergo wandering or retain these expression changes RR. For this reason, we believe that the mid-third instar pulse is what triggers the mid-third instar event. By developing a detailed synchronization scheme, we have studied the mid-third instar event on a genomic level.

We analyzed 25% of the entire Drosophila genome. Of 600 genes that were differentially regulated throughout the timecourse we studied, we found that nearly 160 genes are repressed, approximately 150 are activated at the mid-third instar event. Of these genes, only ~80 were previously known to be ecdysone targets. We have shown that many known ecdysone targets are responding to a signal that occurs at the precise time of the mid-third intar pulse of ecdysone.

A developmental profile of the Drosophila transcriptome was reported recently. While they show the profile for each life cycle stage, the resolution of the larval stages (10 hour intervals) was not sufficient to investigate the mid-third

instar event. Our study lends a higher resolution (3 hour intervals) to the mid-third instar event, allowing us to see the complete patterns of expression transitions during this event.

Consequently, we would expect there to be a set of genes during the mid-third instar event that mimic the Ashburner Model set of gene expression patterns. This model defines the cascade effect of any pulse of ecdysone, via the Ecdysone Receptor complex. Accordingly, we find that there are specific subsets of genes that reveal a pattern of expression that reflects the Ashburner Model. In Figure 3.12, Panel A we have shown the expression patterns of the *EcR-A* and *EcR-B* isoforms. These two isoforms are known to be directly regulated by ecdysone. The fact they show a transition in expression, indicates there is a pulse of ecdysone occurring. In panel B of this same figure, we show a battery of genes that are positively correlated with *EcR-B1* and therefore are possible activated by this isoform. In Panel C of this figure we show known "late" genes (as defined by the Ashburner Model) and the patterns of this set of genes is in concordance with the expected trends of late gene expression transitions for a pulse of ecdysone at the mid-third instar event. This suggests that the pulse of ecdysone, that some speculate does not exist, does in fact occur.

Ultimately, this genomics study was a success in the isolation of novel ecdysone target genes. As we have shown there to be a signal at the mid-third instar event that affects ecdysone target genes, we also present a set of unknown genes that are affected in the same manner. Additionally, we have implicated several genes of known function to be ecdysone regulated. This work has laid the

foundation for further analysis of ecdysone signaling and elucidation of the component of the ecdysone pathway.

Future Directions

Confirmation of Gene Candidate Gene Targets

Because of software constraints, we were unable to utilize all data obtained for the genomic studies. For this reason, we have a very limited set of genes to investigate, as well as low correlations for the timepoint repeats. In order to definitively characterize the expression patterns we have observed, we must show a higher rate of reproducibility within our data set. We intend to repeat several of the genomic comparisons because of the low correlations and borderline T-test results obtained upon statistical analysis of the data integrity. After the data sets have been improved, we will then seek to confirm the expression patterns we have found, and ideally add additional genes to each set, as we increase the percentage of the genome analyzed. Subsequently, we will seek more supportive evidence of these candidate ecdysone target genes being directly regulated by ecdysone.

We also undertook a second functional genomics approach to independently isolate a set of ecdysone regulated genes, which we have not presented in this dissertation. In this project we are utilizing the *ecdysoneless (ecd¹)* temperature sensitive mutant (Henrich et al., 1993; Warren et al., 1996). When shifted to the restrictive temperature of 29 degrees C, these mutants no longer have proper release of the ecdysone hormone. Therefore, when comparing the transcriptional profiles of mutants at the restrictive temperature with

mutants at the permissive temperature of 22 degrees C, we should see the genomic effects of removing ecdysone. Specifically, we expect that upon removal of the hormone, the genes that are regulated by the hormonal signal will fail to be activated or repressed.

We have chosen the pupariation pulse of ecdysone to use as a data collection point for this project. Presumably the pupariation pulse, the largest pulse of ecdysone, will reflect the greatest response in gene regulation. Upon the onset of metamorphosis, the pulse of ecdysone is not only at its highest concentration, but responses to the pupariation pulse are remarkably diverse (D'Avino and Thummel, 2000; Jiang et al., 2000; Kozlova and Thummel, 2000; Riddiford et al., 2000). Whereas previous pulses trigger molting and growth, the pupariation pulse triggers apoptosis of larval tissues, as well as differentiation of imaginal tissues and other morphological remodeling. These distinct morphological responses are the results of diverse genes and biochemical pathways responding accordingly to the ecdysone signal. Because so many tissues respond diversely to ecdysone at this time, there should be a large number of genes regulated by ecdysone isolated from this experiment. We expect to see previously defined ecdysone sensitive genes revealed in this data set, as well as a plethora of novel ecdysone targets. This profiling project is the next step toward compiling the complete set of all ecdysone target genes.

Ecdysone regulation confirmation

Ecdysone regulation confirmation includes such endeavors as characterization of EcRE's within the promoter regions of these genes, as well as determining loss of expression transition in a mutant background. For the

mutant analysis we will utilize EcR mutants as well as ecdysoneless mutants, both of which should remove the ability of the ecdysone pulse to regulate target genes.

We are also considering a supplementary approach of ecdysone regulation confirmation, which is another functional genomics study using the specific EcR isoform mutants. This study would be another approach in determining ecdysone target genes. Because the ecdysone signal is transduced by the receptor disruption of the receptor function would remove the ability of ecdysone to regulate the target genes. Additionally, an isoform-specific study would give supportive evidence in determining the molecular distinction between EcR isoforms functionality.

Isoform-Specific Functionality

In a recent study, Cherbas et al. (2003) show that when using a dominant negative EcR construct in a tissue specific manner, different EcR isoforms have distinct effects in specific tissue types. This implies that the isoforms have unique molecular functions and interactions. To address whether these different effects are due to differences in target genes between the isoforms, we could employ this dominant negative- tissue specific technique, with the tissue specific analysis seen in Li et al. (2003). By undertaking the same type of isoform specific rescue, and incorporating a genomics perspective, we can determine whether the different isoforms regulate a unique set of target genes in these specific tissue types. The outcome of such an investigation would give the most definitive evidence of the isoforms having unique gene targets.

In closing, we believe the work presented in this dissertation is a significant contribution to the field of Drosophila ecdysone research. Upon completion of the impending future projects, the application of these studies to other model systems will be an asset to several other fields of biomedical research.

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