REGULATION OF ADULT PHYSIOLOGY AND BEHAVIOR

IN Drosophila melanogaster

A Dissertation

by

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ABSTRACT

The physiological responses involved in mediating adaptive change due to varying environmental conditions or social interactions are complex and involve integration of numerous signaling pathways. With *Drosophila melanogaster*, I can investigate the responses to varied environmental and social stimuli through quantification of signaling activity, stress resistance, and changes in gene expression and behavior. My work focuses on investigating signaling pathways that adult insects use to regulate homeostasis. The steroid hormone 20-hydroxyecdysone (ecdysone) and its receptor (EcR/USP) are vital during arthropod development for coordinating molting and metamorphosis. However, recent adult studies in *Drosophila melanogaster* indicate that the hormone and receptor influence many processes. I characterized the wild-type expression patterns and activity of ecdysone receptors in individual tissues during early adult life. I found that receptor components EcR and *usp* were expressed in numerous adult tissues, but receptor activation varied depending on tissue type and adult age. EcR/USP activity did not detectably change in response to environmental stimuli but is reduced when a constitutively inactive ecdysone receptor is present. The current state of our understanding of this signaling system is reviewed with reference to my findings. I discuss future directions focusing on identifying locations of hormone synthesis, metabolism, and storage, isoform-specific roles of EcR, and functional roles of gene repression and activation to link hormone receptor activity with physiological responses.

Adult physiology is also regulated by interactions between adipose tissue and the central nervous system. Genes expressed in the insect fat body are involved in regulating nutrient homeostasis, stress resistance, immunity, reproduction, and behavior. Of particular interest is *female-specific independent of transformer (fit)*. Several studies indicate that fat body expression of *fit* may influence responses to environmental change by altering adult behavior or

physiology. Our lab created *fit* mutants that I used to assess the effects of these mutations on adult Drosophila physiology and behavior. I found that *fit* mutant adults survive longer without food, have increased nutrient levels, are more active, and feed extensively. My findings indicate that the fat-biased gene *fit* influences multiple aspects of adult physiology that affect appetite modulation, metabolism, and behavior.

DEDICATION

The work I have done was held to the high standards set by an excellent P.I. Dr. Ginger Carney has always supported my work, offering her expertise when she had some available and making opportunity for me to take advantage of the expertise of a whole network of her colleagues. I know that the direction of my research has never paralleled the main focus of the lab… in fact it probably was almost tangential. Despite my being almost entirely ineffective in attacking aims of your grant proposal, I never felt like a burden. Your encouragement to find a way for a protocol to work or get the real answer was sincere motivation.

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NOMENCLATURE

ACPs	Accessory peptides, peptides produced in the accessory glands of males that are present in the ejaculate and associate with sperm tails
CNS	Central nervous system, including the brain and ventral nerve cord
EcR	Ecdysone receptor, one component of a nuclear hormone receptor that functions as an ecdysone activated transcription factor
EcR^{DN}	A dominant-negative EcR allele that is not activated by ecdysone
fit	*female-specific independent of transformer*
Gal4	Yeast transcription factor that activates *UAS* sites, commonly referred to as “driver”
$Gal80^{ts}$	A mutant allele of yeast Gal80 that inhibits Gal4 activation of *UAS* sites at low temperatures but not at elevated temperatures, essentially a temperature switch
NHR	Nuclear hormone receptor, a class of proteins based on sequence homology that act as ligand activated transcription factors
PMR	Post mating response, regards to changes in female behavior following mating
RNAi	RNA interference, a cellular gene regulation mechanism where double stranded RNA leads to degradation of mRNA transcripts matching a specific sequence
qPCR	quantitative polymerase chain reaction, is a technique that is used to quantify relative amounts of transcript abundance
SP	Sex peptide, a component of the male ejaculate that alters female physiology, behavior, oogenesis, and appetite
UAS	Yeast gene promoter sequence which regulates the expression of transgenes in a Gal4-dependent manner, referred to as the responder
wt	Wildtype, refers to an organism that contains functional alleles

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CHAPTER I
INTRODUCTION*

1.1 The model organism *Drosophila melanogaster*

Drosophila melanogaster are tiny frugivorous insects that are amenable to laboratory study because they pose no threat to humans, reproduce rapidly, require very limited resources to maintain, and are easily handled following brief CO_2 anesthesia. The genomes of multiple Drosophila species have been sequenced and they contain naturally occurring mobile and immobile transposable p-elements. The controlled introduction and manipulation of foreign constructs using this p-element system has been used to manipulate the function of a large proportion of the flies' genes- forming the basis of modern Drosophila genetics. Massive libraries of flies containing unique genetic manipulations are readily available and can be combined to manipulate the function of almost any gene. By using these modern genetic techniques to investigate signaling pathways in Drosophila we gain a general understanding of similar pathways in arthropod species as well as vertebrate systems.

1.1.1 Drosophila genetics - Gal4/UAS

The availability of molecular and genetic techniques make *Drosophila melanogaster* a powerful tool for investigating the function of signaling systems used by animals to regulate adult physiology during reproduction, feeding, and stressful conditions. In addition to classical extirpation and replacement surgeries or cell ablation techniques used in other organisms, Drosophila adult gene and cell function can be genetically manipulated by the Gal4/UAS two-component gene activation system (Brand and Perrimon, 1993). This two-

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component system consists of an activator or “driver” which expresses yeast Gal4 transcription factors under an endogenously regulated Drosophila gene promoter. This promoter can be specific to a certain cell type or life stage, be responsive to environmental condition such as high temperature or a chemical, or have a very broad to ubiquitous expression profile. Gal4 activates the expression of genes linked to a yeast-specific promoter sequence referred to as a UAS responder. When a fly line containing a Gal4 driver is crossed to a fly line containing a UAS responder the resulting progeny will contain both the Gal4 and UAS components. Gal4 expression in the progeny will reduce the expression of a specific gene via UAS-hairpin constructs that activate RNA inhibition (Fire et al., 1998) or over express functional or mutated alleles of a *UAS*-linked gene of interest. Expression of a specific gene using the Gal4/UAS system in a mutant background allows for assessment of rescue functionality. In this case, the expression of a gene of interest is restricted to just the cell type of interest so its functionality in just that cell type can be assessed. These techniques provide a more precise means of manipulation compared to standard mutant analysis by allowing for tissue-specific or stage-specific analysis of gene function.

1.1.2 Adult reproductive behaviors

The main focus of the Carney lab is characterizing genes that regulate adult physiology and behavior. Drosophila adults exhibit stereotypical courtship behaviors that are innately present at eclosion (Reviewed by Voshall, 2007; Dickson, 2008). Adult males seek out conspecific females possessing a unique chemical signature originating from volatile and non-volatile pheromones secreted from the cuticle (Wicker-Thomas et al., 2009). Receptive females have both a mature female pheromone cocktail (7-11-nonacosadiene, 7-11-ND and 7-11-heptacosadiene, 7-11-HD) and also lack inhibitory pheromones left behind by previous male mates (cis-vaccenyl acetate, cVA and 7-tricosene, 7-T) (Miyamoto and Amrein, 2008; Everaerts et al., 2010).

Drosophila males rarely engage in male-male courtship because mature males produce male-specific inhibitory pheromones (Ferveur et al., 1997). Once a receptive female is identified the male will orient toward the rear or side of the female and proceed to deliver a series of species-specific courtship behaviors (Reviewed by Spieth, 1974). The main component is a wing extension which includes the lateral extension and vibration of one wing to make a species-specific song. The male taps the female abdomen to collect additional pheromone cues with special gustatory receptors present on the tibia segments of the forelegs. The male licks the genitalia of the female with his proboscis to collect more chemical information about the female's mating status and signal his intent to copulate. Lastly the male mounts the female by approaching the rear of the female, bending his abdomen forward toward her genitals, and initiating copulation. These behaviors are genetically encoded but remain plastic-changing in response to social experience and environmental conditions (Reviewed by Sokolowski, 2001). I used these extensively characterized behaviors as bioassays to quantify behavior change in response to manipulations of signaling pathways used by adults to regulate their physiology and respond to the environment.

1.2 Ecdysone signaling

Twenty-hydroxyecdysone, commonly referred to as "ecdysone", is one of several polyhydroxylated steroid signaling molecules collectively known as ecdysteroids that function as crucial coordinators of cell proliferation, differentiation, and apoptosis during arthropod development. Ecdysone was first isolated in 1954 by Butenandt and Karlson (Butenandt and Karlson, 1954). Ecdysone and its canonical heterodimeric receptor encoded by the *Ecdysone Receptor* (*EcR)* and *ultraspiracle (usp)* genes have been identified and functionally characterized during development (Koelle et al., 1991; Yao et al., 1992; Yao et al., 1993; Thomas et al., 1993). In the model organism *Drosophila*

melanogaster, *EcR* encodes splice variants EcR-A, EcR-B1, and EcR-B2, which share common ligand binding and DNA binding sequences (Koelle et al., 1991; Talbot et al., 1993) but have varied amino-termini that influence receptor activation and repression properties (Hu et al., 2003) (Figure 1). Like vertebrate nuclear hormone receptors (NHR's), EcR/USP receives steroid signals and transmits a transcriptional response from within the nucleus (Bertrand et al., 2000). EcR/USP bind to palindromic sequences called ecdysone-responsive elements (***EcRE's***) (Dobens et al., 1991) which are present in promoter regions of ecdysone-responsive genes. Once bound to an *EcRE*, recruitment of co-activators (when activated by ecdysone) or co-repressors (in the absence of ecdysone) facilitates a transcriptional response. This creates a system in which ecdysone-responsive genes regulated by EcR have three distinct activities (Table 1) (Dobens et al., 1991). Functional analysis of the three isoforms indicates that EcR-A is a weak activator but strong repressor, while EcR-B1 and

Table 1. Predicted activities of ecdysone-responsive genes in response to the EcR/USP regulatory environment.

Ecdysone + EcR/USP + EcRE	
Activity 1 Activation	EcR/USP dimers bind ligand and associate with EcRE's with high affinity, recruiting co-activators specific to the isoform of EcR, and activate transcription of ecdysone-responsive genes. EcR regulated gene expression is responsive to ecdysone signals.
EcR/USP + EcRE - Ecdysone	
Activity 2 Repression	Inactive EcR/USP dimers weakly bind to EcREs and repress gene expression by recruiting co-repressors and inhibiting ecdysone-responsive gene expression. Ecdysone-responsive gene activity is silent in this ecdysoneless condition
Ecdysone + EcRE - EcR	
Activity 3 Deregulation	EcR/USP is absent, does not bind to EcREs, and does not regulate transcription of ecdysone-responsive genes. Gene transcription is not activated by EcR/USP, exists at basal levels, or is regulated by other elements of the promoter. Ecdysone-responsive genes are no longer activated by EcR in *EcR* mutants.

EcR-B2 are weak repressors and strong activators (Hu et al., 2003). In summary ecdysone regulated genes are expressed when EcR/USP is activated and are repressed when ecdysone is absent.

Tremendous progress has been achieved in understanding developmental roles of ecdysone and its receptors. During metamorphosis, the relative expression of *EcR* splice forms influences cell fate (Robinow et al., 1993; Talbot et al., 1993; Truman et al., 1994; Bender et al., 1997; Kraft et al., 1998; Schubiger et al., 1998; Rusten et al., 2004), and disrupting EcR function during development leads to severe morphological defects and lethality (Davis et al., 2005). Ecdysone titers increase prior to developmental transitions. This activation of EcR/USP stimulates expression of ecdysone-responsive gene cascades that lead to organism-wide changes in cell proliferation, differentiation and apoptosis (Reviewed by Thummel, 1996; Riddiford et al., 2000). Yet our understanding of this system in the adult life stage has received considerably less attention. In the following sections I will provide a succinct analysis of adult ecdysone signaling and identify major gaps in our knowledge that I will address in my study.

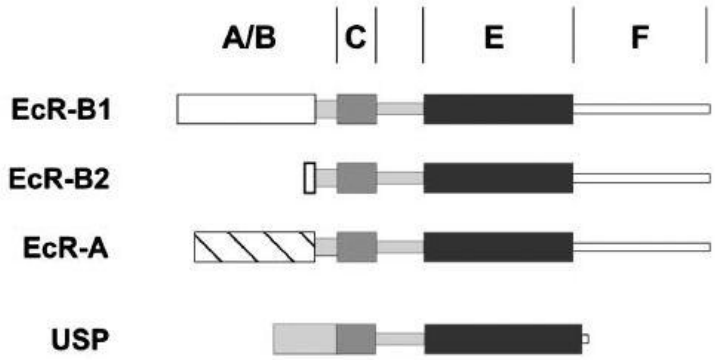

Figure 1. A representation of EcR and USP functional domains. A/B domains confer transcriptional activity; C domain confers DNA binding; E/F domains confer dimerization and ligand binding. (Hu et al., 2003)

1.2.1 Adult ecdysone levels

Whole-animal ecdysone levels in *Drosophila melanogaster* adults are greatly reduced and relatively less dynamic compared to those present in juvenile insects (Bownes et al., 1984). Adult female ecdysone titers increase in response to mating (Harshman et al., 1999) and starvation (Bownes, 1989; Terashima et al., 2005), but little variation is observed in whole bodies up to 16 days post eclosion (Handler, 1982). While the ovary is widely accepted as an ecdysteroidogenic tissue in females, the identity of tissues involved in male ecdysteroid production remains unclear. Ecdysone is a polar steroid that is thought to easily disperse through an insect's open circulatory system (Bownes et al., 1984; Gilbert et al., 2002). Although the location(s) of adult ecdsyone production are not clear, efforts have been made to quantify the amount of ecdysteroid present in whole animals as well as in specific tissues (Table 2 and Table 3).

A variety of strategies can be employed to determine ecdysteroid content, but the most widely used method that binds to a subset of ecdysteroids is radioimmunoassay (RIA). The H-22 antiserum used in common RIAs is most reactive to 20-hydroxyecdysone and α-ecdysone (the precursor of 20-hydroxyecdysone), but H-22 also has limited reactivity to other ecdysteroids (Borst and O'Connor, 1974). Data from RIA analysis using the H-22 antibody are indicative of combined 20-hydroxyecdysone and α-ecdysone levels but are not informative for determining relative levels of each or levels of less reactive conjugates. High pressure liquid chromatography (HPLC) combined with RIA has been used to better characterize ecdysteroid content by quantifying RIA signal from fractions with different column motility. Using this method, RIA reactivity is present in fractions other than those represented by 20-hydroxyecdysone or α-ecdysone, but the molecular amounts and/or reactivity are low (Smith and Bownes, 1985).

Enzyme immunoassay (EIA)-based detection methods have been developed that offer excellent sensitivity and accuracy, relief from handling radioactive material, streamlined high-throughput sampling, and near ubiquitous availability of detection equipment (Kingan, 1989; Porcheron et al., 1989; Shiotsuki et al., 2005). EIA uses the same anti-ecdysone antibody as the RIA method and therefore suffers from a similar lack of specificity of ecdysteroid detection.

Table 2. Adult ecdysone levels. Amounts presented are approximations based upon data reported as pg of ecdysteroid equivalents per mg of adult Oregon R tissue. Flies were raised at 20–25 on standard fly media. All data were collected by radioimmunoassay with the H-22 antiserum (Borst and O'Connor, 1974) for detection of ecdysteroids. The Handler, (1982) data are assumed to be from mixed populations of flies as the methods did not indicate if males were kept separately from females.

Age (day)	Gender	Ecdysone (pg/mg)	Publication
0-1	Male*	7-27	Handler, 1982
0-1	Female*	12-35	
2-14	Male**	5-15	
2-14	Female**	5-15	
1-2	Male/female	15-25	Hodgetts et al., 1977
3-4	Male/female	15	
0-1	Male*	13	Bownes et al., 1984
0-1	Female*	19	
1-2	Male*	4	
1-2	Female*	10	
2-4	Male**	4-7	
2-4	Female**	5-6	

* Animals are likely virgin due to reported age.
** Animals are of unknown sexual status although most are sexually mature and are likely to have mated since they likely were kept in mixed groups.

Although it is not generally used in insects, gas chromatography–mass spectrometry (GC–MS) is an alternate strategy for identifying a variety of small molecules, including ecdysteroids, at concentrations as low as 10–100 pg/mg

(Nirde et al., 1984; Evershed et al., 1987). GC–MS combined with HPLC may provide the resolution required to quantify individual ecdysteroid conjugates, but does not scale as well in high throughput applications as previous methods.

Using RIA, several research groups examined adult Drosophila ecdysone titers under various conditions. These experiments showed that ecdysone is present in adult Drosophila, albeit at lower levels than in earlier developmental stages (Borst et al., 1974; Richards, 1981; Handler, 1982; Berreur et al., 1984; Schwartz et al., 1985). Newly eclosed adults contain approximately 25 pg of ecdysone per mg of tissue, a value that drops approximately two-fold within 24 h (Table 3) (Borst et al., 1974; Hodgetts et al., 1977; Handler, 1982). Little is known about tissue-specific localization of ecdysone in adults, but ecdysone is detected in all 3 body segments, in hemolymph, and in specific tissues such as testes, gut, and Malpighian tubules (Handler, 1982; Bownes et al., 1984) (Table 3).
Male *Anopheles gambiae* have high levels of ecdysone in the accessory glands, but not testes, and transfer ecdysone to females during copulation (Pondeville et

Table 3. Ecdysone content of adult tissues. Numbers indicate percentage of ecdysteroids detected in specific tissues relative to the total amount of ecdysteroid. Summarized data from Oregon R adults as reported in Bownes et al., (1984).

	0-1 Day	1-2 Day	2-3 Day
Male			
Head and thorax	37%	51%	46%
Testes	11%	16%	25%
Abdominal body wall	34%	19%	15%
Gut and Malpighian tubules	18%	14%	14%
Total (pg/adult)	2.84	1.72	4.68
Female			
Head and thorax	34%	21%	26%
Ovaries	16%	35%	30%
Abdominal body wall	28%	22%	26%
Gut and Malpighian tubules	22%	22%	18%
Total (pg/adult)	4.96	5.15	7.84

al., 2008). In Drosophila, ecdysone also was detected in ovaries by Bownes et al., (1984) and Schwartz et al., (1985) but not by Handler, (1982), who suggested that ovaries may rapidly secrete ecdysone or contain inactive forms that cannot be detected via RIA.

Adult ecdysone levels are responsive to changes in environment. Conditions such as starvation (Terashima et al., 2005) and sleep deprivation (Ishimoto and Kitamoto, 2010) appear to increase adult ecdysone content. Similarly, titers are elevated in mated females (Harshman et al., 1999) and males whose courtship advances are rejected by previously mated females (Ishimoto et al., 2009). Changes in nutrient status mediated by the insulin signaling pathway can also impact ecdysone levels (Tatar et al., 2003). The importance of these fluctuations in ecdysone titers due to environmental changes remains unclear, yet the evidence presented suggests that adults integrate and respond to environmental information through subtle changes in ecdysone content. My concern is that these studies have not addressed the impact adult ecdysone content has on gene expression, making it difficult to predict the activity of this signaling system.

1.2.2 Ecdysone metabolism, modification and storage

Ecdysteroid variants share a similar basic structure, but hormone modifying enzymes convert ecdysteroids to different forms through addition or removal of side groups. These modifications influence the affinity of the molecules to hormone binding proteins and their receptors, as well affect the mobility of the molecules within the insect by altering their ability of the molecules to traverse cell membranes.

Adults convert exogenous ecdysone into numerous metabolites which are rapidly eliminated to return ecdysone to wild-type levels (Smith and Bownes, 1985; Grau and LaFont, 1994). The exogenous ecdysteroids are metabolized in most tissues, with the exceptions of head and Malpighian tubules, into apolar

conjugates that are ultimately eliminated in the feces (Grau and LaFont, 1994). Adult tissues also produce other transiently detectable metabolites which retain reactivity to the ecdysone-specific immunogen (Smith and Bownes, 1985).

Ecdysteroids that are not metabolized exist as various conjugate forms which have different activational properties and molecular mobilities. Eckinase was identified in *Bombyx mori* ovaries and found to phosphorylate ecdysone to ecdysone 22-phosphate (Sonobe et al., 2006). This inactive, immobile, and storable form can be localized and retained in Eckinase-expressing cell types. Cells expressing Eckinase potentially act as an ecdysteroid sink and accumulate inactive hormone from surrounding tissues. Stored ecdysone 22-phosphate can be re-activated by ecdysteroid-phosphate phosphatase (EPP) (Yamada and Sonobe, 2003; Sonobe and Ito, 2009). It is postulated that these enzymes work in concert to load Bombyx embryos with inactive ecdysteroids that are used to initiate various developmental processes post fertilization (Sonobe and Ito, 2009). Eckinase-like enzymes may also be used to vary the response a tissue has to increased ecdysone levels. Tissues with Eckinase-like activity may trap excess ecdysteroids and facilitate their metabolism, protecting other adult tissues from potentially disruptive ecdysone signals.

Tissue-specific ecdysone content has been analyzed in adults to identify possible sites of ecdysone localization (Table 3), though it is likely that stored ecdysteroids do not react with the antiserum used in RIA detection techniques. These conjugated forms of ecdysone do not influence ecdysone-responsive gene expression and are not detectable by ecdysone-responsive reporters. A detection method that is capable of quantifying ecdysone conjugates was used to more accurately assay tissue-specific ecdysteroid content (Grau and Lafont, 1994). Together, the results suggest that ecdysteroids are found in many tissues and exist in a variety of forms and biological activities. These mechanisms of ecdysone metabolism and conversion may inhibit an adult's responsiveness to exogenous ecdysone treatment and lead to what may be perceived as ecdysone

insensitivity. Characterizing ecdysone-responsive gene expression in adult tissues would be a good way of identifying physiological responses to adults following ecdysone treatment.

1.2.3 Ecdysone signaling function influences adult behavior

Adult ecdysone signaling modulates sleep, memory formation, and aspects of male courtship behavior (Table 4 and 5). Mature flies supplemented with ecdysone have increased sleep bout durations whereas females carrying a single *l(3)DTS-3*^{1} mutation, an allele linked with reduced ecdysone levels, sleep for shorter durations (Ishimoto and Kitamoto, 2010). Similarly, adults with mushroom body over-expression of *EcR-A* or *EcR-B1* sleep significantly more than controls (Ishimoto and Kitamoto, 2010). Total sleep and sleep bout lengths are reduced in males carrying EcR mutations, but the phenotypes are not rescued by ecdysone treatment. Female *EcR* mutants also have a decrease in total sleep and sleep bout length, but the effect in females can be rescued by ecdysone supplement (Ishimoto and Kitamoto, 2010). This gender-specific response suggests that the gonad may be an influential tissue in governing ecdysone-mediated regulation of activity or that the sensitivity to ecdysone content is different in males and females.

Alterations in EcR function also affect courtship memory formation and choice of courtship object. Wild-type male Drosophila learn to avoid courting non-receptive females. However, a deficit in long-term courtship memory occurs in males with reduced EcR function, a phenotype which can be rescued by ecdysone treatment (Ishimoto et al., 2009). This inability to quickly identify a non-receptive female is also observed in males carrying the *l(3)DTS3*^{1} mutation, which regain the ability to form and recall behavioral memory when supplemented with ecdysone during training (Ishimoto et al., 2009). Due to the nature of EcR and ecdysone manipulation it is difficult to predict what tissues are involved in this response.

Table 4. Adult *EcR* mutant phenotypes. EcR^{fs} is an EcR null mutant representing the frame shift allele $EcR\text{-}M554^{fs}$ or $EcR\text{-}V559^{fs}$ (Bender et al., 1997). EcR^{ts} represents the temperature sensitive *EcR-A483T* allele which has reduced functionality at 29 ºC (Bender et al., 1997). $EcR^{\{p\}}$ is a p-element insertion allele which causes reduced EcR expression and homozygous lethality (Ishimoto and Kitamoto, 2010). EcR^{DN} represents an *EcR* allele that is not activated by ecdysone (Cherbas et al., 2003). *EcRi* represents an allele that produces a double-stranded RNA that reduces EcR levels through RNA interference (Colombani et al., 2005).

Manipulation	Gender	Tissue affected	Phenotype	Response	Publication
EcR^{fs}/EcR^{ts}	Male	Adult only	Male-male courtship	Increased	Ganter et al., 2007
EcRi	Male	*fru* neurons	Male-male courtship	Increased	Dalton et al., 2009
$EcR^{ts}/+$ $EcR^{ts/+}$ *EcRi*	Male	Whole body Adult only Adult Neurons Mushroom body	Long-term memory	Decreased	Ishimoto et al., 2009
$EcR^{ts}/+$	Male Female	Whole body	Symptoms of aging	Decreased	Simon et al., 2006
$EcR^{ts}/+$	Male Female	Adult only	Sleep	Decreased	Ishimoto and Kitamoto, 2010
$EcR^{ts}/EcR^{\{p\}}$	Male Female				
UAS-EcR-A *UAS-EcR-B1*	Female	Mushroom body	Sleep	Increased	
$EcR^{fs}/+$	Male Female	Whole body	Lifespan	Increased	Simon et al., 2003
	Male Female		Stress resistance		

Table 4. Continued.

Manipulation	Gender	Tissue affected	Phenotype	Response	Publication
Mild *EcRi*	Male	Whole body	Lifespan	Increased	Tricoire et al., 2009
Strong *EcRi*				Decreased	
Mild EcR^{DN}				Increased	
EcRi		Fat body		Increased	
EcR^{DN}				None	
EcRi		Neuronal		None	
EcR^{DN}				None	
EcRi	Female	Whole body		Decreased	
EcR^{DN}				Decreased	
EcRi		Fat body		None	
EcR^{DN}				None	
EcRi		Neuronal		None	
EcR^{DN}				Decreased	
EcRi		Whole body	Egg laying	Decreased	
EcR^{ts}/EcR^{ts}	Female	Adult only	Egg laying	Decreased	Carney and Bender, 2000
EcR^{DN}	Female	Stage 8+ follicle cells	Chorion and appendages	Defective	Hackney et al., 2007
EcRi-B1	Female	Stage 8+ follicle cells	Follicle cell polarity	Decreased	Romani et al., 2009
EcRi-B1		Early stage follicle cells	Follicular epithelium and cell survival	Decreased	
EcR^{fs}	Female	Germline clone	Egg chamber progression	Arrested	Buszczak et al., 1999

Table 5. Adult ecdysone signaling mutant phenotypes that are rescued by treatment. e*cdysoneless*^{1} *(ecd*^{1}*)* and *lethal (3) DTS3*^{1} *(l(3)DTS3*^{1}*)* are temperature-sensitive mutations that decrease ecdysone titers (Garen et al., 1977; Gaziova et al., 2004; Holden et al., 1986). Wild-type (wt) adults were fed with 0.1-1.0mM 20-hydroxyecdysone enriched food (Ishimoto and Kitamoto, 2010).

Manipulation	Gender	Phenotype	Response	Publication
l(3)DTS3^{1}/+	Female	Lifespan	Increased	Simon et al., 2003
l(3)DTS3^{1}/+	Male	Long-term memory	Decreased	Ishimoto et al., 2009
l(3)DTS3^{1}/+	Female	Total sleep + duration	Decreased	Ishimoto and Kitamoto, 2010
wt + ecdysone			Increased	
l(3)DTS3^{1}/+	Female	Sleep deprivation response	Decreased	
Ecd^{1} (29ºC)	Male	Male-male courtship	Increased	Ganter et al., 2011
		Male chaining		

Wild-type males devote their courtship efforts toward females and spend very little time performing courtship toward other males. In contrast, males carrying mutant *EcR* alleles display increased male–male courtship (Ganter et al., 2007), a phenotype also identified in males with decreased EcR function in *fruitless (fru)*-expressing neurons (Dalton et al., 2009). Interestingly, male–female courtship interactions were not reported to be compromised in *EcR* mutant males. Adult males carrying *ecd*^{1} mutations engage in male–male chaining behavior and display increased male–male courtship (Ganter et al., 2011). Together, these results suggest that ecdysone-responsive gene expression, specifically in the CNS, is required for inhibiting male–male courtship but not male–female interactions. More thorough analysis of tissue-specific mutants and adult-only manipulations are required to better characterize the interaction between EcR and these behaviors.

1.2.4 Stress and lifespan

Adult lifespan and resistance to unfavorable environmental conditions are influenced by ecdysone signaling. Male and female adults carrying a single EcR null mutation (which presumably halves EcR levels) survive longer

under normal laboratory conditions as well as in high temperature, dry starvation, or oxidative stress conditions (Simon et al., 2003). As they age, *EcR* mutants experience a less rapid physical decline than control flies (Simon et al., 2006). Male lifespan also increases when adult EcR is reduced to approximately 50% of wild-type expression via *EcRi* or when EcR/USP signaling is disrupted by EcR^{DN} expression (Tricoire et al., 2009). This longevity increase is linked to EcR function in the fat body (Tricoire et al., 2009). However, reducing EcR by more than 50% of wild-type levels decreases male lifespan. The male dose response to EcR suggests that Drosophila maintain a tightly regulated balance between expression of genes promoting somatic maintenance and those with alternate functions expressed at the cost of longevity.

Tricoire et al. (2009) varied activating ligand dosage of geneswitch drivers (Osterwalder et al., 2001) to modulate the intensity of *EcRi* and expression of EcR^{DN}. Modest reduction in EcR signaling by *EcRi* or EcR^{DN} expression decreases female lifespan (Tricoire et al., 2009), conflicting with the findings of Simon et al. (2003) where 50% EcR dose in females increases lifespan by 40–50%. One explanation for this discrepancy is that in one set of experiments EcR levels were reduced throughout development (Simon et al., 2003), whereas Tricoire et al., (2009) reduced EcR specifically in the adult stage. It is possible that decreases in EcR expression during development account for lifespan-enhancing effects observed in *EcR*/+ females, although a link to EcR functions in female ovaries was also postulated (Tricoire et al., 2009). Since mild reduction of male EcR levels had similar effects in both sets of experiments, it appears EcR regulates lifespan genes differently in males compared to females, an effect that is possibly linked to reduced female ovarian function in regulating longevity. $l(3)DTS3^{1}$/+ mutant females, which experience reduced ecdysone levels, are long-lived- a phenotype that is reversed by ecdysone supplement (Simon et al., 2003). Females may be able to live longer by reallocating resources freed up from reduced ovarian investment. In contrast to the gender-dependent

differences in lifespan phenotypes observed, EcR mutation increases stress resistance in both sexes (Simon et al., 2003). These varied phenotypes associated with EcR dosage and gender suggest that adult ecdysone-responsive gene expression and repression are regulated within tight thresholds to adapt to changes associated with stress and aging in a gender-specific manner. It is important to note that with the exception of Carney and Bender (2001), Dalton et al., (2009), and Ishimoto and Kitamoto (2010), all of these experiments that identified adult *EcR* mutant phenotypes were conducted without attempting to characterize EcR or USP in adult tissues.

1.2.5 Characterizing adult EcR/USP expression and activity

Adult sensitivity to ecdysone is conveyed by EcR and USP expression. Available antibodies allow detection of all three proteins via the common carboxyl terminus or isoform-specific detection of EcR-A and EcR-B1 (Koelle et al., 1991; Talbot et al., 1993). While EcR protein expression and functions have been well characterized in numerous larval and pupal tissues (Robinow et al., 1993; Talbot et al., 1993; Truman et al., 1994; Bender et al., 1997; Schubiger et al., 1998), EcR adult protein expression has only been investigated in the ovary and central nervous system (CNS) (Carney and Bender, 2000; Dalton et al., 2009; Ishimoto et al., 2009). I predicted that other adult tissues express EcR as well given the breadth of ecdysone and *EcR* mutant phenotypes (Table 4 and 5). USP is an insect nuclear hormone receptor homologous to the mammalian retinoid X receptor (RXR) (Oro et al., 1990). USP is expressed in many larval and imaginal disc tissues prior to molting and metamorphosis (Henrich et al., 1994), but little is known about USP expression or function in adults.

1.2.6 Adult ecdysone signaling goals

To address gaps in our knowledge concerning EcR and USP expression, I have characterized the adult tissue-specific expression of these nuclear hormone

receptors. While whole body mutation of *EcR* results in mutant phenotypes, we lack understanding of which tissues require EcR function. To address this gap in our knowledge I have manipulated EcR function in multiple tissue types in attempt better characterize how tissue-specific or isoform-specific expression of EcR affects male courtship and female egg-laying behaviors. There is evidence that both ecdysone and ecdysone receptors are present in adults, but we have no understandingof tissue-specific EcR/USP signaling activity in wild-type adult tissues. To address this issue, I have characterized the expression of an ecdysone-responsive reporter, as a proxy for EcR/USP activation in adults and qualified the activity of this reporter in adults experiencing multiple environmental conditions.

1.3 Insect nutrient homeostasis

The primary function of animals in the adult lifestage is reproduction and dispersal, both energetically demanding tasks. Insects use energy stores obtained during larval feeding, and many complement their energy demands with appropriate adult feeding behaviors. The primary energy storage tissue is the fat body. The fat body cells, adipocytes, contain lipid droplets that store fats in the form of triglyercides, glycerol, and free fatty acids (Reviewed by Arrese and Soulages, 2010). Insect adipocytes also store carbohydrates in the form of glycogen. Glycogen is readily accessible polymer of glucose used for maintaining circulating sugar levels in the form of the disaccharide trehalose. When circulating nutrient levels fall, nutrient sensing neurons in the brain release adipokinectic hormone (AKH). AKH targets the AKHR expressing cells in the fat body activating lipid and protein mobilization (Van der Horst, 2003; Lee and Park, 2004; Grönke et al., 2007). Nutrient influx stimulates target of rapamycin (TOR) signaling pathways in the gut, fat, and muscle. TOR activation leads to increases in cellular glucose uptake, transcription, and translation activity (Reviewed by Wullschleger et al., 2006; Geminard et al., 2009). Through an unknown pathway,

fat body signals the release of Drosophila insulin-like peptides (DILPs) from neurosecretory median neurons in the brain (Geminard et al., 2009). DILPs signal through insulin-like receptors (InR) to inhibit Forkhead box O (FOXO) upregulating metabolism and growth and suppressing autophagy.

1.3.1 Adult fat body and appetite

The fat body of the model organism *Drosophila melanogaster* is analogous to vertebrate white adipose tissue and is regulated by conserved mechanisms (Reviewed by Baker and Thummel, 2007; Teleman, 2010). The adult fat body is a loosely organized sheath of cells, primarily comprised of adipocytes, that exists in close proximity to the epidermis as well as to the CNS and visceral organs of the fly (Reviewed by Arrese and Soulages, 2010). These adipocytes are bathed in hemolymph, the insect circulatory system, and produce and secrete many hemolymph components, including immune response peptides (reviewed by Hoffman, 2003), insulinotropic factors (Geminard et al., 2009), and neuromodulators (Meunier et al., 2007; Lazareva et al., 2007). The fat body is also the primary producer of vitellogenin, which is taken up by developing eggs as the main component of the yolk (Jowett and Postlethwait, 1980; Bownes et al., 1996; Isaac and Bownes, 2005). Simpy put, the fat body is an important tissue for metabolism, storage, mobilization, and production of many vital molecules.

Given the extremely high rates of obesity in the United States and across the world, it is imperative to understand more about signaling pathways that regulate appetitive behaviors as well as nutrient metabolism and storage. The effective assessment of nutrient demands and reserves, mobilization of nutrients, and regulation of feeding behaviors requires the coordinated function of adipose and nervous system tissues. Adipose tissue serves as a repository for lipid and glycogen stores, but it also is an endocrine gland that produces and releases factors known as adipokines that affect a variety of physiological processes such as neural circuit development, hormone levels, and central nervous system

(CNS) response to environmental signals. For example, adipokines such as leptin and adiponectin modulate inflammation, reproduction, obesity, and motivated eating behavior (Reviewed by Bouret and Simerly, 2007; Lago et al., 2007; Pan and Kastin, 2007). Many problems affecting appetite control and nutrient regulation stem from aberrations in signaling between the CNS and adipose tissue.

Similarly to vertebrate adipocytes, insect adipocytes contain lipid droplets and glycogen which can be mobilized during periods of increased energy demand or reduced nutrient availability. Nutrient levels are monitored by carbohydrate and amino acid transporters in the fat body and neurosecretory cells in the CNS. Sugar and protein absorption by the fat body following feeding is regulated by the conserved TOR and insulin signaling pathways (Attardo et al., 2005; Mirth and Riddiford, 2007; Masumura, 2000; Brogiolo et al., 2001). Nutrient mobilization is also regulated by similar signaling pathways in vertebrates and invertebrates. During periods of elevated nutrient demand such as flight, courtship, and reproduction, AKH is released by neurons in the corpora cardiaca to mobilize stored lipid, protein, and carbohydrates from the fat body (Lee and Park, 2004; Grönke et al., 2007). Neuropeptide F (NPF) production in the gut and CNS is also nutrient responsive and stimulates orexigenic neurons to initiate feeding behavior (Krashes et al., 2009). Another signal that affects feeding in female Drosophila is the orexigenic signal sex peptide (SP), which is transferred to the female via the male's ejaculate. SP, together with other components of the male ejaculate, stimulates oogenesis and oviposition behaviors in mated females (Carvalho et al., 2006; Yapici et al., 2008). These changes are accompanied by SP-controlled increases in feeding and changes in food preference (Ribeiro and Dickson, 2010) to meet the energetic demands of oogenesis and egg laying. Correlated with these post-mating behavioral and physiological changes are increases in locomotor behavior that are expected to be important for finding ovipostion sites and food sources (Isaac et al., 2010). From these findings it is

apparent that the fat body is both the source and target for many signaling molecules that regulate homeostasis.

1.3.2 Investigating a fat-biased gene's influence on feeding and nutrient homeostasis

My primary goal is to understand the adult fat body's role in regulating nutrient homeostasis and behavior in *Drosophila melanogaster*. Of great interest are the signaling pathways that are responsible for mediating responses to changing environmental circumstances. Recent studies aimed at identifying genes involved in adult responses to environmental change have utilized microarray technology to detect changes in transcript abundance in adult tissues. Of the identified loci, the most intriguing candidate was the fat body-expressed gene *female-specific independent of transformer* (*fit*), which increases in expression in courting or mated adults (Lawniczak and Begun, 2004; Carney, 2007; McGraw et al., 2008; Dalton et al., 2010; Ellis and Carney, 2010; Ellis and Carney, 2011) and decreases in starved animals (Fujikawa et al., 2009; Boyd et al., 2011). However, the nature of *fit*'s involvement in behavior, reproduction, and nutrient homeostasis remains unclear. Based on these findings I hypothesized that *fit* is involved in metabolic processes and regulates behaviors that involve nutrient uptake. To address this hypothesis, I investigated the influence *fit* expression in fat body has on adult behavior, starvation resistance, and nutrient homeostasis.

CHAPTER II
ADULT ECDYSONE RECEPTOR SIGNALING FUNCTION*

2.1 Introduction

Multiple behavioral phenotypes reported in ecdysone and ecdysone receptor mutant animals were identified in chapter I (Table 4 and 5). The manipulations used to identify these phenotypes are coarse and likely lead to gross changes in ecdysone-responsive gene expression. These mutants have the functionality of all three EcR isoforms reduced in multiple tissues making identification of functional roles of EcR and interpretation of the results difficult. In this section, I will characterize adult ecdysone receptor expression by immunostaining for EcR proteins and quantifying *usp* transcripts in specific tissues. At the time we began this work, EcR was only known to influence female egg-laying behavior and male-male courtship (Table 4) so I focused on addressing the influence of specific isoforms of EcR and the involvement of specific tissues in regulating these behaviors.

2.2. Methods

2.2.1 Experimental animals

The flies used in this study were reared at 25 °C on a 12 h light:12 h dark cycle on standard cornmeal media in glass vials or plastic bottles unless otherwise indicated. Isogenized *Canton-S* adults were used for EcR immunostaining. *EcR* mutant behavior was assayed in adults carrying combinations of the following *EcR* mutant alleles*: EcR^{m554fs}/SM6B, y^1w^1; EcR^{V559fs}/CyO, y^+,EcR^{Q50st}/SM6B, EcR^{94}/SM6B, EcR^{A483ts}/SM6b*, and y^1w^1;EcR^2/CyO,y^+. EcR/USP activity was assayed in the *w*;P{w+mC =EcRE.lacZ}SS4* strain, which was generously provided by Carl S. Thummel.

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The ubiquitously expressing *actin-Gal4* element was obtained from the *yw;actin-Gal4/CyO,y*$^{+}$ stock provided by Vlad Panin. *3.1Lsp-Gal4/TM3,Sb* was graciously provided by Bridgette Dauwalder. Gal4 activity was regulated by the temperature sensitive Gal80 element from a *w;;tubulin-Gal80*^{ts} stock provided by Paul Hardin. The EcR dominant-negative strains *w;;P{w+mC=UAS-EcR.B2.F645A}TP1, w;;P{w+mC=UAS-EcR.B2.W650A}TP5, w;;P{w+mC=UAS-EcR.B1.F645A}TP1, w;;P{w+mC=UAS-EcR.B1.W650A}TP1-9, w;;P{w+mC=UAS-EcR.A.F645A}TP2, w;;P{w+mC=UAS-EcR.A.W650A}TP5,* and RNAi lines *w;P{w+mC=UAS-EcR-RNAi}97, w*^{1118}*; P{w[+mC]=UAS-EcR.B1.dsRNA}168, w*^{1118}*; P{w[+mC]=UAS-EcR.A.dsRNA}91/TM3, P{w[+mC]=ActGFP}JMR2, Ser1* (referred to as *EcRi* in the text) were acquired from Bloomington Drosophila Stock Center. Dicer2 was expressed using *P{UAS-Dcr-2.D}1* from the VDRC*. w*^{1118}*; l(2)c805-Gal4/CyO; TM2/TM6B,Tb* was used to express Gal4 in testis and male accessory glands and *P{w[+mW.hs]=GawB}elav[C155]* was used to express Gal4 in neurons.

2.2.2 EcR immunostaining

I used monoclonal anti-EcR antisera that target isoform-specific regions of EcR-A (15G1A) or EcR-B1 (AD4.4) or regions common to all EcR isoforms (DDA2.7 and AG10.2) (Koelle et al., 1991 and Talbot et al., 1993) (BDRC). Adults were collected within 8 h of eclosion and aged in mixed populations of fewer than 20 individuals for 4 h (0 day), 5 days (5 day), or 10 days (10 day). Tissues were dissected in phosphate buffered saline (PBS) on ice, fixed in 4% paraformaldehyde for 20 min, washed in PBS + 0.1% Triton X-100 (PBST), and incubated in a 1:5 dilution of anti-EcR antisera in PBST with 5% normal goat serum overnight at room temperature. Tissues were then washed in PBST and incubated in 1:500 biotinylated goat anti-mouse secondary antisera (Pierce Immunopure) for 2 h. Immunoreactivity was visualized by incubating tissues in a solution of diaminobenzidine (DAB) and H2O2/urea (Sigma) with bound HRP-biotin/avidin complex (Vectastain ABC Kit). Tissues from 0, 5, and 10 day adults

were immunostained with anti-EcR antisera (DDA2.7 and AG10.2) that target regions of EcR common to all isoforms, and 5-day adult tissues also were immunostained with isoform-specific antisera for EcR-A (15G1A) and EcR-B1 (AD4.4). Staining patterns were imaged with a Zeiss Axio Imager Z1 microscope.

2.2.3 usp *mRNA detection*

Total RNA was extracted from 0-, 5- or 10-day (n = 15) male and female dissected CNS, abdominal cuticle tissue (containing epidermis, muscle, fat body, and oenocytes), gut and Malpighian tubules, as well as individual male reproductive tract tissues (testes, seminal vesicles, accessory glands, ejaculatory duct, ejaculatory bulb). Samples were treated with DNase I (Invitrogen) and converted to cDNA using a Superscript II cDNA Synthesis Kit (Invitrogen). qPCR was performed as described previously (Ellis and Carney, 2009). In short, cDNAs were amplified using the SYBR green qPCR mastermix (Applied Biosystems) and *usp*-specific primers. Reactions were run in the ABI7700 using default run parameters, and relative amounts of *usp* in each reaction were determined by the Relative Standard Curve Method (Applied Biosystems) using *Rps15a* primers as the control for amplification. I included negative and positive control reactions on each plate and used melting curve analysis to confirm primer specificity.

2.2.4 Male courtship behavior assays

Male-male courtship was assayed for various naïve males individually aged 5 days. If these adults carried any temperature sensitive alleles (*EcR*^{ts} or *tub-Gal80*^{ts}), they were raised at 20-22° and then shifted to 29° following collection at eclosion. Behavior testing occurred at 29° for adults containing temperature sensitive alleles and 23-25° for those that do not.

Male-male courtship was quantified as an Attraction Index (A.I.) for each male. The A.I. is a ratio of the total amount of time a male spent orienting,

extended one or both wings, tapping, licking, and abdomen bending during the 10 minute period.

2.2.5 Female egg-laying behavior

Female mutants were collected at eclosion and housed in like-gender groups at 29° for 3 days. On day 3, females were housed in individual vials along with 2 *Canton-S*. males. Females were then transferred to new vials each day for 3 days, so that numbers of eggs laid could be recorded. Total number of eggs laid was compared between mutants and control by Student's *t*-test analysis.

2.2.6 Adult ecdysone manipulation

Adult ecdysone levels were manipulated to better understand how this signaling pathway influence adult behavior. The ecdysone hypomorphic allele *ecdysoneless (ecd^{1})* is a temperature sensitive allele that reduces ecdysone levels (Garen et al., 1977). Adults carrying this allele develop at 20-22° and then are aged after eclosion at 29°. Adults aged at 29° are considered ecdysone mutants.

Ecdysone supplementation was performed to try and stimulate ecdysone regulated gene expression in adults. Ecdysone supplement of 1mM was found to reverse the phenotype of ecdysone deficient *l(3)DTS3^{1}* adults (Simon et al., 2003) so I aged wild-type males in vials containing 1mM ecdysone in yeast paste for 5 days and assayed male-male courtship behavior as described in section 2.2.4.

2.3 Results

2.3.1 Adult EcR expression

Disruption of adult EcR results in deficits in reproduction, behavior, and learning, and increases in lifespan and stress resistance. These adult effects remain weakly linked to EcR/USP function and activity. To understand the extent

to which ecdysone signaling can function in the adult I first needed to identify the tissues that express and activate EcR/USP. I used antisera that recognize all 3 isoforms of EcR to examine EcR expression in wild-type adults under standard conditions. EcR protein was detected in numerous tissues in newly eclosed (0 day), 5-day, and 10-day adults (Figure 2). EcR protein was in the nuclei of cells in the brain, ventral nerve cord, fat body, oenocytes, gut (cardia/midgut/hindgut/ rectum), Malpighian tubules and ovarian follicle cells (Figure 2). These observations confirm other reports of EcR expression in the adult tissues (Carney and Bender, 2000; Dalton et al., 2009; Ishimoto et al., 2009).

I also detected EcR in male reproductive tissues including the accessory glands, testes, seminal vesicles, ejaculatory bulb, and ejaculatory duct (Figure 2). In some instances I observed a shift in EcR cell number and sub-cellular localization in male seminal vesicles (compare Figure 2a and b). Cells of the seminal vesicles had two distinct staining patterns with isoform-specific as well as common antisera. In one state, EcR localized outside the nucleus (Figure 2b), but in other instances EcR expression was present in outer sheath cell nuclei (Figure 2a). Although the images are from animals of different ages, I observed both types of patterns when I examined males of multiple ages and mating statuses, suggesting that this phenomenon is not related to these variables.

I did not detect obvious differences in EcR staining patterns between tissues that are present in both sexes such as the CNS, fat, oenocytes, gut, and Malpighian tubules. Adult age did not appear to affect EcR expression since EcR was detected in each tissue examined in 0-, 5- and 10-day animals. The only exception was developing eggs, which have little EcR in day 0 females. Five-day tissues positive for EcR contained EcR-A and EcR-B1 isoforms, although CNS expression of EcR-B1 appeared lower than that of EcR-A, confirming a previous report by Dalton et al., (2009). EcR is an essential regulator of basic cellular processes and so may be functioning in the adult to maintain adult somatic and germline tissues.

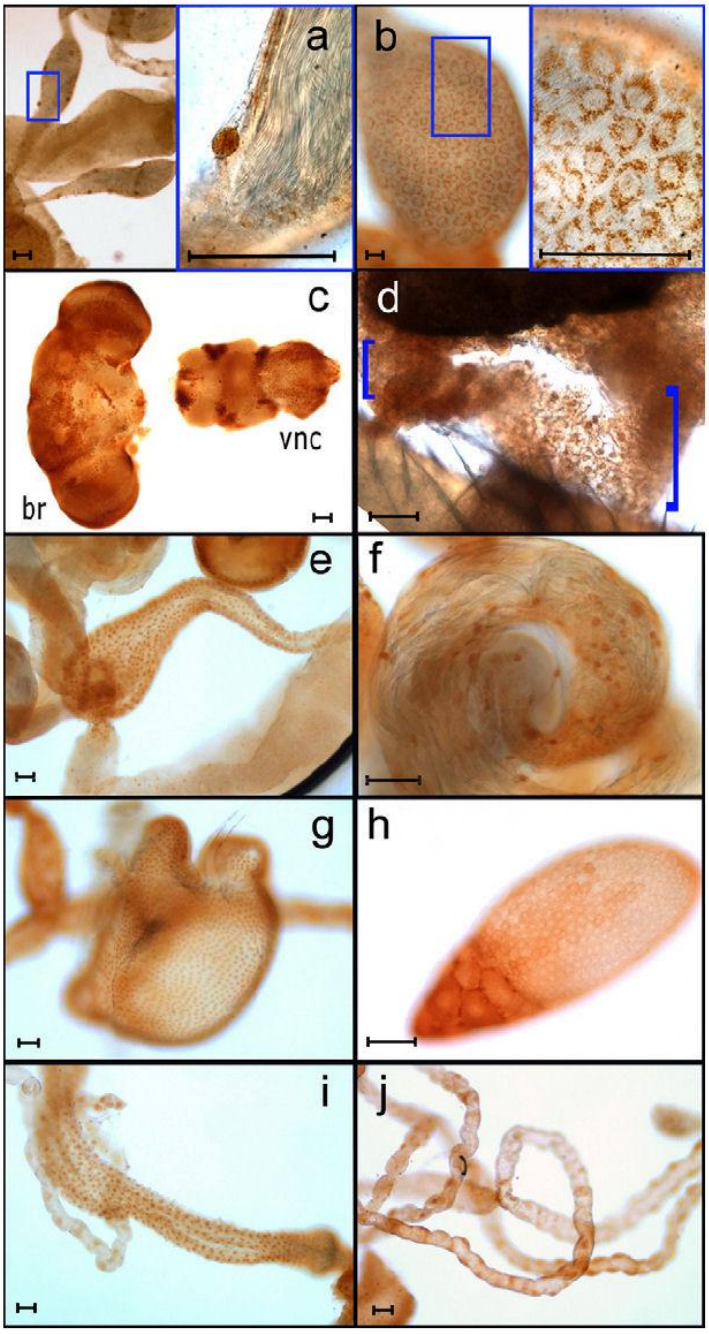

Figure 2. EcR protein expression in adult tissues. Nuclear EcR immunoreactivity (dark brown) is present in a variety of tissues. (a and b) seminal vesicles (10× and 100× inset); (c) central nervous system (10×); (d) abdominal cuticle fat (brackets) (20×); (e) ejaculatory duct (10×); (f) testes (20×); (g) ejaculatory bulb (10×); (h) developing egg (20×); (i) midgut (10×); (j) Malpighian tubules (10×). Panels (a, d, f, g and j) are 5-day tissues and panels (b, c, e, and i) are 10-day tissues. Panel (h) shows a stage 11 developing egg. Although the seminal vesicles in panels (a; 5 days) and (b; 10 days) have different EcR patterns, I observed both types of patterns with equal frequency in animals of both ages. 100*u*m scale bars are imbedded for reference.

2.3.2 Adult usp *expression*

EcR is only one component of the functional ecdysone receptor, which is comprised of both the EcR and the USP proteins. An antiserum for detection of USP protein is not available, but recent data indicate that *usp* is expressed in a variety of adult tissues (Chintapalli et al., 2007; Celniker et al., 2009). I was interested in understanding how *usp* varies over time in particular tissues. I used qPCR to assay *usp* expression relative to *ribosomal protein* (*rp49)* in tissues expressing EcR, including the CNS, abdominal cuticle, gut and male reproductive tissues. Since the *usp* gene does not contain introns, I verified that my mRNA preparations were free of genomic DNA contamination by PCR amplification across an intron in *rp49* (Figure 3). *usp* is present in EcR-expressing tissues (Table 6), and *usp* levels appear to fluctuate in some of these tissues but lack an identifiable trend. FlyAtlas data also indicate that *usp* as well as EcR transcripts are expressed in 7-day adults raised at 22 °C (Chintapalli et al., 2007), a time point similar to my 5 day. While the presence of transcripts does not guarantee protein I found it likely that functional EcR/USP ecdysone receptor dimers exist in these tissues.

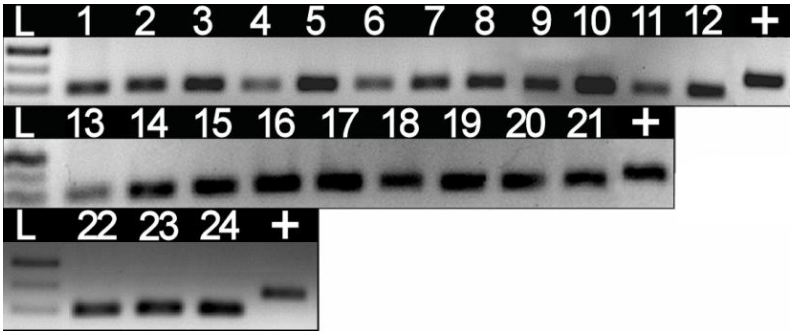

Figure 3. Confirmation that cDNA samples lack genomic DNA contamination by amplification of intron-containing *rp49*. Samples 1- 5 (0 day), accessory gland, ejaculatory bulb, ejaculatory duct, seminal vesicle, gut/tubule; 6-12 (5 day), CNS, testis, ejaculatory bulb, ejaculatory duct, seminal vesicle, gut/tubule, abdominal cuticle; 13-20 (10 day), CNS, testis, accessory gland, ejaculatory bulb, ejaculatory duct, seminal vesicle, gut/tubule, abdominal cuticle; 21 (0 day), CNS; 22-23 (0 day), testis, abdominal cuticle; 24 (5 day), accessory gland.

Table 6. *usp* expression during early adult life. Relative levels of *usp* expression with SEM values indicated. Abdominal cuticle tissues contain fat body, oenocytes, muscle and epidermis.

	0 Day	5 Day	10 Day
CNS	1.21±0.17	0.42±0.05	0.55±0.13
Abdominal Cuticle	0.40±0.04	9.03±2.60	1.09±0.29
Gut and Tubules	0.16±0.03	2.36±0.75	0.51±0.02
Testes	0.28±0.15	1.84±0.16	0.68±0.14
Seminal Vesicles	0.26±0.01	1.30±0.11	0.79±0.05
Male Accessory Gland	0.14±0.06	0.31±0.06	0.31±0.07
Ejaculatory Duct	0.36±0.05	0.19±0.01	0.22±0.06
Ejaculatory Bulb	0.27±0.12	0.21±0.05	0.38±0.09

2.3.3 Analysis of EcR's influence on adult behavior

The goal of the following work was to better characterize the influence specific isoforms of EcR as well as the tissues which require EcR for wild-type behavior. Removing a strong repressor most likely will cause a different response compared to removing a strong activator. The balance between EcR repression and activation during development is accentuated by massive changes in ecdysone concentration, but in adult tissues ecdysone level changes are much more subtle. For this reason I have focused on increasing our understanding on the behavior responses due to isoform-specific manipulations. Additionally the functional role of EcR in adult tissues is likely to vary and differentially influence behavioral responses to EcR manipulation.

My work expanded on existing investigations that used conditional *EcR* null mutants to quantifying behavioral response to reduction of EcR function (Carney and Bender, 2000; Ganter et al., 2007). I investigated male courtship behavior and female egg-laying as these phenotypes are of principal reproductive importance and had only received preliminary investigation. Using a combination of isoform-specific hypomorphic alleles, tissue-specific Gal4 drivers and a variety of UAS responders I attempted to identify tissues of major effect.

2.3.4 Egg-laying behavior response to adult EcR manipulation

Oogensis and female egg-laying behavior requires EcR function (Table 4), but the influence of individual isoforms remains a mystery. To address this gap in our knowledge I assayed the egg-laying abilities of conditional *EcR* mutants that affect expression of *EcR-A*, *EcR-B1*, or *EcR-B1* and *-B2*. Egg-laying behavior of 3-6 day females was then assessed at non-permissive temperatures. My hypothesis was that reduction of an *EcR* isoform necessary for egg-laying behavior would result in fewer eggs being laid. I found that females capable of only expressing *EcR-B1* and *EcR-B2* laid significantly fewer eggs (*t*-test P=0.002) than the controls (Figure 4). This phenotype was not observed in females that could express EcR-A or EcR-A and EcR-B2. As previously reported by Carney and Bender (2001), I was able to confirm that the egg-laying rates of females carrying lesions that affected all EcR isoforms had reduced egg laying compared to controls (*t*-test P<0.05). I found that EcR-A may provide a unique regulatory influence that is not functionally redundant with EcR-B1 and EcR-B2. To further understand this phenotype I needed to investigate the influence of EcR in specific adult tissues.

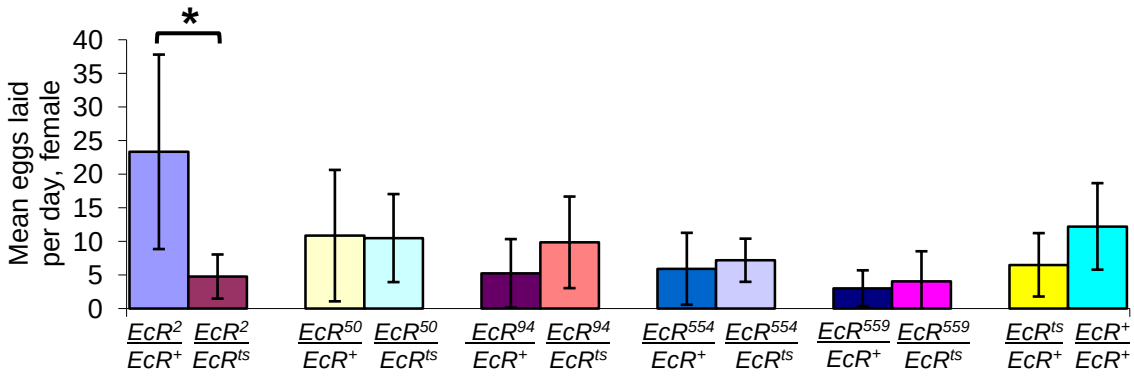

Figure 4. Female *EcR* mutant egg laying rates at 29°. Key for *EcR* mutations: EcR^{2}, allele with EcR-B1 and EcR-B2 lesion; EcR^{50}, allele with EcR-B1 lesion; EcR^{94}, allele with EcR-A lesion; EcR^{ts}, temperature sensitive hypomorph; EcR^{554} and EcR^{559}, alleles with early frame shifts that are EcR nulls. Adults sampled for each genotype n>10. * denotes *t*-test significance of P=0.002.

Using tissue-specific Gal4 drivers, I manipulated EcR functionality in the fat body and adult CNS with *UAS-EcRi* constructs that knockdown *EcR* or individual *EcR* isoforms. The fat body is the primary source of vitellogenin synthesis, a principal component of egg yolk, hemolymph, and an important tissue for regulating nutrient homeostasis (Roy et al., 2007; reviewed by Lemaitre and Hoffmann, 2007; Arrese and Soulages, 2009). Egg production is a nutrient demanding process that I predicted might be influenced by EcR function. I inhibited EcR by expressing *UAS-EcRi* constructs in the fat body of females and quantified their egg-laying rates for 3 days at 29°. Fat body *EcR-B1* knockdown resulted in increased egg laying rates (*t*-test P=0.028), while reduction of *EcR* or just *EcR-A* levels in fat did not influence egg laying rate (Figure 5). This contrasts with my findings that whole-body reduction of *EcR* leads to decreased egg laying. This suggests that egg-laying behavior is potentially positively and negatively influenced by *EcR* function in multiple tissues and that specific isoforms of EcR can impact egg-laying rates.

To further expand my investigation I manipulated EcR function in another tissue important for egg laying, the adult CNS. Genes in specific neurons of the CNS have been reported to influence egg-laying behavior (Monastiriotti et al.,

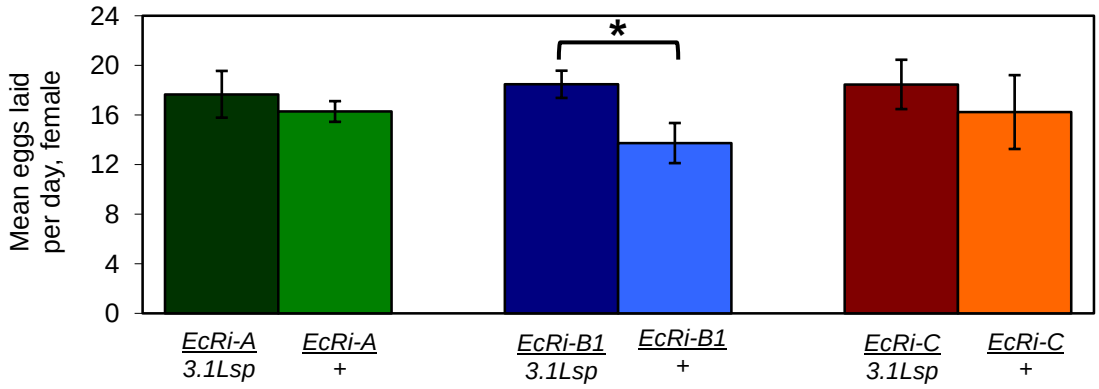

Figure 5. Activation of *EcRi* in the fat body of females influences egg laying. Adults sampled for each genotype n>10. * denotes *t*-test significance of P=0.028.

Table 7. *EcRi* expression in the female CNS does not influence egg-laying rate. Significance determined by Student's *t*-test.

Genotype	Eggs (29°)	n	*t*-test
c155-Gal4,Tub-Gal80ts,UAS-Dicer2, UAS-EcRi-A	14.0	11	
Tub-Gal80ts, UAS-Dicer2, UAS-EcRi-A	18.3	19	0.241
c155-Gal4, Tub-Gal80ts, UAS-Dicer2, UAS-EcRi-B1	16.9	12	
Tub-Gal80ts, UAS-Dicer2,UAS-EcRi-B1	23.4	13	0.180
c155-Gal4, Tub-Gal80ts, UAS-Dicer2, UAS-EcRi-C	14.9	22	
Tub-Gal80ts, UAS-Dicer2, UAS-EcRi-C	12.7	13	0.608

1996; Carney and Taylor, 2003; Boltz et al., 2007) so I predicted that EcR function in the CNS would affect oviposition. I expressed constructs that would activate RNAi targeting common regions of *EcR* or regions specific to *EcR-A* or *EcR-B1* in the CNS of adults. A component of the RNAi machinery, Dicer2, was co-expressed to strengthen the affect of this manipulation. On eclosion females were shifted to 29° to activate *EcRi* expression, aged 3 days and then mated. I found that after 3 days of quantifying egg-laying behavior that EcR function in the CNS does not significantly affect egg-laying rates (*t*-test P>0.24) (Table 7). This evidence suggests that EcR function is not required in adults for wild-type egg-laying behavior.

2.3.5 Male-male courtship behavior response to adult EcR manipulation

Male-male courtship was identified in males containing an *EcR* null and a temperature-sensitive hypomorph (Ganter et al., 2007). While this report identified that EcR is involved in regulating this behavior, we lack an understanding of what isoforms and what tissues caused this behavioral change. To address this, I investigated the male-male courtship levels of males lacking specific EcR isoforms. I was interested in the isoform-specific function of EcR that was responsible for inhibiting this male-male courtship. I expanded on the previous study by investigating the behavioral responses of males lacking the

functionality of specific isoforms of EcR in addition to reducing the function of EcR in specific tissue types.

I assayed male-male courtship in males carrying *EcR* null alleles as well as isoform-specific lesions for *EcR-A*, *EcR-B1*, or *EcR-B1*/B2. I expected that males carrying a combination of *EcR* mutant alleles would express increased levels of male-male courtship. Unfortunately, males mutant for *EcR-A*, *EcR-B1*, *EcR-B1/B2*, or all three forms of *EcR* showed no significant male-male courtship (Figure 6). When I could not reproduce the reported increase in male-male courtship I considered that perhaps modifiers had built up in our stocks. I out-crossed our mutant alleles to a wild-type background to reduce the influence of these predicted modifiers and repeated the analysis. Male-male courtship in these outcrossed *EcR* mutants was also not detectable (Figure 7).

The potency of each mutant allele was not in question because when raised at non-permissive temperatures these animals experience developmental arrest as expected. I took into account that many factors could potentially alter attributes of courtship and concluded that some environmental variable in our lab was preventing the *EcR* mutations from causing male-male courtship as was

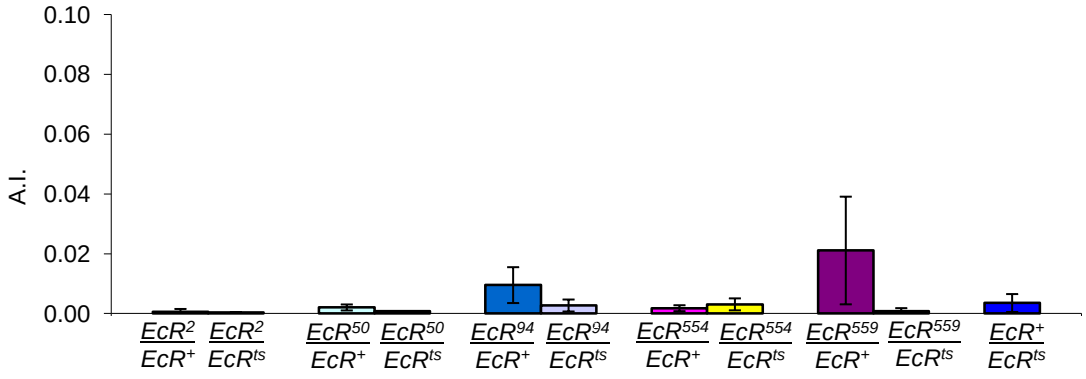

Figure 6. Male-male courtship of *EcR* mutants and controls. Key for *EcR* mutations: EcR^{2}, allele lacking EcR-B1 and EcR-B2; EcR^{50}, allele lacking EcR-B1; EcR^{94}, allele lacking EcR-A; EcR^{ts}, temperature sensitive hypomorph; EcR^{554} and EcR^{559}, alleles are EcR nulls due to early frame shifts.

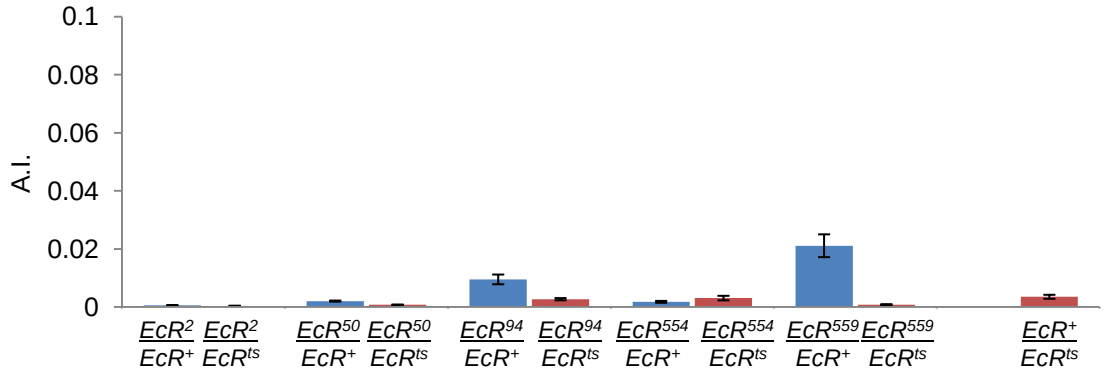

Figure 7. Male-male courtship levels of *EcR* mutant adults in a Cantonized background. Key for *EcR* mutations: EcR^{2}, allele lacking EcR-B1 and EcR-B2; EcR^{50}, allele lacking EcR-B1; EcR^{94}, allele lacking EcR-A; EcR^{ts}, temperature sensitive hypomorph; EcR^{554} and EcR^{559}, EcR nulls due to early frame shift.

previously reported. The influence of *EcR* on male-male courtship inhibition was most likely also due a function in a specific tissue which may be masked by a lack of functionality in other tissues. For this reason I shifted to characterizing the behavior of males using tissue-specific Gal4/UAS-based manipulations.

fru-neurons in the CNS strongly regulate male courtship behaviors (Manoli et al., 2005). Increased male-male courtship behavior was observed in *EcR* mutants (Ganter et al., 2007), potentially due to EcR's influence on this candidate tissue. To test this hypothesis, I expressed *EcRi* in the adult male CNS and assayed male-male courtship. However, I found that reduction of *EcR* function in the CNS of males did not result in increased male-male courtship (Table 8a). Perhaps EcR functions in another adult tissue to regulate this behavior.

A recent study found that male-male courtship can be influenced by genes expressed in the fat body (Lazareva et al., 2007), exemplifying that the fat body, in addition to the CNS, regulates reproductive behavior. My prediction was that a portion of the previously reported male-male courtship behavior (Ganter et al., 2007) was due to EcR function in the fat body. I targeted EcR function in the fat body using fat body-specific *EcRi* to assay EcR's influence on male-male

Table 8. Male-male courtship in response to adult *EcR* reduction. Reduction of *EcR* in adult neurons (a); fat body (b), gonad (c), and all tissues (d). Significance determined by Student's *t*-test.

	Manipulation	A.I. 29C	n	P-value
(a)	*c155-Gal4, Tub-Gal80ts, UAS-EcRi-A*	0.06	20	
	Tub-Gal80ts, UAS-EcRi-A	0.09	12	0.54
	c155-Gal4, Tub-Gal80ts, UAS-EcRi-B1	0.01	20	
	Tub-Gal80ts, UAS-EcRi-B1	0.18	18	0.001
	c155-Gal4, Tub-Gal80ts, UAS-EcRi	0.02	11	
	Tub-Gal80ts, UAS-EcRi	0.14	17	0.03
(b)	*3.1Lsp-Gal4 , Tub-Gal80ts, UAS-EcRi*	0.24	21	
	Tub-Gal80ts, UAS-EcRi	0.26	23	0.763
(c)	c805-Gal4, Tub-Gal80ts, UAS-EcRi	0.08	12	
	Tub-Gal80ts, UAS-EcRi	0.07	21	0.866
(d)	*Act-Gal4, Tub-Gal80ts, UAS-EcRi*	0.12	10	
	Tub-Gal80ts, UAS-EcRi	0.22	7	0.43
	Tub-Gal4, Tub-Gal80ts, UAS-EcRi	0.07	19	
	Tub-Gal80ts, UAS-EcRi	0.03	20	0.39

courtship behavior. Initially I found that fat body reduction of *EcR* resulted increase male-male courtship (Table 9a). Unfortunately, this phenotype was not robust and was not repeatable when I increased the sample size (Table 9b). I also targeted EcR function in the fat body after eclosion using *EcRi* in the expectation that male-male courtship would be increased, but a change in behavior was not identified (Table 8b). A more specific manipulation of EcR function involves the expression of EcR^{DN}. EcR^{DN} will mask the activity of endogenous EcR exclusively with repression and potentially block the expression of male-male courtship behavior inhibiting gene expression. I observed no change in male-male courtship behavior in adults expressing EcR^{DN} in the fat body (Table 10). This suggested that either this manipulation had not targeted a tissue that strongly influences behavior or that repression is the wild-type role of EcR in the adult fat body. If EcR in the fat body acts mainly as a repressor than

Table 9. Male-male courtship of 5 day adults with reduced *EcR* in the fat body. *UAS-EcRi-A* and *UAS-EcRi-B1* specifically target the degradation of *EcR-A* and *EcR-B1* respectively. Signifigance determined by Student's *t*-test.

	Genotype	A.I. 25°	n	*t*-test
(a)	*3.1Lsp-Gal4, UAS-EcRi*	0.34	23	
	UAS-EcRi	0.06	22	0.0001
	3.1Lsp-Gal4 , UAS-EcRi-A	0.27	34	
	UAS-EcRi-A	0.22	39	0.355
	3.1Lsp-Gal4 , UAS-EcRi-B1	0.17	35	
	UAS-EcRi-B1	0.10	44	0.102
(b)	* *3.1Lsp-Gal4, UAS-EcRi*	0.00	15	
	* *3.1Lsp-Gal4*	0.06	10	0.231

* Denotes a separate later assessment of male-male courtship behavior

a more potent manipulation would be to express a constitutively active allele of EcR, but this construct is unavailable.

Ecdysone receptors are broadly expressed in the gonad of adult males (Figure 2 and Table 6). The accessory glands of males produce potent behavior modifying peptides (Reviewed by Gillott, 2003). I wanted to quantify male behavior change in response to *EcR* reduction in the male testis and accessory glands. I did not identify any increase in male-male courtship in adults with reduced *EcR* in testis and accessory glands (Table 8c). Since reducing EcR in the main EcR-expressing tissues did not cause a male-male courtship phenotype, I reduced *EcR* in adults with ubiquitously expressed Gal4 drivers. Male-male courtship levels were expected to increase similar to previously reported *EcR* conditional mutants (Ganter et al., 2007). However, ubiquitous reduction of *EcR* in adult tissues did not result in increased male-male courtship (Table 8d). These findings suggest that the male-male courtship previously identified in EcR^{554fs}/EcR^{A483t} adults (Ganter et al., 2007) is dependent on unknown factors.

I did not identify consistent behavior change due to reduction of EcR function in adult tissues. Male courtship phenotypes that should have been

Table 10. Male-male courtship of 5 day mutant and control males expressing EcR-B2 or EcR^{DN} in the adult fat body. Signifigance determined by Student's *t*-test.

Genotype	A.I. (22°)	n	A.I. (29°)	n	*t*-test
UAS-EcR-A^{DN645}, Tub-Gal80^{ts}, 3.1Lsp-Gal4	0.17	6	0.28	10	0.55
UAS-EcR-A^{DN650}, Tub-Gal80^{ts}, 3.1Lsp-Gal4	0.19	20	0.19	20	0.99
UAS-EcR-B1^{DN645}, Tub-Gal80^{ts}, 3.1Lsp-Gal4	0.28	18	0.30	7	0.87
UAS-EcR-B1^{DN650}, Tub-Gal80^{ts}, 3.1Lsp-Gal4	0.39	4	0.26	3	0.65
UAS-EcR-B2^{DN650}, Tub-Gal80^{ts}, 3.1Lsp-Gal4	0.27	14	0.20	15	0.54
UAS-EcR-B2, Tub-Gal80^{ts}, 3.1Lsp-Gal4	0.24	9	0.23	5	0.96
3.1Lsp-Gal4, Tub-Gal80^{ts}	0.29	18	0.22	21	0.45
EcR-A^{DN650}	0.19	20	0.21	14	0.85

observed in *EcR* mutants could not be confirmed but reduction of ecdysone levels could provide the same effect. I assayed the behavior of *ecd1* mutants and wildtype adults exposed to 1mM ecdysone enriched food to identify if these methods would produce the behavior change that was previously reported in *EcR* mutants. Increased male-male courtship relative to controls was not observed in *ecd^{1}* (P=0.81) and ecdysone fed (p=0.41) males. This data suggests that the male-male courtship reported in Ganter et al., (2007) and (2011) are phenotypes that are, at the very least, difficult to reproduce.

2.4 Discussion

Contrary to our understanding of ecdysone/EcR/USP function during development, our understanding of ecdysone/EcR/USP function in adult tissues is still in its infancy. To address this gap in our understanding I showed that ecdysone receptors are broadly expressed in adult tissues. Egg-laying behavior was influenced by EcR-A expression in adults and EcR-B1 in the adult fat body, but not by EcR expression in the CNS. Male-male courtship behavior was not increased due to manipulation of EcR function in any of the adult tissues tested.

As stated in Section 1.2, EcR/USP can have multiple influences on ecdysone-responsive gene expression. The transcriptional activity of EcR/USP in the fat body and other adult tissues remains a mystery. Though I was able to

identify tissue-specific roles for EcR in regulating the previously identified egg-laying phenotype, to further understand the role of EcR in adults I needed to better characterize EcR's transcriptional role in the affected tissues.

It remains unclear why my analysis of male-male courtship was unable to identify behavior levels similar to those published. However, male-male courtship previously identified in *EcR* mutants (Ganter et al., 2007) may be specific to the fly line that was used as courtship targets. *Canton-S* males were used in both Ganter's study as well as my own, but these stocks may contain subtle differences in cuticle hydrocarbons or other identifying attributes after years of genetic isolation. While most behavior studies assay courtship in courtship chambers without food (Dauwalder et al., 2002), Ganter's study assayed male behavior in chambers with food present. However, a later study by Dalton et al., (2009) found increased male-male courtship in adults lacking EcR in *fru*-neurons in chambers lacking food suggesting that the presence or absence of food during the courtship assay is not a factor. Ganter et al. (2007) used a temperature-sensitive allele of *EcR* to reduce function in adults. One possibility is that this allele is leaky and also reduces *EcR* function to some extent during development. In this case, it may be pre-adult reduction of *EcR* that led to the effects on male-male courtship described by Ganter et al. (2007).

In parallel to my work, recent studies in other labs have identified functions for EcR and ecdysone in adult reproduction, behavior, learning, aging, and stress resistance (Dalton et al., 2009; Ishimoto et al., 2009; Tricoire et al., 2009, Ganter et al., 2011), yet these studies provide a fragmented understanding of endocrine involvement during adult life. After multiple attempts to characterize tissues of major effect, it became clear that in order to make better predictions as to how EcR was influencing adult behavior I needed to characterize ecdysone-responsive gene expression in adult tissues. The goal of my investigation shifted to characterizing endogenous ecdysone receptor regulated gene expression in tissues relevant to adult *EcR* mutant phenotypes. This information will provide a

more comprehensive understanding of adult endocrine activity and identify EcR's transcriptional role in adult tissues of interest.

CHAPTER III
ADULT ECDYSONE RECEPTOR ACTIVITY*

3.1 Introduction

Ecdysone content of adult tissues was observed to be maintained at 5-25pg/mg (Table 2 and 3) and ecdysone receptors were detected in many adult tissues (Figure 2 and Table 6). I hypothesized that endogenous activity of these receptors in wild-type adults would be relatively low in most adult tissues. Previous studies focusing on ecdysone-regulated genes during development and metamorphosis utilized an *EcRE-LacZ* construct as a reporter for EcR/USP-dependent gene expression *in vivo* (Kozlova and Thummel, 2003). *EcRE-LacZ* contains seven EcR/USP binding motifs from the *Hsp27* promoter fused to the *β-galactosidase* gene (Koelle et al., 1991). This construct is useful for assaying EcR/USP regulated gene expression under various environmental conditions because it is regulated by endogenous EcR/USP, does not require heat shock or exogenous stimuli for activation, and does not cause detectable morphological defects or lethality. I characterized EcR/USP regulated gene expression in early adult tissues using the *EcRE-LacZ* reporter.

3.2 Methods

3.2.1 EcRE-LacZ *detection*

Adults containing *EcRE-LacZ* reporter were aged 0, 5, or 10 days at 25 °C and then dissected in PBS on ice, fixed in 4% paraformaldehyde for 10 min, washed with PBST, and incubated for 1-2 h in reaction buffer consisting of 0.2% X-gal (Invitrogen) in PBS containing 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, and 2.5 mM magnesium chloride. In each experiment flies lacking the *EcRE-LacZ* reporter construct were used as negative controls,

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and white pre-pupae from the *EcRE-LacZ* strain were used as positive controls. Only those tissues containing robust reporter expression throughout the tissue were counted as positive for activity if corresponding control tissues were negative.

3.2.2 Ecdysone feeding

EcRE-LacZ adults were aged for 5 days post eclosion on standard media at 25 °C then transferred to vials containing a film of yeast paste containing 0 mM, 0.01 mM, 0.1 mM, or 1.0 mM 20-hydroxyecdysone and red food dye (Simon et al., 2003). Presence of red dye in the guts of flies indicated that the control or ecdysone-containing food was consumed. Adults were aged on this media for 5 additional days and then dissected and assayed for β-gal protein content 10 days post eclosion as described in Section 3.2.1.

3.2.3 Ecdysone tissue culture

Mid third instar larvae and 10 day *EcRE-LacZ* adults were dissected in chilled complete medium composed of Schneider's insect medium, penicillin/ streptomycin, and fetal bovine serum solution (Sigma) then incubated for 1 h at 25 °C. Tissues were transferred to fresh medium containing 0 μM, 5 μM, or 10 μM 20-hydroxyecdysone (Alexis Biochemical) and incubated at 25 °C for an additional 5 h as previously described (Hackney et al., 2007; Kozlova and Thummel, 2002). Cultured tissues were assayed for reporter activity in X-gal solution as described in Section 3.2.1. In additional experiments I extended the incubation and reaction periods for up to 18 h each.

3.2.4 EcRE-LacZ *expression in response to stress*

Reporter response to acute heat stress (36 °C for 4–6 h), dry starvation (empty vial for 18 h at 25 °C), or mating on day 4 was assayed in 5-day *EcRE-LacZ* male and female adults. The age of the animals tested is consistent with

those of Simon et al. (2003), which reported that *EcR/+* animals have increased longevity and stress resistance. This time point is also a good choice because *EcRE-LacZ* expression is low or absent in many tissues (exceptions include gut and male accessory glands, see Table 11), so stress-induced reporter expression increases would be readily detectable. Relative levels of reporter signal were assayed using methods described in Section 3.2.1, and treated animals were compared to controls that did not mate or experience a stress condition.

3.2.5 Inhibition of EcRE-LacZ *expression*

A dominant-negative form of EcR (referred to as EcR^{DN}) (*UAS-EcR.B2.F645A*, Hu et al., 2003) was used to repress expression of *EcRE-LacZ*. EcR^{DN} is an allele of EcR that is not responsive to ecdysone and does not induce EcR/USP regulated gene expression. When overexpressed, EcR^{DN} masks the function of endogenous EcR and function only as a repressor. Adults of genotype *w;act-Gal4/CyO;tub-*$Gal80^{ts}$ were crossed with *w;EcRE-LacZ;UAS-*EcR^{DN} at 25 °C in vials for 1–2 days. These vials were then incubated at 20 °C for the duration of development. Newly eclosed adults of genotype *w;act-Gal4/EcRE-LacZ;tub-*$Gal80^{ts}$*/UAS-*EcR^{DN} were aged for 5 days at either 20 °C or 29 °C. At 20 °C *UAS-*EcR^{DN} is not expressed due to the ability of $Gal80^{ts}$ to inhibit Gal4 activation. Incubation at 29 °C inhibits $Gal80^{ts}$ functionality, allowing expression of EcR^{DN}. Control *w;EcRE-LacZ/CyO;tub-*$Gal80^{ts}$*/UAS-*EcR^{DN} were collected and aged for 5 days at 29 °C. This group serves as a temperature control for *EcR-LacZ* activity at 29 °C under conditions in which EcR^{DN} is not expressed. On day 5 animals from each of the 3 groups were dissected for X-gal staining as described in Section 3.2.1.

3.3 Results

3.3.1 Adult EcRE-LacZ *expression*

To identify adult tissues with active EcR/USP, I assayed *EcRE-LacZ* reporter expression in 0-, 5-, and 10-day adult tissues. Reporter expression was not ubiquitous, but was specific to tissue type and age (Table 11 and Figure 8). *EcRE-LacZ* expression in the Malpighian tubules and male accessory glands was observed in the majority of tissues examined at all three time points. The CNS, abdominal cuticle fat, and seminal vesicles rarely had detectable EcR/USP activity after day 0, whereas adult oenocyte *EcRE-LacZ* expression increased after day 0. Within the first few hours of eclosion *EcRE-LacZ* expression was observed in the testes, but by day 5 activity was absent. The ejaculatory duct and ejaculatory bulb varied in *EcRE-LacZ* expression patterns throughout the time period assayed. *EcRE-LacZ* expression was also detected in the follicle cells of developing eggs, including follicle cells present in the nurse cell compartment, but not the nurse cells themselves (Figure 8e). Regression analyses indicated that significant ($p < 0.05$) age-dependent changes in expression occurred in the CNS, fat, oenocytes, Malpighian tubules, seminal vesicles, and ejaculatory ducts.

Table 11. *EcRE-LacZ* expression in various tissues during early adult life. *EcRE-LacZ* tissues were assayed for β-galactosidase activity. The percentage of positive tissues is indicated for each tissue type with sample sizes are in parentheses.

Adult Tissue	0 Day		5 Day		10 Day	
CNS	92%	(13)	12%	(17)	0%	(9)
Fat	87%	(15)	7%	(56)	10%	(28)
Oenocytes	0%	(15)	7%	(56)	79%	(28)
Gut	83%	(18)	48%	(27)	55%	(11)
Malpighian Tubules	100%	(24)	45%	(22)	100%	(10)
Testes	14%	(14)	0%	(14)	0%	(11)
Seminal Vesicles	62%	(13)	18%	(11)	18%	(11)
Male Accessory Gland	83%	(12)	63%	(19)	81%	(16)
Ejaculatory Duct	75%	(16)	11%	(19)	75%	(12)
Ejaculatory Bulb	17%	(12)	0%	(14)	22%	(11)

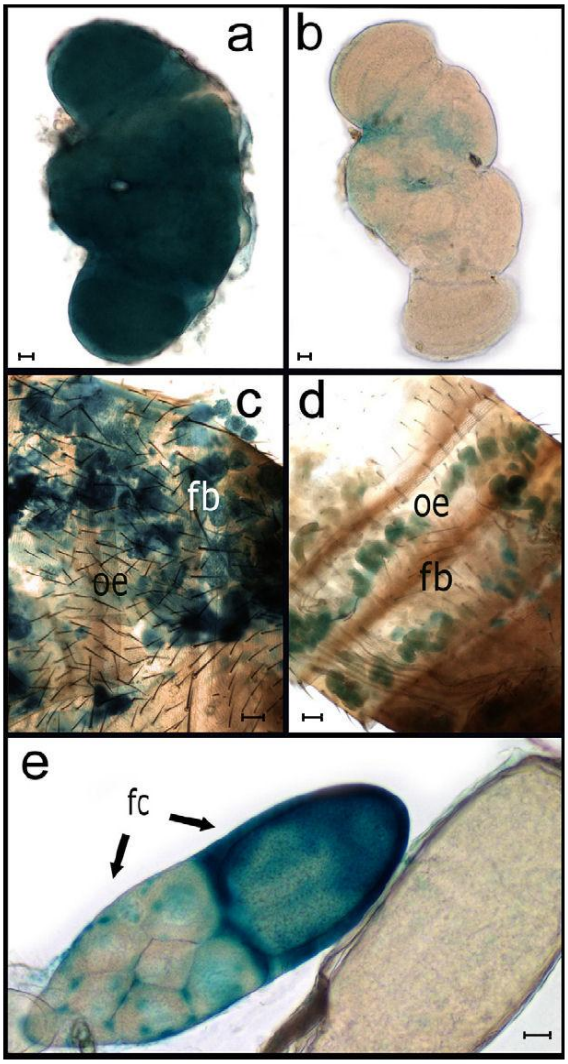

Figure 8. *EcRE-LacZ* expression in adult tissues. Representative images of β-galactosidase activity (blue) in adult *EcRE-LacZ* tissues. (a) 0-day brain (10×); (b) 10-day brain (10×); (c) 0-day abdominal cuticle with fat body (fb) and oenocytes (oe) (10×); (d) 10-day abdominal cuticle (10×); (e) follicle cells (fc) of developing and mature eggs (20×). Images contain 100*u*m scale bars.

Significant expression differences were not detected in gut, testes, male accessory glands or ejaculatory bulbs. Overall, these results differed from my expectation that reporter activity in adult EcR/USP expressing tissues would decline in concert with the falling adult ecdysone titers reported previously (Handler, 1982).

EcR heterozygous mutants (*EcR/+*) are resistant to heat and dry starvation stressors (Simon et al., 2003), so I investigated *EcRE-LacZ* expression in response to stress conditions. I compared *EcRE-LacZ* expression in adults experiencing dry starvation or heat stress to non-stressed controls to determine if there was an endocrine response to these conditions. *EcRE-LacZ* expression in 5-day adult tissues was not affected by heat (n = 10) or dry starvation (n = 8) (data not shown). There was no difference in the identity and proportion of tissues with *EcRE-LacZ* expression nor was there a detectable difference in the level of activity in stressed animals compared to controls.

Mating elevates ecdysone levels and alters ecdysone localization in mature adult females (Harshman et al., 1999). Therefore I expected that mating would change the pattern of EcR/USP activity. However, I did not detect increases in *EcRE-LacZ* expression in any tissues from 5-day mated adults (n = 21). My results suggest that if EcR/USP-mediates a cellular response to these stressors or to mating, it occurs in the gut or Malpighian tubules where increases in *EcRE-LacZ* expression would be harder to detect rather than in the CNS or fat body where *EcRE-LacZ* expression was absent.

3.3.2 EcR regulation of adult EcRE-LacZ *expression*

I used the inducible EcR dominant-negative (EcR^{DN}) construct *UAS-EcR-F645A* to block EcR/USP activity to confirm that adult *EcRE-lacZ* reporter activity is dependent upon EcR/USP activation. The product of *EcR-F645A* dimerizes with USP and binds DNA and ligand but does not activate transcription (Cherbas et al., 2003). Tissue-specific expression of *EcR-F645A* causes developmental defects that can be rescued by overexpressing EcR (Cherbas et al., 2003). I limited expression of *UAS-EcR-F645A* to adult tissues using $Gal80^{ts}$, which prevents Gal4-mediated expression at the non-permissive 20 °C. Five-day adults containing the *EcRE-LacZ* reporter and *UAS-EcR-F645A* under control of the ubiquitously expressing *act-gal4* were assayed for reporter activity at the

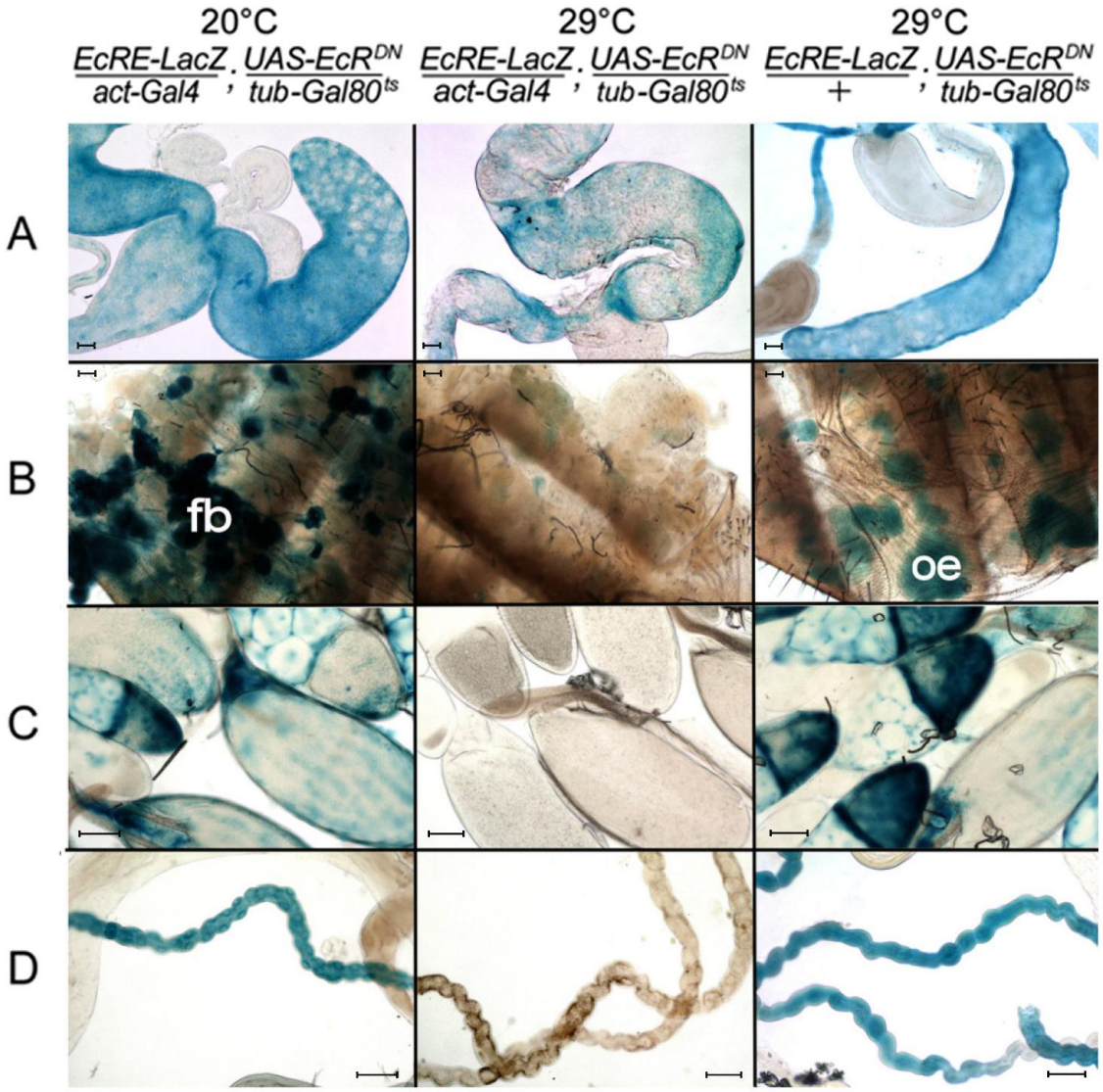

Figure 9. *EcRE-LacZ* expression in response to adult EcR^{DN} expression. β-Galactosidase activity (blue) in adult *EcRE-LacZ* tissues was reduced at 29 °C in adult tissues expressing EcR^{DN} (center column). Genotypes: *EcRE-LacZ/act-Gal4;UAS-EcR^{DN}/tub-Gal80^{ts}* at 20° (left column), *EcRE-LacZ/act-Gal4;UAS-EcR^{DN}/tub-Gal80^{ts}* at 29° (middle column), *EcRE-LacZ/+;UAS-EcR^{DN}/tub-Gal80^{ts}* at 29° (right column), (A) Accessory glands; (B) abdominal cuticle; (C) egg; and (D) Malpighian tubules (all 10x). In the rightmost panels the *UAS-EcR^{DN}* construct is present but is not activated at 29 °C because *act-Gal4* is not present in these animals. fb, fat body; oe, oenocytes. Images contain 100*u*m scale bars.

permissive or non-permissive temperatures. Reporter activity was reduced or absent at 29 °C in male accessory glands, oenocytes, developing eggs, and

Malpighian tubules (Figure 9, middle column). However, reporter activity was high in these tissues when EcR^{DN} was not activated (Figure 8, Table 11, Figure 9 left and right columns). Activity was detected in fat tissue from animals containing EcR^{DN} raised at 20 °C (no EcR^{DN} expression) but not in those lacking EcR^{DN} and raised at 29 °C (Figure 9b, left and right columns respectively). This EcR/USP activity is consistent with my results showing that fat activity decreases with physiological maturity (Table 11) since adult maturation rates at 29 °C would be accelerated by the increased temperature. In contrast, oenocyte activity increased with physiological maturity (Table 11), and *EcRE-LacZ* expression was detected in oenocytes from animals raised at 29 °C that lack EcR^{DN} expression (Figure 9b, rightmost column); this activity was dramatically reduced in 29 °C animals expressing EcR^{DN} (Figure 9b, middle column). These results indicate that the characterized *EcRE-lacZ* reporter expression in adults requires EcR/USP activity.

3.3.3 Ecdysone feeding

Though it appears that most tissues express ecdysone receptors, it remained unclear what components of the ecdysone signaling system determine which tissues contained active EcR/USP. My initial hypothesis was that receptor-expressing adult tissues with little or no detectable EcR/USP activity have lower sensitivities to hormone. Therefore, I expected that increasing circulating hormone levels would increase EcR/USP activity.

To test this hypothesis, I assayed changes in EcR/USP activity in *EcRE-LacZ* adult tissues from 10-day animals that had been fed a diet supplemented with 20-hydroxyecdysone for 5 days starting on day 5 post eclosion. I began feeding at day 5 because tissues such as CNS, fat, seminal vesicles and testes have little or no EcR/USP activity by day 5. By day 10, activity is fully eliminated in the CNS and is low in many other tissues in unfed control animals, simplifying my ability to determine if increased levels of ecdysone activate receptors during

this period. I found that adults feeding on 0.01 mM (n = 14), 0.1 mM (n = 16) nor 1 mM (n = 23) ecdysone supplemented food did not have detectably increased EcR/USP activation in the CNS, abdominal cuticle fat, seminal vesicles, or testes (data not shown). These same concentrations of ecdysone supplement were reported to increase adult ecdysone levels over a multi-day period (Simon et al., 2003). My findings suggest either that supplemental ecdysone did not reach the tissues of interest or ecdysone was inactivated in some adult tissues, preventing EcR/USP activation.

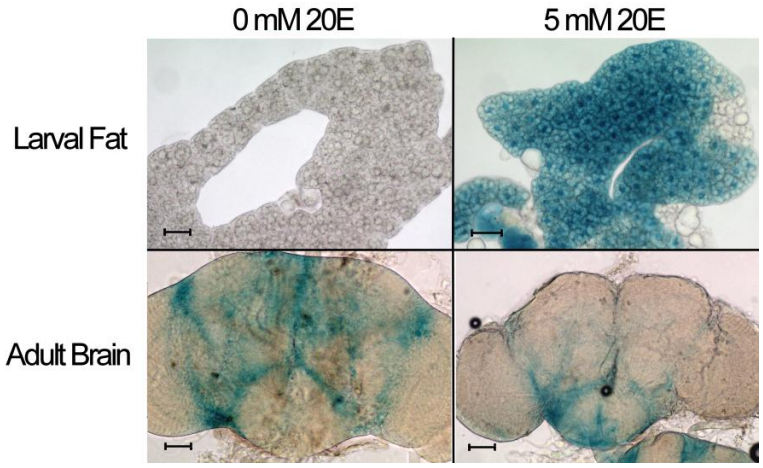

Figure 10. *EcRE-LacZ* expression in larval and adult tissues in response to ecdysone (20E) incubation. Larval fat tissue was dissected from wandering 3rd instar larvae, adult brains were dissected from 10 day adult virgins. Images contain 100*u*m scale bars.

3.3.4 Ecdysone tissue culture

To ensure that supplementary ecdysone reached tissues that contain EcR/USP receptors, I incubated dissected tissues in standard medium or in medium containing 5 µM ecdyone. Third instar larval tissues or CNS, fat body, and male gonad tissues from 10-day *EcRE-LacZ* adults were incubated in medium for 5 h and then assayed for EcR/USP activity by X-gal staining.

EcR/USP was activated in 3rd instar larval tissues incubated with ecdysone (n = 7) (Figure 10). However, adult CNS, abdominal cuticle fat, seminal vesicles, or testis tissues (n = 21) incubated in medium containing ecdysone did not show reporter activation (Figure 10). Reporter activity was not detected even when these tissues were incubated with 10 μM ecdysone for longer periods (up to 18 h) or when the enzymatic development period was extended to 18 h. Since precocious receptor activation occurred in ecdysone-treated larval tissues, these observations suggest that adult *EcRE-LacZ* expression is not solely dependent on tissues having access to ecdysone but upon some other mechanism.

3.4 Discussion

3.4.1 Differential EcRE-LacZ *expression in adult tissues*

Somewhat surprisingly, I regularly observed EcR/USP activity in many tissues (Table 11) despite low levels of ecdysone in *D. melanogaster* adults (Bownes et al., 1984). EcR proteins were present in numerous male and female adult tissues (Figure 2). *usp* also was present in these EcR-expressing adult tissues (Table 6) suggesting that functional ecdysone receptors are present. Fluctuations in *usp* expression were detected in adult tissues but differences in *usp* abundance were not useful predictors of change in *EcRE-LacZ* expression over time. My results are consistent with those from other studies assessing *EcR* and *usp* expression (Chintapalli et al., 2007; Celniker et al., 2009). FlyAtlas data, based upon microarray analysis of dissected tissues, indicate that *usp* expression is consistently higher than *EcR* expression in adult tissues (Chintapalli et al., 2007). Therefore, *usp* may not be the limiting factor for functional receptor complexes in adults. Since we do not know the half-life of EcR or USP in adults, I currently cannot determine how changes in mRNA levels may affect protein abundance and EcR/USP activation.

3.4.2 Regulation of EcRE-LacZ *expression in adults*

The activity patterns of EcR/USP varied by tissue type and changed with age in many tissues. I did not detect an adult endocrine response to stress, mating, or ecdysone treatment by assaying *EcRE-LacZ* expression, suggesting that these conditions do not detectably influence *EcRE-LacZ* expression. Initially I suspected that USP availability could be limiting *EcRE-LacZ* expression since I regularly detected EcR protein in adult tissues for all three time points. *usp* also was expressed in all of the tissues examined, although the levels fluctuated. However, changes in *usp* expression did not correlate with *EcRE-LacZ* expression over time, suggesting that these changes do not fully account for altered *EcRE-LacZ* expression. Furthermore, ecdysone incubation reliably activated EcR/USP in larval tissues but consistently failed to detectably increase *EcRE-LacZ* expression in adults. As a consequence of these findings, I hypothesize that adult *EcRE-LacZ* expression is regulated by additional, unidentified factors.

EcR/USP activation of gene expression requires recruitment of coactivators such as Taiman (Tai), Trithorax-related (Trr), and Dek (Bai et al., 2000, Sedkov et al., 2003 and Sawatsubashi et al., 2010). While a complete adult characterization of these components is unavailable, *tai*, *trr*, and *Dek* transcripts are detected in all analyzed adult tissues obtained from adults raised at 22 °C for 7 days (Chintapalli et al., 2007). (These animals are roughly equivalent to my 5-day animals raised at 25 °C.) Transcripts are present in the CNS, fat body, gut, and male reproductive tissues, although testis expression of all three genes is slightly lower (Chintapalli et al., 2007).

Conversely, in an inactive conformation EcR/USP recruits corepressors to down regulate gene expression. SMRTER (Tsai et al., 1999) and ALIEN (Dressel et al., 1999) are two nuclear receptor corepressors that interact with EcR, and their transcripts are also expressed at moderate levels in all adult tissues examined (Chintapalli et al., 2007). Together these data suggest that observed

tissue-specific differences in EcR/USP activity are not due to variation in availability of coactivators and corepressors.

3.4.3 Adult ecdysone signaling

Adult ecdysone levels in Drosophila are relatively low and less dynamic compared to the fluctuations experienced in metamorphic pupae or autogenous insect adult females, such as mosquitoes. The ovaries of female mosquitoes increase ecdysone levels to activate oogenesis shortly after feeding on a blood meal (Hagehorn et al., 1975). *D. melanogaster* females produce ecdysone in the ovaries (Bownes et al., 1984) which I detected as EcR/USP activity in follicle cells that diminished as eggs matured (Figure 8). *EcRE-LacZ* expression in the follicle cells, but not the nurse cells, of late stage eggs is consistent with previously reported observations of EcR expression (Carney and Bender, 2000; Hackney et al., 2007).

The accessory glands of adult male Aedes (mosquito) are also ecdysteroidogenic, but the ecdysone produced in these tissues is transferred to the female during copulation to further stimulate the production of eggs in the female (Pondeville et al., 2008). Male *D. melanogaster* have not been reported to produce or store ecdysteroids in the accessory glands, but my reporter data indicated that *EcRE-LacZ* expression is robust in this tissue, suggesting that either the male accessory glands are ecdysteroidogenic or are able to activate EcR/USP with sequestered ecdysone.

I did not detect an adult endocrine response to stress, mating, or ecdysone treatment by assaying *EcRE-LacZ* expression. Tissues that did not have *EcRE-LacZ* expression remained inactive after treatment and tissues containing active EcR/USP remained active with no detectable increase in activity. I expected that increased levels of ecdysone found in mated females (Harshman et al., 1999), would result in an increase in *EcRE-LacZ* expression in at least one female tissue. Since I did not detect increased *EcRE-LacZ*

expression in mated females, it is likely that the increased ecdysone in these females is used mainly by the ovary to augment egg production.

Additionally, EcR/+ heterozygotes are resistant to stressors such as dry heat and starvation (Simon et al., 2003). This effect was hypothesized to be due to either reduced repression or decreased activation of *EcRE*-containing genes. My experiments indicate that these stress conditions do not visibly affect *EcRE-LacZ* expression in wild-type animals, suggesting that the stress resistance observed in adult EcR/+ mutants is related to effects on EcR developmental functions or is due to adult EcR function in tissues such as the gut where I detect the most variation in *EcRE-LacZ* expression.

Modified ecdysteroids have altered activational properties and mobility. Perhaps the Drosophilla orthologues to the ecdysone modifiers Eckinase (Eck) and ecdysone-22-phosphate (E22P) (Sonobe et al., 2006) discussed in Section 1.2.2 are important regulators of adult ecdysone responsive gene expression. Functional homologs for Eck and EPP in Drosophila have not been identified, but my BLAST searches (Altschul et al., 1997) indicate that CG1561 (26% identity and 44% similarity), CG13813 (23% identity and 42% similarity), and CG31974 (25% identity and 41% similarity) encode proteins that share high sequence similarity with Eck, and the gene product of CG13604 (38% identity and 58% similarity) shares high similarity with EPP.

These two enzymes allow for compartmentalized storage, inactivation, and controlled activation of an otherwise freely circulating steroid. Tissues expressing Eck could inactivate ecdysone signals and reduce the sensitivity of that tissue to *EcRE-LacZ* expression without having to restrict EcR or USP expression. This would result in a tissue that is ecdysone resistant and reduce the impact of potentially disruptive EcR/USP activation. Conversely, tissues which express EPP would be able to reactivate E22P and use the resulting ecdysone to activate EcR/USP. Tissues expressing EPP should have more robust EcR/USP activity than tissues expressing Eckinase. Further investigation

into the function of these genes in D. melanogaster is required to determine if they have ecdysone modification functionality and if they contribute to the observed tissue-specific changes in EcR/USP activity.

3.4.4 EcR/USP activity and function in adult tissues

EcR protein is extensively expressed in adult CNS where it may function to potentiate memory formation and recall (Ishimoto et al., 2009). Specific *fruitless*-expressing neurons require EcR to regulate aspects of male courtship (Dalton et al., 2009). Interestingly, reduction of EcR by RNAi in *fruitless*-expressing neurons caused an increase in male–male courtship while adult-only expression of EcR^{DN}, a nonfunctional EcR allele that binds EcREs but cannot be activated by hormone, did not (Dalton et al., 2009). I found that EcR/USP present in the brain is rarely active after eclosion (Table 11), suggesting that male courtship circuit development initiated prior to eclosion requires EcR/USP activation, while EcR/USP represses gene expression in the adult CNS.

Drosophila fat tissue modulates behavior, metabolism, appetite, immunity, and female yolk protein synthesis (Lazareva et al., 2007; Roy et al., 2007; reviewed by Lemaitre and Hoffmann, 2007; Arrese and Soulages, 2009). EcR reduction in male fat and gut tissues increases lifespan, while expressing EcR^{DN} has no effect (Tricoire et al., 2009). Conversely, no change in female lifespan is observed when either *EcRi* or EcR^{DN} is expressed in the fat (Tricoire et al., 2009). The fat body of both males and females expressed EcR and *usp*, but EcR/USP activity was rarely detected in fat body shortly after eclosion (Figure 2, Figure 8c,d and Table 6, Table 11). These results suggest that EcR/USP represses genes in male fat body which influence longevity. Deregulation of male gene expression due to reduced numbers of EcR/USP complexes may be responsible for observed differences in lifespan between males with wild-type or reduced EcR.

The male accessory glands contain EcR protein and were consistently positive for EcR/USP activity throughout early adult life (Figure 2 and Table 11). This tissue produces many of the components present in the male ejaculate that are responsible for initiating the female post mating response (Ram and Wolfner, 2007). However, male *EcR* mutants do not experience detectable deficits in fertility or fecundity (Ganter et al., 2007). A recent report showed that the accessory gland lumen in the male Anopholes mosquito is enriched with ecdysone (Pondeville et al., 2008). Ecdysteroids in the Anopholes ejaculate are transferred during copulation, functioning as allohormones to stimulate oogenesis in the mated female. While this functionality has not been reported for the male accessory glands of D. melanogaster it is interesting to note that this tissue contains active EcR/USP during all three time points tested.

Adult oenocytes also expressed EcR and developed robust EcR/USP activity by 10 days after eclosion. This tissue facilitates lipid conversion and hydrocarbon synthesis (Diehl, 1975) and is required for species and mate recognition. Drosophila with genetically ablated oenocytes elicit strong courtship from wild-type flies due to the elimination of many species and gender-specific courtship-inhibiting pheromones (Billeter et al., 2009). EcR function is probably not necessary for sex-specific pheromone regulation since EcR mutations do not alter male or female attractiveness (Ganter et al., 2007). Continued investigation is required to identify the function of EcR protein and age-dependent increases in *EcRE-LacZ* expression in oenocytes.

3.4.5 Summary

I characterized wild-type ecdysone receptor expression and activity in individual adult insect tissues during the early adult life stage. Many tissues expressed EcR and *usp*, but *EcRE-LacZ* expression was observed in only a subset of adult tissues. Those tissues in which EcR/USP was not active may require repression of EcR/USP-regulated genes for wild-type functionality since I

did not activate ecdysone receptors with any of multiple environmental and pharmacological stimuli. While environmental conditions can lead to endocrine responses in other taxa (reviewed by Denver, 2009), I found that adult *EcRE-LacZ* expression is unresponsive to exogenous ecdysone treatment, stress, or mating status. However, adult age is influential in determining *EcRE-LacZ* expression in some tissues, though the mechanisms involved in shifting receptor activity remain a mystery. My results indicate that ecdysone signaling in adult Drosophila is regulated in a complex manner potentially involving localization of hormone, modification of hormone activity and mobility, regulation of complementary transcriptional elements, and regulatory protein interactions with EcR/USP. Further investigation of this endocrine system should yield clues necessary to understand how ecdysone signaling in the adult contributes to maintaining homeostasis and help identify mechanisms responsible for the previously reported adult *EcR* mutant phenotypes.

CHAPTER IV
THE FAT BIASED GENE *fit* INFLUENCES ADULT FEEDING AND NUTRIENT HOMEOSTASIS IN *Drosophila melanogaster*

4.1 Introduction

Given the extremely high rates of obesity in the United States and across the world, it is imperative to understand more about signaling pathways that regulate appetitive behaviors as well as nutrient metabolism and storage. The effective assessment of nutrient demands and reserves, mobilization of nutrients, and regulation of feeding behaviors requires the coordinated function of adipose and nervous system tissues. Adipose tissue serves as a repository for fat and glycogen stores, but it also is an endocrine gland that produces and releases factors known as adipokines that affect a variety of physiological processes such as neural circuit development, hormone levels, and central nervous system (CNS) response to environmental signals. For example, adipokines such as leptin and adiponectin modulate inflammation, reproduction, obesity, and motivated eating behavior (Reviewed by Bouret and Simerly, 2007; Lago et al., 2007; Pan and Kastin, 2007). Many problems affecting appetite control and nutrient regulation stem from aberrations in signaling between the CNS and adipose tissue.

The fat body of the model organism *Drosophila melanogaster* is analogous to vertebrate white adipose tissue and is regulated by conserved mechanisms (Reviewed by Baker and Thummel, 2007; Teleman, 2010). The adult fat body is a loosely organized sheath of cells, primarily comprised of adipocytes, that exists in close proximity to the epidermis as well as to the CNS and visceral organs of the fly (Reviewed by Arrese and Soulages, 2010). These adipocytes, which are bathed in hemolymph, the insect circulatory system, produce and secrete many hemolymph components, including immune response peptides (reviewed by Hoffman, 2003), insulinotropic factors (Geminard et al.,

2009), and neuromodulators (Meunier et al., 2007; Lazareva et al., 2007). The fat body is also the primary producer of vitellogenin, which is taken up by developing eggs as the main component of the yolk (Jowett and Postlethwait, 1980; Bownes et al., 1996; Isaac and Bownes, 2005).

Similarly to vertebrate adipocytes, insect adipocytes contain lipid droplets and glycogen which can be mobilized during periods of increased energy demand or reduced nutrient availability. Nutrient levels are monitored by carbohydrate and amino acid transporters in the fat body and neurosecretory cells in the CNS. Sugar and protein absorption by the fat body following feeding is regulated by the conserved target of rapamycin (TOR) and insulin signaling pathways (Attardo et al., 2005; Mirth and Riddiford, 2007; Masumura, 2000; Brogiolo et al., 2001). Nutrient mobilization is also regulated by similar signaling pathways in vertebrates and invertebrates. During periods of elevated nutrient demand such as flight, courtship, and reproduction, adipokinetic hormone (AKH) is released by neurons in the corpora cardiaca to mobilize stored lipid, protein, and carbohydrates from the fat body (Lee and Park, 2004; Grönke et al., 2007). Neuropeptide F (NPF) production in the gut and CNS is also nutrient responsive and stimulates orexigenic neurons to initiate feeding behavior (Krashes et al., 2009).

Another signal that affects feeding in female Drosophila is the orexigenic signal sex peptide (SP), which is transferred to the female via the male's ejaculate. SP, together with other components of the male ejaculate, stimulates oogenesis and oviposition behaviors in mated females (Carvalho et al., 2006; Yapici et al., 2008). These changes are accompanied by SP-controlled increases in feeding and changes in food preference (Ribeiro and Dickson, 2010) to meet the energetic demands of oogenesis and egg laying. Correlated with these post-mating behavioral and physiological changes are increases in locomotor behavior that are expected to be important for finding ovipostion sites and food sources (Isaac et al., 2010).

My primary goal is to understand the adult fat body's role in regulating nutrient homeostasis and behavior in *Drosophila melanogaster*. Of great interest are the signaling pathways that are responsible for mediating changes in adult nutrient homeostasis and behavior in response to changing environmental circumstances. Recent studies aimed at identifying genes involved in adult responses to environmental change have utilized microarray technology to detect changes in transcript abundance in adult tissues. Of the identified loci, the most intriguing candidate was the fat body-expressed gene *female-specific independent of transformer (fit*), which increases in expression in courting or mated adults (Lawniczak and Begun, 2004; Carney, 2007; McGraw et al., 2008; Dalton et al., 2010; Ellis and Carney, 2010; Ellis and Carney, 2011) and decreases in starved animals (Fujikawa et al., 2009; Boyd et al., 2011). However, the nature of *fit*'s involvement in behavior, reproduction, and nutrient homeostasis remains unclear. Given its expression in the fat body, I hypothesized that *fit* is involved in the physiological changes in metabolism, feeding, and nutrient storage that occur in mated adults. To test this hypothesis, I assessed the effects of *fit* mutations on adult Drosophila starvation resistance, metabolism, and behavior.

4.2 Methods

4.2.1 Strains

Three *UAS-fit RNAi* lines w^{1118}; fit^{GD6217}/*TM3, Sb* (stock #'s 14433 and 14434) and w^{1118}; $fit^{KK106812}$ (stock #109482) were obtained from the Vienna Drosophila RNAi Center (VDRC); hereafter referred to as *UAS-*fit^{v14433}, *UAS-*fit^{v14434} and *UAS-*$fit^{v109482}$. The *Cg-Gal4* stock was obtained from Carl Thummel, and the *PromE-Gal4* stock was generously provided by Joel Levine. y^1 w^{1118}; *P{GawB}*ap^{md544}/*CyO* was acquired from Bloomington Drosophila Stock Center (stock #3041). Strains for generating *fit* mutants were provided by Keith Maggert,

Texas A&M University. All stocks and crosses were maintained on standard cornmeal media in a 25^0C incubator on a 12 hr light/dark cycle.

4.2.2 fit *mutants*

fit mutant lines were produced by the ends-out knockout technique (Maggert et al., 2008). Primers were designed to amplify a 3968 bp sequence immediately upstream of the *fit* locus and a 4219 bp sequence beginning 32 bp downstream of *fit*. These flanking sequences were cloned into pCR-BluntII-TOPO cloning vectors (Invitrogen, Carlsbad, CA, USA) for subsequent subcloning into the pW25.2 vector. The 3968 bp upstream amplification product was first cloned between the BsiWI and AscI restrictions sites in pW25.2, and then the 4219 bp downstream product was cloned between the NheI and MluI sites. The construct was injected into w^{1118} embryos by Genetic Services (Cambridge, MA) following standard protocols. Resulting transgenic lines were crossed to *FLPase, I-SceI* flies for recombination between the donor and endogenous chromosomes. Crossing to FLPase flies confirmed that recombination occurred in the germline at the desired locus. Two independent fly lines designated w;; fit^{NNN}/*TM6* and w;; fit^{T}/TM6 with properly targeted recombination events were validated both phenotypically and by PCR amplification. These two lines were then crossed to *CreRec* flies for removal of the w^+ marker. The resulting strains used in my study are *w;;* fit^{NNN1}*/TM3, Sb and w;;* fit^{T15}. Sequencing across the *fit* locus verified that the deletion of *fit* in each strain.

4.2.3 UASp-fit *strains*

UASp-fit expression constructs used in this study were created by subcloning the *fit* cDNA sequence from RH40291 (Drosophila Genome Resource Center, DGRC) into *NotI* and *BamHI* sites downstream of the UAS sequence in the *pUASp* vector. The construct was sequenced and then injected into w^{1118} embryos by Duke University Model System Genomics (Durham, NC).

4.2.4 RT-PCR

Individual tissues from were dissected from 5-day, non-virgin adults in phosphate buffered saline (PBS) on ice. mRNA was extracted from these tissues or from wandering 3rd instar larvae with Trizol reagent (Invitrogen), and cDNA templates for PCR amplification were prepared using the Superscript First-Strand Synthesis Kit (Invitrogen). The *fit* gene does not contain introns, so PCR amplification across the intron-containing gene *rp49* was used to confirm that there was no genomic DNA contamination in the samples.

4.2.5 Humid starvation

Adult virgins were collected as they eclosed within 4 hrs of lights ON. These adults were separated by genotype and gender and aged for 3 days at 25^{0}C on standard food in groups of fewer than 30 flies per vial. On day 3 at 3 hrs after lights ON, adults were transferred to clean vials containing only moist filter paper disks and assayed for mortality every 1 hr (starting at +18 hrs since no deaths were observed within the first 18 hrs of humid starvation) until the last fly was expired. Individual survival durations were then calculated and compared by χ^2 analysis using JMP software. Each of the three *UAS-fit-RNAi* lines was used to investigate the influence of *fit* expression on starvation resistance. However, the *UAS-fit* ^{v109482} line was found to be inherently starvation resistant (without GAL4-mediated activation) and so was not used in my analyses.

4.2.6 Glycerol and triglyceride (TAG) analysis

Newly eclosed virgins were collected from 1-4 hrs after lights ON and grouped in vials containing standard cornmeal media. Adults housed in vials containing fewer than 30 individuals were either immediately sampled for the 0-day time point or aged for the 3-day and later time points. Adults aged for 3 days were transferred to empty vials with moist filter paper disks starting at 3 hrs after

lights ON. Samples consisting of 8 adults each were taken immediately for the 0 hr time point. Remaining flies were starved for a period of 12 hrs, 24 hrs, or 36 hrs and then sampled. Sample preparation was conducted as described by Song et al., (2010). For each replicate, groups of 8 adults were collected, homogenized in lysis buffer (0.05% Tween 20, 0.01% leupeptin), heated for 5 min at 70^0C, and centrifuged at 14,000 RPM for 10 min. Supernatant from this sample was then assayed for free glycerol and TAG using glycerol detection reagents (Sigma Aldrich). Samples were then analyzed by spectrometry (540 nm) with a plate reader to quantify glycerol levels relative to glycerol standards. Sample protein content was quantified by Bradford assay to normalize both glycerol and TAG content. Statistical analysis was completed by Student's *t*-test using JMP software.

4.2.7 Carbohydrate analysis

Samples collected for glycerol and TAG analysis were used to quantify relative levels of whole body glycogen and trehalose as described previously (Song et al., 2010). These assays depend on glucose quantification by colorimetric analysis with glucose reagent (Sigma Aldrich G3293). Glycogen content was determined after completing separate reactions with amylase (Sigma Aldrich G3285) at 37^0C for 1 hr to break down the glycogen to glucose. Detection of trehalose was determined after incubating the samples with trehalase enzyme (Sigma Aldrich T8778) in acetate buffer (pH 5.5) at 37^0C overnight. Glucose content of glycogen and trehalose reactions was then quantified by spectrometry (340nm) via a plate reader using glucose standards. Statistical analysis of the resulting data was completed by Student's *t*-test using JMP software.

4.2.8 Adult feeding rate

Newly eclosed adults were collected within 4 hrs of lights ON and housed in same-sex groups of fewer than 20 adults per vial on standard cornmeal media at 25^{0}C. Groups of 10 adults were then transferred to vials containing 15% sucrose, 1% agar, and 0.5% brilliant blue FCF dye (Wako) for 1 hr. I found that 1 hr feeding periods were sufficient to achieve accurate quantification of sample dye consumption while reducing variability due to excretion of labeled food. During my analysis I did not observe dye-containing feces in the feeding chambers, confirming that the 1 hr assay was quantifying rate of ingestion, and not capacity or egestion rate. Adult feeding rate was assayed every 6 hrs from 24 hrs to 66 hrs after eclosion. Adults were anaesthetized under CO_2 at the end of the 1 hr feeding period, homogenized in PBS, and centrifuged at 13,000 RPM for 25 min. Samples were analyzed by spectrometry (625nm) with a plate reader and were normalized to control samples prepared from adults fed food that did not contain dye. Statistical analysis was completed by Student's *t*-test using JMP software.

4.2.9 Adult locomotor activity

Adult activity was measured using the Drosophila activity monitoring system (DAM). Newly eclosed adults were collected by gender and genotype within 3 hrs of lights ON and aged overnight in like-groups of fewer than 20 adults per vial on standard cornmeal media at 25^{0}C. Beginning on day 1, adults were monitored for activity for 3 days under 12 hr light/dark cycles at 25^{0}C as described (Benito et al., 2007). Total activity was statistically compared by Student's *t*-test with JMP software.

4.3 Results

4.3.1 Adult fit *expression*

fit was first identified due to its female-biased expression in adult head fat body (Fuji and Amrein, 2002). An additional microarray study found that *fit* expression is female-biased in the fat body as well as the CNS (Goldman and Arbeitman, 2007). One conclusion of these studies was that sex-biased expression of *fit* is regulated by components of the sex-determination pathway, a gene hierarchy that controls Drosophila reproductive behaviors. A recent analysis of socially-responsive gene expression showed that *fit* expression increases in male heads following exposure to females or other males (Ellis and Carney, 2011), and increased *fit* expression is maintained in males at least 2 hrs after mating (Ellis and Carney, 2010). Females also up-regulate *fit* for at least 3 hrs post-mating (Lawniczak and Begun, 2004; McGraw et al., 2002; Goldman and Arbeitman, 2007). These findings and the reported fat-body biased expression of *fit* suggest that *fit* plays a role in physiological changes associated with reproduction.

The earlier studies indicated that *fit* is not expressed in larvae but is expressed in adults, predominantly in the fat body (Fuji and Amrein, 2002; Chintapalli et al., 2007). Microarrays also detected moderate levels of *fit* in the

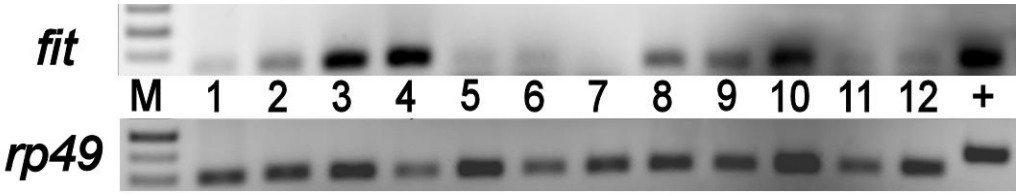

Figure11. *fit* expression in Drosophila tissues. Tissues are from adults unless otherwise indicated. Lanes 1-6 contain male tissues. Lanes 7-12 contain female tissues. M, molecular weight marker; 1, larvae; 2, brain; 3, head cuticle (includes fat body but not brain); 4, abdominal cuticle (epidermis, fat body, muscle); 5, reproductive tract; 6, gut; 7, larvae; 8, brain; 9, head cuticle (includes fat body but not brain); 10, abdominal cuticle (epidermis, fat body, muscle); 11, ovary; 12, gut; +, genomic DNA control. *rp49* expression is shown as a control for DNA contamination since the *fit* gene does not contain an intron.

female CNS (Chintapalli et al., 2007; Goldman and Arbeitman, 2007). I independently confirmed these results with RT-PCR by examining *fit* expression in larvae and in specific adult tissues. Trace levels of *fit* were detected in wandering 3rd instar larvae as well as adult gut and reproductive tissues (Figure 11), although transcripts were not detected in these tissues by *in situ* (data not shown). *fit* expression was detected in adult brains and head cuticles as well as in abdominal cuticles (Figure 11). The head cuticle preparations contain fat body, eyes, and sensory tissues but lack the brain. Based upon previous studies (Fuji and Amrein, 2002; Chintapalli et al., 2007) and our own *in situ* analyses (Ellis and Carney, 2011), I attribute the majority of head cuticle expression to fat body and eyes. My tissue expression data confirm the microarray-based findings of Chintapalli et al. (2007) available on FlyAtlas. Since the predominant location of *fit* expression in both sexes is the fat body, which is the major storage organ for lipid and carbohydrate, I predicted that *fit* functions in regulating nutrient homeostasis of adult Drosophila.

4.3.2 fit *expression in fat body influences adult starvation resistance*

Mutations that affect Drosophila nutrient homeostasis impact survival under starvation conditions. For example, Drosophila mutants with increased triglyceride (TAG) and carbohydrate content often show increased resistance to starvation (Reviewed by Baker and Thummel, 2007). In contrast, mutants for the fat body expressed gene *lipid storage droplet-2 (Lsd2)* have decreased TAG content and die more rapidly as a consequence of starvation than starved control flies (Grönke et al., 2003; Teixeira et al., 2003). To assess ***fit's*** involvement in adult nutrient homeostasis I starved *fit* mutant adults and determined their survival durations. After eclosion, adult fat body requires approximately 48 hrs to develop by absorbing circulating nutrients left over from dissociated pupal fat (Aguila et al., 2007). To get an accurate assessment of adult nutrient regulation I

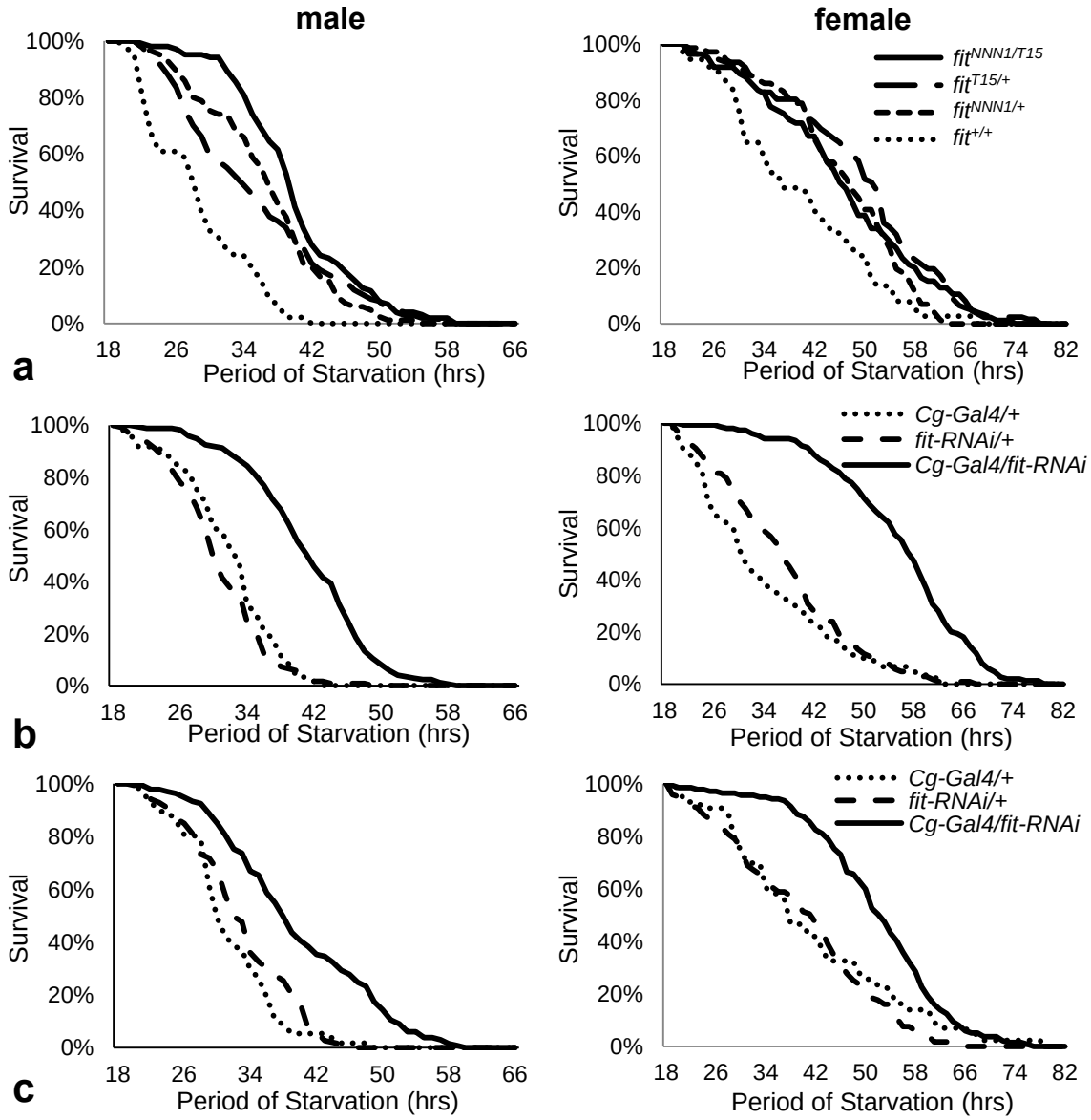

Figure 12. Influence of reduced *fit* expression on adult survival under starvation conditions. (a) fit^{NNN1}/fit^{T15} mutant (solid) male $\bar{x}$ =39 female $\bar{x}$ =48, fit^{T15}/+ (long dashed) male $\bar{x}$ = 33 female $\bar{x}$ =52, fit^{NNN1}/+ (dashed) male $\bar{x}$ = 36 female $\bar{x}$ =48, +/+ (dotted) male $\bar{x}$ = 27 female $\bar{x}$ =37. fit^{NNN1}/fit^{T15}, fit^{T15}/+, and fit^{NNN1}/+ adults have extended survivorship compared to +/+ controls ($p<0.001$). Data shown represents survivorship of 88-109 male and 56-131 female adults per genotype. (b) *Cg-Gal4*/+ (dotted) male $\bar{x}$ = 31 female $\bar{x}$ = 30, *UAS-fit* ^{v14433}/+ (dashed) male $\bar{x}$ =29 female $\bar{x}$ =36, *UAS-fit* ^{v14433}*/Cg-Gal4* (solid) male $\bar{x}$ =41 female $\bar{x}$ =57 . *UAS-fit* ^{v14433}*/Cg-Gal4* adults have extended survivorship compared to *UAS-fit* ^{v14433}/+ and *Cg-Gal4*/+ controls ($p<0.0001$). Data shown represents survivorship of 56-175 male and 57-149 female adults per genotype. (c) *UAS-fit RNAi* ^{v14434}*/Cg-Gal4* (solid) male $\bar{x}$ =38 female $\bar{x}$ =52, *Cg-Gal4*/+ (dotted) male $\bar{x}$ =29 female $\bar{x}$ =37, *UAS-fit RNAi* ^{v1443}/+ (dashed) male $\bar{x}$ =31 female $\bar{x}$ =41. *UAS-fit RNAi* ^{v14434}*/Cg-Gal4* extends survivorship compared to *UAS-fit RNAi* ^{v14434}/+ and *Cg-Gal4*/+ controls ($p<0.0001$). Data represents 57-153 males and 43-136 females per genotype.

allowed 72hrs post eclosion so that the adult fat body could form before assaying starvation resistance. Adult survival was quantified by recording survivorship every hour while the flies were experiencing humid starvation conditions. Adult males mutant for *fit* (fit^{NNN1}/fit^{T15}, fit^{T15}/+, or fit^{NNN1}/+) survived starvation conditions for 22-44% longer than wild-type (*fit* +/+) controls ($p<0.001$) (Figure 12a).

I determined if decreased *fit* expression in the fat body was responsible for the increased starvation resistance by assaying the survivorship of adults expressing *UAS-fit-RNAi* in the fat body under the control of the *Cg-Gal4* driver. *Cg-Gal4/UAS-*fit^{v14433} males survived over 32% longer than controls, while females survived over 58% longer ($p<0.0001$) (Figure 12b). A similar increase in survivorship was observed in adults expressing an alternate *fit-RNAi* construct (Figure 12c), therefore, I attributed the starvation resistance of *fit* mutants to reduced *fit* expression in the fat body.

To confirm that the starvation resistance phenotype of *fit* mutants was due to reduced *fit* levels in the fat body, I expressed a *UASp-fit* transgene in *fit* mutants under the control of *Cg-Gal4*. My expectation was that animals with *fit* expression restored in fat body would die more rapidly under starvation conditions, thereby rescuing the starvation resistance phenotype. Fat body expression was sufficient to rescue the starvation resistance phenotype of *fit* mutant males ($p<0.0001$) as expected (Figure 13a). Contrary to my prediction, starvation resistance was not rescued in mutant females (Figure 13a) suggesting that *fit* expression was not able to simulate wildtype *fit* levels in female *fit* mutants.

Since animals with reduced levels of *fit* were more starvation resistant, I assayed the effects of increasing *fit* expression in adults. Surprisingly, *fit* over-expression in the fat body of non-mutants resulted in a 31% increase in the starvation resistance of males and a 26% increase in females ($p<0.0001$) (Figure 14b). Therefore, I conclude that mis-regulation of *fit* expression in the fat body leads to increased adult starvation resistance.

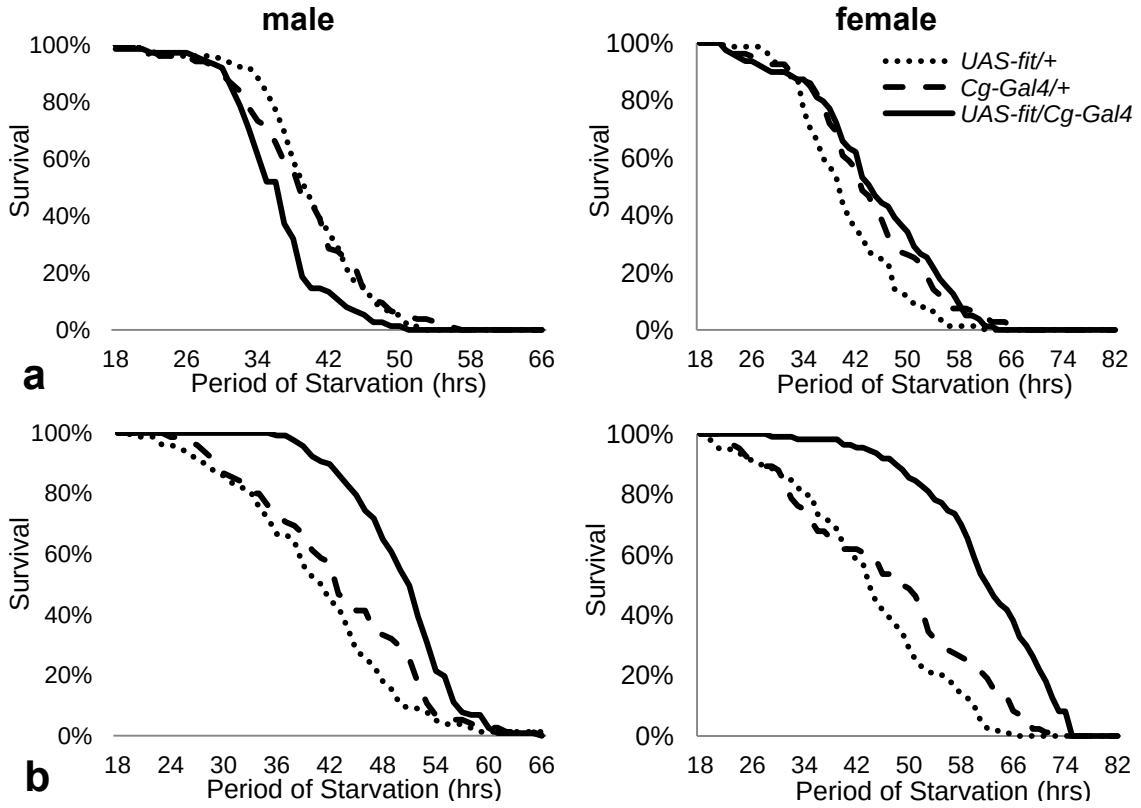

Figure 13. Influence of *UASp-fit* expression in wildtype and *fit* mutant backgrounds on adult survival under starvation conditions. (a) For rescue of *fit* expression in the fat tissue, all animals are fit^{NNN1}/fit^{T15} mutants. *UASp-fit/Cg-Gal4* (solid) male $\bar{x}$ =36 female $\bar{x}$ =43, *Cg-Gal4/+* (long dashed) male $\bar{x}$ =38 female $\bar{x}$ =42, *UASp-fit/+* (short dashed) male $\bar{x}$ =39 female $\bar{x}$ =39. *fit* mutant males carrying *UASp-fit/Cg-Gal4* die more rapidly during starvation than fit mutant mutants containing a single UAS/Gal4 component ($p<0.0001$). *UASp-fit/Cg-Gal4* female survivorship was not significantly reduced compared to *UASp-fit/+* and *Cg-Gal4/+* controls ($p=0.55$). Data shown represents survivorship of 76-105 male and 77-103 female adults per genotype. (b) *fit* overexpression in a wild-type background. *UASp-fit/Cg-Gal4* (solid) male $\bar{x}$ =51 female 62, *Cg-Gal4/+* (dashed) male $\bar{x}$ =42 female $\bar{x}$ =50, *UASp-fit/+* (dotted) male $\bar{x}$ =39 female $\bar{x}$ =43. *UASp-fit/Cg-Gal4* adult survivorship significantly increased compared to *UASp-fit/+* and *Cg-Gal4/+* controls ($p< 0.0001$). Data shown represents survivorship of 62-112 male and 79-110 female adults per genotype.

4.3.3 CNS or oenocyte expression of fit *does not affect mutant starvation resistance*

fit expression in the fat body could alter starvation responses within fat body or FIT protein could be secreted from the fat into the hemolymph to affect a

secondary tissue. The predicted FIT protein is 121 amino acids long and has a candidate signal sequence, indicating that FIT may be a secreted protein. Since *fit* is expressed in non-adipose tissues such as the CNS (Figure 11), and expressing *fit* in the fat body was not sufficient to rescue female mutant starvation resistance, it is possible that *fit* expression or activity in an alternate tissue also influences the ability of animals to withstand starvation.

The CNS regulates behavior in response to signals from the peripheral tissues (Meunier et al., 2007; Reviewed by Gronke and Partridge 2010). To quantify the influence CNS *fit* expression had on starvation resistance I assayed the survival duration of *fit* mutants expressing *fit* in the CNS with the pan-neural *ap-Gal4* driver (Ellis and Carney, 2011). I found that there was no difference between control and experimental animal survivorship meaning that CNS expression of *UASp-fit* in *fit* mutants did not reduce mean survival duration (Figure 14a). This result suggests that either CNS expression of *fit* was not effective at simulating wild-type levels of FIT or that CNS does not require *fit* expression for wild-type starvation resistance.

If secretion of FIT modulates response to starvation in wild-type animals, I expected that *fit* expression in oenocytes, another secretory tissue, would rescue the starvation resistance identified in *fit* mutants. Oenocytes are adult secretory cells that exist near tergite boundaries in close proximity to the epidermis and fat body. Oenocytes produce and secrete cuticular hydrocarbons (Billeter et al., 2009) and play a role in lipid mobilization during starvation (Gutierez et al., 2007). Expression of the fat body-secreted TAKEOUT (TO) protein in oenocytes rescues the courtship defects of *to* mutants (Lazareva et al., 2007), so I asked if expression of *fit* in oenocytes similarly rescues starvation resistance. I did not detect *fit* transcripts in wild-type adult oenocytes (data not shown), and expressing *fit* in oenocytes via *PromE-Gal4* did not affect mutant survivorship (Figure 14b). These data show that *fit* expression in the CNS or oenocytes of *fit* mutants is insufficient to rescue starvation resistance.

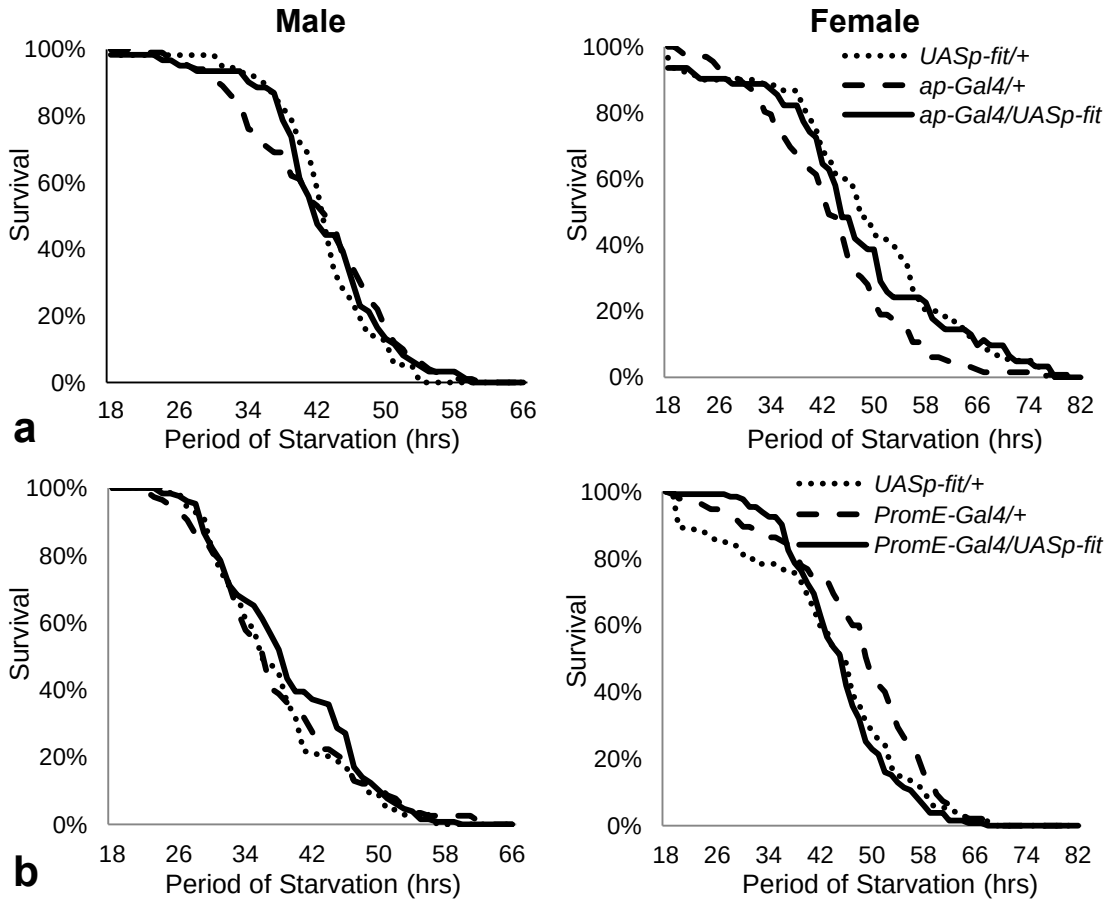

Figure 14. Survival under starvation conditions of *fit* mutants expressing *fit* in CNS or oenocytes. All flies are fit^{NNN1}/fit^{T15} mutants. (a) *UASp-fit/ap-Gal4* (solid) male $\bar{x}$ =42 female $\bar{x}$ =43, *ap-Gal4/+* (dashed) male $\bar{x}$ =41 female $\bar{x}$ =42, *UASp-fit/+* (dotted) male $\bar{x}$ =42 female $\bar{x}$ =43. Data represent 57-98 males and 59-134 females per genotype. (b) *UASp-fit/PromE-Gal4* (solid) male $\bar{x}$ =38 female $\bar{x}$ =45, *PromE-Gal4/+* (dashed) male $\bar{x}$ =35 female $\bar{x}$ =46, *UASp-fit/+* (dotted) male $\bar{x}$ =35 female $\bar{x}$ =46. Data represents 72-121 males and 66-128 females per genotype.

4.3.4 Adult nutrient content

Adult nutrient stores positively correlate with variation in starvation resistance in wild populations (Ballard et al., 2008). Like vertebrates, Drosophila store surplus energy in the form of triglyceride (TAG) and glycogen. Trehalose is the primary circulating carbohydrate in insects (Wyatt and Kalf, 1957), and it is mobilized from fat body glycogen stores to power energetically demanding

activities such as flight and reproduction (Wigglesworth, 1949). TAG is contained in lipid droplets within fat body adipocytes and is hydrolyzed to release glycerol and fatty acids to meet energetic requirements (Reviewed in Arrese and Soulages, 2010).

Given the correlation between available nutrients and the ability to survive starvation, I predicted that the observed increase in adult *fit* mutant survival duration under starvation conditions was due to increased adult energy content prior to starvation. I characterized adult nutrient content by quantifying carbohydrate and fat stores. To determine carbohydrate content, I quantified whole-body glycogen and trehalose in *fit* mutants and controls. Adult fat content was determined by quantifying whole-body TAG and glycerol in these same genotypes.

I assayed carbohydrate and fat levels in 0-day newly-eclosed adults to determine nutrient content due to accumulation during development. I did not identify significantly increased levels of carbohydrate or fat content in *fit* mutants compared to controls at eclosion (data not shown). This result was not surprising because *fit* is only weakly expressed in larvae and so was not predicted to strongly influence an animal's physiology prior to adulthood (Figure 11) (Chintapali et al., 2007; Graveley et al., 2011).

The carbohydrate and fat content of 3-day adults was assayed since this was the initial age of the animals that were tested for starvation resistance. I found that *fit* mutant females contained nearly twice the amount of glycogen detected in wild-type controls ($p=0.0396$) but had similar trehalose levels (Figure 15). In contrast, *fit* mutant male carbohydrate levels were not significantly different from those of wildtype (Figure 15). Identifying gender specific differences in glycogen content was not surprising given the differences in male and female energetic requirements and the higher levels of *fit* expression in female fat body.

TAG levels were elevated in male and female *fit* mutants, although the differences were not significant (male P= 0.143, female p=0.054). However, a significant effect of *fit* mutation on glycerol levels was not detetced. Mutant adult glycerol content was 49% higher in males (p=0.001) and 66% higher in females (p=0.002) compared to wild-type controls (Figure 15). My findings indicate that during the first three days of adulthood, *fit* mutants accumulate additional nutrients in the form of glycogen and glycerol that likely contribute to their ability to survive starvation.

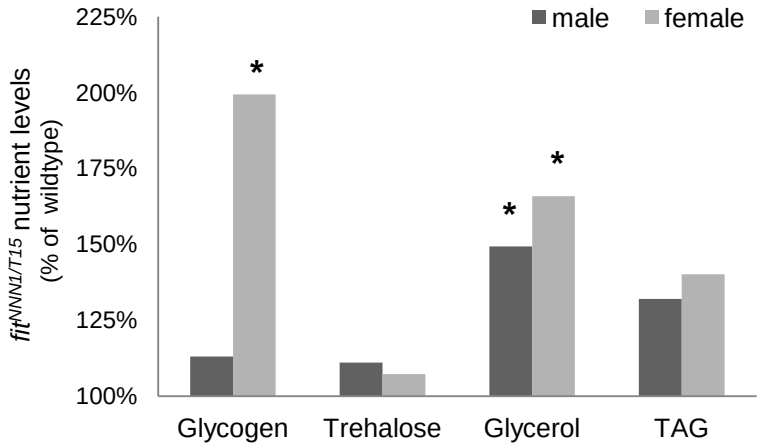

Figure 15. Whole body nutrient levels of 3 day *fit* mutant adults. *fit* mutant nutrient levels were normalized to *fit* +/+ levels and are presented as percentages of wildtype. Data representative of 64 – 80 adults. "*" denotes p<0.05 by Student's *t*-test.

4.3.5 Adult feeding and locomotor activity

Starvation resistant animals typically have increased energy stores and/or decreased metabolism. During starvation, glycogen and TAG levels declined at similar rates in *fit* mutants and controls (Figure 16), suggesting that *fit* mutants do not have defects in nutrient mobilization and metabolism. Therefore, the most likely possibility is that *fit* mutants eat more than controls, which provides the mutants additional energy stores to draw upon during starvation stress. To test

this hypothesis, adult feeding rate of *fit* mutants and controls was assayed during the initial 3 days of adult life (every 6 hrs from 24-66 hrs after eclosion) to assess food intake during the period prior to when starvation resistance was examined.

Feeding rate in very young adults is not well characterized since published studies on adult feeding behavior have tested animals that were at least 4 days old (Xu et al., 2008; Chatterjee et al., 2010). The majority of feeding behavior in these animals occurs during the lights ON/OFF transitions (Xu et al., 2008; Chatterjee et al., 2010). In my feeding assay, lights OFF took place during the 36 hr and 60 hr timepoints. Little diurnal variation in feeding behavior was identified in control adults (Figure 17a) but *fit* mutants appear to have precociously acquired feeding behavior patterns of older adults. *fit* mutant male feeding was

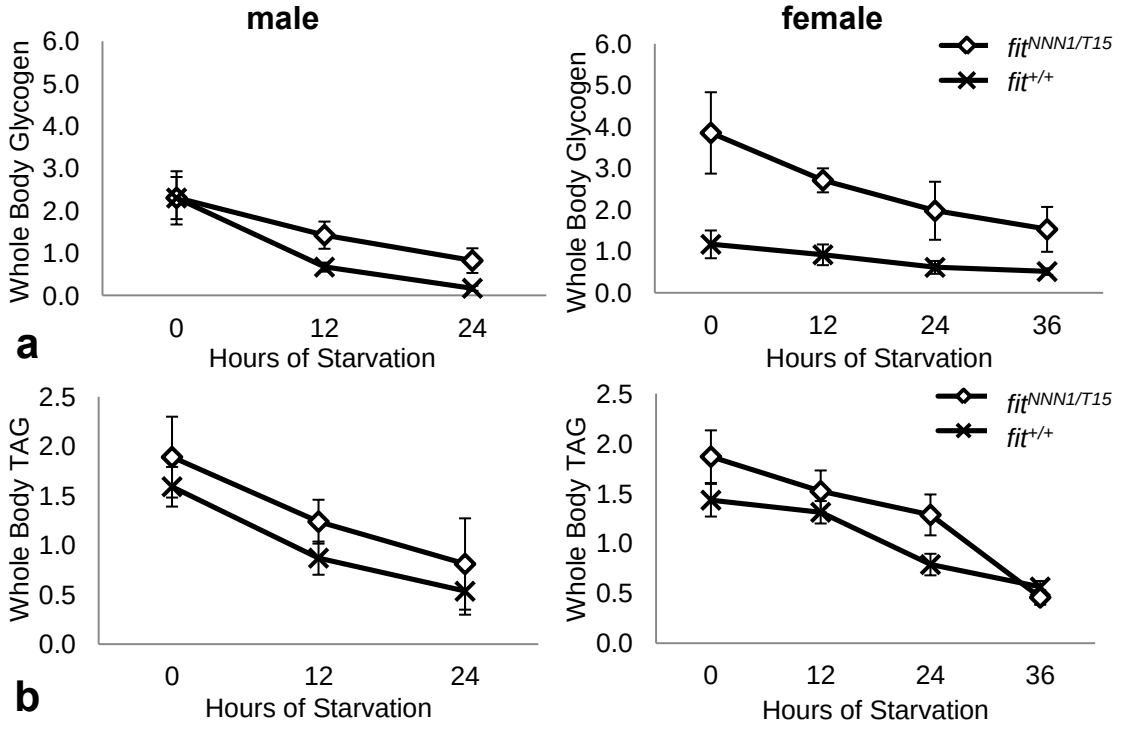

Figure 16. Whole body stored nutrient content of 3 day adults in response to starvation conditions. fit^{NNN1}/fit^{T15} mutant (diamond) and *fit*+/+ (cross). (a) Representative glycogen content. (b) Representative TAG content. Data shown represents 64 males and 66 female adults per genotype.

greater at the 36 hr time point (p=0.004) (Figure 18a) but this single timepoint of increased feeding did not lead to a change in cumulative food uptake (p=0.19) (Figure 17b). Female *fit* mutant feeding was greater than control animals from 36- 66 hr (p<0.05) (Figure 17a). This increase in feeding axcross multiple timepoints resulted in a 52% increase in cumulative feeding (p<0.001) in *fit* mutant females. These data suggest that *fit* may function in a gender–specific manner to regulate adult appetite. Increased eating likely accounts for the detected increases in energy stores, providing a means for prolonged survivorship under starvation conditions.

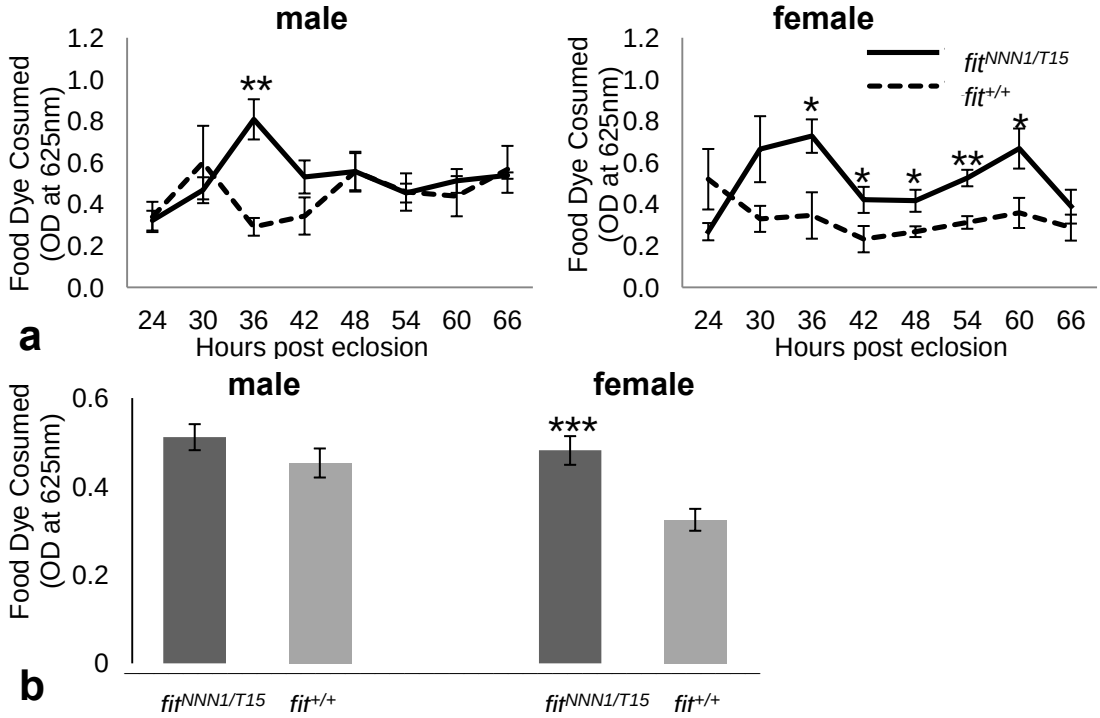

Figure 17. Adult feeding rate of *fit* mutants. (a) Average feeding rate for each genotype at each time point. fit^{NNN1}/fit^{T15} mutant (solid) and *fit*+/+ (dashed). Data points represent the average values from 40 adults sampled per genotype. (b) Cumulative average food consumption of 320 adults per genotype from all time points tested. fit^{NNN1}/fit^{T15} mutant (dark grey) and *fit*+/+ (light grey). "*" denotes p< 0.05; "**" denotes p<0.005, "***" denotes p<0.001 by Student's *t*-test.

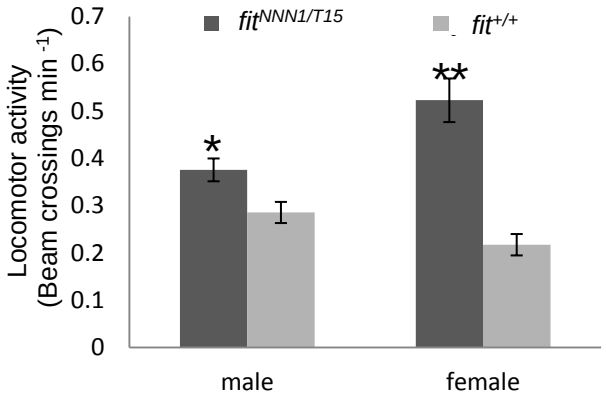

Figure 18. Adult *fit* mutant locomotor activity. Activity of 1-3 day fit^{NNN1}/fit^{T15} mutant (dark grey) and *fit* +/+ (light grey) adults. Data shown represent the average activity for 28-32 adults per genotype. "*" denotes p=0.008; "**" denotes p<0.0001 by Student's *t*-test.

I expected that the increase in food consumption would be accompanied by an increase in activity levels so the Drosophila Activity Monitoring (DAM) system was used to characterize relative activity *fit* mutants and controls. Mutant male and female virgin locomotor activity during the initial 3 days of adult life was increased (p=0.008 and p<0.001 respectively) (Figure 18). This uptick in activity correlates well with elevated food consumption and may be indicative of increased food-seeking behavior. Increased activity is also typical of mated females (Isaac et al., 2010) and is associated with increased searching for oviposition sites and food sources. Since my *fit* mutants were virgins, reduction of *fit* levels may result in changes in nutrient homeostasis and appetite that mimic changes in appetite that occur in recently mated animals.

4.4 Discussion

4.4.1 Nutrient sensing

Efficient nutrient management is required to survive periods of poor food availability, maximize reproductive output, and effectively allocate available nutrients to meet metabolic requirements. Insects regulate nutrients by perceiving ingested food quality through gustatory receptor neurons (Melcher

and Pankratz, 2005) and alter feeding behavior to optimize nutrient uptake (Bell et al., 1985; Carvalho et al., 2005; Min and Tatar, 2006; Lee et al., 2008). Disrupting appetite regulating neurons or communication between peripheral tissues and the CNS leads to aberrations in feeding and/or nutrient storage (Ikeya et al., 2002; Al-Anzi et al., 2009; Meunier et al., 2007; Chatterjee et al., 2010). *fit* expression is reduced in response to prolonged nutrient deprivation (Fujikawa et al., 2009; Boyd et al., 2011) which I predicted functions to promote feeding behavior. In adults lacking *fit* expression I observed increased nutrient accumulation and feeding behavior (Figure 15 and 17), suggesting that *fit* gene products are required to modulate appetite or communicate internal nutrient state in Drosophila. Further analysis of nutrient preference should substantiate the preliminary feeding results I have presented. If *fit* mutants show a change in food preference then a link between *fit* expression and function can be further investigated more mechanistically to understand how this gene alters adult feeding behavior.

4.4.2 fit *mutant starvation resistance and nutrient content*

Nutrient levels are higher in *fit* mutants (Figure 15), which provides an explanation for the increased starvation resistance of these animals. Female mutants have significantly higher levels of glycogen, a type of stored carbohydrate (Figure 15). I linked these nutrient increases to fat body expression of *fit*, since reduced expression of *fit* in fat body increases starvation resistance (Figure 12b,c). Significantly elevated levels of glycerol were detected in mutants of both sexes. Glycerol is an important metabolic intermediate and product of TAG hydrolysis that is capable of entering protein, sugar, and lipid synthesis pathways. I expected that increased glycerol would be a consequence of increased available TAG in *fit* mutants and its breakdown during starvation. Levels of TAG are elevated in *fit* mutants, but the levels are not significantly different than those of controls, and I did not find evidence that TAG was utilized

differently during starvation. Compared to wild-type controls, *fit* mutants consistently had higher levels of TAG at 3 days and during starvation, so although the differences in TAG levels are not statistically significant they may reflect true physiological differences between the mutant and control genotypes. Additionally, my whole-animal measures of nutrient levels would not allow us to detect localized differences in storage or mobilization that may be affected by *fit* mutations. Perhaps more exaggerated differences in TAG content would be observed in older *fit* mutant adults that have experienced prolonged periods of uninhibited feeding. This scenario would be similar to some nutrient homeostasis and metabolic conditions in humans where chronic misregulation of appetite or nutrient storage leads to obesity.

4.4.3 Sex-specific effects of fit expression

Phenotypes identified in *fit* mutants such as nutrient content, activity, and feeding rate were consistently stronger in females. Female *fit* mutants consume more food during the first 3 days of adulthood (Figure 17), while mutant males eat more only at 36 hrs post eclosion. Although locomotor activity levels are increased in mutants of both sexes, the increase is more striking in females (Figure 18) and is maintained over a 10 day period (CCS and GEC, unpublished results). These observations are consistent with female-biased *fit* expression (Fuji and Amrein, 2002), therefore, reduced *fit* expression might be expected to affect some female phenotypes disproportionately. If FIT modulates appetite as I propose, it is not unexpected that *fit* mutant female foraging phenotypes would be stronger than those of males. Although both sexes require influxes of nutrients to fuel their day-to-day activities, because of their unique reproductive capabilities and their generally larger size, females require more nutrients than males. Therefore, females, particularly those that are mated, should eat more than males to ensure sufficient resources can be allocated to egg production.

Restoring fat body *fit* expression reduces the starvation resistance of mutant males, making their survival more similar to that of wild-type controls. However, female mutants expressing *fit* in the fat body survive food deprivation similarly to mutants that have not had *fit* expression restored (i.e., they are starvation resistant) (Figure 13a). The most likely explanation for the inability of fat body expression to restore wild-type starvation resistance to females is the level of *fit* that is required to do so. Adult females express higher levels of *fit* than males (Chintapalli et al., 2007), so *Cg-Gal4*-mediated fat body expression may not produce sufficient *fit* activity to overcome the defect. Males, on the other hand, express little *fit* during adulthood, and fat body expression was sufficient to rescue the phenotype (Figure 13a).

4.4.4 Mating-responsive changes in physiology

Mating causes significant changes in female metabolism, activity, feeding, and energy allocation. These physiological changes result from components of the male ejaculate stimulating the neurons that regulate feeding behavior and activate oogenesis and oviposition (Carvalho et al., 2006; Yapici et al., 2008; Wong and Wolfner, 2006). The increased feeding, activity, and nutrient accumulation I identified in virgin *fit* mutants partially mimic those of mated females suggesting that *fit* may be involved in mating-responsive appetite change. Mating also activates oogenesis and the associated increase in nutrient demands of egg production. Components of the male ejaculate stimulate female feeding rate (Carvalho et al., 2006) and increase preference for protein rich food (Riberio and Dickson, 2010). The increase in nutrient uptake observed in mated females is thought to increase adult starvation resistance (Rush et al., 2007). Additionally, starved females can halt oogenesis and absorb nutrients from the breakdown of immature eggs which are rich in nutrients. Therefore, immature eggs can function as a secondary energy reserve for females to use to satisfy nutrient demands and temporarily maintain circulating nutrient levels.

Mated females increase egg laying and have increased mobility and food-seeking behaviors, all of which are likely modulated by male accessory peptides, including SP (Yapici et al., 2008; Ram and Wolfner, 2007; Wong and Wolfner, 2006). Once animals of either sex anticipate a potential mating, they begin up-regulating *fit* (Carney, 2007; Lawniczak and Begun, 2004). This increase is maintained in mated animals (Ellis and Carney, 2010; Dalton et al., 2010; McGraw et al., 2004), but *fit* levels decline to baseline in males if mating does not occur (LLE and GEC, unpublished results); females have not been assayed for this effect. A simple interpretation is that increases in *fit* are preparative for modulating increased foraging and eating post-mating, a phenomenon that is documented in females but not males (Carvalho et al., 2006). My assays were performed on virgin *fit* mutants. Since they have heightened activity and feeding, *fit* mutant females are behaving similarly to mated females. Therefore, one likely scenario is one in which *fit* expression modulates appetite so that increases in *fit* serve as a "brake" in an attempt to inhibit post-mating increases in feeding due to SP. Therefore, FIT may be functioning directly or indirectly as an anorexigenic signal by modulating the brain feeding centers affected by SP.

The *fit* mutant males are also more active, but I was only detected a transient increase in feeding rate that does not affect overall food intake during the first three days of adulthood (Figure 17 and 18). Males, similarly to females, should need to increase food uptake post-mating to restore nutrients. However, the only study we are aware of that addresses this issue indicates that mating does not increase male feeding behavior in the 24 hr period following mating (Carvalho et al., 2006). Despite this result, it remains likely that there are transitory increases in feeding similar to the one I detected in *fit* mutants that induce males to seek food sources post-mating. Such an increase would be concordant with the courtship and mating-induced changes in male *fit* expression.

4.4.5 FIT, an anorexigenic signal?

Appetite is a complexly regulated behavior in insects and vertebrates. Nutrient sensing cells in the gut, fat, and CNS communicate internal nutrient state to appetite regulating centers in the brain. Neurons in the vertebrate hypothalamus regulate appetite in response to positive and negative signals (Reviewed by Dietrich and Horvath, 2009; Klok et al., 2007), including adipokines- a group of signaling molecules produced in adipose tissue that regulate various aspects of physiology (Reviewed by Rondinone, 2006). One vertebrate adipokine, leptin, negatively regulates appetite by altering the Neuropeptide Y (NPY) activity of neurons expressing leptin receptors in the hypothalamus (Reviewed by Oswal and Yeo, 2010). NPY is one of many appetite regulating molecules identified in vertebrates (Sahu and Kalra, 1993).

Insect feeding behavior is regulated by molecules orthologous to vertebrate peptides as well as other neuromodulators. For example, Neuropeptide F (NPF) is an insect ortholog of NPY and functions to elicit feeding behavior (Wu et al., 2003). AKH functions similarly to vertebrate glucagon by regulating nutrient mobilization from the fat body and influencing feeding behavior (Konuma et al., 2012). DILPs, functional orthologs of vertebrate insulin, are responsive to feeding and reduce appetite (Brogiolo et al., 2001). Similarly to vertebrates, insect feeding is negatively regulated by neuromodulators such dopamine (Allen et al., 2011) and serotonin (Falibene et al., 2012). Insect feeding behaviors are also regulated by molecules with no clear vertebrate orthologs. Feeding behavior is influenced by SP, an allohormone transferred to the female in male ejaculate (Carvalho et al., 2006; Isaac et al., 2010), and the fat body expressed gene *to* (Meunier et al., 2007). My work identified another such molecule, FIT, which influences starvation resistance, activity, feeding, and nutrient accumulation. What I observed in the *fit* mutants are phenotypic hallmarks of animals lacking an appetite-regulating molecule.

MNSTLVILLLSALALVQARNIRWSEEDNSSQGPSLSHPHPHSVNWPCDVGHFPEAFILM**HK**
VD**KR**LERIDNEST**KKR**IENYAVNQ**LR**QCILDGQMDVHCV**RR**SIGYTMSFI**HH**QMSQANGM

Figure 19. Predicted FIT sequence. The predicted signal sequence is highlighted in bold and underlined, candidate peptide processing sites are highlighted in bold, and FIT peptide sequences detected in eggs (Nakahara et al., 2005) are underlined.

4.4.6 Secretion of FIT and models for function

While the cellular activity of FIT remains a mystery, the starvation phenotype resulting from my misexpression of *fit* suggests that *fit* may be altering fat activity or is secreted to target tissues such as the CNS or gut. The predicted FIT protein has a signal sequence as well as potential peptide cleavage sites (Figure 19). These features suggest two intriguing possibilities for FIT function. The first is that FIT may be secreted and function as an adipokine, similarly to the well-known vertebrate feeding modulator leptin. The second observation of candidate dibasic residues that mark sites for protease cleavage indicate the possibility that FIT is cleaved into 5-6 small peptides that could each have different activities and functions (Figure 19). The fact that FIT peptides are present in eggs (Nakahara et al., 2005), although only trace amounts of transcript are detected in this tissue (Figure 11 and Chintapalli et al., 2007), further supports the secretion hypothesis. This MALDI-TOF mass spec analysis detected peptides corresponding to only three of the potential cleavage products (Nakahara et al., 2005). Therefore, FIT may be secreted and individual peptides may have alternate functions in regulating appetite and behavior that are separate than functions in oocytes.

If, indeed, FIT functions as a secreted ligand, I expected that production and secretion of FIT from a secretory tissue (other than fat body) would be able to compensate for loss of *fit* activity in mutants. Ectopic expression of *fit* in the oenocytes of *fit* mutants did not suppress starvation resistance (Figure 14b), whereas expression of *fit* in fat body did (Figure 13a). One possibility is that

oenocyte expression of *fit* did not provide high enough levels of secreted protein to cause physiological change. I was surprised that *fit* overexpression in the fat body of wild-type animals caused starvation resistance similarly to reducing levels of *fit* (Figure 13b). In this case it appears that *fit* expression outside some physiological threshold causes changes to adult physiology and behavior. If FIT is an anorexogenic signal as I propose, how can I reconcile my data showing that loss-of-function mutants and flies overexpressing *fit* have similar phenotypes, i.e., survive longer during periods of starvation? FIT could function as a ligand to affect appetite-regulating neurons. Overexpression of *fit* may produce excess ligand, forcing appetite-regulating neurons that are usually responsive to FIT to reduce their sensitivity to protect themselves from overstimulation.
Ligand/receptor interactions often have negative feedback mechanisms that vary the activation of the target cells in response to exposure to a specific signal. This phenomenon is best exemplified in Type 2 diabetes and certain cases of obesity in humans where peripheral tissues become insensitive to insulin stimulation or when responses to leptin are decreased due to chronic overexposure (Benoit et al., 2004; Davis et al., 2009). Additional characterization of *fit* gene product(s) is required to determine if FIT is secreted and targets the CNS or functions within the fat body to regulate appetitive behaviors. If FIT does function as a signaling molecule than the identity and characterization of the receptor(s) should be investigated as well. I predict that the adult phenotypes I identified in *fit* mutants will also be visible in FIT receptor mutants. Nonetheless, my study has contributed toward a broad effort in understanding mechanisms that regulate appetite and nutrient homeostasis by characterizing the anorexigenic gene *fit* in *Drosophila melanogaster*.

CHAPTER V
SUMMARY AND CONCLUSIONS*

5.1 Regulation of adult physiology and behavior

The primary role of adults is reproduction, a process in insects that is plastic but for the most part genetically programmed. For males to accomplish this goal, they must seek out conspecific receptive mates, execute species-specific courtship behaviors, and successfully deliver gametes and other behavior modifying factors to the female. The physiology and behavior of a recently mated female changes due to potent neuromodulators present in the male ejaculate (Wong and Wolfner, 2006; Meunier et al., 2007). These signaling peptides increase feeding, activate oogenesis, increase egg laying, and reduce receptivity to remating. My work focused on two signaling systems that regulate gamete production, reproductive behavior, feeding dynamics, stress resistance, activity, and nutrient homeostasis.

5.2 Adult ecdysone signaling activity is age and tissue dependent

I identified EcR protein and *usp* mRNA in essentially all adult tissues (Figure 2, Table 6) suggesting that many adult tissues have the potential to respond to ecdysone signals to regulate adult physiology. Surprisingly, EcR/USP activity was rarely detected in a handful of these tissues (CNS, fat body, and testes), while it is consistently present in others (Figure 8, Table 11). EcR activity in the CNS and fat body declined with age while simultaneously increasing in the oenocytes. This finding suggests that ecdysone sensitivity varies with tissue type and is influenced by adult age. While I have characterized adult *EcRE-LacZ* expression for the initial 10 days of adult life, it would be very interesting to

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observe *EcRE-LacZ* activity for a more extended period. Lifespan extension has been reported in *EcR* and ecdysone mutants (Table 4 and 5) but our understanding of how this and other phenotypes are impacted by EcR activity remains weak. The EcR/USP activity reported was provided using a canonical *EcRE* sequence from the promoter of *Hsp27* (Koelle et al., 1991), yet many variations in ecdysone-responsive genes' promoter sequences have been identified, which may influence their resulting expression. Further analysis of a subset of known ecdysone-responsive genes would be useful for directly characterizing ecdysone-responsive gene expression in adult tissues.

5.3 Ecdysone receptors in the fat body and CNS act as repressors

I was surprised to find that EcR in the adult CNS and fat body acted mainly as repressors and did not respond to ecdysone stimulation. Previous studies focused on memory and learning had identified EcR in the CNS (Ishimoto et al., 2009) but never specifically assayed the influence of EcR in the CNS through tissue-specific mutant analysis. In that study, *EcR* mutants were given ecdysone-supplemented media during behavior training. Ecdysone stimulation of *EcR* mutants in this case resulted in memory and learning levels similar to wildtype (Ishimoto et al., 2009). My work shows that this method fails to cause detectable increases in EcR activity in the CNS or fat body (Figure 10), suggesting that learning and memory are influenced by a change in EcR function in alternate tissues. It is my prediction that adult EcR-expressing cells in the CNS and fat body are resistant to ecdysone as a protection mechanism. During metamorphosis ecdysone levels rise and activate EcR in these tissues and result in neuron growth, differentiation, and apoptosis. The effects of changes like these to cells which function as the primary regulator of memory and behavior or the primary site of nutrient storage as an adult would be detrimental. I predict that these tissues express components that either inhibit EcR activation by ecdysone,

inactivate ecdysone, block recruitment of coactivators by activated EcR, or some combination of the these conditions.

5.4 Robust adult ecdysone receptor activity

Adult gut, tubules, male accessory glands and developing oocytes tissues were consistently positive for *EcRE-LacZ* expression (Table 11 and Figure 9a,c,d). Male accessory glands secrete protein components of the ejaculate that influence sperm storage, activate oogenesis, and alter female behavior (Reviewed by Avila et al., 2011). Robust *EcRE-LacZ* expression was detected in the primary cell type, accessory gland main cells, but not the secondary cells, which are a small population of binucleate cells at the distal tip of the gland (Bertram et al., 1992). The role of EcR in male accessory glands has not been formally investigated, but EcR may play a role in influencing accessory peptide (ACP) production or secretory cell maintenance. If EcR activation is required for ACP production perhaps serially mated adult *EcR* mutant males would have reduced ability to consistently induce strong behavioral or physiological changes in mated females.

EcRE-LacZ expression in the gut and tubule was consistently detected throughout the time period assayed. While the function of the identified EcR activity and the mechanism supporting this level of activation remain unclear, I predict that EcR activity in these cells is involved in cellular regeneration due to EcR's known role in cellular differentiation and growth. The *EcRE-LacZ* expression (Table 11) is very similar to the Notch signaling activity in the intestinal stem cells of the gut (Biteau et al., 2008) and to the expression pattern of a differentiation factor *teashirt* in multipotent stem cells of the tubules (Singh et al., 2007). These same patterning genes are regulated by ecdysone signaling (Li and White, 2003), suggesting that gut regeneration in *EcR* or ecdysone mutants could be inhibited and influence gut function or nutrient homeostasis.

Developing oocytes require ecdysone stimulation for follicle cell proliferation (Gaziova et al., 2004) and egg shell gene expression through *Eip74EF* and *Eip75B* (Terashima and Bownes, 2005; Terashima and Bownes, 2006). I see a common theme in EcR activity that correlates nicely with adult cells that are differentiating like the regeneration of gut and tubule cells from stem cells (Ohlstein and Spradling, 2005) or the rapid proliferation of follicle cells in the developing egg (Spradling et al., 2001). I have shown that tissue-specific differences in EcR activity are due to distinct differences in transcriptional environment and not simply EcR or ecdysone availability. Identification of the components determining if an adult tissue type is responsive to or protected from endogenous or pharmaceutical ecdysone activation is an area of study that needs further investigation.

5.5 Relating ecdysone signaling activity and mutant phenotypes to EcR function

Many of the previously described phenotypes illustrate cases where ecdysone stimulation and EcR regulate multiple processes in adult tissues. It appears that minimal levels of activation are required since whole-body levels of ecdysone (Table 3) are a fraction of the levels found in metamorphic pupae when organism-wide EcR/USP-mediated gene expression is occurring (Borst et al., 1974; Hodgetts et al., 1977; Handler, 1982; Kozlova and Thummel, 2002; Hu et al., 2003). By considering the type of EcR mutation involved, the resulting phenotype, and the tissue-specific activity of EcR/USP, I can identify potential mechanisms responsible for reported changes in adult physiology. Here I will discuss the proposed involvement and function of ecdysone signaling in three major tissue types.

5.5.1 EcR in the CNS

The CNS regulates many aspects of adult physiology, behavior, and reproduction. EcR and usp are expressed in the CNS throughout adult life, but EcR/USP activity in the CNS is present only in young animals, after which activity significantly diminishes and becomes unresponsive to environmental stimuli. Male–male courtship increases when EcR function is reduced in *fru*-expressing neurons by expression of *EcRi* or EcR^{DN}, suggesting a role for EcR in establishing wild-type courtship (Dalton et al., 2009). The fru^{gal4} driver used in the study is active in both pupal and adult neurons (Stockinger et al., 2005), but the main requirement for EcR in preventing male–male courtship appears to be during development since expression of EcR^{DN} only in the adult stage does not induce male–male courtship (Dalton et al., 2009). My work limiting EcR reduction to the male CNS post-eclosion did not result in increased male–male courtship behavior (Table 8a-d), suggesting that EcR activation is only required for male behavior circuit development prior to eclosion. These observations are consistent with neural EcR/USP activity being high only in newly eclosed adults (Figure 8 and Table 11) and suggest that EcR-mediated gene activation in *fru* neurons during metamorphosis and not the adult stage inhibit male–male courtship behaviors.

Adult sleep patterns are influenced by ecdysone signaling, responding to both changes in ecdysone levels and EcR expression (Ishimoto and Kitamoto, 2010). Ecdysone supplement increases sleep duration, presumably through activation of EcR/USP in the mushroom body neurons since mushroom body over-expression of either EcR-A or EcR-B1 isoforms leads to increased sleep (Ishimoto and Kitamoto, 2010). Ecdysone levels increase in sleep deprived animals, suggesting that ecdysone production somehow alters neuronal function (Ishimoto and Kitamoto, 2010).

Similarly, neurons involved in the creation, storage, and retrieval of memories require ecdysone and EcR activity. Wild-type males form long-term courtship memories (LTM) in a process that likely involves neural signaling in the mushroom body and other brain regions. This memory consolidation requires both ecdysone and EcR since LTM formation does not occur in males with reduced whole-body levels of hormone or receptor, and ecdysone feeding during the training period rescues both types of mutants (Ishimoto et al., 2009). Therefore, it appears that ecdysone regulates genes involved in memory consolidation via the activity of its receptor, but the tissues which require EcR/USP activation to govern these processes have not been identified.

In the assay, males were trained with courtship objects when they were 3–5 days old and tested for LTM when they were 8–10 days old. Since neural EcR/USP activity decreases rapidly within 5 days of eclosion (~90% of animals have CNS activity at day one; only ~10% have activity at day 5) and ecdysone feeding does not activate EcR/USP in animals aged similarly to those in the LTM study, it remains unclear how ecdysone signaling modulates LTM formation. It is likely that the spike in ecdysone levels in response to the LTM training paradigm (Ishimoto et al., 2009) is accompanied by a transient increase in EcR/USP activity. During the training, extensive male–female social interactions result in ecdysone increases in male adults (Ishimoto et al., 2009). The importance of this spike in ecdysone levels in response to social stimulation is not known (Ishimoto et al., 2009). However, adults aged in social groups of 15–20 animals do not show signs of EcR/USP activation. In the study, an increase in neural EcR/USP activity due to ecdysone feeding or tissue incubation was not detected, but the researchers did not examine immediate effects of exogenous hormone. It is possible that continual feeding or long-term social interaction may have de-sensitized the system by the time activity was measured or that the *EcRE-lacZ* reporter is not sensitive enough to detect small changes in expression from ecdysone signaling.

Another possibility is that transient whole-body increases in ecdysone act on one or more non-neural tissues to modulate memory consolidation indirectly. An intriguing candidate tissue to exert such an effect is the fat body. The fat body modulates reproductive behavior, possibly by secreting factors that interact with neurons to influence their activity (Dauwalder, 2008). The fat body also expresses EcR (Figure 2) as well as many genes that are responsive to changing social conditions (Ellis and Carney, 2011).

5.5.2 EcR in the fat body

Fat body tissue lines the interior of the cuticle in all 3 adult body segments, existing in close proximity to the CNS, gut, and gonads. Genes expressed in the fat body influence behavior, metabolism, immunity, appetite, and yolk protein synthesis (Lazareva et al., 2007; reviewed by Lemaitre and Hoffmann, 2007; Roy et al., 2007; Arrese and Soulages, 2009). EcR null mutants have defects in oogenesis and oviposition (Carney and Bender, 2000), yet fat body EcR/USP activity does not detectably increase in mated females, which should initiate ecdysone-dependent yolk synthesis.

EcR/USP activity is rarely detected in the fat body shortly after eclosion (Table 11), suggesting that EcR protein mainly functions as a repressor of gene expression in adult fat body. Reducing EcR levels by *EcRi* or by expressing EcR^{DN} concomitantly in male fat body, gut, and testis sheath increases longevity, implicating one or more of these tissues in regulating lifespan (Tricoire et al., 2009). *EcRi* should decrease overall numbers of receptors, while EcR^{DN} should affect receptor response to hormone without affecting receptor number. Both types of manipulations are expected to result in reduced activation of EcR/USP-regulated gene expression if the principal tissue affected mainly contains active receptors. Because the Gal4 driver's expression is not limited to fat, the increase in lifespan maybe due to reduced EcR/USP-activated gene expression in the gut (where EcR/USP activity is moderate) and not from altered EcR function in the

fat (which should contain mainly inactive receptors). More careful investigation of the effects on longevity from each strategy will help clarify the relationship between gene expression and gene repression in regulating this important trait.

5.5.3 EcR in the ovary

The ovary houses maturing oocytes and acts as an emergency energy reserve for females experiencing brief nutrient deficit. Adult female reproduction requires ecdysone signaling for wild-type oocyte maturation and oviposition (Buszczak et al., 1999; Carney and Bender, 2000; Hackney et al., 2007), a characteristic reported in other insect species as well (Wang et al., 2000; Roy et al., 2007; Takeuchi et al., 2007; reviewed by Swevers and Iatrou, (2009)). Ecdysone synthesis mutations cause severe defects in oocyte maturation and oviposition rates (Audit-Lamour and Busson, 1981; Walker et al., 1987). Similarly, oocyte expression of EcR^{DN} produces eggs with thin shells (Hackney et al., 2007), while *EcRi-B1* in follicle cells induces apoptosis (Romani et al., 2009). EcR expressed in the ovary (Carney and Bender, 2000), activates ecdysone-responsive genes since EcR/USP activity is consistently detected in developing eggs, but not in mature eggs (Figure 8). I found that adult containing EcR^{TS} and EcR^{2} (one containing a lesion spanning the *EcR-B1* and *EcR-B2* locus) had reduced egg-laying rates (Figure 4) but that reduction of EcR-B1 in the fat body increased egg laying rates (Figure 5). This activity compares well with previous studies which focused on egg development and gene expression (Buszczak et al., 1999; Terashima and Bownes, 2005; Terashima and Bownes, 2006). Female *EcR* mutants are starvation resistant (Simon et al., 2003), potentially because increased egg degeneration and reduced egg maturation frees up nutrients to compensate for the deficit in feeding.

5.6 Gene activation versus repression

One area that has received little attention, with the exception of a few developmental studies (Schubiger and Truman, 2000; Schubiger et al., 2005), is the relative contribution of EcR/USP in mediating gene activation compared to repression. Receptors that are not bound to hormone repress genes; upon binding hormone, receptor conformational change allows recruitment of cofactors necessary for activating target genes (reviewed by King Jones and Thummel, 2005). Different phenotypic consequences may arise depending upon whether or not receptors are completely eliminated (EcR null), partially eliminated (*EcR*/+ or *EcRi*), or cannot be activated by hormone (EcR^{DN}) yet the phenotypes themselves are due to the resulting changes in EcR regulated gene expression. In the simplest circumstances (discounting potential autoregulatory effects), loss or partial loss of receptor expression is expected to affect gene activation as well as repression, whereas receptors that do not respond to hormone (EcR^{DN}) are expected to function exclusively as repressors.

In the only experiments that compared phenotypes from adult-specific *EcRi* and EcR^{DN}, there is not a clear difference in effects on lifespan (Tricoire et al., 2009). These results are surprising since many tissues express receptors that are not activated, indicating that they repress expression under the assay conditions (Table 11). The lack of phenotypic difference between the two strategies may be a consequence of the assay method, which relies upon identifying a strong phenotypic difference between the treatments. It is possible that the *EcRE-LacZ* reporter will be useful for distinguishing tissue-specific effects from the two methods. However, a more specific question may need to be asked. For example, lifespan is likely to be affected by positive as well as negative effects from hormone signaling, so it will be more important to identify the specific loci that are activated or de-repressed due to changes in EcR/USP activity.

5.7 Linking hormone to activity

Ecdysone treatment in adults leads to rapid metabolism and excretion of ecdysteroid metabolites but does not alter EcR/USP activity (Smith and Bownes, 1985) (Figure 10). In many tissues these receptors appear to have a repressive role in restricting genes that control cellular reorganization during development so that they do not disrupt adult physiology. In some tissues EcR/USP consistently activates gene expression (Table 11), but it is unclear how this gene expression is regulated. There is evidence that EcR and USP are phosphoproteins (Sun and Song, 2006). Phosphorylation state changes EcR/USP's ability to recruit coactivators such as *Taiman* (Bai et al., 2000) and *Bonus* (Beckstead et al., 2001), which are required during development and reproduction.

A recent study in salivary glands showed that *Eip75A* nuclear receptors replace EcR/USP at *EcREs* when ecdysone is undetectable during the larval-to-pupal developmental transition. In this environment EcR/USP remains outside of the nucleus and *Eip75A* appears to function as a repressor in the place of the EcR/USP heterodimer (Johnston et al., 2011). However, in adult tissues EcR is almost exclusively observed in the nucleus (Figure 2), indicating that levels of ecdysone are sufficient for EcR nuclear localization or that Eip75A is not able to outcompete EcR/USP. The repressive effects of *Eip75A* are also dependent upon nitrous oxide (NO) activity (Johnston et al., 2011), which is known to affect nuclear receptor localization and gene regulation (Cronauer et al., 2007). *Nitric oxide synthase (NOS)* is expressed at low levels in adult tissues and is enriched only in CNS and testis (Chintapalli et al., 2007). Further study is warranted to strengthen our understanding of adult ecdysone-responsive gene repression by EcR/USP or other factors such as Eip75A.

Induction of EcR/USP-regulated gene expression is detected when nanomolar levels of ecdysone are present (Hu et al., 2003). If a subset of adult tissues experiences sufficient levels of ecdysteroid signals to activate EcR/USP

under standard laboratory conditions, gene expression could only occur through hormone enrichment in specific tissues, as whole-body ecdysone content is a fraction of that found in pupae (Handler, 1982). To determine whether specific adult tissues sequester ecdysteroids, radio labeled ecdysone was administered to adults and later detected in the individual tissues, before being quickly eliminated (Grau and Lafont, 1994). This evidence leads us to predict that unknown transcriptional components are silencing EcR/USP in specific adult tissues as a form of endocrine protection.

It remains unclear what information is being transduced by ecdysteroid signals in the adult. Wild-type adult tissues are unresponsive to ecdysone stimulation (Figure 10), but utilizing a more sensitive and quantifiable technique such as transcriptional analysis of ecdysone-responsive genes could identify subtle changes in expression associated with changes in physiology. For example, linkages between insulin signaling and ecdysone signaling have been proposed (Tu et al., 2002; Simon et al., 2003; reviewed by Tatar et al., (2003); Giannakou and Partridge, 2007; Francis et al., 2010), suggesting that elevation of ecdysteroid levels is linked to nutrient deficit. To identify genes which could alter the responsiveness of a tissue, one could focus on genes which differ in expression in fat body early in adult life when EcR/USP is active and compare gene expression to fat body at day 10 when EcR/USP activity is reduced.

5.8 Characterization of adult fat body functions

Like the ecdysone signaling system, the insect fat body influences multiple aspects of adult physiology, behavior, feeding, and stress resistance. I investigated the function of a fat body-biased gene, *fit*, whose expression is responsive to reproductive behaviors as well as nutrient availability. In my analysis I found that adult *fit* mutant feeding behavior, activity, starvation resistance, and nutrient content are all increased. I found that changes in

starvation resistance were influenced by *fit* expression in the fat body, but it remains unclear what signaling pathways *fit* influences to alter adult physiology.

5.9 Adult feeding behavior

I found that feeding rate increased in virgin *fit* mutant females- a phenotype I am currently following up on by characterizing food preference. Perhaps the increased feeding is due to a shift in nutrient demands. Virgin wild-type adults prefer a diet rich in carbohydrates unless deprived of protein for extended periods of time (Ribiero and Dickson, 2010). Mated wild-type females prefer protein rich diets (Carvalho et al., 2006), but this shift in nutrient preference is poorly understood. Investigating food preference in *fit* mutants would provide evidence for *fit's* involvement in changing gustatory neuron function. The increased feeding in *fit* mutants could be due to a compensatory response in feeding because a sensory system is not properly detecting ingested nutrients.

Currently, I have starvation resistance data from testing fat body overexpression and RNAi in adults, but lack a complete understanding of the feeding behaviors and nutrient levels of these adults. Expanding my feeding and nutrient content assays to include these adults will strengthen our understanding of *fit's* influence on nutrient homeostasis and better characterize responses to altered *fit* expression in multiple backgrounds. I expect that adults with non-wild-type expression of *fit* in the fat body will share many similarities with *fit* mutants with regard to nutrient content, feeding behavior, and activity. Identifying these phenotypes in flies experiencing non-wild-type *fit* expression would suggest that *fit* functions in the fat body to regulate these processes.

5.10 Adult nutrient homeostasis

Increased adult feeding behavior in *fit* mutants may be due to perceived nutrient stress. In this case the animal fails to accurately assess internal nutrient

state and will likely respond by increasing food uptake behaviors and/or reducing the activity of pathways such as TOR or insulin signaling. Insulin signaling has shown to be nutrient responsive and results in activation of downstream targets such as TOR and S6K (Ribiero and Dickson, 2010), or downregulation of FOXO (Gershman et al., 2006). These factors represent well characterized regulators of cell activity and growth. Characterizing insulin, TOR, S6K or FOXO activity in *fit* mutants would then be a tangible means of assaying an animal's perceived nutrient state and help identify interactions with known metabolic, growth, and nutrient responsive signaling pathways. I expect that *fit* mutants would have elevated TOR, S6K activity due to the increased feeding activity and resultant nutrient availability but it is unclear if this change will result in altered insulin signaling.

5.11 FIT characterization

To better understand how *fit* gene product(s) influence adult physiology these peptides need to be characterized in adult tissues. Our lab has developed new antisera that target 3 different epitopes of the predicted FIT sequence. These epitopes were targeted in order to detect potential cleavage products in both tissues where mRNA expression has been identified, mainly CNS and fat body, but also other tissues such as eggs where peptide fragments matching FIT sequences have been detected by mass spectroscopy (Nakahara et al., 2005). Being able to track the FIT peptide(s) will aid in characterizing molecular functionality by providing cellular localization information useful for identifying a receptor or other associated molecules.

5.12 Summary

My work has characterized ecdysone receptor expression and activity in adult tissues under standard laboratory and stressful conditions. EcR, a prolific regulator of adult gene expression, functions as a repressor in some tissues (fat

body, CNS) and an activator in others (male accessory glands, tubules, and gut). EcR activity is strictly regulated in the mature adult fat body and CNS. EcR/USP controlled gene expression in these tissues failed to respond to ecdysone stimulation perhaps to protect these tissues from genes which induce changes in cell activity that are inappropriate for adult function. Knowing the wild-type activity of EcR in adult tissues gives investigators an advantage when designing experiments aimed at characterizing a tissue's role in specific *EcR* mutant phenotypes. However, adult ecdysone content remains poorly understood, the location of synthesis remains elusive, and mechanisms influencing EcR regulated gene expression all require further investigation.

Another means of regulating adult behavior and nutrient homeostasis involves the activity of the fat body. My work shows that the fat body-biased gene *fit* influences starvation resistance by an increase in feeding behavior and nutrient accumulation. *fit* expression is responsive to nutrient availability and reproductive state and is gender biased. It is also very intriguing that mated wild-type females and virgin *fit* mutants share so many phenotypic similarities. Although the gene products are molecularly uncharacterized this anorexigenic fat signal in many ways parallels the vertebrate appetite regulating peptide leptin. Future investigations should focus on identifying FIT mode-of-action so that FIT function can be integrated into the context of existing nutrient homeostasis pathways.

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