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THE RELATIVE QUANTIFICATION OF *DROSOPHILA BROAD COMPLEX* EXPRESSION IN *βFTZ-F1* LOSS-OF-FUNCTION MUTANTS DURING METAMORPHOSIS

by

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‘যদি গহন পথে যাবার কালে কেউ ফিরে না চায়---
তবে পথের কাঁটা
ও তুই রক্তমাখা চরণতলে একলা দলো রে’

- রবীন্দ্রনাথ ঠাকুর

Nana this thesis is dedicated to you.

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ABSTRACT

Steroid hormones play a crucial role in the development of higher organisms. In the fruit fly *Drosophila melanogaster,* fluctuating levels of the one functional steroid hormone 20-hydroxyecdysone (ecdysone) control important developmental changes throughout the life cycle. In the salivary gland a pulse of ecdysone triggers the expression of the early genes *BR-C* (Broad Complex)*, E74A*, and *E75A* at the beginning of metamorphosis or 0 hours after puparium formation (APF). Roughly 10 hours APF another ecdysone pulse triggers the prepupal to pupal transition mediated by the *βFTZ-F1* gene, which is expressed at 6 hours APF. At 10 hours APF, the *βFTZ-F1* gene provides the early genes *BR-C*, *E74A, E75A* and *E93* the competence to be induced by ecdysone in certain tissues (Woodard, 1994). I am trying to determine which tissues require *βFTZ-F1* for proper genetic response to ecdysone. In my experiments *βFTZ-F1* loss-of-function mutants and wild type flies (*w1118*) have been used to examine the difference in the expression of *BR-C* mRNA in various tissues in mutants compared to controls. My results thus far indicate that *βFTZ-F1* mutant salivary gland contained less *BR-C* mRNA compared to the wild type salivary gland at 10 hours APF while mutant hindgut expressed an increased level of *BR-C* compared to controls at 10 and 12 hours APF.

INTRODUCTION

Over the past century molecular genetic studies of *Drosophila melanogaster* have led to advances in the understanding of the regulation of developmental processes (Arbeitman, 2002). Large scale changes in gene expression accompany development (Levy and Manning, 1981), a fact which is demonstrated in the fruit fly, *D melanogaster*. Over 5000 to 7000 different polyadenylated RNA species are produced at each stage of the *Drosophila* life cycle and the composition of this set of RNAs shift during development through metamorphosis (Levy and Manning, 1981).

The *Drosophila* genome encodes 18 nuclear receptors, compared to 48 in humans and 284 in *C. elegans*. The fruit fly possess the smallest complete set of nuclear receptors known in any genetic model system, providing a good model for studying nuclear receptor regulation and function (King-Jones and Thummel, 2005).

Lipophilic hormones such as steroids serve as regulators of development, cell differentiation and organ physiology (Mangelsdorf, 1995). In *Drosophila*, the one functional steroid hormone, 20-hydroxyecdysone (hereafter referred to as ecdysone) controls important developmental processes including embryogenesis, organogenesis, and metamorphosis. *Drosophila* is an ideal organism with which

developmental processes can be analyzed and is the organism of choice for the purposes of this study.

Lifecycle of *Drosophila melanogaster*

The fruit fly, *Drosophila melanogaster* completes its life cycle (Fig. 1) in approximately 8 days at 25^{o}C and approximately 19 days at 18^{o}C, as temperature is one of the factors that affect the duration of the life cycle. Lower temperature increases the number of days it takes for the larva to reach the adult stage. Pulses of the steroid hormone ecdysone direct each of the major developmental transitions in the fly life cycle (Riddiford, 1993).

The eggs laid by female flies hatch after 12-15 hours at 25^{o}C. The resulting larvae then go through three phases of growth termed the first, second and the third larval instar stage. At each stage ecdysone pulses trigger molting of the cuticle, a response restricted largely to the epidermis (Riddiford, 1993) and the resulting larvae from each consecutive stage are bigger than the previous ones. A high titer ecdysone pulse at the end of the third larval instar stage triggers widespread changes throughout the animal, signaling the onset of prepupal development (Robertson, 1936). At this stage the larval salivary glands secrete a mixture of glue proteins that affix the animal to a solid surface for puparium formation or entry into the prepupae stage. The beginning of puparium formation marks entry into metamorphosis for *Drosophila*. Afterwards the animal shortens its body and the larval cuticle tans and hardens to form a protective pupal case.

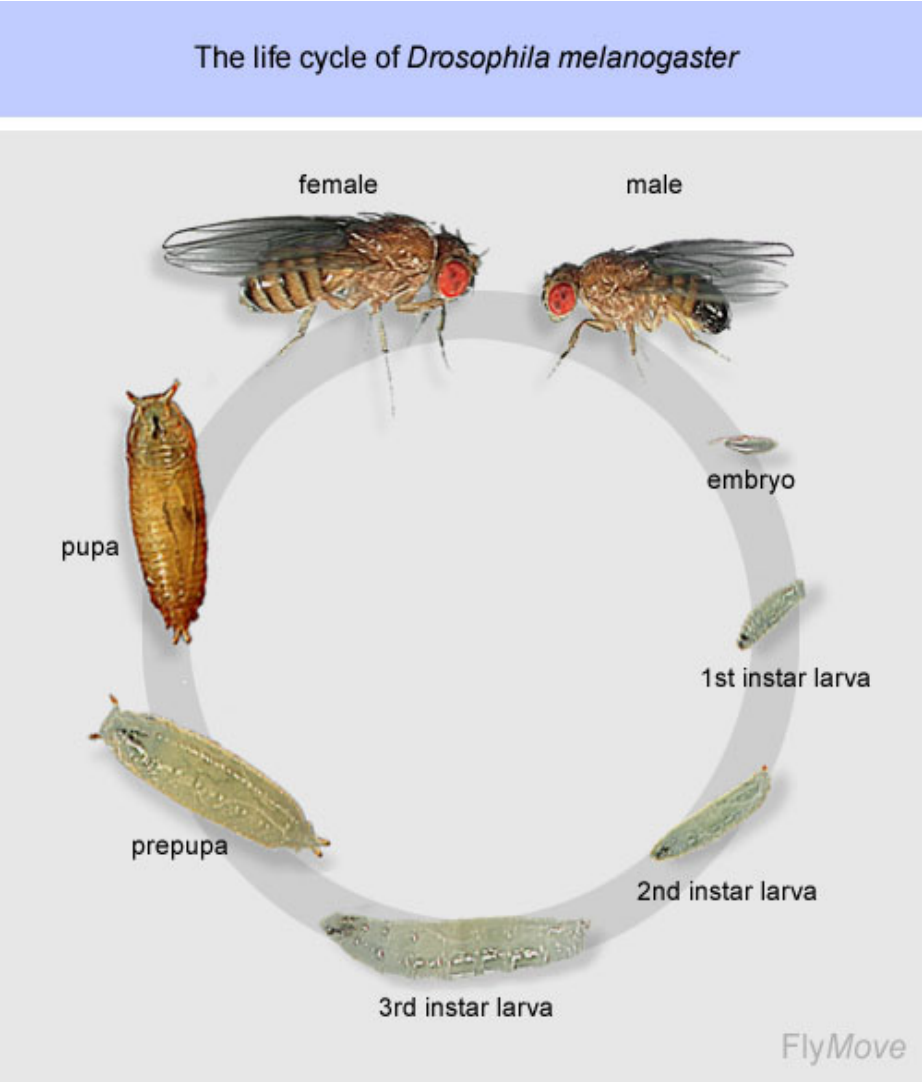


Figure 1. Lifecycle of *Drosophila melanogaster.* The fruit fly completes its life cycle in approximately 8 days at 25°C and goes through six different stages before eclosing as an adult fly. The stages consist of an embryonic stage which lasts for 0.5 days , three larval stages punctuated by the molting of the cuticle lasting approximately 6.5 days, a prepupal stage lasting 0.5 days and a 3.5 day long pupal stage after which an adult fly is ecloses. The short lifecycle of *Drosophila* is advantageous for research purposes because many generations can be cultivated in a short period of time (Ashburner and Thompson, 1978; Ashburner *et al*., 2005).

Throughout development subsets of larval tissues, including the midgut, initiate programmed cell death (Robertson, 1936) whiles the legs and wing imaginal discs evert and elongate to form rudiments of adult appendages.

A subsequent ecdysone pulse, ~ 12 hour after puparium formation (APF) triggers the prepupal-pupal transition. In response to this signal the head everts out of the anterior end of the puparium. This pulse also triggers the final extension of the wings and legs as well as the destruction of the remaining larval tissues including the salivary glands (Robertson, 1936).

These events form the basic body plan of the adult insect, with a head, thorax, abdomen and appendages. Terminal differentiation then occurs over ~3.5 days (25^{o}C) of pupal development after which the adult fly ecloses.

Ecdysone: The Lipophilic Steroid Hormone

Lipophilic hormones such as steroids serve as regulators of development, cell differentiation and organ physiology (Mangelsdorf *et al*., 1995). Unlike the water-soluble peptide hormones and growth factors which bind to cell surface receptors, the fat-soluble steroid hormones can pass through the lipid bilayer of the cell membrane and interact with their receptors. Historically, they were isolated in the early part of the 20th century based on their abilities to affect development, differentiation, metamorphosis, and physiology.

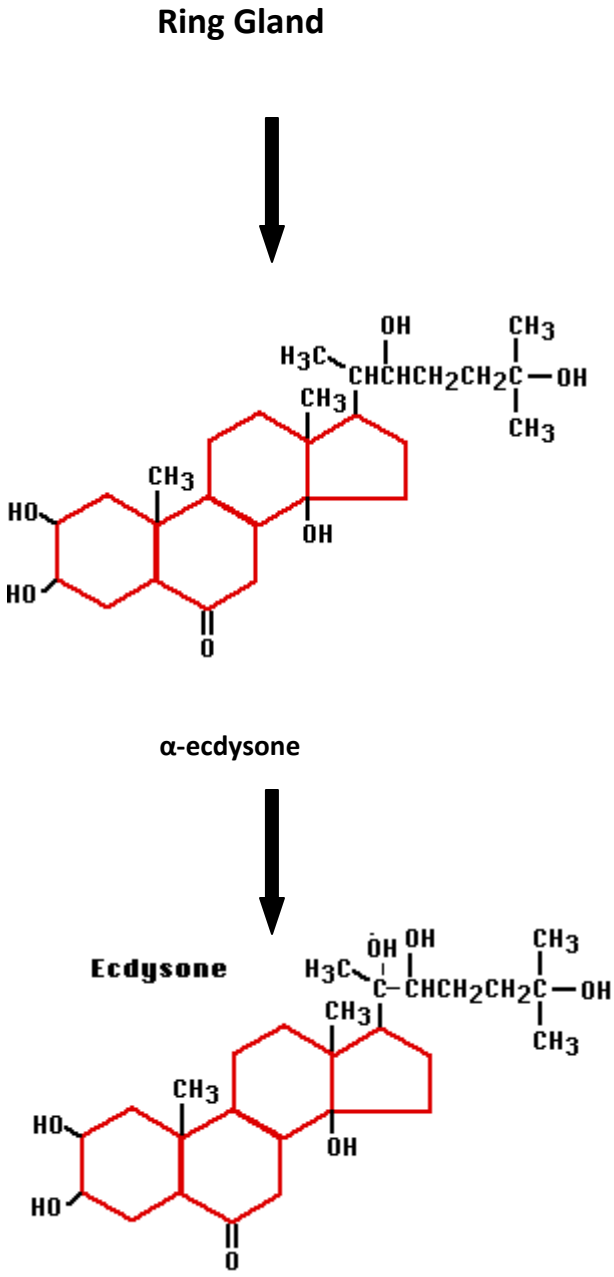


Figure 2. Conversion of α-ecdysone to functional 20-hydroxyecdysone. The *Drosophila* ring glad secretes the prohormone α-ecdysone and 20-deoxymakisterone (not shown in this figure). α-ecdysone is converted into 20-hydroxy ecdysone (ecdysone) upon reaching target tissues. Ecdysone possess the characteristic 3, six member ring and 1 five member ring that defines steroid hormones and is lipid soluble. (Modified from Riddiford, 1993)

Ecdysone is a steroid hormone found in invertebrates. It originates in the prothoracic glands of insects and in ovarian follicles. Prothoracic hormone (PTTH) is a neuropeptide which works as the key signal that initiates the release of the precursor steroid ecdysone (Henrich *et al*., 1999). The release of PTTH is controlled by various environmental and internal cues such as photoperiod, temperature, crowding and abdominal stretch (Randall *et al*., 2001). In *Drosophila* melanogaster the action of PTTH on the prothoracic ring gland results in a rise in prohormone α-ecdysone hemolymph concentrations and 20-deoxymakisterone (Riddiford, 1993). When α-ecdysone reaches its target tissue, it is converted to β-ecdysone or 20-hydroxyecdysone. Target tissues in this scenario include the epidermis, fat body, and imaginal discs during metamorphosis. 20-deoxymakisterone produces the hormone Makisterone A (Redfern, 1984) which is detectable in *Drosophila* larvae and may contribute to physiologically molting hormone levels in *Drosophila* grown on cornmeal-yeast media (Redfern, 1984).

Hormonal Regulation of Development

The ability of lipophilic hormones to diffuse from a source and permeate to a target, enable them to serve as regulators of developmental processes. Along with developmental regulation these hormones, including the steroids, retinoids, thyroid hormones, and vitamin D, act as regulators of cell differentiation, and organ physiology. While the mediators of the action of these hormones remain elusive, development of radiolabeled ligands have allowed the identification of

binding proteins that were shown to translocate from the cytoplasm to the nucleus, leading to a suggested link between transcriptional control and physiology (Jensen et al., 1966).

This link was first elaborated by the work of Ashburner et al. (1974), which demonstrated that ecdysteroids, the metamorphic hormones of insects, trigger chromosomal puffing at specific sites in the *Drosophila* salivary gland polytene chromosome. In the mid-1970s, steroids were shown to be targeted to their responsive tissues by the presence of specific high affinity receptor proteins. Unlike the water-soluble peptide hormones and growth factors, which bind to cell surface receptors, the fat-soluble steroid hormones can pass through the lipid bilayer of the cell membrane and interact with their cognate receptors. The subsequent identification of hormonally responsive target genes within these tissues completed the initial characterization of a steroid hormone signaling pathway. Yamamoto (1985) provided a model of steroid hormone action which postulated that the binding of hormone to its receptor induces an allosteric change that enables the hormone-receptor complex to bind to high affinity sites in chromatin and modulate transcription.

The Role of Ecdysone in Metamorphosis

Metamorphosis in holometabolous insects is the developmental process during which changes in gene expression trigger the remodeling of the larval form of the

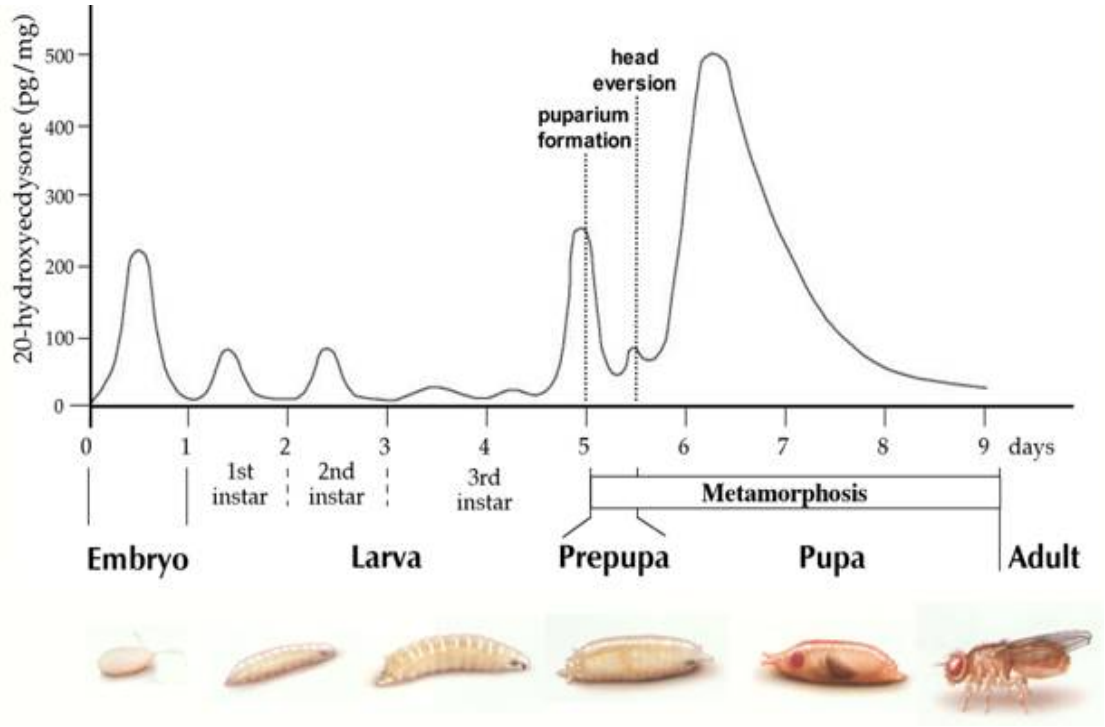


Figure 3. Levels of 20-hydroxyecdysone at the different stages of *Drosophila* development. Ecdysone level varies during the lifecycle of the fruit fly. Each important developmental change in its life cycle is controlled by varying level of ecdysone. The ecdysone pulse at the end of the third larval instar stage triggers puparium formation or the beginning of metamorphosis through which the larva begins the process of transformation into an adult animal. Approximately 10 hours after puparium formation (APF) another pulse of ecdysone marks the beginning of the pupal stage. In the beginning of the pre-pupal stage early genes important for larval tissue histolysis and formation of adult parts are expressed while *βFTZ-F1* is expressed right before head eversion when the ecdysone titer is low. Once development of the organism is completed ecdysone level goes down. (Modified from Riddiford, 1993).

animal to an adult. In *Drosophila* this process is governed by systemic signals from ecdysone (Fig. 3). During metamorphosis ecdysone elicits divergent responses in different target tissues. As can be seen from figure 3, ecdysone pulses trigger major developmental changes including but not limited to embryogenesis, puparium formation, and head eversion. The beginning of puparium formation is referred to as 0 hours after puparium formation (0 hours APF) and marks the beginning of the prepupal stage. 12 hours APF another ecdysone pulse triggers prepual-pupal transition. At the beginning of puparium formation the genes *E74, E75* and *BR-C* are induced by the ecdysone pulse while at 6-8 hours APF the gene *βftz-f1* is induced and re induces the early genes and the stage specific early gene *E93* at 10-12 hours APF.

In *Drosophila* many larval tissue such as the salivary glands, respond to ecdysone first by initiating expression of new genes and later by undergoing histolysis (Robertson, 1936). Imaginal discs, imaginal histoblasts, and imaginal rings are induced by ecdysone to undergo morphogenetic changes to form adult structures such as epidermis, appendages, and internal organs even as the larval tissues degenerate (Fristrom and Fristrom, 1993).

Ashburner's Model of Ecdysone Action

Ecdysone induces hormone responsive puffs in the salivary gland polytene chromosome designated as intermolt, early, early-late and late puffs. These are sequentially activated during the larval period preceding puparium formation at

which stage the larva stops moving and begins cuticular tanning (Huet *et al*., 1995). The intermolt puffs are active at the beginning of the response to ecdysone, encode genes that code for salivary gland glue proteins and thereafter regress. Afterwards, at the beginning of puparium formation (Fig. 3), the regression of the intermolt puff genes occur. A small number of early puffs become active within minutes shortly after the increase in hormone titer (Ashburber 1967, 1972) and require the continuous presence of ecdysone for puffing (Ashburber and Richards, 1976). These puffs remain active for approximately 4 hours and then regress (Ashburner, 1967, 1972). Induction of these early puffs by ecdysone is insensitive to the inhibition of protein synthesis suggesting that the early gene set is a primary target for the steroid hormone.

The early-late puffs appear with a delay of two hours or so after the early puffs regress, and the late puffs, which appear from three hours onwards (Huet *et al*., 1995). The early-late puffs are considered a subclass of the late puffs. (Ashburner *et al*., 1974). However, they differ from the late puffs in that they regress immediately after hormonal withdrawal in culture, a characteristic shared with the early puffs.

The early puffs repress their own function while inducing the expression of many late puff genes including the secondary cell death genes (DiBello *et al*., 1991). Each of the four sets of puffs code for genes that play important roles in fly development.

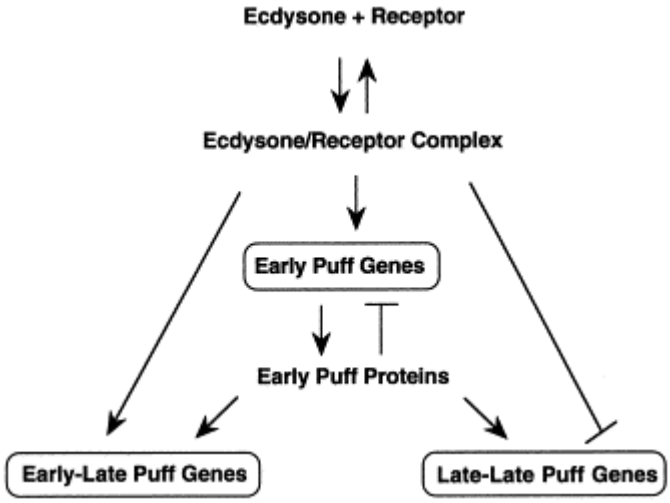


Figure 4. The Ashburner model for ecdysone-regulated puff gene expression. A modification of the Ashburner model is depicted in which the early, early late and late-late puffs have been included (Ashburner and Richards, 1976). Ecdysone, bound to its receptor, directly induces the early puff genes. These genes express proteins that perform dual regulatory functions — they repress their own expression and they induce the late puff genes. The early-late puff genes are also dependent on direct induction by the ecdysone–receptor complex, resulting in their expression earlier than the late-late puff genes, which are repressed by the ecdysone–receptor complex. (Modified from Ashburner *et al*., 1974)

Ashburner (1974) proposed a model that explained this hormone initiated and sequential induction of two coordinately expressed gene sets arising from the early and the late puffs (Fig. 4). A central feature of the model held that early gene products resulting from the early puffs act in *trans* to activate the late genes in a temporally delayed, hormonally dependant, and hierarchical manner.

Ashburner *et al*. (1974) proposed a model for the activation of the early and late puffs (Fig. 4). Ecdysone, complexed with a receptor, activates directly and rapidly a few early puffs while repressing the late puffs. When the proteins encoded by the early puffs become sufficiently abundant they both repress their own promoters and activate the late genes (Huet *et al*., 1995). The early genes are expressed in all tissues studied in this experiment, suggesting that the general features of the Ashburner model apply tissues other than the salivary gland (Andres and Thummel, 1992; Huet *et al*., 1993).

Although the early genes are potential regulators for the late class of genes or for their own repression, the specific functions of each early gene product remain to be determined (Huet *et al*., 1995).

Imaginal discs, precursors of adult epidermal structures, also exhibit a hierarchical set of ecdysone dependant developmental and transcriptional responses that are similar to those of the salivary glands (Fristrom *et al*. 1985). The molecular characterization of several early genes provides support for the Ashburner model (Fristrom *et al*. 1985).

Drosophila **Ecdysone Receptor**

Ecdysone plays a key role in development via its interaction with the ecdysone receptor, the only ligand dependant nuclear receptor that has been identified in *Drosophila* (Thummel, 1995). The ecdysone receptor consists of a heterodimer that resembles a vertebrate retinoic acid receptor more than a steroid receptor.

Half of the ecdysone receptor is encoded by the *EcR* gene (Koelle *et al*., 1991). *EcR* is induced directly by ecdysone, providing an autoregulatory loop that increases the level of receptor protein in response to its ligand. The three protein isoforms that are encoded by *EcR*, are termed *ECR-A, ECR-B1,* and *ECR-B2* (Talbot *et al*., 1993). These proteins differ in their amino terminal sequences but contain identical DBD and LBD sequences. Two of these isoforms are expressed in patterns that correlate with the different developmental fates of the larval and adult tissues. The *ECR-B1* isoform is expressed primarily in larval cells fated to die while *ECR-A* is expressed in developing adult structures and tissues (Talbot *et al*., 1993).

All three *ECR* isoforms bind DNA after heterodimerization with *Ultraspiracle* (*USP*) (Yao *et al*., 1992). *USP* is the only known *Drosophila* homolog of the vertebrate RXRs (Table 1) (Oro *et al*., 1992; Shea *et al*., 1990).

Table 1. *Drosophila melanogaster* nuclear recptors.(King-Jones and Thummel, 2005)

D. melanogaster receptor (ligand)	Subfamily	Nomenclature	20E-regulated	No. of isoforms (PR/PP/RNA)	Human orthologue (ligand)	DBD/LDB identity
E75	1D/E	NR1D3	+	3/4/4	*REV-ERBA	80/25
E78	1D/E	NR1E1	+	2/3/2	*REV-ERBA	69/23
DHR3	1F	NR1F4	+	1/3/3	*RORB (all *trans* retinoic acid)	76/35
EcR (20-hydroxyecdysone)	1H	NR1H1	+	3/3/5	FXR (chenodeoxycholic acid) *LXR (22(R)-hydroxycholesterol)	72/28 64/37
DHR96	1I/J	NR1J1	(+)	1/1/2§	*VDR (1α, 25-dihydroxyvitamin D_3)	55/20
HNF4	2A	NR2A4	?	1/3/3	*HNF4A	89/61
USP	2B	NR2B4	–	1/1/1	RXRA (9-*cis*-retinoic acid)	84/43
DHR78	2C/D	NR2D1	(+)	1/2/4‡	*TR2	67/23
Tailless	2E	NR2E2	?	1/1/1	TLX	80/34
Dissatisfaction	2E	NR2E4	?	1/1/1	TLX	74/35
DHR83/CG10296	2E	NR2E5	?	0/1/1	PNR	60/20
DHR51/CG16801	2E	NR2E3	?	0/2‡/2‡	PNR	70/47
Seven up	2F	NR2F3	?	1/3/3	*COUP-TF1	89/92
ERR	3B	NR3B4	?	0/2/2	*ERRb (diethylstilbestrol)	88/34
DHR38	4A	NR4A4	–	2/2/2	*NURR1	93/59
FTZ-F1	5A	NR5A3	+	2/2/3§	LRH-1 (phospholipid) SF-1 (phospholipid)	89/35 88/28
DHR39	5A	NR5B1	+	2/2/2	LRH-1 (phospholipid) SF-1 (phospholipid)	62/25 60/26
DHR4	6A	NR6A2	+	1/2‡/2§	GCNF	61/21

*The boxed receptors have been discussed in this paper.

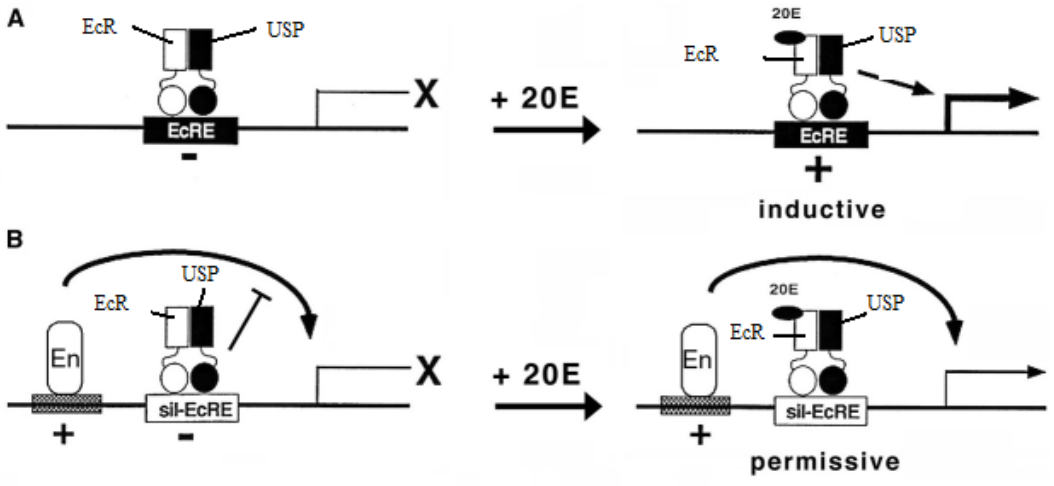


Figure 5. Simple working model for activation and silencing of hormone responsive genes. (A) Inductive *EcR*E. A 20E (ecdysone) inducible gene is repressed when its *EcR*E is bound by the unliganded receptor, but is strongly activated when the *EcR*E is bound by the liganded receptor. (B) Permissive *EcR*E. A signal at the mid-third instar induces an enhancer 'En', which binds to a response element of the gene. However, because of the presence of the unliganded receptor on the silencing *EcR*E (sil-*EcR*E), the gene remains suppressed. As the hormone titer rises, the liganded receptor no longer silences and leads to a permissive state, allowing transcription of the gene by the enhancer 'En'. (Modified from Schubiger and Truman, 2000)

Consistent with this sequence conservation, *USP* can heterodimerize with a variety of vertebrate RXR partners, including retinoic acid receptor, thyroid hormone receptor, and vitamin D receptor (Yao *et al*., 1992).

Although *ECR* can bind ligand weakly on its own, this binding is enhanced by the addition of *USP* (Koelle, 1992; Yao *et al*., 1993). Ligand binding both stabilizes the *ECR*-*USP* heterodimer and also increases its affinity for binding to ecdysone responsive elements (*EcR*Es) (Fig. 5). Two naturally occurring *EcR*Es, from the ecdysone inducible hsp27and fip28/29 promoters, are bound by *ECR*-*USP* (Yao *et al*., 1992). In addition, *ECR*-*USP* can bind to direct repeats of AGGTCA (Fig. 6) sequences separated by 3, 4, or 5 bp, much like vertebrate RXR heterodimers (Horner *et al*., 1995). Although it remains possible that *ECR* and *USP* may exert distinct functions as homodimers, in vivo evidence indicates that these proteins function as a heterodimer. Antibody stains of larval salivary gland polytene chromosomes support this, as all sites occupied by *ECR* also contain bound *USP* protein (Talbot, 1993; Yao *et al*., 1993).

The *USP* gene was originally identified based on its lethal mutant phenotypes, which suggest a role in mediating ecdysone responses during development (Oro *et al*., 1992). *USP* functions in abdominal cuticle synthesis during mid embryogenesis and larval cuticle molting. However, adult thoracic and abdominal metamorphosis can occur in the absence of *USP*, indicating that these

responses are either not regulated by ecdysone or are dependent on the activity of another nuclear receptor (Thummel, 1995).

Nuclear Receptor Superfamilly

The term nuclear receptor superfamily is used to encompass all of the known nuclear hormone receptors. The nuclear receptor superfamily is further divided into the steroid receptor family and the thyroid, retinoid, vitamin D (or nonsteroid) receptor family (T'sai and O'malley, 1994).

The steroid hormone receptor superfamily consists of a large number of genes (Mangelsdorf *et al*,,1992; Koelle *et al*., 1991), and represents the largest known family transcription factors in eukaryotes. It includes receptors for the steroids, estrogen (ER), progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and androgen (AR) as well as receptors for thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR), *9-cis* retinoic acid (RXR), and ecdysone (*EcR*). Since the ligands for these genes are not known, they have been termed "orphan receptors."

Amino acid sequence analysis and mutational dissection of intracellular receptors indicate that they can be subdivided into several domains as indicated in figure 6. The N-terminal A/B domain is highly variable in sequence and in length.

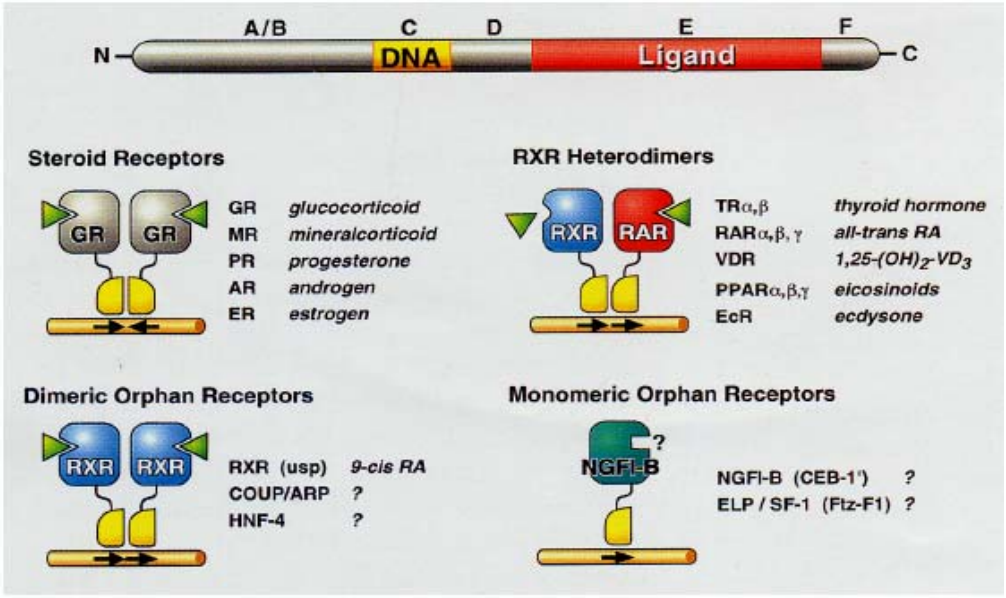


Figure 6. Nuclear Receptors Share Common Structure/Function Domains. A typical nuclear receptor contains a variable N-terminal region (A/B), a conserved DBD (C), a variable hinge region (D), a conserved LBD (E), and a variable C-terminal region (F). Nuclear receptors can be grouped into four classes according to their ligand binding, DNA binding, and dimerization properties: steroid receptors, RXR heterodimers, homodimeric orphan receptors, and monomeric orphan receptors. Shown are representative receptors for each group. Question marks refer to orphan receptors for which ligands are not known. (Mangelsdorf, 1995)

Usually, this domain contains a transactivation function (AF) (Fig. 7), which activates target genes presumably by interacting with components of the core transcriptional machinery, coactivators, or other transactivators (T'sai and O'malley, 1994). This region may be important also for determining target gene specificity for receptor isoforms, which recognize the same response element (T'sai and O'malley, 1994).

The C region contains two type II zinc (Zn) fingers, which are responsible for DNA recognition and dimerization (Mangelsdorf *et al*., 1995, T'sai and O'malley, 1994).

Downstream of the C region, a variable hinge region exists (D region). This region may allow the protein to bend or alter conformation, and often contains a nuclear localization domain (GR, PR) and/or transactivation domain (TR, GR) (T'sai and O'malley, 1994). The ligand-binding domain or E region is located carboxy-terminal to the D region.

The E region is relatively large (~250 aa) and is functionally complex. It usually contains regions important for heat-shock protein association, dimerization, nuclear localization, transactivation, intermolecular silencing (TR, RAR, COUP-TF, etc), intramolecular repression (PR) and, ligand binding . Although most of these functions require only small stretches of amino acid sequence, ligand binding appears to involve a majority of the E region (Fig. 6), as most of the mutations identified in the LBD compromise the ability of the altered

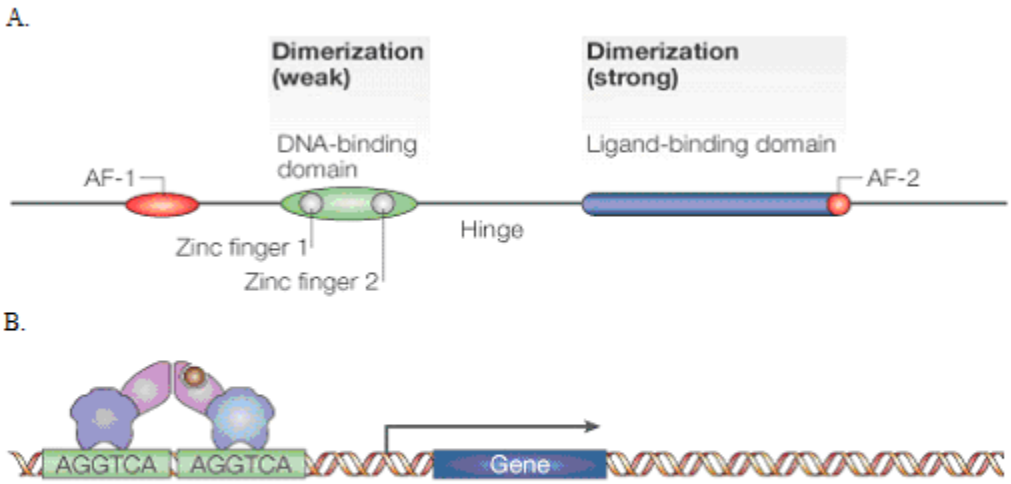


Figure 7. Nuclear receptors are defined by the presence of common structural elements: a highly conserved DNA-binding domain (DBD) and a ligand-binding domain (LBD) are joined by a flexible hinge region (panel A). The DBD comprises two C_4 zinc fingers. The first zinc finger provides DNA-binding specificity through a stretch of five amino acids known as the P-box. The second zinc finger possesses a relatively weak dimerization interface that allows DBDs to dimerize, but only in the presence of a target DNA molecule. The less conserved LBD is located C-terminal to the DBD, and constitutes the principal dimerization interface of this protein family. The LBD allows different receptors to dimerize, thereby vastly expanding the repertoire of potential DNA target sequences and regulatory functions. The activation function 1 (AF-1) domain can act in a ligand-independent fashion and is located at the N-terminus, whereas the AF-2 activation domain is located at the C-terminal end of the LBD and is often ligand-dependent. Each nuclear receptor binds to a half site that is derived from, or identical to, the archetypal AGGTCA sequence (panel B). These half sites can be arranged as direct repeats, inverted repeats or everted repeats, and display a dimer-specific spacing that generally ranges from 0–6 bp. The sequence and arrangement of the half sites contributes to receptor binding specificity.(Modified from King-Jones and Thummel, 2005)

receptor to bind hormones. The major dimerization domain of receptors has been localized in the C-terminal half of the LBD (T'sai and O'malley, 1994). This region contains leucine-rich sequences that may form coil-coil interactions as the receptor dimerizes.

Finally, located at the C-terminal end of certain receptors is the variable F region, for which no specific function has been identified. The nuclear receptors are characterized by a central DNA-binding domain (DBD) (Fig. 7) which targets the receptor to specific DNA sequences known as hormone response elements. The DBD is composed of two highly conserved zinc fingers that set the nuclear receptors apart from other DNA-binding proteins (Klug and Schwabe, 1995). The C-terminal half of the receptor encompasses the ligand-binding domain (LBD), which possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiologic response. The LBD thus functions as a molecular switch that, upon binding ligand, shifts the receptor to a transcriptionally active state (Mangelsdorf *et al*., 1995).

Nuclear Receptor Function in *D. melanogaster*

Drosophila melanogaster has provided an ideal model system for defining receptor function in the context of a developing animal. 18 genes of the nuclear receptor superfamily have been isolated in the fruit fly (King Jones and Thummel, 2005). In spite the small number, the fly nuclear receptors represent all major subclasses of human receptors and include orthologs of key human receptors as

demonstrated in table 1 (King-Jones and Thummel, 2005). The fly genes encode members of heterodimeric and orphan class of receptors (Mangelsdorf and Evans, 1995). Some Superfamily members function specifically during embryonic stages while others are required during multiple stages of development. Half of the known *Drosophila* superfamily members are regulated by ecdysone and contribute to the developmental pathway associated with metamorphosis (Thummel, 1995).

Receptor function during embryogenesis

Embryogenesis in *Drosophila* occurs during the first day of the life cycle and involves the initial segmentation of the animal followed by organogenesis. Of the *Drosophila* superfamily members, 8 appear to perform specific functions during embryonic development while some act at later stages as well. Two of the orphan receptors FTZ-F1 and DHR39 are related to one another within the DBD (63%) and are both related to the murine SF1 orphan receptor (Thummel, 1995). The *ftz-f1* gene encodes two protein isoforms with distinct amino terminal sequences joined to identical DBD and LBD sequences (Lavorgna *et al*., 1993). *αftz-f1* is expressed during early embryogenesis while *βftz-f1* is expressed in late embryos and during metamorphosis.

Importance of Early Genes

The small number of early puffs observable at the beginning of puparium formation in the salivary gland encodes genes that have been termed early genes.

These puffs are located at the cytogenetic loci 2B5, 74EF and 75B and code for *BR-C, E74,* and *E75* respectively. These three early ecdysone-inducible genes are large (>60 kb) and complex, with multiple overlapping transcription units, each encoding a family of proteins structurally related to transcription factors (Andres and Thummel, 1992). These transcription factors serve to transmit the ecdysone signal to downstream target genes (Burtis *et al*., 1990; Segraves and Hogness, 1990; DiBello *et al*., 1991).

Early Gene: *E75*

E75 is a complex early ecdysone-inducible gene, originating at the 75B puff that directs the synthesis of three protein isoforms, E75A, E75B and E75C, by the use of nested promoters (Segraves and Hogness, 1990). E75 proteins are members of the nuclear receptor superfamily. These proteins contain the canonical DNA binding domain and ligand binding domain that define members of the nuclear receptor superfamily, although they are referred to as orphan nuclear receptors because a corresponding hormonal ligand has not yet been identified (Mangelsdorf and Evans, 1995). *E75A* and *E75C* are orphan receptors that have distinct amino terminal sequences joined to an identical DBD and LBD. In contrast *E75B* retains only one zinc finger, suggesting that it is incapable of binding DNA. Antibody staining of polytene chromosomes has revealed that E75A protein is bound to both early and late gene loci, suggesting that it functions at both level of hierarchy (Hill *et al*., 1993). Also, studies have shown a role for

E75 in embryonic gut morphogenesis (Bilder and Scott, 1995). Several *ECR-USP* binding sites have also been identified upstream of the *E75*A promoter (Talbot, 1993).

E75B mutants are viable and fertile, suggesting that this gene functions in a redundant pathway during development, while *E75C* mutants die as adults. In contrast, most *E75A* mutants die as delayed second instar larvae with a reduced ecdysteroid titer, or arrest during the molt to the third instar. Remarkably, some *E75A* mutant second instar larvae express genes characteristic of the third instar and pupariate without progressing through a molt, indicating that molting can be uncoupled from the onset of metamorphosis (Bialecki et al., 2002).

Early gene: *E74*

The ecdysone-inducible early gene *E74* is a member of the ets proto-oncogene family and plays a key role in metamorphosis and larval tissue destruction. *E74* is encoded within the 74EF early puff and consists of two overlapping transcription units, *E74A* and *E74B*. Mutations in *E74* are predominantly lethal during prepupal and pupal development, indicating that *E74A* and *E74B* have essential functions during metamorphosis. *E74B* mutants are unable either to form a normal puparium or to evert their cephalic complexes, and die as prepupae or early pupae. In contrast, many *E74A* mutants survive the prepupal period and die as pharate adults.

Phenotypic analysis has revealed that *E74* function is required for both pupariation and pupation, and for the metamorphosis of both larval and imaginal tissues. *E74A* activity is also essential for the maximal induction of a subset of late puffs at puparium formation, confirming the results obtained by Walker and Ashburner (1981) and implicating *E74A* as one function in the 74EF/75B interval that is necessary for proper late puff regulation (Thummel *et al*., 1990).

E74 also plays a wide role in reshaping the insect during metamorphosis, affecting tissues other than the salivary gland in which it was originally identified. Overlapping *E74A* and *E74B* transcription units that make up the *E74* gene (Burtis *et al*., 1990) are ecdysone-inducible in late third instar tissues by direct activation of their promoters, and each is transcribed in association with all six major ecdysone pulses during development (Thummel *et al*., 1990). They are also widely expressed in late third instar larvae (Thummel *et al*., 1990; Karim and Thummel, 1991; Huet *et al*., 1993), providing the capacity to transduce the ecdysone signal in a variety of target tissues.

The E74A and E74B proteins are composed of distinct N-terminal regions and a common C-terminal region containing an ETS DNA binding domain (Burtis *et al*., 1990). E74A protein binds to a subset of late puff loci on the polytene chromosomes (Urness and Thummel, 1990), suggesting that *E74A* may directly regulate the corresponding late genes during metamorphosis.

Early gene: *BR-C*

The 2B5 region is a focal point for an ecdysone inducible, genetically complex early puff which contains up to four complementation groups *[broad(br), reduced bristles on palpus (rbp), l(l)ZBc, and l(f)ZBd]* and a group of *non-pupariating (n p r l)* alleles that do not complement these functions (Kiss *et al*. 1988). This genetic region is collectively known as the *Broad-Complex (BR-C*) . The genetic analyses of Kiss *et al*. (1988) suggested that the complementing *BR-C* functions might be of a similar nature and that they might complement for each other, *e.g.*, br+ might compensate for rbp-.

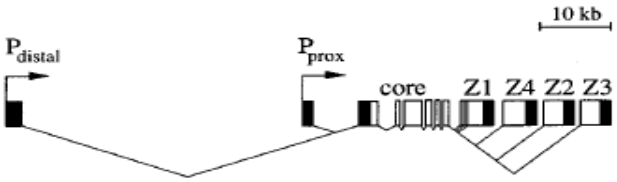
The products of *BR-C* are necessary for the normal ecdysone triggered puffing progression in the fruit fly. *BR-C* alleles autonomously arrest development of the salivary glands and the imaginal discs during late larval through pupal periods, indicating that normal *BR-C* function within these tissues is essential for ecdysone dependant development (Kiss *et al*. 1988). Also animals made deficient for the *BR-C* region are defective in their salivary gland ecdysone response as the late puffing sequence fails to take place (Kiss *et al*. 1988).

The regulatory role of *BR-C* products is also apparent at other levels since their absence affect intermolt puffs (Belyaev *et al*, 1981), impair the puffing response of the 74EF and 75B early puffs, prevents puffing of the 63F early puff (Belyaev *et al*. 1981), and blocks transcription of the 71E late puff genes (Guay and Guild, 1991).

An autoregulatory role is also observed in *BR-C*. Although the 2B5 early puff is fully induced by ecdysone, its regression takes much longer in in glands from *BR-C* mutants. It has thus been concluded that *BR-C* affect intermolt puffing, autoregulation of its own puff, and induction of both members of the early and late puff sets (DiBello, 1991).

BR-C encodes a family of transcription factors that are defined by unique pairs of C_2H_2 zinc fingers (Bayer *et al*., 1997) (Fig. 8). All BR-C proteins contain a common core exon (amino terminus) which contains a highly conserved amino-terminal motif (the BTB or the POZ domain) widely distributed in metazoans (Zollman *et al*., 1994). The core is alternatively spliced to one of four pairs of zinc-finger DNA binding domains, generating four classes of proteins, the Z1, Z2, Z3, and Z4 isoforms (DiBello *et al.,* 1991; Bayer *et al.,* 1996). Three variants of the Z1 class have been identified. These isoforms differ in the linker region between the common region and the DNA binding motif (Fig. 8). Although a variety of proteins are produced at the Z1 locus, individual BR-C protein isoforms are not tissue-specific. Most tissues examined express all *BR-C* isoforms. However, the relative level of each isoform varies among tissues according to their metamorphic stage (Bayer *et al*., 1996). Analyses of *BR-C* expression patterns show that all *BR-C* isoforms are induced at the beginning of metamorphosis and are detected in most tissues during this period (Emery *et al*., 1994; Bayer *et al*., 1996). However in the late pre-pupal period or the pupal period Z1 isoform is expressed the most (Emery *et al.*, 1994; Bayer *et al*., 1996).

A.



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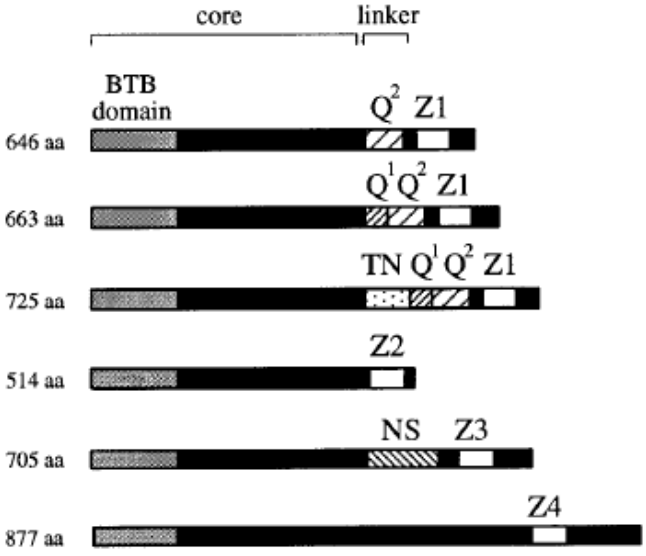


Figure 8. The Broad-Complex locus and protein isoforms. (A) *BR-C* transcripts encode four different pairs of C2H2 zinc finger domain (Z1, Z2, Z3, AND Z4) that are linked by alternative splicing to the common core exons (DiBello, 1991). DNA sequence published in DiBello *et al*. (1991) indicated that the core exon is divided into six exons. Open boxes indicate open reading frames and filled boxes represent untranslated regions of *BR-C* transcripts. (B) The first 113 aa of the 431 aa core region shared by all *BR-C* proteins encodes the conserved BTB (or POZ) domain believed to mediate protein-protein interactions. Each *BR-C* protein isoform is distinguished by its unique linker and zinc-finger containing domains. Thee TNT, Q1, Q2 sequences found in Z1 isoforms are contiguous in the Z1 exon in the order of 5'-TNT, Q1, Q2, 3'. Thus only three Z1 isoforms can be generated by alternative splicing from the 3' end of the core to the three alternative splice acceptor sites within the Z1 exon. These are TNT-Q1-Q2-Z1, Q1-Q2-Z1, and Q2-Z1. (Bayer *et al*., 1997)

BR-C Z1 isoforms also complement the transcriptional defects seen in *rbp* mutants but the Z2, Z3, and Z4 isoforms could not. Also the over-expression of *BR-C* Z3 or Z4 isoforms in salivary glands represses *L71* expression, indicating that BR-C proteins might also function as transcriptional repressors (Crossgrove 1996) of other genes as well.

Early gene: *E93*

The stage specificity of the early 93F puff 10-12 hours APF resulted in the isolation of the early gene *E93*. The E93 gene consists of a single transcription unit that spans at least 55 kb of genomic DNA and encodes a novel protein (Baehrecke and Thummel, 1995).

E93 is directly induced by the prepupal ecdysone pulse in the larval salivary gland but does not respond to the late larval hormone pulse. Broadus *et al* (1999) have demonstrated that *βftz-f1* provides *E93* with the competence to respond to the pre-pupal pulse of ecdysone in the salivary gland.

E93 also plays a key role in programmed cell death which will be discussed later. It should be noted however, that ectopic expression of *βftz-f1* is sufficient to prematurely induce salivary gland cell death and *βftz-f1* mutant salivary glands fail to undergo programmed cell death. Also, ectopic expression of *βftz-f1* transgene in late third instar larval stage is sufficient to allow ecdysone to prematurely activate transcription of the pupal-specific *E93* early gene.

The Role of *βftz-f1* in Development

FTZ-F1 is a member of the steroid receptor superfamily, implicated in the activation of the homeobox segmentation gene *fushi-tarazu* during *Drosophila* embryogenesis (Lavorgna, 1991). The gene encoding FTZ-F1 has been mapped to the 75CD mid prepupal puff (Lavorgna, 1991). *Ftz-f1* encodes two isoforms of mRNA and protein that differ in electrophoretic mobility and temporal expression patterns during embryogenesis (Lavorgna *et al*., 1993).

The FTZ-F1 isoform that is expressed in early embryos is designated as αFTZ-F1, and encodes a polypeptide of 1043 aa (Lavorgna, 1991). The second FTZ-F1 isoform, designated βFTZ-F1 is expressed later during embryogenesis and during prepupal development. The cDNA sequence for *βftz-f1* reveals an open reading frame of 816 codons, encoding a protein of predicted molecular mass of 88 kDa (Fig.9). Like *αFTZ-F1*, this protein is also a steroid receptor superfamily member, identical to *αFTZ-F1* in the carboxyl-terminal two-thirds of the protein, including the DNA- and ligand-binding domains (Fig.9); the amino-terminal portions of the proteins, however, are unique. As shown in figure 9, the sequence of the unique amino-terminal portion of αFTZ-F1 is enriched for glutamine, asparagine, and serine residues, which has a limited similarity to the corresponding portion of βFTZ-F1, which is rich in serine, alanine, and threonine residues. An analysis of *ftz-f1* transcription during larval and prepupal development showed the appearance of the 5.6- and 4.8-kb *βftz-f1* RNAs after 6-8

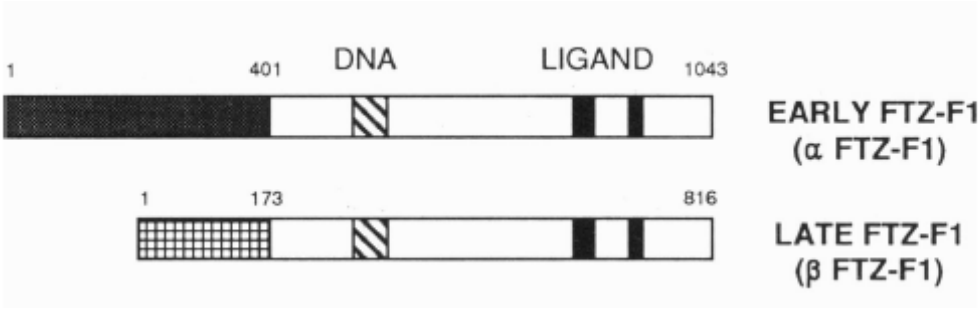


Figure 9. Arrangement of conserved and unique domains of the early and late isoforms of *FTZ-F1* protein. DNA and ligand binding domains are represented by hatched and filled sections, respectively. The shaded section the early *FTZ-F1* isoform, and the checked section in the late *FTZ-F1* isoform represent regions unique to the α *FTZ-F1* and β*FTZ-F1* isoforms. (Lavorgna *et al*., 1993)

hours of prepupal development, which is an important fact for the purpose of this study (Lavorgna *et al*., 1993).

Experiments with cultured larval salivary glands have demonstrated that *βftz-f1* transcription is repressed by ecdysone (Woodard *et al*. 1994). Consistent with this regulation, *βFTZ-F1* is expressed during the brief interval of low ecdysone titer in mid prepupae, following *E78B* and *DHR3* . Ectopic expression of *βftz-f1* can enhance the ecdysone induction of certain early genes. In addition, *βftz-f1* is sufficient to direct the stage-specific ecdysone induction of the *E93* early gene in prepupal salivary glands at 10 hours APF, along with the re-induction of *E74, E75* and *BR-C* (Woodard *et al*., 1994).

Thus, *βftz-f1* expression in mid prepupae re-sets the system, allowing the reinduction of the early/late genetic response to ecdysone as well as providing the competence for stage-specific transcriptional responses to the hormone. Interestingly, *βftz-f1* represses its own transcription, ensuring that the competence it provides is of short duration. The identity of βFTZ-F1 as an orphan receptor hints at the possibility that it may interact with the *ECR-USP* heterodimer, allowing the receptor to activate a set of stage-specific promoters in prepupae. This interaction could be manifested either by binding to adjacent sequences in the DNA or through the formation of novel heterodimer combinations.

Programmed Cell Death of Larval Tissues

Programmed cell death (PCD) is essential for the normal development of most metazoans (Chike Cao, 2007). Multicellular organisms remove obsolete or damaged cells through PCD and it is initiated inside the cell through an evolutionarily conserved genetic regulatory pathway characterized by distinct morphological changes (Chike Cao, 2007).

Hormones play a key role in controlling PCD. The term was first used in the study of steroid regulated intersegmental muscle degeneration during silk moth metamorphosis. During amphibian metamorphosis, thyroid hormone signals cell death that leads to the re-absorption of the tadpole tail and other larval tissues. During vertebrate limb development, separation of the limb digits requires the death of the inter-digital regions which is controlled by BMP signaling (Zuzarte-Luis and Hurle, 2005). Steroid hormones also control cell death in a wide range of mammalian tissues including neurons, mammary glands, testes, the prostrate, ovaries and the uterus.

In *D. melanogaster* ecdysone triggers cell death of larval tissues during different stages of metamorphosis and provides an ideal system to define the molecular mechanisms of steroid regulated cell death (Jiang *et al*., 2000). In response to sequential ecdysone pulses obsolete larval tissues are destroyed in a stage specific manner as adult tissues and structures develop from small clusters of progenitor cells (Fig. 10).

In *Drosophila*, increases in ecdysteroids trigger cell death in larval midgut and salivary glands (Jiang *et al*., 1997). Salivary gland cells die in a rapid and synchronous manner in response to the pulse of ecdysone that peaks 12 hours APF while the larval midgut initiates cell death at the beginning of puparium formation in response to the late larval pulse of ecdysone.

Markers of apoptosis including nuclear acridine orange staining and DNA fragmentation are detected 14 hours following puparium formation in salivary glands. Also salivary glands along with the larval midgut cultured in a physiologically elevated level of ecdysone undergo programmed cell death (Jiang *et al*., 1997) elucidating the relationship between steroids and cell death (Fig. 11).

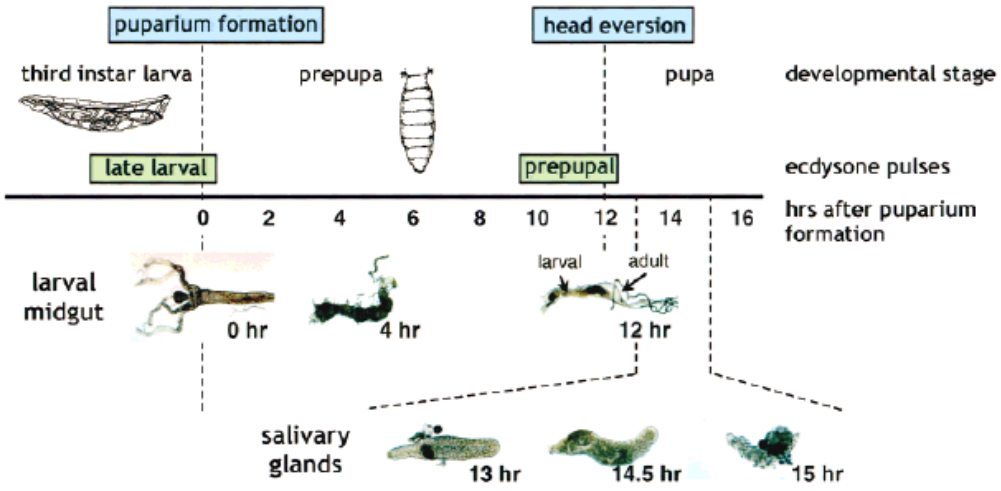


Figure 10. Ecdysone triggers the stage-specific programmed cell death of larval tissues. Green boxes indicate the two ecdysone pulses that influence major developmental transitions. The late larval ecdysone pulse triggers puparium formation, defining the larval-prepupal transition. 10 hours after puparium formation ecdysone pulse that triggers adult head eversion and the prepupal-pupal transition. The larval midgut begins to undergo cell death in newly formed prepupae, in response to the late larval ecdysone pulse. By 12 hours after puparium formation, the larval midgut cells condense to form the yellow body, surrounded by an intact adult gut developed from adult progenitor cells. The larval salivary glands survive the late larval ecdysone pulse but die rapidly following the prepupal pulse of ecdysone. (Jiang *et al*., 1997)

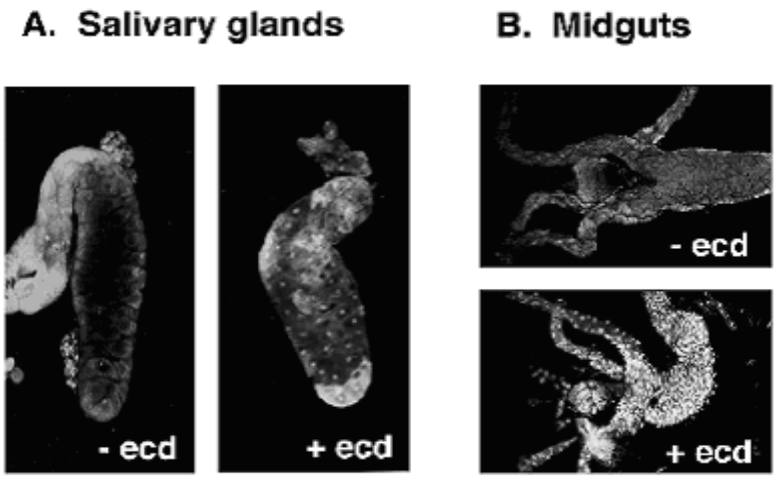


Figure 11. Ecdysone triggers programmed cell death in larval salivary glands and midguts. (A) Salivary glands dissected from 8 hour prepupae cultured in the absence (-ecd) or presence (+ecd) of 5 x 10^{-6}M 20-hydroxyecdysone for 5 hours and tested for viability by incubation with acridine orange. Only salivary glands cultured in presence of ecdysone undergo cell death as indicated by nuclear staining. (B) Mid third-instar larvae were injected with ecdysone(+ecd) or solution lacking ecdysone(-ecd), dissected after 8-10 hours, and tested for viability by incubation with acridine orange. Midguts were dissected 10 hours later and midguts from ecdysone injected larvae began cell death as indicated by nuclear staining. (Jiang *et al*., 1997)

Genetic and molecular studies in *Drosophila* have identified homologs of most of the genes involved in mammalian and *C. elegans* programmed cell death (Song and Steller, 1999). These include an *APAF-1/CED-4* homolog, *ark* (Zhou *et al*., 1999) , the ecdysone-inducible caspase *dronc* (Dorstyn *et al*., 1999), and two key death-inducer genes, *reaper (rpr)* and *head involution defective (hid)* (White *et al*., 1994). The *Df(3L)H99* deletion that removes both *rpr* and *hid*, along with a related death inducer, *grim*, results in a complete block in embryonic programmed cell death (White *et al*., 1994). In addition, ectopic expression of either *rpr*, *hid* or *grim* is sufficient to trigger cell death, indicating that these genes are central players in the cell death pathway. Recent studies have shown that *rpr, hid,* and *grim* exert their effect by binding to *IAPs* (Inhibitor of apoptosis), preventing *IAP* association with caspases and thereby allowing the caspase cascade to initiate apoptosis (Goyal *et al*., 2000). The vertebrate gene *SMAC/DIABLO* performs a similar role in triggering the death response, and thus is likely to represent a functional homolog of *rpr, hid,* and *grim* (Green, 2000) Finally, *croquemort (crq)*, a *CD36* receptor homolog, is expressed in macrophages that engulf apoptotic corpses during *Drosophila* embryonic development. This gene is required for engulfment and is regulated by the amount of apoptosis that is occurring in the organism (Franc *et al*., 1999). Interestingly, *ark, dronc, rpr, hid,* and *crq* are all induced in larval salivary glands immediately before the onset of cell death, implicating a direct role in this response.

Several steroid regulated genes function in programmed cell death (Fig. 12). The *EcR*, *USP*, *βFTZ-F1*, *BR-C*, *E74*, and *E93* genes have been implicated in the regulation of programmed cell death in a variety of tissues including the midgut, salivary glands and nervous system (Jiang *et al*, 2000; Broadus *et al*, 1999; Lee *et al*, 2000; Robinow *et al*, 1993; Lee *et al*., 2002). *E93* expressed after the prepupal pulse of ecdysone around 10-12 hour APF, appears to function specifically in the destruction of larval tissues (Lee *et al*, 2000). *E93* encodes a novel nuclear protein that is expressed in the larval midgut and salivary glands immediately prior to ecdysone induction of their death. *E93* mutants have defects in larval salivary gland cell destruction, and expression of *E93* is sufficient to induce programmed cell death. (Lee *et al*., 2000). Mutations in *E93* result in decreased levels of *rpr, hid, ark, dronc*, and *crq* RNA transcription. *E93* protein binds to sites in the salivary gland that contain both steroid regulated genes and cell death genes (Lee *et al*., 2000).

The ecdysone receptor complex *EcR/USP* and *βFTZ-F1* regulate *BR-C*, *E74*, and *E93* transcription in larval salivary glands immediately prior to the destruction of larval cells (Broadus *et al*., 1999). In larval midgut and salivary glands, *rpr*, and *hid* transcription increase prior to ecdysone regulated cell death (Jiang *et al*., 1997).

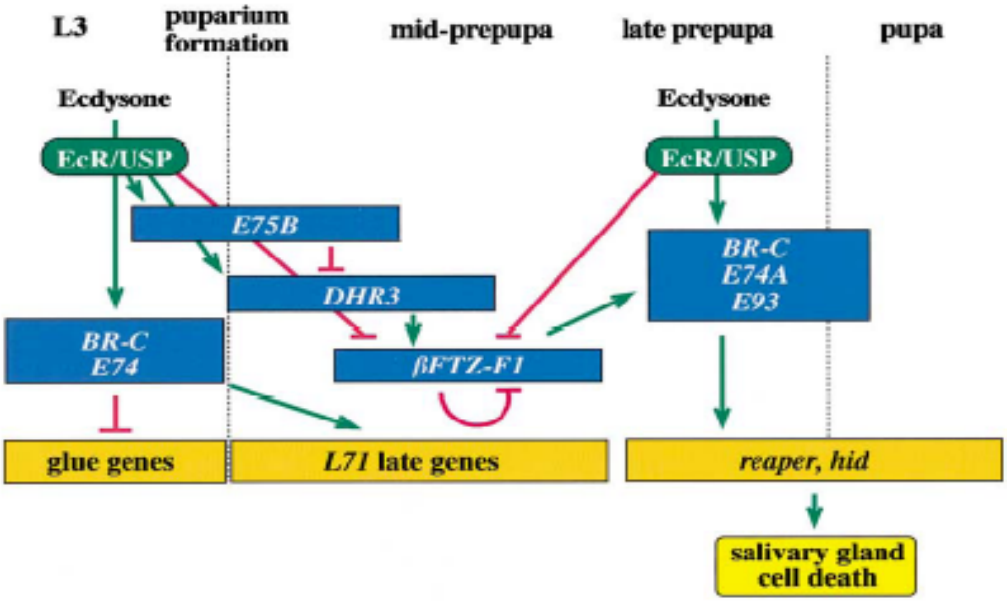


Figure 12. A model for the ecdysone triggered regulatory interactions leading to the destruction of the larval salivary gland. The blue boxes represent genes that encode ecdysone inducible transcription factors. The yellow boxes represent secondary response target gene. The green lines in this figure characterize inductive effects while the red lines show repressive effects. (Thummel, 2001)

The core cell death machinery is also involved in ecdysone regulated programmed cell death. Caspase activity is required for the destruction of larval salivary glands. Inhibition of caspases by expression of the baculovirus inhibitor p35 blocks midgut and salivary gland cell death (Jiang *et al*., 1997). In addition *ark*, and *dronc* transcription increases immediately prior to salivary gland cell death (Lee *et al*., 2000).

Jiang *et al*, (2000) have shown that *rpr* transcription is directly regulated by the ecdysone receptor complex, but *BR-C* function is also required for maximum levels of *rpr* mRNA transcript in dying larval salivary glands. While mutations in the *E74A* gene do not impact *rpr* transcription, both *BR-C* and *E74A* genes are required for proper transcription of *hid* in dying larval salivary glands (Jiang *et al*, 2000).

Salivary gland cell death exhibit hallmark features of autophagy, including increase in acid phosphatase activity and increased number and size of autophagic lysosomal vacuoles (Thummel, 2001). The same manner of cell death is observed in the midgut as well, because the larval midgut cells possess vacuoles that contain cellular organelles, indicating that these cells die by autophagy.

Following an increase in the steroid 20-hydroxyecdysone (ecdysone) at the end of larval development (beginning of puparium formation), future adult midgut epithelium is formed, and the larval midgut is rapidly destroyed. Mutations in the steroid-regulated genes *BR-C* and *E93* differentially impact

larval midgut cell death but do not affect the formation of adult midgut epithelia. In contrast to *BR-C* and *E93*, mutations in the ecdysone-regulated *E74A* and *E74B* genes do not hamper midgut development during metamorphosis. While mutations in the *BR-C*, *E74*, and *E93* genes do not impact DNA degradation during this cell death, mutations in *BR-C* inhibit destruction of larval midgut structures, including the proventriculus and gastric caeca, and *E93* mutants exhibit decreased formation of autophagic vacuoles. Dying midguts express the *rpr*, *hid*, *ark*, *dronc*, and *crq* cell death genes, suggesting that the core cell death machinery is involved in larval midgut cell death much like the salivary gland. The transcription of *rpr*, *hid*, and *crq* are altered in *BR-C* mutants, and *E93* mutants possess altered transcription of the caspase *dronc*, providing a mechanism for the disruption of midgut cell death in these mutant animals.

An increase in *rpr*, and *grim* transcription foreshadows ecdysone induction of neuronal cell death (Robinow *et al*., 1997) and mutations in these genes prevent the cells from dying.

These studies indicate that ecdysone triggers a two-step hierarchy composed of steroid-induced regulatory genes and apoptosis genes that, in turn, regulate the autophagic death of midgut cells during development.

Purpose of the Project

The goal of this project can be perceived from two different perspectives. At the molecular level, this project tries to comprehend how ecdysone regulates the expression of *BR-C* and whether this regulation by ecdysone is dependent on *βftz-f1* as a competence factor or not. In this study *βftz-f1* loss-of-function mutants and wild type flies (w^{1118}) have been utilized to examine the change in the expression of *BR-C* mRNA in different *βftz-f1* mutant tissues such as the salivary gland, fat, midgut and hindgut using Real Time PCR. Furthermore, to acquire an understanding of the temporal relationship between *βftz-f1* and *BR-C* Real time PCR has been done on the specified tissues at different time points (10hours APF, 12 hours APF).

On a broader level, this project aims at deciphering a part of the molecular pathway through which metamorphosis in *Drosophila* is controlled by the functional steroid hormone ecdysone. Although it is known that ecdysone induces different responses in different tissues at different time points, the mechanism through which the differential responses are generated are not clearly understood. The answer to the question, how a repetitive hormonal signal can direct different biological responses at different times remain elusive. It is understood however that specific transcription factors play an important role in conferring this temporal and stage specificity. *Bftz-f1* acting as a competence factor at the mid prepupal stage (Woodard *et al*., 1994) in the salivary gland, is in an ideal position

to regulate the responses to ecdysone. Through this research I have tried to define the hierarchical position of *BR-C* in insect metamorphosis. A clear comprehension of the pathway that leads to metamorphosis will not only help us understand the role steroids play in the development of holometabolous insects but also in other higher organisms.

MATERIALS AND METHODS

Unless otherwise stated all procedures other than the fly stock maintenance performed for the purpose of this experiment were done with gloved hands to avoid skin DNA contamination. Also the user's manual was followed through the course of this study unless otherwise specified.

Maintenance of Fly Stocks

For the purpose of this experiment three different genotypes of fruit flies were maintained on standard cornmeal agar at 25^{o} C in 200mL bottles. The fly stocks were changed every 4-5 days while the stocks were being built up and afterward changed every two weeks. Before transferring to a new bottle a small amount of yeast was sprinkled into the bottles to facilitate the laying of fly eggs. The three genotypes that were maintained were the control w^{1118} which was characterized by the presence of white eyes and thin, long prepupae and the mutants *Ftz-F1*^{17}*/Tm6B,Hu e Tb* also characterized by white eyes and the red eyed mutant *Df(3L)Cat*^{DH104}*/Tm6B, Hu e Tb*.

Periodically, *Df(3L)Cat*^{DH104}*/Tm6B, Hu e Tb* virgin females were collected by emptying the bottle and storing either at 18^{o} C for less than 18 hours or at 25^{o} C for less than 8 hours. The newly eclosed flies were collected from these bottles before they were mature enough to mate ensuring the collection of virgin females.

Cultivation of *βftz-f1* Loss-of-Function Mutants

As the experiment focused on the role *βftz-f1* plays in the induction of *BR-C* in specific tissues at different time points, *βftz-f1* mutants were cultivated by crossing *Ftz-F1*^{17}*/Tm6B,Hu e Tb* males with *Df(3L)Cat*^{DH104}*/Tm6B, Hu e Tb* virgin females. This cross produced four different genotypes of flies out of which *Ftz-F1*^{17}*/Df(3L)CatDH*^{104} animals were collected. This genotype of mutants possess a hypomorphic mutation in its only copy of *Ftz-F1*gene (Broadus et al., 1999) and lacks the *Tubby(Tb)* marker which results in long thin prepupae. Thus the *Ftz-F1*^{17}*/Df(3L)CatDH*^{104} prepupae can be easily identified in the bottle as the other genotypes give rise to short, fat pre-pupae which contains the *Tb* allele. Figure 13 demonstrates the cross performed to produce the desired mutants

Virgin females		**Males**
Df(3L)Cat^{DH104}	X	*FtzF1^{17}*
Tm6B, Hu e Tb		*Tm6B,Hu e Tb*

Tm6B, Tb	*FtzF1^{17}*	*Df(3L)Cat^{DH104}*	***FtzF1^{17}***
Tm6B,Tb	*Tm6B, Tb*	*Tm6B, Hu e Tb*	***Df(3L)CatDH^{104}***
(Lethal mutation)	(Tubby pre-pupae)	(Tubby pre-pupae)	**(Desired *βftz-f1* mutant pre-pupae)**



Figure 13. A diagram of fly crosses performed to generate the desired *βftz-f1* mutant animals. The diagram demonstrates the short and tubby pre-pupal phenotype of two of the genotypes of the progeny and the desired long, thin pre-pupal phenotype of βFtz-*F1* mutants resulting from a cross between virgin *Df(3L)Cat^{DH104}* females and *FtzF1^{17}* males. Both the parental genotypes possesses the *Tb* allele. The *Tm6B, Tb/Tm6B,Tb* mutants generated from the cross carry lethal mutations and do not reach the pre-pupa stage.

Tissue Dissection

The control w^{1118}, and the mutant *Ftz-F1*^{17}*/Df(3L)CatDH*^{104} 0 hour prepupae were collected and placed on a damp filter paper in a petri dish and incubated at 25^{o} C for 10 or 12 hours. After the incubation period the prepupae were dissected in 1x Robb's solution under a dissection microscope using Dumstar#5 tweezers. The salivary gland, midgut, hindgut, and fat body were collected from each of the time periods mentioned and stored in separate RNase-free 1.5 mL microfuge tubes containing 30µL of 1x Robb's solution. After dissection 300µL of TRIzol reagent (Life Technologies/Sigma-Aldrich) was added and homogenized by pipetting up and down several times with sterile pipette tips. The samples were then stored at -80^{o} C until RNA isolation.

Isolation of Small Amounts of RNA from Dissected Tissues

Tissues homogenized in TRIzol were allowed to incubate at room temperature (15-30^{o} C) for five minutes before being transferred to a pre-spun Phase Lock Gel-Heavy 2mL tubes. Afterwards 60µL of chloroform was added, the tube shaken to mix the contents and centrifuged at 12,000 x g for 10 minutes at 4^{o} C. After centrifugation the clear aqueous phase was transferred to a sterile RNase-free tube. Approximately 0.7-0.8 volume of isopropanol (160µL) was added to the tubes, vortexed and precipitated overnight at -20^{o} C.

The samples were then spun at 14,000g for 30 minutes at 4 ^{o} C, after which the supernatant was removed and the pellet was washed once with 500uL 75% ethanol. The tubes were then spun at 15,000 x g for 5-10 minutes. The supernatant from this step was discarded and the pellet was air dried for 1-2 minutes. The pellet was then redissolved in 5µL of RNase-free water through incubation at 55-60 ^{o} C and stored at -80 ^{o} C.

Quantification of isolated RNA

The concentration of the isolated RNA was determined using the Nano Drop^{TM} ND- 1000 spectrophotometer. 1 -2µL of undiluted RNA sample was placed on the pedestal and the concentration and the 260/280 values were recorded from the machine. For this experiment samples with 260/280 values higher than 1.70 were selected.

Treatment with DNAfree

To ensure that the isolated RNA contained no contaminating agents (e.g. DNA), it was DNased using RNeasy® MiniKit. 0.1 volume10x DNase I buffer and 1 µL rDNase I (2U/ µL) was added to the RNA and incubated at 37°C for 30 minutes. Afterwards, 0.1 volume of DNase inactivation reagent was added and incubated at room temperature for 2 minutes. The sample was then centrifuged for 1.5 minutes at 10,000x at room temperature and the supernatant was transferred into a new tube. This procedure was repeated twice before performing cDNA synthesis.

Synthesis of Complementary DNA (cDNA)

Prior to the conduction of polymerase chain reaction (PCR), complementary DNA (cDNA) copies of the isolated RNA were performed. cDNA synthesis was carried out using the SuperScript™ First Strand Synthesis System for RT-PCR kit from Invitrogen™.

Isolated mRNA treated with DNAfree isolated from w1118 and *βFtz-F1* mutants were used for cDNA synthesis. For each sample a control and a no-Reverse Transcriptase (no RT) control was prepared. The pipetting scheme as described in table-2A was combined, vortexed and incubated at 65 ^{o} C for 5 minutes. Afterwards the reaction mixture was cooled in ice for at least 1 minute and 9 µL of the reaction mix as described in table-2B was added to the previous mixture. This solution was then incubated for 2 minutes at 42 ^{o} C. After the incubation 1µL of SuperScript II RT was added to all the tubes except the no-RT controls and incubated again at 42 ^{o} C for 50 minutes followed by incubation at 70 ^{o} C for 15 minutes. The samples were then chilled on ice and centrifuged. 1 µL of RNase-H (2units/µL) was added to each reaction and incubated at 37 ^{o} C. RNaseH served to degrade the template mRNA thereby producing pure cDNA copies of the mRNA. The synthesized cDNA was stored at -80 ^{o} C.

Table 2A. Pipetting scheme for the first stage of cDNA synthesis

Component	Volume (µL)
RNA treated with DNAfree	3.5
10mM dNTP mix	1
Oligo dT (0.5microgram/ µL)	1
Random hexamer (50ng/ µL)	1
DPEC treated water	3.5
Total volume	**10 µL**

TablE 2B. Pipetting scheme for the second stage of cDNA synthesis after incubation at 65 ^{o} C for five minutes

Component	Volume (µL)
10X RT buffer	2
25mM MgCl2	4
0.1M DTT	2
RNaseout recombinant rnase inhibitor	1
Total	**9 µL**

Designing of Primers and Probes

The *BR-C* primers and the *β-actin* primers were designed using Primer3®, version v.0.4.0 (http://frodo.wi.mit.edu) and ordered from Integrated DNA Technologies®, Inc, while the probes were designed and ordered from Integrated DNA Technologies®, Inc. To design the *BR-C* primers mRNA transcript of the BRC Z1 protein isoform was utilized which lies in exon 9. The sequences for this purpose were obtained from Flybase in which *BR-C* is designated as *br*.

The *β-actin* primers were designed from the sequence of the gene actin-5C on Flybase. The *β-actin* primers spanned an exon intron boundary (Figure.14) and were designed as such to ensure that genomic DNA would not be amplified during the PCR reaction.

The probes that were designed in this experiment lay between the forward and the reverse primers designed for the specific gene. Figure 14 shows the location of the forwad and reverse primers for *BR-C* along with the location of the probe. Table 3, and table 4 demonstrates the primer and probe names and sequences for *BR-C* and *β-actin* respectively.

Table 3. Sequence and name of PCR primers and probe designed for *BR-C*

Primer name	Primer sequence (5'-3')
BRCQ1Z1 left	AGGAGCCCGTGTGCAATA
BRCQ1Z1 Right	CTGGTGCTGCTGGTGATAGA
Name set 1 Probe	AATAGCCTGCGCAACCACAAGAG

Table 4. Sequence and name of PCR primers and probe designed for *β-actin*

Primer name	Primer sequence (5'-3')
B-actin left primer	TCTACGAGGGTTATGCCCTT
B-actin right primer	GCACAGCTTCTCCTTGATGT
β-actin Probe	TGGTCGCGATTTGACCGACTACCT

Polymerase Chain Reaction

The target DNA was amplified in 0.2 mL thin walled sterile PCR tubes. Each PCR reaction contained 50µL of the reaction mixture prepared by adding the reagents stated in table 5. The primers used in this reaction were diluted in nuclease free water to obtain 100µM stock solution and a working concentration of 10µM (See appendix).

Table 5. Pipetting scheme for PCR reaction

Reagent	**1 rxn (50 µL)**	**Final concentration**
10x PCR buffer without $MgCl_2$	5	
50mM Mg Cl_2	1.25	
10mM dNTP mix	1	
Taq DNA polymerase	0.4	
Forward BRC Q1Z1 primer	2	400nM
Reverse BRC Q1Z1 primer	2	400nM
cDNA sample	2	
Nuclease free water	36.35	
Total volume	**50 µL**	

PCR was carried out using the Progene and Techgene machines from Techne. The PCR program was set up as described in table 6. The reaction consisted of 35 cycles of denaturation, annealing and extension steps.

Table 6. PCR program for *BR-C* and *ß-actin*

Stage	Step	Temperature	Time	No. of cycles
Denaturation	1	95°C	30sec	35
Annealing	2	60.2°C	30sec	
Extension	3	72°C	30sec	
	4	72°C	5min	1
	5	72°C	1sec	
	6	4°C	∞	

Gel Electrophoresis to Ensure cDNA Quality

1.6% agarose gels were used to visualize PCR products to ascertain that desired cDNA was synthesized. The agarose gels were prepared by heating 75mL TAE buffer, 1.2gm agarose and 3.5µL of EtBr. The mixture was then poured into the mold of a gel electrophoresis machine. Prior to loading the prepared cDNA, the gels were submerged in 1x TAE buffer containing 10µL of 10mg/mL EtBr. 50 bp ladder from New England Biolabs was used to determine the product size. The gel was run at 124 volts for approximately 40 minutes and visualized using a UV illuminator and photographed using the FujiFilm LAS-3000 machine.

Setup of Real Time PCR reaction

Real time PCR was conducted in a 7300 Real-Time PCR system from ABI using *BR-C* primers and probe. The components used in the reaction are specified in table 7. Each reaction in the 96 well Real Time PCR plate consisted of 50µL of the reaction mix (table 7). Every reaction was performed in three replicates as described in figure 15 and figure 16.

Table 7. Components of a Real Time PCR reaction

Component	**1 reaction (µL)**	**22 reactions (µL)**
TaqMan Master Mix	25	550
Forward primer	2	44
Reverse primer	2	44
Probe	2	44
Nuclease free water	16	352
cDNA	3	
Total	**50 µL**	

Unlike reverse transcriptase PCR the reaction plate needed to be removed from the machine as soon as the reaction was completed. In the Real Time PCR reaction BR-C was the gene of interest while b-actin was the endogenous control.

Standard curves for the Real Time PCR reaction was set up as demonstrated in figure. RNA isolated from control prepupae 10 hours APF were used to make 6, 10 fold dilutions. Each dilution was tested with b-actin and BR-C primers and probes. The data generated from this experiment was used in the validation experiment.

Figure 16. demonstrates the set up of a Real Time PCR reaction while analyzing certain tissues. Salivary gland, midgut, hindgut and fat body were the tissues being analyzed. All the tissues mentioned were tested at 10 hours APF and 12 hours APF for the reference gene and the endogenous control.

Data Analysis for Real Time PCR Experiments

Relative quantification was used in this experiment and the difference in gene expression for each tissue at the specified time points were calculated using ΔΔCt method or the comparative Ct method.

Calculation of fold change using the comparative Ct method

To analyze the data, the threshold for the Real Time PCR reaction was set up following the instructions provided in the Applied Biosystems User Manual for relative quantification. The correct setting of the threshold is imperative in determining the Ct values which are the PCR cycle number where the PCR reaction crosses the threshold. As each sample was tested in triplicates, for each tissue at the mentioned time point, the average Ct values were determined and the ΔCt value was obtained by subtracting the average Ct value for *β-actin* expression from the average Ct value of *BR-C* expression (Livak and Schmittgen, 2001). The control genotype w^{1118} was used as the calibrator for the experiment and was used in the final equation required for the calculation of ΔΔCt. (See appendix for sample calculation).

The efficiency of the primers were determined using the formula, $10^{-(1/slope)}$-1. An efficiency between 90% and 110% is considered acceptable when using the Taqman gene expression mastermix.

The equation used to calculate the fold change in gene expression was $2^{-\Delta\Delta Ct}$. A positive ΔΔCt value indicates a decrease in gene expression. In such cases the inverse of decimal values were used to demonstrate the fold change. When ΔΔCt was negative (increase in gene expression) the fold change was calculated directly from the formula $2^{-\Delta\Delta Ct}$.

Calculation of Standard Error

The standard error for ΔΔCt was calculated using the standard errors for the mutant and the control tissues. To calculate the standard error for the control, standard error was determined from the averages of BR-C Ct values and the averages of b-actin Ct values. The individual standard errors were then placed in the formula $SE(control) = \sqrt{((SE_{BRC})^2+(SE_{\beta actin})^2)}$. The standard errors for the mutant tissues were calculated in the same way as was the standard error for ΔΔCt. The SE for ΔΔCt was the square root of the summation of the squared standard error values for the control and the mutant for the timepoint and tissue considered. The error was converted to linear form using the equation $SE(fold\ change)=2^{SE\Delta\Delta Ct}$.

The students T test was performed by dividing the ΔΔCt value by the standard error for ΔΔCt. The value generated was used to determine the P value, where the degree of freedom was 2 (number of replicates -1).

2984 CGTTCACATGCGCCCCACCA**AGGAGCCCGTGTGCAATA**TCTGCAAGCGCGTCTACAG
3042 CTCCTTG**AATAGCCTGCGCAACCACAAGAGCAT**CTACCACCGCAACCTCAAGCAGCC
3099 GAAGCAGGAGCCAGGCGTCGGCGCAACCCAGGCGGCGGCCAATAGCT**TCTATCACC**
3156 **AGCAGCACCAG**CAGCAGCAGCTCAATCACCACTCCTCTTCATAGGGCTTCATGTACT
3213 TTGATTACCACCAGCAGCATCAGGCCCAGCACCACCCACACCCGCACCTGCATCCGC
3270 ACTCGCATCCGCGCCCGCATCCGCACCACGGGGGGTTCTCCA

Figure 14. Part of BR-C mRNA transcript from exon 9. The **red** sequence designates the primers while the **blue** sequence shows the location of the probe. The expected product size from this reaction is 161bp. It should be noted here that the probe lies between the forward and the reverse primers. When Taq polymerase extends along the sequence the reporter fluorochrome on the 5' end of the probe is degraded and fluoresces. The amount of fluorescence detected initially increases as the cycle number gets higher.

	β-actin					*BR-C*						
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	W1118 10 fold dilution					W1118 10 fold dilution						
B	W1118 100 fold dilution					W1118 100 fold dilution						
C	W1118 1000 fold dilution					W1118 1000 fold dilution						
D	W1118 10000 fold dilution					W1118 10000 fold dilution						
E	W1118 100000 fold dilution					W1118 100000 fold dilution						
F	W1118 1000000 fold dilution					W1118 1000000 fold dilution						
G												
H	No template control					No template control				H_2O		H_2O

Figure 15. Schematic representation of a Real time PCR plate set up to construct the standard curve for analysis of data using the comparative CT method.

	β-actin					***BR-C***						
	1	**2**	**3**	4	5	**6**	**7**	**8**	9	10	11	12
A	w1118 Salivary Gland 10 hours APF					W1118 Salivary gland 10 hours APF						
B												
C	*Ftz-F1*^{17}*/Dfcat* Salivary Gland 10 hours APF					*Ftz-F1*^{17}*/Dfcat* Salivary Gland 10 hours APF						
D												
E												
F												
G												
H	No template control					No template control				H_2O		H_2O

Figure 16. Schematic representation of a Real Time PCR plate to comprehend BR-C gene expression in the salivary glands of control and *Ftz-F1*^{17}*/Dfcat* mutants at 10 hours APF. Columns 1,2, and 3 contains cDNA template samples tested with beta-actin primer and probes while columns 6,7, and 8 contain cDNA template with BR-C primers and probes. The no template controls contain all the ingredients necessary for a real time PCR reaction except cDNA. Row A has cDNA from the salivary glands of control prepupae at 10 hours APF while row C contains cDNA from the salivary glands of the mutant prepupae aged to 10 hours.

RESULTS

Use of Agarose Gel to Ensure Quality of cDNA

For the purpose of this experiment cDNA was synthesized using sample mRNA produced from the control and mutant animals aged to 10 hours APF and 12 hours APF. A No RT control cDNA sample was also prepared to detect whether amplification of genomic DNA was taking place or not. Figure 17 demonstrates an agarose gel which was utilized to ensure the quality of cDNA synthesized from mRNA collected from prepupae aged to 10 hours APF. As can be seen from the figure, no bands are visible in the no RT control indicating that Superscript II reverse transcriptase was necessary for the production of the desired cDNA template. It can also be seen that the bands for the control and the mutant animals are 161 bp in length, confirming that the primers were amplifying the desired sequence.

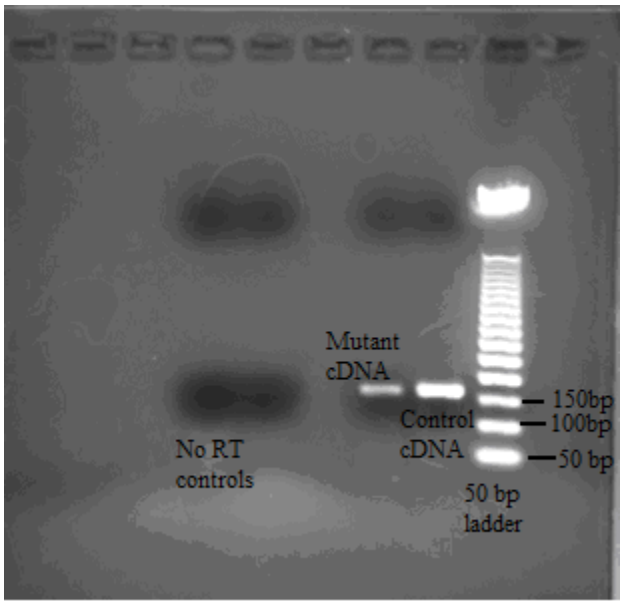
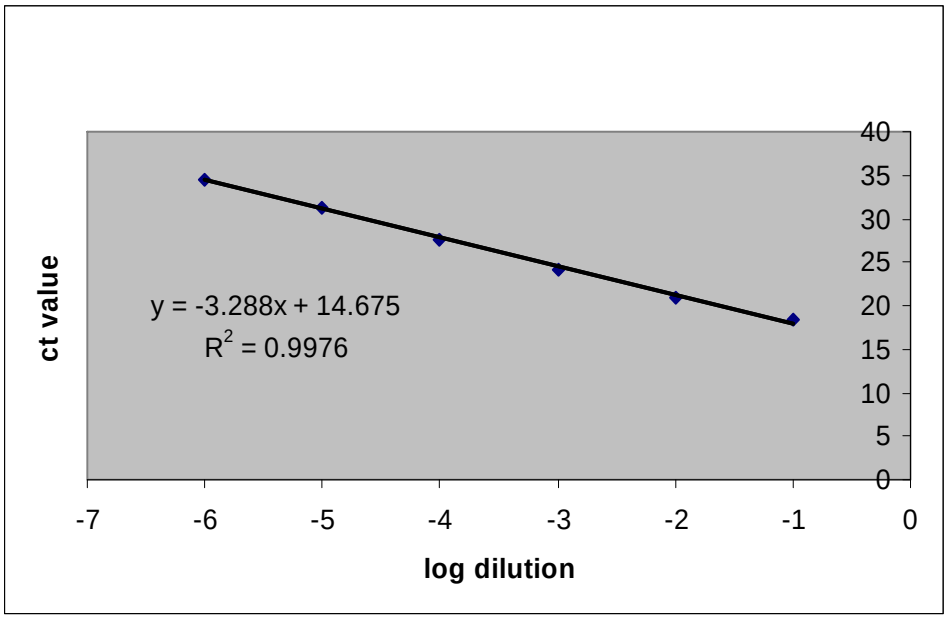


Figure 17. Image of gel confirming the quality of cDNA and the *BR-C* primers. On the rightmost side of the gel, 50 bp ladder is visible, after which the two lanes contained cDNA from w^{1118} prepupae and *βftz-f1* loss-of-function mutant prepupae respectively, aged to 10 hours APF. The desired and the resultant product size was 161 bp. The two lanes after the *βftz-f1* mutant were the No RT controls for the mutant and w^{1118} respectively and do not show the presence of PCR product, ensuring the quality of the primers and the cDNA.

Amplification Efficiency of the Real Time PCR Experiment

To determine the amplification efficiency of the Real Time PCR experiment standard curves were generated using the Ct values from different 10-fold dilutions, plotted against the respective log value of the dilutions for *β-actin* and *BR-C.* A slope of -3.32 indicates a 100% efficient reaction. Thus a Ct value of -3.32 demonstrates a reaction in which the PCR product doubles after each cycle. Figure 18a and figure 18b demonstrates the standard curves generated for *β-actin* and *BR-C*. The slope of the standard curve for *β-actin* was -3.28 and the slope of the standard curve for *BR-C* was -3.23. The calculated amplification efficiency for both *BR-C* and *β-actin* was a little over a 100% so the formula $2^{-\Delta\Delta Ct}$ could be used to calculate the fold change. Also, as the amplification efficiency of both primers were similar, the ΔΔCt method of calculating fold change in gene expression could be used.

A.



B.

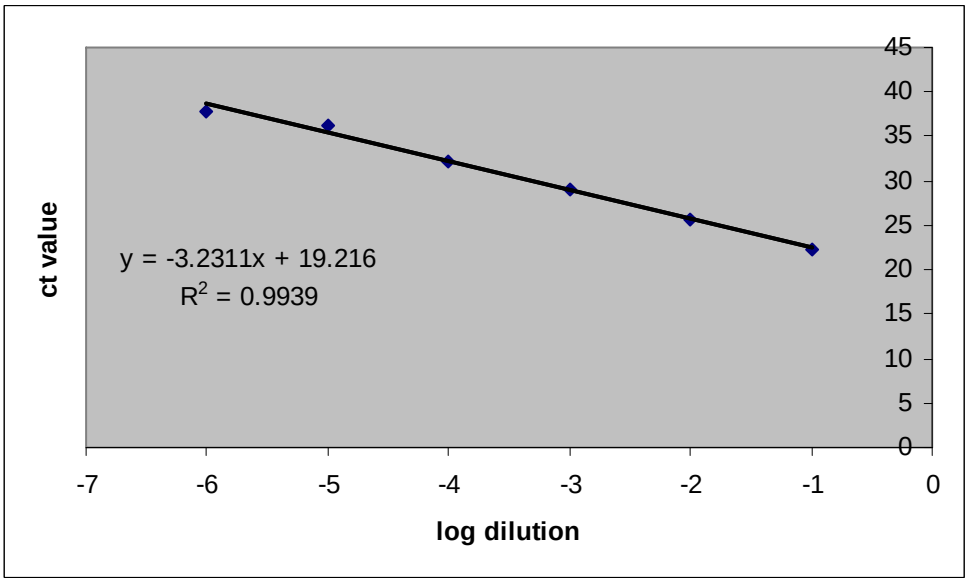


Figure18. Standard curves generated for *β-actin* and *BR-C* using prepupae aged to 10 hours APF. (A) Figure A demonstrates the standard curve for *β-actin* which provides a slope of -3.28. (B) Figure B shows a standard curve with a slope of -3.23 for *BR-C*. Both standard curves generated in this experiment indicate that the PCR reaction was being performed at near 100% efficiency.

Validation of the Comparative Ct Method of Data Analysis

To use the comparative Ct method of data analysis the difference between the Ct values of *BR-C* and *β-actin* for different dilutions were plotted against their respective log value of dilution. 6, 10 fold dilutions were made in this experiment resulting in 6 data points (Fig.19). The best fit line generated from the graph had a slope of 0.05. A slope less than 0.1 is necessary to use of the comparative Ct method. The slope in this case being less than 0.1 validated the use of the comparative Ct method.

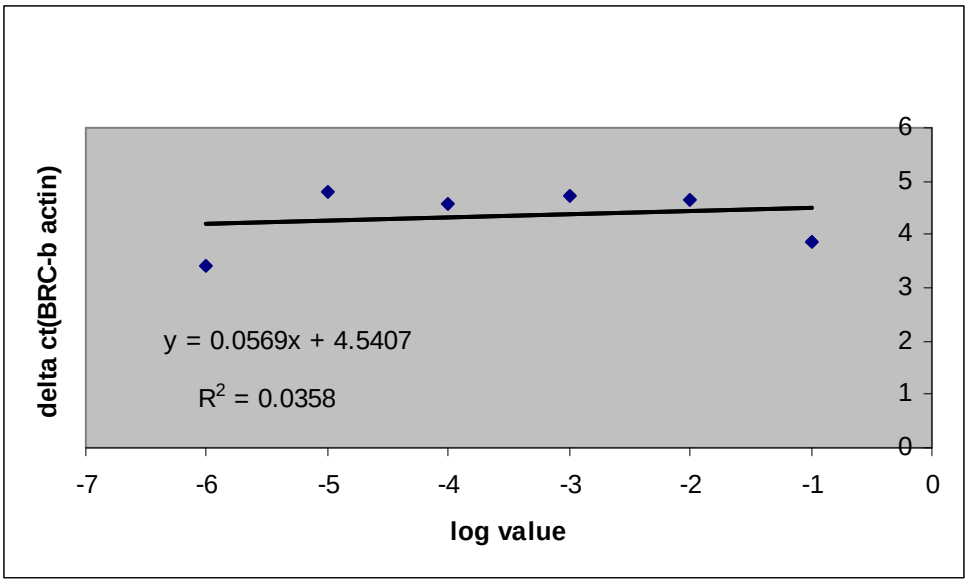


Figure 19.Validation of the comparative CT method using *BR-C* as the target gene and *β-actin* as the endogenous control. The validation experiment was performed to ensure that the comparative CT method of data analysis could be utilized in this case. The difference in CT values for *BR-C* and *β-actin* was plotted against their respective log values of 6, 10 fold dilutions. The value of the slope generated in this experiment was 0.05.

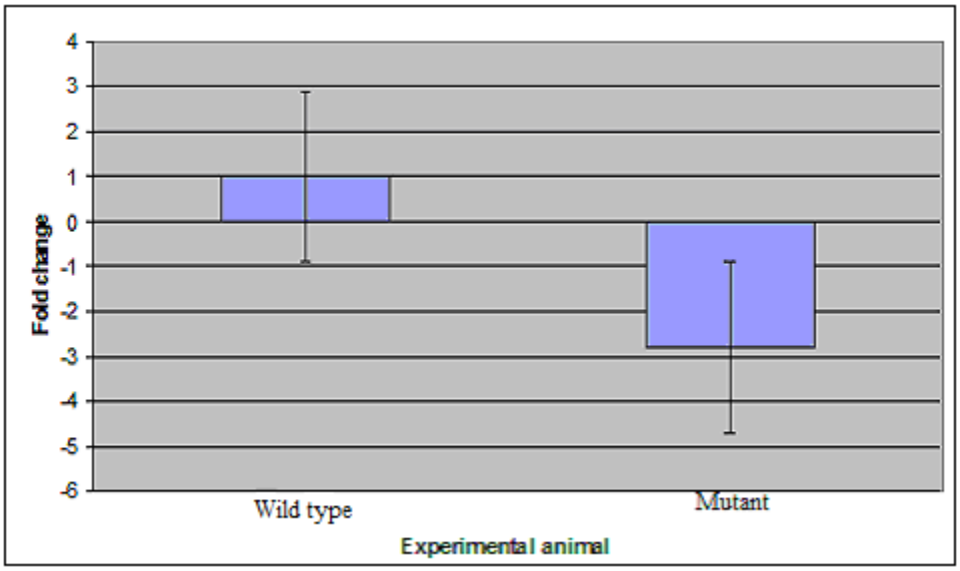
Relative Expression in Different Tissues of Mutant Prepupae Compared to Control

Through the use of the comparative CT method of analysis the gene expression in different tissues (salivary gland, midgut, hindgut and fat) were determined in the mutant compared to the same tissues from the control prepupae. In the graphs depicted in this section, the expression of *BR-C* in control animals is shown as being 1 while the expression in the mutants are indicated relative to the expression in the control tissue. An over-expression of *BR-C* in a certain tissue at a certain time point indicates that *BR-C* was expressed more compared to the control while an under-expression indicates that *BR-C* was expressed less.

Salivary Gland:

In the salivary gland at 10 hours APF the expression of *BR-C* decreased 2.8 fold compared to the control salivary gland at the same time point (Fig. 20A). The standard error for the fold change was 1.13 where the P-value for the T test was 0.0072. At 12 hours APF the expression of *BR-C* increased 64.45 fold with a standard error of fold change value of 1.35. The P- value in this case was 0.0025. The low P-value in both cases indicated that the fold change in gene expression in the *βftz-f1* mutant salivary gland was significant at 10 hours APF and at 12 hours APF.

A.



B.

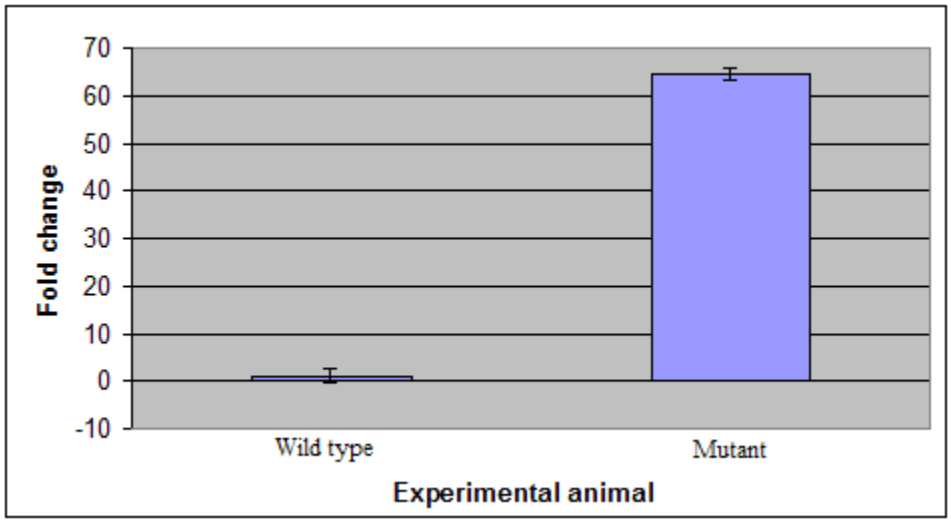


Figure 20. Comparison of *BR-C* expression in *βftz-f1*mutant salivary glands compared to *BR-C* expression in the control salivary gland.(A) Figure A demonstrates that *BR-C* expression decreased by 2.8 fold in the mutant salivary gland at 10 hours APF.(B) *BR-C* expression increased by 64.45 fold in the mutant salivary gland at 12 hours APF. The standard errors for the fold change in gene expression were 1.13 and 1.35 respectively.

Midgut:

The data analysis performed for the midgut at 10 hours APF indicated that the mutant midgut showed a slight over-expression of 1.08 fold (Fig. 21A). The standard error for the fold change in this case was 1.10 but the P- value from the T test was 0.54 (higher than 0.05) indicating that the fold change was not significant.

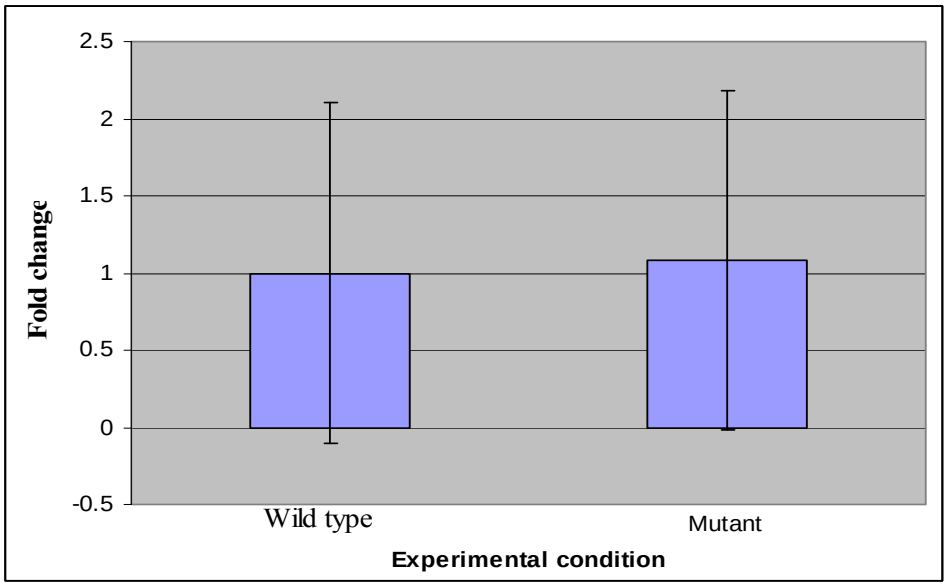
The analysis done for midgut at 12 hours APF compared to the control indicated an over-expression of 2.69 fold (Fig. 21B). The standard error was calculated to be 1.19 with a P-value of 0.029. Thus the increase in fold change observed for the mutant midgut at 12 hours APF was significant compared to the control.

Hindgut:

In the mutant hindgut *BR-C* over-expression of 13.36 fold was observed at 10 hours APF compared to the control hindgut at 10 hours APF (Fig. 22A). The standard error for the fold change was 1.82 while a P-value of 0.04 was generated from the T test. This indicated that the increase in gene expression in the mutant hindgut compared to the control hindgut was significant at 10 hours APF.

In the mutant hindgut at 12 hours APF an over expression of 36.76 fold of *BR-C* was observed with standard error for fold change being 2.03 (Fig. 22B). The P-value from the T test being 0.03 indicated that the results from this experiment were significant.

A.



B.

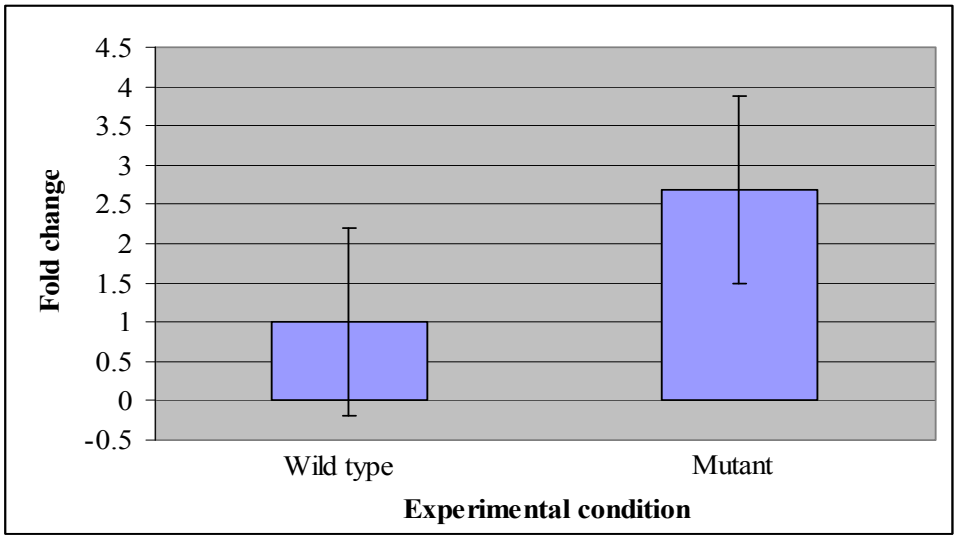
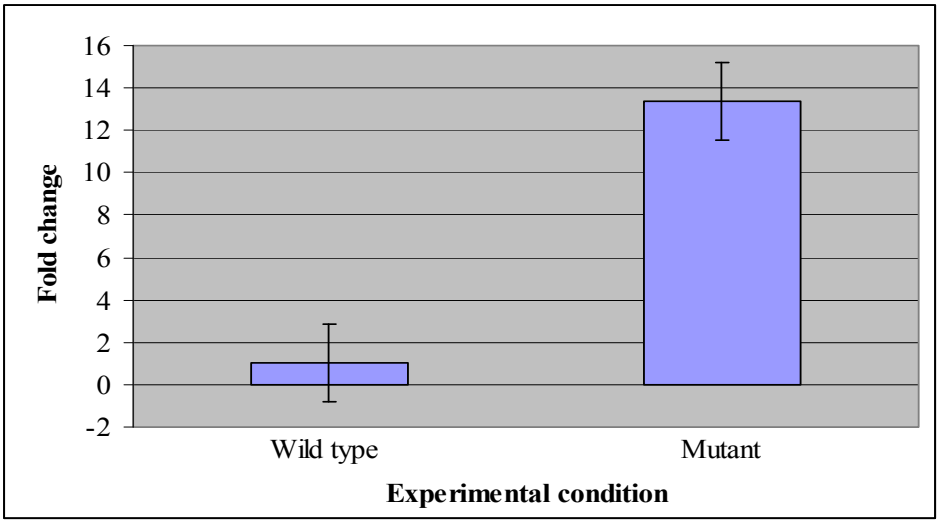


Figure 21. Comparison of *BR-C* expression in the *βftz-f1* mutant midgut compared to the control midgut at different time points. (A) *BR-C* expression in the midgut of *βftz-f1*mutants at 10 hours APF showed a slight overexpression with a standard error value for fold change 1.10. A high P-value indicated that this change was not significant.(B) However, *BR-C* was overexpressed 2.69 fold in the mutant midgut at 12 hours APF compared to the control midgut. The standard error in this case was 1.19. A low P-value indicated that the change was significant.

A.



B.

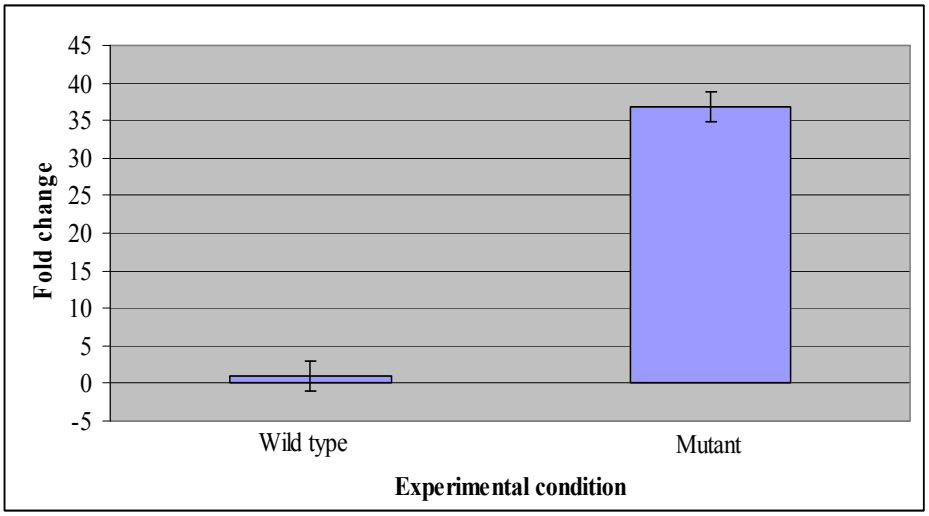


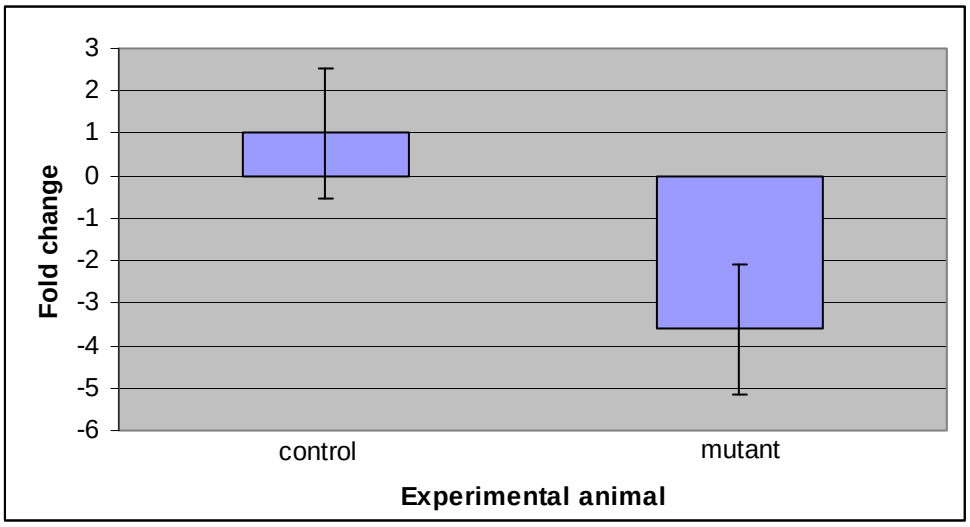
Figure 22. *BR-C* expression in *βftz-f1*mutant hindgut compared to control hindgut at different time points. (A) This figure demonstrates that *BR-C* was overexpressed 13.36 fold in the *βftz-f1*mutant hindgut at 10 hours APF. In this case the standard error for fold change was calculated to be 1.82. (B) At 12 hours APF, *BR-C* was overexpressed 36.76 fold in the mutant hindgut compared to the control hindgut at the same time point. The standard error for fold change was calculated to be 2.03. Low P-values for both the experiments indicated that the over expression was significant.

Fat Body:

In the *βftz-f1*mutant fat cells at 10 hours APF an underexpression of 3.6 fold was observed (Fig. 23A). The standard error was calculated to be 1.53 while the student's T test resulted in a P-value of 0.05. The high P-value indicated that the underexpression was not significant.

However, in the *βftz-f1*mutant fat cells at 12 hours APF a significant underexpression of 3.12 fold was observed. The P-value in this case was 0.002 and the standard error was 1.07. Even though at both time points the underexpression was similar the differing standard error values generated varying P-values which indicated that the underexpression of *BR-C* at 12 hours APF was significant while *BR-C* underexpression at 10 hours APF was not.

A.



B.

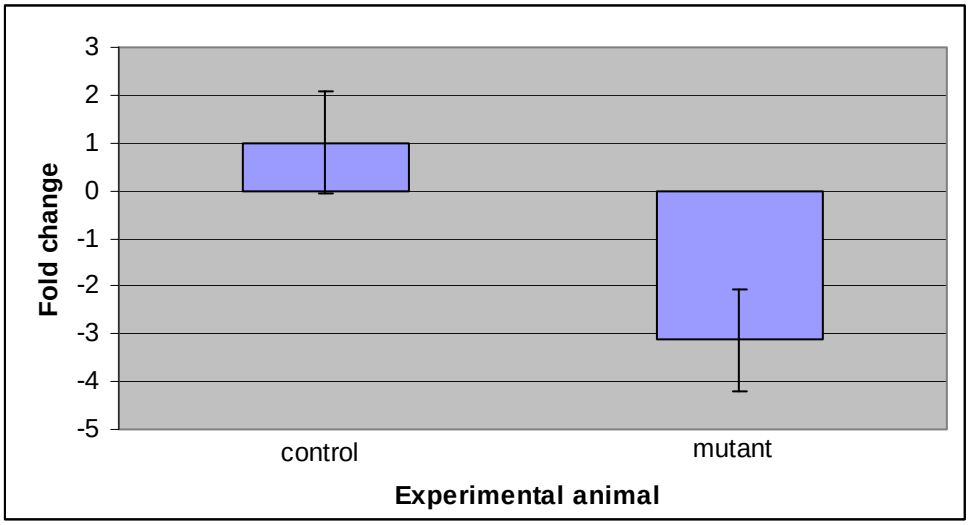


Figure 23. *BR-C* expression in *βftz-f1*mutant fat cells at 10 and 12 hours APF. (A)*BR-C* was under expressed in the mutant fat cells at 10 hours APF. But a P-value of 0.05 indicated that the fold change was not significant. (B) At 12 hours APF *BR-C* was under expressed 3.12 fold with a standard error of 1.07. The P-value in this case suggested that the fold change in gene expression was significant.

DISCUSSION

BR-C Expression in *βftz-f1* Mutant Midgut

Experimental data from the midgut, at 10 hours APF demonstrated that a slight over expression of *BR-C* occurred in the midgut of *βftz-f1* mutants compared to the *BR-C* expression in the control midgut. But statistical analysis indicated the over expression to be insignificant. However, at 12 hours APF the 2.69 fold increase in the expression of *BR-C* in the mutant midgut was deemed significant.

The results of analysis performed on *βftz-f1* mutant midgut thus suggest that although the gene expression remains the same in the control and mutant midgut at 10 hours APF, at 12 hours APF *βftz-f1* may actually repress the expression of *BR-C*.

βftz-f1 is expressed during a period of low ecdysone concentration, at 6-8 hours APF after the larval pulse of ecdysone. As *βftz-f1* is expressed after the initiation of programmed cell death in the midgut, it was expected that the expression of *BR-C* mRNA would not change. This was partially true for the results of this experiment where *BR-C* expression did not change in the *βftz-f1*mutant midgut at 10 hours APF. Curiously, the results also indicated that *BR-C* expression increased in the *βftz-f1*mutant midgut at 12 hours APF.

BR-C is a gene whose products play an essential role in the remodeling of larval tissues through programmed cell death. The ecdysone receptor complex, along with *βftz-f1* regulates the transcription of *BR-C, E74* and *E93* immediately

prior to larval cell histolysis (Broadus *et al.*, 1999). The expression of certain death genes such as *rpr,* and *hid* transcription also increase prior to ecdysone regulated cell death in the midgut (Jiang *et al.,*1997). Even though *rpr* transcription is directly regulated by the ecdysone receptor complex, *BR-C* is necessary for the maximum levels of *rpr* expression.

The larval midgut undergoes cell death at puparium formation (0 hours APF) in response to the late larval pulse of ecdysone (Jiang *et al.*, 1997) while *βftz-f1* is expressed much later around 6-8 hours APF. As cell death in the midgut occurs prior to the expression of *βftz-f1*, *BR-C* expression is possibly turned off in the control midgut after the expression of *βftz-f1.* A similar mechanism might be in place to shut off *BR-C* expression in the midgut 12 hours earlier at the beginning of puparium formation. Lee *et al* (2002) hypothesized that the ecdysone receptor complex activates *BR-C* and *E93* independently of *βftz-f1* in the midgut. There might be unrelated transcriptional regulators that induce these early genes (Lee et al., 2002). But at 12 hours APF, *βftz-f1* may be acting as a repressor of *BR-C.*

The results of this study of *βftz-f1*mutant midgut thereby raise two vital questions. The first one asks whether *βftz-f1* has the ability to repress the expression of certain early genes in a stage and tissue specific manner, and what mechanism it employs for the repression of the early genes. Secondly, the experiment raises the question of whether there are other key regulators that play a role in the repression of the early genes.

BR-C Expression in *βftz-f1* Mutant Hindgut

In the hindgut of *βftz-f1* mutants *BR-C* is over expressed at 10 hours APF and 12 hours APF. At 10 hours APF, *BR-C* expression increases by 13.36 fold in the *βftz-f1* mutant hindgut compared to *BR-C* expression in the control hindgut at the same time point. At 12 hours APF, *BR-C* expression increases to 36.76 fold compared to the control hindgut. The results of the T-test indicate that the calculated fold change is significant for both time points. Thus, as in the case of *BR-C* expression in the control midgut at 12 hours APF, *βftz-f1* may be repressing the expression of *BR-C* in the control hindgut.

The key factor that should be kept in mind while discussing gene expression in the hindgut of a developing prepua/ pupa is that, the hindgut does not undergo cell death in the same manner salivary glands and midgut do. The remodeling that occurs in the midgut happens through autophagic cell death whereas the remodeling in the hindgut is more a result of rearrangement of larval gut tissue (Lengyel and Iwaki, 2002). Thus, a key cell death regulatory gene such as *BR-C* may be repressed by *βftz-f1* in the hindgut during the prepupal-pupal transition.

It should also be noted that *BR-C* is not one of the genes involved in *Drosophila* hindgut development (Lengyel and Iwaki, 2002) and perhaps the key function of this gene in the control hindgut is to be repressed in order for the hindgut to develop properly in response to the prepupal ecdysone pulse.

BR-C Expression in *βftz-f1* Mutant Fat Body

Data from the Real Time PCR experiment performed on *βftz-f1* mutant fat body indicated an under expression of *BR-C* at 10 hours APF. However, the high standard error coupled with the result from the T-test suggested that the under expression was insignificant. This suggests that *βftz-f1* is not necessary for *BR-C* expression in the hindgut at 10 hours APF, as fat body from both the *βftz-f1*mutant and the control animal expressed *BR-C* at the same level. However, at 12 hours APF, fat cells showed a significant under expression of 3.12 fold which indicates that *βftz-f1* is necessary for optimal *BR-C* expression in fat body at 12 hours APF. The under expression indicates that mutant fat cells express *BR-C* at a lower level, compared to the control fat cells at 12 hours APF.

Unlike the midgut and the salivary gland which are remodeled through programmed cell death during metamorphosis, the fat body is remodeled by undergoing tissue dissociation. Tissue dissociation results in the redistribution of the individual fat cells throughout the body of the pupa and is essential for the completion of pupal development (Cherbas et al., 2003). The remodeling of the fat body takes place during early stages of metamorphosis and following the prepupal ecdysone pulse at 10-12hours APF anterior fat body cells physically detach from each other (Nelliot et al., 2006).

The results of this study indicate that *βftz-f1* is necessary for the optimal expression of *BR-C* in the prepupal fat body at 12 hours APF but not at 10 hours APF. It can be hypothesized based on the results of this experiment that *βftz-f1*

being expressed prior to the prepupal-pupal transition provides *BR-C* the ability to be induced in the fat cells 12 hours APF in response to the prepupal pulse of ecdysone.

***BR-C* Expression in the *βftz-f1* Mutant Salivary Glands**

The ΔΔCt analysis of the salivary glands showed *BR-C* under expression of 2.8 fold in the salivary glands of *βftz-f1* mutants at 10 hours APF. This indicates that the mutant salivary glands expressed less *BR-C* mRNA compared to the salivary glands of the control animals. In the control salivary gland, at 10 hours APF, *βftz-f1* serves as a competence factor for the re-induction of a set of early genes in response to the prepupal pulse of ecdysone (Woodard *et al.*, 1994). The under expression observed from this study hence indicates that *βftz-f1* is necessary for the expression of *BR-C* in this tissue at 10 hours APF.

BR-C was also expected to be under expressed in the mutant salivary glands 2 hours later at 12 hours APF. However, at 12 hours APF *βftz-f1* mutant salivary glands over expressed *BR-C* by 64.45 fold. This may be attributed to the hypomorphic *βftz-f1*mutation. While *βftz-f1* was expressed at the optimal level in the control salivary gland at 10 and 12 hours APF, in the *βftz-f1*mutant salivary gland there might have been insufficient copies of *βftz-f1* mRNA transcripts at 10 hours APF which accumulated by 12 hours APF. It can be hypothesized that at 12 hours APF a delay in *BR-C* expression was observed. The reduced level of *βftz-f1* transcript may have elicited a delayed *BR-C* response.

It should be noted that the expression of *BR-C* is induced in the salivary gland prior to puparium formation (Fig. 24), while it is expressed again at 10 hours APF in response to the prepupal ecdysone pulse. However, at 12 hours APF *BR-C* expression cannot be detected in the salivary glands of control animals. It can be concluded that *BR-C* is rapidly shut off after 10 hours APF in the control animal, a fact which may be mediated by *βftz-f1*. Hence, a delay in *BR-C* response may account for the over expression of *BR-C* in the mutant midgut at 12 hours APF.

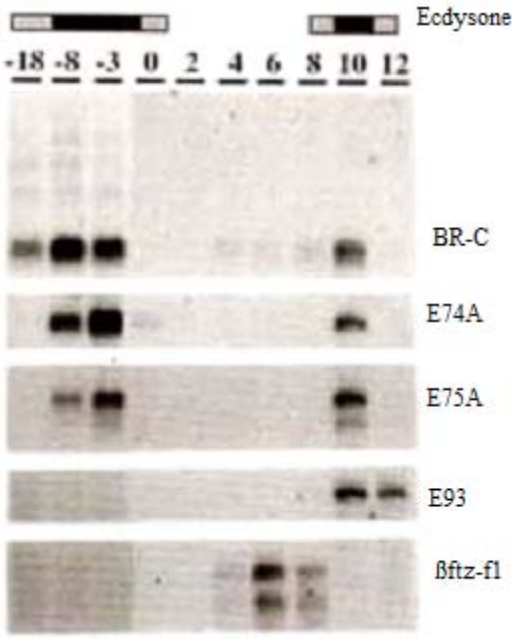


Figure 24. Northern blot demonstrating the expression of the early genes in response to ecdysone. The figure demonstrates the levels of mRNA transcript of the early genes *BR-C, E74A, E75A, E93* and that of *βftz-f1* in the salivary glands of the fruit fly at different stages during development. The top bars show the presence of ecdysone while the numbers designate development in terms of puparium formation. *BR-C* is expressed before 0 hours APF and then again at 10 hours APF while *βftz-f1* is expressed around 6-8 hours APF (Modified from Woodard *et al*., 1994).

Future Directions

The results of this study have so far suggested that *βftz-f1* is required for the optimal expression of *BR-C* in the mutant salivary glands at 10 hours APF and at 12 hours APF. It has also suggested that *βftz-f1* possibly represses the expression of *BR-C* in tissues where programmed cell death does not occur (hindgut 10 and 12 hours APF) and also in the tissues after programmed cell death occurs (midgut 12 hours APF).

To further understand the role of *βftz-f1* on the expression of *BR-C* further Real Time PCR trials must be performed on the tissues discussed in this paper. For the purposes of this study each tissue at a specific time point was utilized once to determine the change in gene expression. Studies performed with further replicates may be helpful in explaining the data better. The study should also include other tissues such as the central nervous system and the imaginal discs.

While the two specific time points studied in this paper provide a basic comprehension of the expression of the early gene *BR-C,* a study at different stages of metamorphosis is necessary to understand the role of *BR-C* on a broader level.

This study should also be extended to study the role of the other early genes (E74, E75, and E93). This will provide a more detailed understanding of the regulatory hierarchy of ecdysone action in *Drosophila.*

Furthermore, it should be borne in mind that the results of this study could be analyzed using other statistical methods of data analysis. Different statistical

methods employed to interpret Real Time PCR data may provide dissimilar results, thus to verify the validity of the results higher levels of statistical analysis should be used.

APPENDIX

Primer Dilution

1µL of water was initially added for each nM of primer resulting in a stock concentration of 100µM. To make a working concentration of 10 µM, 1 µL of primer from the stock concentration was added to 99µL of nuclease free water.

Sample Calculation of Real Time PCR Data

Comparative Ct Method (Salivary gland 10 hours APF)

Genotype	Gene	Average Ct value	Standard Deviation	Standard Error
w^{1118}(control)	*β-actin*	24.49	0.14	0.08
w^{1118}(control)	*BR-C*	25.58	0.31	0.18
β -FTZ-F1 mutant	*β-actin*	25.47	0.01	0.01
β -FTZ-F1 mutant	*BR-C*	28.03	0.18	0.1

ΔCt control = (Average Ct, control, *BR-C*) – (Average Ct, control, *β-actin*)
=25.58-24.49 = **1.08**
Standard deviation for ΔCt control = $\sqrt{((0.14)^2 + (0.31)^2)} = 0.35$
Standard error for ΔCt control = $\sqrt{((0.08)^2 + (0.18)^2)} = 0.19$

ΔCt mutant = (Average Ct, mutant, *BR-C*) – (Average Ct, mutant, *β-actin*)
=28.03-25.47 = **2.56**
Standard deviation for ΔCt (mutant) = $\sqrt{((0.01)^2 + (0.18)^2)} = 0.17$
Standard error for ΔCt (mutant) = $\sqrt{((0.01)^2 + (0.1)^2)} = 0.1$

ΔΔCt = ΔCt mutant- ΔCt control = 2.56-1.08 = **1.48**
Standard deviation for ΔΔCt = 0.17
Since the standard deviation for the mutants and the control is an arbitrary number the standard deviation for the mutant can be considered as the total standard deviation.
Standard error for ΔΔCt= $\sqrt{((0.19)^2 + (0.1)^2)} = 0.21$

Fold change in gene expression = $2^{-\Delta\Delta Ct} = 2^{-(1.48)}$ = **0.35**
= 1 / 2.8 fold underexpression.

Standard error for fold change = $\mathbf{2}^{\mathbf{SE\ \Delta\Delta Ct}} = \mathbf{2}^{\mathbf{(0.21)}} = \mathbf{1.16}$ (here SE stands for standard error)

T test

The students T test was performed to check whether the calculated fold change was significant or not.

T value = ΔΔCt/ SE ΔΔCt
= 1.48 / 0.21
= 7.05

The degree of freedom is 2 in this case as there were 3 replicates of each sample.

A P value generator found on the web was used to calculate the P value and the one tailed P value was considered.

P value (one tailed) = 0.01 < 0.05; indicating that the fold change is significant.

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