Understanding phenotypic plasticity: the effect of temperature and the roles of posterior Hox genes on the development of abdominal melanic pigmentation in the milkweed bug, Oncopeltus fasciatus

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<td>EcR</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
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<td>TH</td>
<td>Tyrosine Hydroxylase</td>
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<tr>
<td>Ubx</td>
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ABSTRACT

Complex traits, such as body pigmentation, often exhibit phenotypic plasticity, the ability of a genotype to produce contrasting phenotypes in different environments. Yet the mechanism underlying phenotypic plasticity is not well understood. To begin to understand the regulation of phenotypic plasticity in abdominal melanic pigmentation of the milkweed bug, *Oncopeltus fasciatus*, the effects of dynamic environment and the RNA interference mediated knock-down of the posterior Hox-genes, *abdominal-A* and *Abdominal-B*, and the effector gene, *Tyrosine hydroxylase*, (*TH*) were investigated. Higher temperature induced lighter abdominal melanic pigmentation, while lower temperature induced darker pigmentation in milkweed bugs. The temperature sensitive period for abdominal melanic pigmentation was found to occur during final nymphal stage of *O. fasciatus*.

While the knock-down of *abd-A* resulted in an almost-melanin-less ventral abdomen, *Abd-B* knock-down resulted in extra melanic pigmentation in the A6 segment of *O. fasciatus* that normally lacks pigmentation. The knock-down of *TH* resulted in the lack of melanic pigmentation in the abdomen as well as the whole body. The relative expressions of *abd-A*, *Abd-B* and *TH* in fifth instar nymphs at 20°C and 33°C were found to be temperature-independent. Preliminary expression profile of *E75A* suggested an involvement of ecdysone in phenotypic plasticity observed in *O. fasciatus* ventral abdominal melanism. Investigation of adaptive significance of abdominal melanism in *O. fasciatus* revealed that abdominal melanism does not confer desiccation resistance. Based on the findings of this study, *O. fasciatus* will provide an interesting opportunity for future studies on how evolution might act to shape the developmental systems underlying adaptive polyphenisms and developmental robustness. Future studies involving ecdysone titers, genes affected by *abd-A* and *Abd-B* and knock-down of *abd-A* and *Abd-B* at extreme temperatures would help us understand the complete pathway for cuticular melanization in *O. fasciatus* and establish the mechanisms underlying phenotypic plasticity in general.
INTRODUCTION

I. PHENOTYPIC PLASTICITY

Phenotypic variation can arise from either phenotypic plasticity or genetic polymorphism (Lorenzon et al., 2001). Phenotypic plasticity, the ability of a given genotype to produce variable phenotypes in different environments (Agarwal, 2001; Debat and David, 2001; Stearns, 1989), has recently emerged as important target of study because of its potential implications for understanding evolutionary biology and disease onsets. Virtually any trait ranging from the morphological traits, which are discussed more commonly (Schlichting and Pigliucci, 1998; Woltereck, 1909), to physiological and behavioral traits, can show phenotypic plasticity (Whitman and Agrawal, 2009). Twentieth century evolutionary biologists lacked explanations for the mechanisms underlying phenotypic plasticity because it was considered to be of lesser importance with limited contribution from genes (Agarwal, 2001). However, the modern view of phenotypic plasticity rejects the lack of genetic basis. It proposes the adaptive plasticity hypothesis that phenotypic plasticity evolves to maximize fitness in variable environments (Agarwal, 2001) and can facilitate evolutionary change and speciation (West-Eberhand, 2005).

Although phenotypic plasticity has been well discussed in ecological contexts, how organisms’ genes respond to different environmental stimuli to produce the various phenotypes and conditions remains poorly understood. Additionally, why some traits are more phenotypically plastic than others also remains unclear from a mechanistic perspective. Recognizing phenotypic plasticity and its genetic basis have important implications for
understanding life on earth, the events that facilitated evolution, diseases and disorders that
are triggered by environmental conditions and morphological polyphenisms (Whitman and
Agrawal, 2009). Additionally, a better understanding of phenotypic plasticity can better
establish the link between trait variability and adaptation (Lorenzon et al., 2001).

Whitman and Agarwal propose that phenotypic plasticity can be passive, anticipatory,
instantaneous, delayed, continuous, discrete, permanent, reversible, beneficial, harmful,
adaptive or non-adaptive (Whitman and Agrawal, 2009). While some of the physiological,
morphological or developmental traits can switch back and forth between alternative states in
response to changes in an organism’s environment, other changes, typically the
morphological ones are irreversible (Callahan et al., 1997). Phenotypic plasticity, as a form
of interaction between genotypes and their heterogeneous environment, can also buffer a
population against natural selection through the maintenance of genetic variation within
populations (Gillespie and Turelli, 1989; Nijhout, 2003; Wright, 1931).

Phenotypic plasticity has been documented in many species. For example, a barnacle
(Chthamalus anisopoma), in presence of a predatory snail, grows in a bent-over form that is
resistant to predation but has a lower reproductive capacity (Lively, 1986a; Lively, 1986b).
However, in absence of a predator in the surroundings, the same barnacle develops into a
typical morph, which has a high reproductive output (Lively, 1986a; Lively, 1986b).
Moreover, environmental sex determination (ESD) is a phenomenon in which an
environmental variable has an effect on the sex phenotype in vertebrates like the snapping
turtle, Chelydra serpentina (Rhen and Lang, 1995). The sex ratio is 100% male at
intermediate temperatures but switches to 100% female at lower or higher temperatures in
these turtles (Rhen and Lang, 1995; Willmer et al., 2005). Although phenotypic plasticity has
been documented in many species, its underlying regulation remains unclear. In many cases, endocrine regulation has been shown to underlie adaptive polyphenisms (Nijhout, 1994; Nijhout, 2003; Oostra et al., 2011). However, little is known about how hormonal regulation interacts with patterning mechanisms to regulate the plasticity of traits.
II. INSECT PIGMENT DEVELOPMENT

Insects are the most diverse group of animals, representing approximately 80 percent of the world’s species. At any time, it is estimated that there are some 10 quintillion (10,000,000,000,000,000,000) individual live insects (Sabrosky, 1952). Insect pigmentation is a highly variable trait with diversity between species, life stages of a single individual, sexes and body parts (Wittkopp and Beldade, 2009). There is no adaptive role that by itself can account for the diversity of pigmentation observed in insects (Wittkopp and Beldade, 2009). Because it is often highly plastic, insect pigmentation provides an excellent system for understanding processes underlying phenotypic plasticity.

a. Melanism phenomenon and its significance

The melanism phenomenon is one of the most commonly cited examples of natural selection in evolutionary biology (Koch et al., 2000) as a model for visible variation in insects (True, 2003; Zhan et al., 2010). The most common function provided by melanism in insects is protection against predators via crypsis, the ability to avoid observation of detection (True, 2003). Melanism also helps insects become aposematic by having warning coloration (True, 2003). In terms of non-visual functions, melanism also contributes to thermoregulation (Majerus, 1998; True, 2003; Watt, 1968; Watt, 1969), higher desiccation resistance and higher fecundity (Parkash et al., 2009). One of the reasons melanic coloration remains plastic is because of the seasonal variability in climatic conditions that favor melanics (True, 2003). Additionally, melanic patterns are often sexually dimorphic in many insects and allow for the recognition of species and sex (Wiernasz, 1989; Wiernasz and Kingsolver, 1992).
b. Pigmentation pathway in insects

The pigmentation pathway is well understood in various insects and is a convenient target for the study of phenotypic plasticity (True, 2003). Among insects, the developmental mechanisms underlying pigmentation is best understood in fruit fly, *Drosophila melanogaster*, the model organism for the study of insect development (Wittkopp and Beldade, 2009). In *Drosophila melanogaster*, the biochemical link between the regulatory genes, which affect the developmental patterning, and the genes which ultimately produce the adult pigmentation trait is well studied (Wittkopp and Beldade, 2009). Based on the functions of the genes involved, Wittkopp and Beldade (2009) divide the process of pigmentation in insects into two stages: (1) positioning of pigments in space and time (controlled by patterning genes) and (2) the biochemical synthesis of pigments (controlled by effector genes) (Wittkopp and Beldade, 2009). In *Drosophila melanogaster*, pigment patterning is controlled by pleiotropic regulatory proteins, including sex determination genes, HOX genes (e.g. *Abdominal-B*), signaling pathways (e.g. *wingless* and *decapentaplegic*), and selector genes (e.g. *optomotor-blind*, *bric-a-brac* and * engrailed*) (Gibert et al., 2007; Wittkopp and Beldade, 2009). These factors also regulate multiple other traits in fruitflies (Wittkopp et al., 2003).

Melanin is a complex pigment formed from oxidation of dopa or dopamine (Anderson, 2005). The cellular and molecular mechanisms of cuticular melanization have been extensively studied in *D. melanogaster* and tobacco hornworm, *Manduca sexta* (Gibert et al., 2007; Hiruma and Riddiford, 2009; Riddiford et al., 2003; Wittkopp et al., 2003). Melanins in *D. melanogaster*, *M. sexta* and most other insects are synthesized by a branched biochemical pathway that converts phenylalanine into tyrosine and then
polymerizes modified molecules, such as dopa, dopamine and N-beta-analyl-dopmine (NBAD), into black, brown, and yellow pigments (Wittkopp et al., 2003). Figure 1 delineates an overall summary of the steps involved in the biosynthesis of insect melanin. The first step of cuticular melanization pathway involves Tyrosine Hydroxylase (TH), an essential enzyme which converts Tyrosine to Dopa (Hiruma and Riddiford, 2009; Wittkopp and Beldade, 2009) (Figure 1). Dopa decarboxylase (DDC) converts dopa to dopamine, and Di-phenoloxidase (PO) catalyzes the oxidization of dopa or dopamine (Anderson, 2005; Hiruma and Riddiford, 2009). Dopamine is further processed by PO to finally form Dopamine melanin (Hiruma and Riddiford, 1985) (Figure 1). Another pathway involves the synthesis of Dopa melanin via PO catalyzed processing of Dopa to Dopaquinone, which is subsequently converted to Dopa melanin (Hiruma and Riddiford, 1985).

Cuticular melanization in insects generally involves melanin deposition in either a random dispersion or in definitive granules (Kayser-Wegmann, 1976). In black M. sexta larvae, the epidermis synthesizes and deposits premelanin granules into newly forming cuticle during a molt (Hiruma and Riddiford, 1985). After the molt, enzymes, such as PO, are activated and premelanin granules are oxidized to melanin causing melanization dependent on the amount of melanin granules present (Hiruma and Riddiford, 1985).
Figure 1. Pathway for cuticular melanization in insects (Hiruma and Riddiford, 2009; Wittkopp and Beldade, 2009; Wittkopp et al., 2003). Molecules drawn using ChemDraw Ultra 12.0.
c. Hormonal regulation of melanization

Ecdysone receptor (EcR) has been shown to play major roles in molting, metamorphosis, reproduction, immunity and other aspects of insect life in many arthropods (Brown and Truman, 2009). It is switched on and off by ecdysteroids, such as 20-hydroxyecdysone (20E), the most common active form of molting hormone found in insects (Nijhout, 1994). The ecdysone-ecdysone receptor complex initiates a cascade of activation and inactivation of regulatory factors, which mediate the actions of this hormone (Zhou et al., 1998). In Manduca sexta, ecdysone acts through its heterodimeric receptor comprising of the ecdysone receptor (EcR) and Ultraspiracle (USP) to initiate and orchestrate molt. In the context of melanism, DDC, the essential enzyme involved in the conversion of dopa to dopamine (Figure 1) (Anderson, 2005), is under the control of the two major developmental hormones, ecdysone and juvenile hormone (JH) in M. sexta (Hiruma and Riddiford, 2009). The expression of Ddc in the epidermis is regulated by ecdysone signaling (Davis et al., 2007), and its transcription in the epidermis is controlled by the ecdysone response element (EcRE) (Chen et al., 2002) located between -97 bp to -83 bp from the transcription initiation site (Hiruma and Riddiford, 2009). Additionally, the transcription factor Broad-Z2 (BR-Z2), which is induced by the rising ecdysteroid titer, is also required for the full expression of DDC (Hodgetts et al., 1995). Thus, EcR is most probably involved in insect melanization. In fact, recent studies done on rice planthoppers Nilaparvata lugens and Laodelphax striatellus have found the involvement of EcR in wing morphogenesis and melanization in these species (Wu et al., 2012). Additionally, polyphenisms observed in seasonally polyphenic butterfly Araschnia levana and various other insects have been shown to be under the control of ecdysone (Nijhout, 2003) and ecdysteroids (Rountree and Nijhout, 1995).
Since ecdysone is crucial for molting in most insects, its direct knock-down is lethal (Fletcher and Thummel, 1995). The expression of ecdysteroid-induced transcription factor E75 can be used to give a direct measure of ecdysone without affecting the insect life cycle (Zhou et al., 1998). E75, which has been shown to be required in larval molting and metamorphosis in *Drosophila* (Zhou et al., 1998), has two isoforms – E75A and E75B. Because its RNA transcription is directly induced by 20E, E75A expression in particular can be used as an indicator of the circulating ecdysone levels (Zhou et al., 1998). The measurement of E75A levels in insects with different melanin levels would allow us to determine the involvement of ecdysone in melanism and phenotypic plasticity in general.

### d. HOX genes

The specification of the anteroposterior orientation of different structures of a wide range of animals depends on a group of genes called the HOX complex (McGinnis and Krumlauf, 1992). They have been shown to be involved in pleiotropic regulation of pigment patterning (True, 2003). Their study is crucial in terms of understanding the mechanisms underlying phenotypic plasticity of insect pigmentation. The Hox complex is split into two clusters: bithorax complex (BX-C) and Antennapedia complex (ANT-C) in *Drosophila* (Lewis, 1978). Within BX-C, *Ultrabithorax (Ubx)*, *abdominal-A (abd-A)* and *Abdominal-B (Abd-B)* determine the identities of the various abdominal segments (Tiong et al., 1985).

Both *abd-A* and *Abd-B* are posterior Hox genes that specify the identities of the abdominal segments and terminalia in *D. melanogaster* (Aspiras et al., 2011). Gain-of-function mutations in *abd-A* and *Abd-B* in *Drosophila* have been shown to result in homeosis and transformation of different segments into more posterior ones (Lewis, 1978).
A has been found to be required only in the development of female genitalia of flies (Aspiras et al., 2011), Abd-B, in particular, has been shown to bind to a cis-regulatory sequence controlling the male-specific pigmentation in the Drosophila abdomen, activating the production of black melanin (Wittkopp and Beldade, 2009). Other studies have shown that Abd-B represses several crucial pigmentation enzymes in D. melanogaster (Gibert et al., 2007). Moreover, temperature studies involving phenotypic plasticity in D. melanogaster have shown that plasticity in the abdominal pigmentation of flies is mediated by Abd-B (Gibert et al., 2007). A combination of the study of Hox genes and the physiological mechanisms that interact with the environment should shed light on how patterning and physiology interact to regulate phenotypic plasticity of melanic patterns.

**e. Milkweed Bug, Oncopeltus fasciatus**

The milkweed bug Oncopeltus fasciatus (Heteroptera) is a member of the Paraneoptera, the sister-clade to the Holometabola (Wheeler et al., 2001). It is a medium-sized hemipteran (true bug) of the family Lygaeidae (Wheeler et al., 2001). It is an important model organism that allows us to perform functional analyses of genes and study phenotypic plasticity and developmental stability (Feir, 1974). In particular, the abdominal melanic pigmentation of O. fasciatus has been shown to be affected by temperature (Novak, 1955), while its wing pigmentation remains unaffected. Because of these robust as well as plastic melanic pigmentation traits, O. fasciatus is an ideal model system to investigate phenotypic plasticity and robustness. Ewen-Campen et al. recently completed the 454 sequencing-only transcriptome of O. fasciatus facilitating the use of this organism as a model to study the genetics of phenotypic plasticity (Ewen-Campen et al., 2011).
A large milkweed bug adult is a 9-18 mm long insect and mainly feeds on seeds, particularly those of the milkweed plant in its natural habitat (Kaufman and Eaton, 2007). It reproduces easily and readily adjusts to the research environment, making it a convenient model organism in a lab (Feir, 1974). It can be fed on sunflower seeds in a lab setting. The life cycle of *O. fasciatus* consists of five different nymphal stages. Bright orange nymphs hatch from eggs after about a week and molt five times before becoming adults in a span of about a month. An adult milkweed bug is orange with wings that have black rhomboidal melanin spots at both ends of its body and a black band in the middle. The life of an adult is about 30 days long, during which a female can lay up to 2000 eggs (Feir, 1974).

*O. fasciatus* provides a useful and robust system for highly sequence specific whole organism RNA interference (RNAi, explained in the following section) knock-down of various genes and the study of associated phenotypic variation (Angelini and Kaufman, 2004). Previous studies have successfully performed RNAi in *O. fasciatus* embryos as well as nymphs (Angelini et al., 2005) and have revealed that the expression patterns of different Hox genes in *O. fasciatus* differ from those in *Drosophila* and other insects (Rogers and Kaufman, 1997; Rogers et al., 1997). Posterior Hox-genes *abd-A* and *Abd-B* in *O. fasciatus* have been shown to be involved in proper genital development (Aspiras et al., 2011). The genetic mechanisms underlying phenotypic plasticity of abdominal melanism, however, have not yet been explored in *O. fasciatus*. The study of the effect of temperature and genes on melanism in *O. fasciatus* can allow us to explore the mechanisms underlying phenotypic plasticity.

The study of phenotypic plasticity, developmental stability, genes and their variable expression in *O. fasciatus* can be a powerful tool to understanding epigenetic regulation of
phenotypic plasticity. *O. fasciatus* provides a unique system where the interactions between patterning Hox genes, hormones and phenotypic plasticity associated with variable environment can be investigated in relation to each other. Additionally, its amenability to RNAi increases the scope of research that can be performed using this unique organism. In fact, exploiting RNAi based knock-down at particular developmental stage is much easier in *O. fasciatus* as compared to *D. melanogaster*. This allows us to investigate various interconnected variables using one system in order to better understand phenotypic plasticity.

**f. RNA interference**

The method of RNA interference (RNAi) is a broadly used reverse genetics method (Timmons and Fire, 1998). RNAi is a technique used to interfere with the function of an endogenous gene and involves administration of double stranded RNA (dsRNA) for the disruption of gene expression in biological systems like *O. fasciatus*. After the dsRNA enters the cells, an enzyme called Dicer processes it and cleaves the dsRNA to short fragments called short interfering double-stranded RNAs (siRNAs), which are about 21-23 nucleotides in length (Hannon, 2003). The siRNAs are incorporated into a protein complex called RNA-induced silencing complex (RISC) where one of the strands of double stranded siRNAs is discarded to have RISC attached to a sequence specific single-stranded RNA. This complex recognizes the complementary sequence in the target mRNA, resulting in the cleavage and degradation of the target mRNA by RISC and subsequent silencing of the gene (Hannon, 2003). Thus, RNAi is an excellent tool for addressing gene function without having to knock out the gene of interest at chromosomal level.
III. PROJECT AIMS AND GOALS

The study of phenotypic variation and the factors affecting phenotypic variation have been given considerable attention by evolutionary biologists. Pigmentation of organisms provides an excellent two-dimensional system for understanding processes underlying phenotypic plasticity. In this study, we analyzed the effects of temperature and post transcriptional silencing of posterior Hox genes \textit{abd-A} and \textit{Abd-B} and effector gene \textit{TH} on the abdominal melanic pigmentation pattern in \textit{O. fasciatus}. As established by Novak, we expected to obtain a darker abdominal pigmentation at lower temperature and a lighter pigmentation at a higher temperature in milkweed bug \textit{O. fasciatus} (Novak, 1955). To establish the sensitive period for the determination of abdominal melanic pigmentation, reciprocal transfers of nymphs from one extreme temperature to another were performed. To begin to elucidate the genetic mechanism underlying plasticity, the roles of \textit{abd-A}, \textit{Abd-B} and \textit{TH} were investigated through RNAi, and their expressions were analyzed. At different temperatures, we hypothesized to see a change in expression of different genes involved in pigmentation. Additionally, the role of ecdysteroid was investigated through an examination of relative expression of \textit{E75A}. Finally, the adaptive significance of plasticity was investigated by determining the survival rates of the extreme morphs under dry conditions.

Since the interaction between physiology and patterning to regulate phenotypic plasticity of melanism has been given limited attention, we have begun to investigate the interactions of these factors with the long-term goal of understanding how phenotypic plasticity is regulated and how it might evolve.
MATERIALS AND METHODS

General Method

Animals

Wild type milkweed bugs, *Oncopeltus fasciatus*, were obtained from Carolina Biologicals and raised in plastic containers on organic sunflower seed and water at 26.5°C. For the temperature experiments, the milkweed bugs were raised separately at 20°C and 33°C. The photoperiod was 16 hrs light: 8hrs dark.

Transfer experiment

To determine the temperature sensitive period of pigmentation, *O. fasciatus* raised at 20°C were transferred on every other day of their final (fifth) nymphal stage (Day-0 (D0) through Day-18 (D18)) to 33°C. Similarly, nymphs raised at 33°C were transferred to 20°C on D0 through D5 of the fifth instar.

Imaging and quantification of pigmentation

Whole bodies of *O. fasciatus* were fixed with 3.7% formaldehyde and stored in 80% glycerol at -20°C for up to 1 week. The ventral abdomen of each bug was mounted in 80% glycerol and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The area of melanic pigmentation was analyzed using the public domain ImageJ program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). The pigmentation area was normalized to body size by dividing the area by the ocular distance,
which is used as a proxy for body size (Aspiras et al., 2011). All statistical analyses were performed using JMP Pro 9.

**Desiccation tolerance experiment**

Day 0 adults raised at three different temperatures (20ºC, 26.5ºC and 33ºC) were kept in a container with silica gel packs but no water at 26.5ºC. This set up maintained the humidity in the container at approximately 5%. Organic sunflower seeds were provided as food. The survival of newly eclosed adults in this condition was tracked. All statistical analyses were performed using JMP Pro 9.

**RNA interference**

**mRNA isolation and cDNA synthesis**

Fifth instar *O. fasciatus* nymphs were dissected in 1X phosphate-buffered saline (PBS, 0.02M phosphate, 0.15M NaCl, 0.0038M NaH₂PO₄, 0.0162M Na₂HPO₄; pH 7.4). The gut and fat body were removed, and the remaining tissue was homogenized in Trizol (Invitrogen, www.invitrogen.com). Chloroform was added to the homogenized sample in a chloroform: Trizol mixture at a ratio of 1:5 by volume and centrifuged at 14046 rcf at 4ºC for 15 minutes. The supernatant containing RNA was extracted and precipitated in isopropanol at a 1:1 ratio after centrifugation at 14046 rcf at 4ºC for 10 minutes. The pellet was washed with 75% ethanol in diethylpyrocarbonate (DEPC)-treated MilliQ water (0.0001% diethylpyrocarbonate in distilled water) and resuspended in 13µL DEPC MilliQ water and stored at -80ºC. The isolated RNA was digested in treated with DNase (Promega) and
precipitated in isopropanol. cDNA was synthesized from 1µg of total RNA using the cDNA synthesis kit (Fermentas) following the manufacturer’s instructions.

**Polymerase chain reaction for the amplification of abd-A, Abd-b and TH and analysis by electrophoresis**

The sequences for abd-A, Abd-B and TH were obtained from Ewen-Campen *et. al.* (2011) and Angelini *et. al.* (2005). Primers were designed using the Primer3 program, v.0.4.0 [http://frodo.wi.mit.edu](http://frodo.wi.mit.edu) (Table 1). Polymerase chain reaction (PCR) mixture that contained 2% cDNA, PCR Buffer (1mM Tris, 5mM KCl, 0.15mM MgCl2, pH 8.3), 0.2 mM dNTPs, 0.4µM forward (FW) primer (Table 1) of each of the genes, 0.4µM reverse (RV) primer of each of the genes (Table 1) and 0.1units/µL Taq DNA Polymerase was prepared in distilled water. PCR involved six different steps. The first step of PCR was done by subjecting the reaction to 94ºC for 2 minutes once. For steps 2-4, the reaction was subjected to 94ºC for 30 s, then 55ºC for 30 s and finally subjected to 72ºC for 1 min. Steps 2-4 were repeated 30 times followed by Step 5, which involved incubation at 72ºC for 10 min and end of the reaction at 4ºC. The PCR product was mixed with the loading dye at a 5:1 ratio and was applied to a 1.0% agarose gel containing 0.005% SybrSafe™ DNA stain from Invitrogen in TBE buffer (0.25M Tris, 1.9M Glycine, 13mM EDTA). The PCR product was subjected to electrophoresis for 20 min at 100V to determine the success of each of the gene amplification and to isolate the PCR product.
Cloning reaction, transformation and linearization of plasmid DNA

Using the MinElute Gel Extraction Kit (Qiagen), the amplified cDNA was extracted from the 1.0% agarose gel following the manufacturer’s instructions. The extracted cDNA was cloned into a TOPO® TA vector (Invitrogen). The cloned TOPO® TA vector reaction mixture was added to TOP10 Chemically Competent bacterial cells (Invitrogen) at a ratio of 1:25 and incubated on ice for 20 min. The transformation mixture was then heat shocked at 42°C for 30 s and cold shocked again on ice. Super optimal broth with catabilite repression (SOC) medium (2% Bacto Tryptone, 0.5% yeast extract, 0.01M NaCl, 0.0025M KCl, 0.01M MgCl₂, 0.01M MgSO₄, 0.02M glucose) was added to the transformation mixture at a ratio of 5:1 transformation mix. The transformation was completed by shaking the mixture at 37°C for 1 hr at 250 rpm. In order to generate transformed bacterial colonies, the transformed cells in LB medium were applied to agar solid growth medium (1% Tryptone, 0.5% Yeast Extract, 1% NaCl, 2% agar) containing 0.1mg/ml ampicillin and incubated at 37°C overnight so that only transformed bacteria would be able to grow on the agar surface. Single colonies were picked and cultured at 37°C overnight in Luria broth (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 0.1mg/ml ampicillin followed by bacterial plasmid purification using QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer’s instructions. The identity of the plasmid was confirmed via sequencing. The plasmid DNA was linearized via restriction enzyme digestion.

Double-stranded RNA (dsRNA) synthesis and injection

Each of the dsRNA strands was synthesized using T3 and T7 MEGAscripts (Ambion) following the manufacturer’s instructions. Equal amounts of single-stranded RNAs (ssRNAs)
were hybridized to form 2 µg/µL solution of double stranded RNA (dsRNA) in DEPC water. dsRNA was annealed as described in Hughes and Kaufman (2002) in a thermal cycler. For the first step, the mixture was maintained at 85°C for 3 minutes, followed by 20 min ramp down to 55°C. For the second step, the mixture was maintained at 55°C for 10 min, followed by 10 min ramp down to 40°C. The mixture was then maintained at 40°C for 20 min, followed by 5 min ramp down to 30°C. Finally the mixture was held at 30°C for 10 min and cooled to 4°C. The annealed dsRNA product was analyzed via agarose gel electrophoresis for confirmation of proper annealing.

*Oncopeltus fasciatus* Day-0 (D0) fifth instar nymphs were anesthetized on ice and injected at their dorsal abdominal segment with 1 µg of dsRNA for each gene separately using a 10-µL glass capillary needle connected to a syringe. For *abd-A*, 0.1 µg of dsRNA was injected. Controls were injected with 1 µg of bacterial *ampicillin-resistance (amp)* dsRNA. All RNAi bugs were raised at 26.5°C on sunflower seed and water in a plastic container. For 33°C Abd-B RNAi, D0 fifth instars from 33°C were microinjected and maintained at 33°C after injection.

**RNAi knockdown verification**

*Semi-quantitative Reverse transcriptase PCR and knockdown verification*

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed in order to confirm knockdown of dsRNA-injected animals. *ribosomal protein subunit 3 (rps3)* was used as a control for loading. Randomly selected fourth instar nymphs were injected with 1 µg of dsRNA for *amp*, *abd-A*, *Abd-B* or *TH* dsRNA, and RNA of day 2
fifth instar stage was isolated from day 2 fifth instar nymphs and converted to cDNA as described above (Materials and Methods: mRNA isolation and cDNA synthesis).

Relative expression profiling via Real Time quantitative PCR

To obtain an expression profile of genes, total RNA of nymphs raised at 20°C, 26.5°C and 33°C was collected daily during the entire fifth instar stadium (20°C: Day 0 – Day 18 (Every other day); 26.5°C: Day 0 – Day 6; 33°C: Day 0 – Day 5), and the RNA was DNase treated and converted to cDNA.

Real time quantitative PCR (qPCR)

External Well Factor Solution (#170-8794) was purchased from Bio-rad and used to collect persistent well factor data to calibrate Bio-Rad iCycler iQ™ Real-Time PCR Detection System (#170-8740) following the manufacturer’s instruction. A quantitative PCR reaction mixture containing 2.5% cDNA, 1X SsoAdvanced™ SYBR® Green Supermix (reaction buffer with hot-start Sso7d-fusion polymerase, SYBR® Green dye, dNTPs, MgCl₂, enhancers, and stabilizers from Bio-Rad), 0.25µM forward primer and 0.25µM reverse primer (Table 1) was prepared on iQ™ 96-Well PCR plates (#223-9441, Bio-rad). Following the manufacturer’s protocol recommendation for standard cycling using cDNA, the reaction was subjected to 95°C for 30 s for the first step of the reaction. Followed by the initial denaturation step, the reaction was subjected to 95°C for 6 s and annealed at 55°C for 30 s for 40 cycles. Finally, the reaction was subjected to melt curve from 65°C to 95°C at 0.5°C increments at 6 s per step. Real Time detection of the annealing and melt curve process were done to determine the Cₜ (threshold cycle) for each reaction mixture. Reaction samples were always run in triplicates.
Standard curve generation and data analysis

In order to generate a standard curve, cDNA from 5 µg of RNA was prepared following the procedure described in mRNA isolation and cDNA synthesis. A quantitative PCR reaction mixture containing 5% cDNA, 1X SsoAdvanced™ SYBR® Green Supermix (reaction buffer with hot-start Sso7d-fusion polymerase, SYBR® Green dye, dNTPs, MgCl₂, enhancers, and stabilizers from Bio-Rad), 0.25µM forward primer and 0.25µM reverse primer (Table 1) was prepared on iQ™ 96-Well PCR plates (#223-9441, Bio-rad). Five sets of reactions containing different concentrations of cDNA were run in triplicates for each of the genes abd-A, Abd-B, TH, and housekeeping control gene rps3 following the manufacturer’s protocol recommendation described above. A standard curve for each gene was generated.

After obtaining the C_T values, relative expression ratio of all the genes under study at 20°C and 33°C was obtained using Day-0 (D0) cDNA of 20°C fifth instar nymph as the reference sample and rps3 as the housekeeping gene control. Relative expression ratio was computed using Pfaffal method after calculating the efficiencies of abd-A, Abd-B and TH from the slopes of the standard curves (Pfaffl, 2001). We were unable to obtain consistent data to generate standard curve for E75A, so data analysis was done using comparative C_T method (Schmittgen and Livak, 2008) using preliminary data obtained. Additional standardizations are required to validate the data obtained for E75A.
Table 1. Primer sequences for various genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>dsRNA Preparation Primer sequence</th>
<th>Knock down verification primer sequence</th>
<th>Real time quantitative PCR primer sequence</th>
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<tr>
<td>abd-A</td>
<td>FW: 5’AGGGCGGTGAAGGAGATAA3’</td>
<td>FW: 5’CGGCTCAGTTCTACCACCA3’</td>
<td>FW: 5’TTGATACCCAAACCCCTTG 3’</td>
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<tr>
<td></td>
<td>RV: 5’TCTGGTGGTGCTTGGTG 3’</td>
<td>RV: 5’TTCTGGGGCTGTTCATT 3’</td>
<td>RV: 5’CAACCCCATACACTTGACCT 3’</td>
</tr>
<tr>
<td>Abd-B</td>
<td>FW: 5’GCCAACAACAAACACAGCA3’</td>
<td>FW: 5’GAGTTCTCTTCAACGCTAC3’</td>
<td>FW: 5’TATCACACTCCCCGCTT 3’</td>
</tr>
<tr>
<td></td>
<td>RV: 5’GGTGTTTCATGGCTCCAC3’</td>
<td>RV: 5’CTGCGGTTTTGGTTCTTCT3’</td>
<td>RV: 5’CGTCGGCTTTTCCCTCTTC 3’</td>
</tr>
<tr>
<td>TH</td>
<td>FW: 5’CTTGAGGAAGGCAGCCTAC3’</td>
<td>FW: 5’TTGATACCCAAACCCCTTG 3’</td>
<td>FW: 5’TATCACACTCCCCGCTT 3’</td>
</tr>
<tr>
<td></td>
<td>RV: 5’GCCAACATTGCTTTCGATTAC3’</td>
<td>RV: 5’CAACCCCATACACTTGACCT 3’</td>
<td>RV: 5’AGG ATC AAC TAC ACC GCT GAC 3’</td>
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</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th># of cycles used</th>
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<tbody>
<tr>
<td>abd-A</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Abd-B</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>rps3</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

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RESULTS

I. The temperature sensitive period for *Oncopeltus fasciatus* abdominal melanic pigmentation occurs during the fifth nymphal stage

In order to investigate the effect of temperature on ventral melanic abdominal pigmentation, wild type *Oncopeltus fasciatus* were raised at three different temperatures, 20°C, 26.5°C and 33°C, and analyzed as adults. Higher temperature resulted in faster growth than lower temperature. Adults raised at a higher temperature had less melanic abdominal pigmentation relative to those raised at lower temperatures (Fig. 2). The average melanic pigmentation of *O. fasciatus* males and females raised at 20°C (the lower temperature) was larger compared to the bugs raised at 26.5°C (the intermediate temperature) and 33°C (the higher temperature). An Analysis of Variance (ANOVA) test followed by Tukey-Kramer HSD analysis revealed significant differences between all male and female pairs of normalized melanic pigmentation area (Female ANOVA: p<0.0001, df = 2, F= 138.8087; Tukey-Kramer HSD analysis for females: p<0.001 for all pairwise comparisons. Male ANOVA: p<0.0001, df = 2, F = 173.6233; Tukey-Kramer HSD analysis for males: p<0.0001 for all pairwise comparisons.).
Figure 2. Higher temperature induces lighter pigmentation while lower temperature promotes darker pigmentation in both male and female *Oncopeltus fasciatus*. A. Examples of ventral pigmentation of males and females reared at different temperatures. 

B. 

![Temperature vs Pigmentation Area/Optical Distance](image)

*Figure 2. Higher temperature induces lighter pigmentation while lower temperature promotes darker pigmentation in both male and female *Oncopeltus fasciatus*. A. Examples of ventral pigmentation of males and females reared at different temperatures. The*
resulting ventral abdomen of each bug was mounted in 80% glycerol and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The image was processed using Microsoft Office Picture Manager and Microsoft PowerPoint and scaled using the public domain ImageJ program. **B.** The average normalized melanic pigmentation of males and females reared at different temperatures along with the standard errors. The area of melanic pigmentation was analyzed using the public domain ImageJ program and processed using Microsoft Excel. The normalization was done using the optical distance of each bug (Aspiras et al., 2011) and statistical analysis was done using JMP Pro 9. **Female** One way ANOVA: p<0.0001, df = 2, F= 138.8087; Tukey-Kramer HSD analysis showed significant difference between all pairs with p<0.0001. **Male** One way ANOVA: p<0.0001, df = 2, F = 173.6233; Tukey-Kramer HSD analysis showed significant difference between all pairs with p<0.0001.

*O. fasciatus* strain used in this study took six days to molt to an adult when raised at 33°C, seven days at 26.5°C and 19-22 days at 20°C from the onset of the final nymphal stages (fifth instar). We sought to determine the sensitive period for the determination of ventral abdominal melanic pigmentation in fifth instar *O. fasciatus* raised at the extreme temperatures (20°C and 33°C). The fifth instar nymphs raised in 33°C were transferred to 20°C on Day-0 (D0) through Day-5 (D5). Similarly, fifth instar nymphs raised in 20°C were transferred to 33°C on every other day of their fifth nymphal stage (D0 through D18). The ventral melanic abdominal pigmentation of the transferred adults was measured and analyzed (Figs. 3 and 4).

The data for *O. fasciatus* fifth instar transfers from 33°C to 20°C suggest that D3 of the fifth nymphal stage is the most temperature sensitive in determining the amount of ventral abdominal melanic pigmentation in *O. fasciatus* males and females raised at 33°C (Fig. 3C). Although D3 is the most sensitive, the sensitive period spans from day 2 to day 5.
(Fig. 3). Similarly, the transfers from 20°C to 33°C suggest a broad sensitive period from D10 – D14 for male and D12-D14 for female *O. fasciatus* when raised at 20°C (Fig. 4C).

While the results of this study established the fifth nymphal stage as the sensitive period for the adult abdominal melanic pigmentation, melanism was affected even before the fifth nymphal stage as suggested by the difference in melanism observed between those raised continuously at one of the extreme temperatures throughout the entire life stage and those that were transferred on day 0 of the final instar. Thus, animals raised at 20°C without transfers had more ventral abdominal melanism compared to those raised at 33°C and transferred to 20°C on day 0 as fifth instars (Fig. 3 A and B). The converse was also true: animals raised at 33°C had less melanism than those raised at 20°C and transferred to 33°C on day 0 of the final instar (Fig. 4A and B). Similarly, those transferred on the last day of the final instar also never reached the amount of pigmentation seen in those that were not transferred: an adult raised at 33°C without transfers had less melanism compared to those raised at 33°C transferred on D5 to 20°C (Fig. 3A and B). In the same way, those raised at 20°C continuously had more melanism than those raised at 20°C and transferred on D18 to 33°C (Fig. 4A and B). Overall, the observed plasticity in adult cuticular melanism suggests a temperature sensitive mechanism that determines the level of melanism before the adult cuticle is laid down. One or more factors which affect melanin production must be temperature sensitive.
A.

B.

(Figure Legend on Page 28)
Figure 3. The sensitive period for the determination of ventral melanic abdominal pigmentation during the fifth nymphal instar of *Oncopeltus fasciatus* raised at 33°C. (A, B) Examples of ventral pigmentation of females (A) and males (B) transferred on various days of their nymphal stages from 33°C to 20°C. The resulting ventral abdomen of each bug was mounted in 80% glycerol and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megapixel camera at 10X magnification. The image was processed using Microsoft Office Picture Manager and Microsoft PowerPoint and scaled using the public domain ImageJ program. C. The average normalized melanic pigmentation of adults along with the standard errors. The area of melanic pigmentation was analyzed using the ImageJ program and processed using Microsoft Excel. The normalization was done using the optical distance of each bug (Aspiras et al., 2011). Statistical analyses were carried our using JMP Pro 9. Tukey-Kramer HSD test levels not sharing the same letters are significantly different from each other (p<0.0001). The shaded region represents the sensitive period for the determination of ventral melanic abdominal pigmentation in fifth instar bugs raised at 33°C.
B.

(Figure Legend on Page 31)
Figure 4. The sensitive period for the determination of ventral melanic abdominal pigmentation in fifth nymphal instar *Oncopeltus fasciatus* raised at 20°C. (A,B) Examples of ventral pigmentation of females (A) and males (B) transferred on various days of their nymphal stages from 20°C to 33°C. The resulting ventral abdomen of each bug was mounted in 80% glycerol and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The image was processed using Microsoft Office Picture Manager and Microsoft PowerPoint and scaled using the public domain ImageJ program. C. The average normalized melanic pigmentation of adults along with the standard errors. The area of melanic pigmentation was analyzed using the ImageJ program and processed using Microsoft Excel. The normalization was done using the optical distance of each bug (Aspiras et al., 2011). Statistical analyses were carried our using JMP Pro 9. Tukey-Kramer HSD test levels not sharing the same letters are significantly different from each other (p<0.0001). The shaded region represents the sensitive period for the determination of ventral melanic abdominal pigmentation in fifth instar bugs raised at 20°C.
II. The study of genetic mechanism underlying the ventral abdominal pigmentation in *Oncopeltus fasciatus*

We next sought to identify the genetic mechanism underlying the ventral abdominal melanic pigmentation in *O. fasciatus* using a reverse genetics project implementing post transcriptional knock-down of *abd-A, Abd-B* and *TH* via RNA interference (RNAi).

a. **Knock-down of *abd-A* leads to lighter ventral abdominal pigmentation in both male and female *Oncopeltus fasciatus***.

To investigate the role of *abdominal-A (abd-A)* in abdominal melanism, 0.1µg of *abd-A* double stranded RNA (dsRNA) was injected into Day zero (D0) fifth instar nymphs of *Oncopeltus fasciatus* maintained at 26.5°C. The knock-down of *abd-A*, verified via semi-quantitative RT-PCR (Fig. 8), resulted in lighter ventral abdominal pigmentation with least amount of melanism suggesting a role for *abd-A* in determining ventral abdominal melanism (Fig. 5).

The mortality rate of *abd-A* knock-down *O. fasciatus* was high even when lower concentrations of *abd-A* dsRNA were injected (Table 2). Additionally, the knock-down adults could not eclose properly, and the cuticle had to be peeled manually. Female mortality was higher than males for *abd-A* knock-downs mostly because they were unable to eclose properly. Interestingly, *abd-A* knock-down *O. fasciatus* adults had extensive melanism on the dorsal side of the abdomen which normally has no melanic pigmentation in adults that developed after *amp*’ dsRNA-injection (Fig. 6). Additionally, the general segmentation and shape of the abdomen and the shape of the genitalia were also affected by the knock-down, similar to the phenotype reported previously (Aspiras et al. 2011). This suggests that *abd-A* acts as the repressor of dorsal melanism and a promoter of ventral
melanism in addition to its normal Hox gene function in the patterning of the abdomen and the genitalia.

We also studied the relative expression profile of abd-A in fifth instars raised at 20°C and 33°C using 20°C D0 as the reference (Fig. 9A). The relative expression profile of abd-A at 33°C was mostly similar to 20°C except for the early final instar when the relative expression at 33°C was much higher than at 20°C (Fig. 9A). The relative expression at 20°C peaked at D16 and D18. However, the relative expression during the most temperature sensitive period for the ventral abdominal melanic pigmentation (D3 at 33°C and D12 – D14 at 20°C) was similar. Thus, it can be inferred that the expression of abd-A likely does not contribute to the temperature dependency of the abdominal pigmentation.

b. Abd-B knock-down results in extra melanism in Oncopeltus fasciatus abdominal segment that normally lacks pigmentation

Abd-B knock-down was done via microinjection of 1 µg of Abd-B dsRNA to D0 fifth instar nymphs to investigate whether Abd-B plays a role in regulating the amount of melanism at two different temperatures – 26.5°C and 33°C. Knock-down of Abd-B mRNA at 26.5°C was verified through semi-quantitative RT-PCR (Fig. 8). Removal of Abd-B expression resulted in extra melanic pigmentation in segment A6 that normally lacks pigmentation in ventral abdomen of both male and female O. fasciatus and no pigmentation in the A7 – A10 segments, which normally have melanic pigmentation (Fig. 5), similar to the phenotype reported previously for fourth instar knock-down of Abd-B (Aspiras et al., 2011). To determine whether the temperature sensitive process occurred downstream of Abd-B, Abd-B expression was knocked down at 33°C. Ectopic melanic pigmentation was also
observed at 33°C despite the overall fading of pigmentation in the medial portion of each band (Fig. 7). This suggests that Abd-B represses melanism in O. fasciatus ventral abdomen at all temperatures. Moreover, Abd-B appears to play a role in patterning the abdominal melanism, but it is independent of the temperature sensitive pathway involved in regulating the amount of melanin produced. Both male and female genitalia were also distorted in Abd-B knock-down adults as previously reported (Aspiras et al, 2011; Fig. 5B).

Although the survival rate of Abd-B knock-down was higher than abd-A knock-down adults at both the temperature, many of the Abd-B knock-down adults also had trouble eclosing, and their cuticle needed to be peeled manually. The severity of problem was lower in Abd-B knock-downs compared to abd-A. Additionally, the mortality rate of Abd-B knock-down male adults was higher than the females (Table 2).

The reduced melanism in the mid-abdomen of 33°C Abd-B knock-down adults suggests that the expression of Abd-B is not temperature sensitive and that temperature possibly acts downstream of Abd-B (Fig. 7). This is further illustrated by the relative expression profile of Abd-B for fifth instars nymphs at 20°C and 33°C (Fig. 9B). The relative expression of Abd-B was dramatically low at 33°C on D0 but high on D1 (Fig. 9B). However, as illustrated by small range of y-axis values and high standard error values, a dramatic rise or drop was not noted. In addition, during the most sensitive period for the determination of ventral abdominal melanic pigmentation, noteworthy difference was not observed between the relative expressions of Abd-B at 33°C and 20°C. Thus, Abd-B expression does not appear to be influenced by temperature.
c. **Knock-down of TH leads to a complete lack of melanism in the whole body of Oncopeltus fasciatus.**

The post-transcriptional knock-down of \( TH \) through injection of 1 \( \mu \)g of \( TH \) dsRNA resulted in a complete lack of pigmentation in the abdomen (Fig. 5B) as well as the full body (Fig. 5A) of \( O.\ fasciatus \) adults. Since the \( TH \) knock-down affected the whole body, \( TH \) was established as an upstream enzyme essential for the whole body melanism in \( O.\ fasciatus \) similar to other arthropods (Fig. 1). Although the \( TH \) knock-down bugs also had trouble eclosing, their body architecture was unaffected.

The relative expression profile of \( TH \) in \( O.\ fasciatus \) fifth instars at 20\(^\circ\)C and 33\(^\circ\)C indicated that \( TH \) was mostly temperature independent (Fig. 9C). Although the relative expression of \( TH \) on 33\(^\circ\)C D0 was much lower compared to that at 20\(^\circ\)C, the relative expressions at the two temperatures were similar during the most sensitive period of ventral abdominal melanic pigmentation (Fig. 9C). Surprisingly, an increase in the relative expression of \( TH \) was noted on the last day of the fifth nymphal stage for both the temperatures. Further studies should investigate the reason behind this increase.
A. (Figure Legend on Page 38)

- 36 -
B. female male

*amp*'^' RNAi

*abd-A* RNAi

*Abd-B* RNAi

*TH* RNAi

(Figure Legend on Page 38)
Figure 5. Effect of Day 0 fifth nympha stage RNAi-induced knockdown of *abd-A*, *Abd-B* and *TH* on adult phenotype of *Oncopeltus fasciatus*. A. Full body images of control *amp* (1 µg), *abd-A* (0.1 µg), *Abd-B* (1 µg) and *TH* (1 µg) dsRNA injected adult males and females. The respective dsRNA were microinjected on Day zero (D0) of their fifth nympha stage. The image of *amp* RNAi adults were taken using Canon® LiDE 5600F Color Image Scanner (Model # 2925B002) and the image of rest of the RNAi adults were taken using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The images were processed using Microsoft Office Picture Manager and Microsoft PowerPoint and scaled using the public domain ImageJ program. B. The ventral abdomen of the adults obtained from control *amp* (1 µg), *abd-A* (0.1 µg), *Abd-B* (1 µg) and *TH* (1 µg) dsRNA injection as described in (A). The ventral abdomen of each bug was mounted in 80% glycerol and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The images were processed using Microsoft Office Picture Manager and Microsoft PowerPoint and scaled using the public domain ImageJ program.
Figure 6. Posterior Hox gene *abdominal-A* represses melanism in *Oncopeltus fasciatus* dorsal abdomen. Day 0 fifth instar nymphs were injected with 0.1µg *abd-A* dsRNA and maintained at 26.5ºC. *amp* RNAi is provided for reference. The dorsal abdomen of each bug was submerged in 15% glycerol in ethanol and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The image was processed using Microsoft Office Picture Manager and Microsoft PowerPoint and scaled using the public domain ImageJ program.
Figure 7. Effect of knockdown of *Abd-B* on adult phenotype of *Oncopeltus fasciatus* raised at two different temperatures. Day 0 fifth instar nymphs were injected with 1µg *Abd-B* dsRNA and raised at two different temperatures. Uninjected control animal raised at 33°C is provided for reference. The ventral abdomen of each bug was mounted in 80% glycerol and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The image was processed using Microsoft Office Picture Manager and Microsoft PowerPoint and scaled using the public domain ImageJ program.
Table 2. RNA interference (RNAi) of abdominally expressed genes by injection of double stranded RNA. The Day zero (D0) fifth instar nymphs of wild-type Oncopeltus fasciatus maintained at 26.5°C were injected with Abd-B (1µg), TH (1µg) and abd-A (0.1µg) dsRNA. 1 µg of Abd-B dsRNA was also injected into D0 fifth instar maintained at 33°C. The bugs which survived 2 days post injection were taken into account to compute n, and bugs which molted to the adult stage were used to calculate % survival. Bugs which successfully gave phenotype were used to compute the sex distribution.

<table>
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<th></th>
<th>% Survival (n)</th>
<th>Sex distribution of the phenotype observed</th>
<th>RNAi Phenotypes for melanic abdominal pigmentation</th>
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</thead>
<tbody>
<tr>
<td>WT 20°C</td>
<td>100%</td>
<td>N/A</td>
<td>Darker and more melanic pigmentation</td>
</tr>
<tr>
<td>WT 33°C</td>
<td>100%</td>
<td>N/A</td>
<td>Lighter melanic pigmentation</td>
</tr>
<tr>
<td>amp′ RNAi</td>
<td>87.0% (23)</td>
<td>8F, 12M</td>
<td>WT</td>
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<tr>
<td>abd-A RNAi</td>
<td>40.8% (49)</td>
<td>9F, 11M</td>
<td>Lighter melanic pigmentation</td>
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<tr>
<td>Abd-B RNAi (26.5°C)</td>
<td>64.4% (45)</td>
<td>16F, 13M</td>
<td>Extra melanic pigmentation</td>
</tr>
<tr>
<td>Abd-B RNAi (33°C)</td>
<td>87.5% (8)</td>
<td>4F, 3M</td>
<td>Extra but diminished melanic pigmentation</td>
</tr>
<tr>
<td>TH RNAi</td>
<td>57.1% (35)</td>
<td>7F, 13M</td>
<td>Complete lack of melanic pigmentation in whole body</td>
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Table 3. Scoring system for abdominal melanic pigmentation for RNA interference phenotypes.

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Female Image" /></td>
<td><img src="image2.png" alt="Male Image" /></td>
<td>1</td>
<td>Complete lack of melanic pigmentation in the body.</td>
</tr>
<tr>
<td><img src="image3.png" alt="Female Image" /></td>
<td><img src="image4.png" alt="Male Image" /></td>
<td>2</td>
<td>Lack of melanic pigmentation in abdominal segments.</td>
</tr>
<tr>
<td><img src="image5.png" alt="Female Image" /></td>
<td><img src="image6.png" alt="Male Image" /></td>
<td>3</td>
<td>Normal 26.5ºC WT.</td>
</tr>
<tr>
<td><img src="image7.png" alt="Female Image" /></td>
<td><img src="image8.png" alt="Male Image" /></td>
<td>4</td>
<td>Normal 20ºC WT.</td>
</tr>
<tr>
<td><img src="image9.png" alt="Female Image" /></td>
<td><img src="image10.png" alt="Male Image" /></td>
<td>5</td>
<td>Extra melanic pigmentation in the segment that normally lacks it.</td>
</tr>
</tbody>
</table>
Table 4. Average score of each RNAi phenotype with standard error. Scoring system established in Table 3 was used to score each phenotype. A sex specific mean score was computed for each type of knock-down with at least 20 phenotypes in total.

<table>
<thead>
<tr>
<th>RNAi</th>
<th>Male Score</th>
<th>Female Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>amp’</td>
<td>3.08 ± 0.05</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>abd-A</td>
<td>2.38 ± 0.07</td>
<td>2.49 ± 0.09</td>
</tr>
<tr>
<td>Abd-B</td>
<td>4.6 ± 0.1</td>
<td>4.91 ± 0.06</td>
</tr>
<tr>
<td>(at 26.5°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Figure 8. Knock-down verification for abd-A and Abd-B using semi-quantitative RT-PCR. Using ribosomal protein subunit 3 (rps3) as loading control, expression was analyzed for Day 2 (D2) fifth instars of *O. fasciatus* injected with control amp’ (1µg), abd-A (0.1µg), Abd-B (1µg) and TH (1µg) dsRNA during their fourth nymphal stage. The fourth instars for dsRNA injection were chosen at random and maintained at 26.5°C. The numbers in brackets refer to the number of PCR cycles used for each gene. The image was taken using Kodak photoimaging equipment and processed using Microsoft PowerPoint.
A. Day of 33°C 5th nymphal stage

Expression ratio using Pfaffl method

Day of 20°C 5th nymphal stage

Expression ratio using Pfaffl method

abd-A expression profile

B. Day of 33°C nymphal stage

Expression ratio using Pfaffl method

Day of 20°C 5th nymphal stage

Expression ratio using Pfaffl method

Abd-B expression profile

(Figure Legend on Page 45)
Figure 9. Relative expression profile of *abd-A*, *Abd-B* and *TH* with SEM in fifth instar nymphs raised at 20°C and 33°C using real time quantitative PCR. A. Expression ratio with SEM of *abd-A* using Pfaffl method. B. Expression ratio with SEM of *Abd-B* using Pfaffl method. C. Expression ratio with SEM of *TH* using Pfaffl method. No data point was obtained for D2. Wild-type milkweed bugs, *Oncopeltus fasciatus*, were reared at 20°C, 26.5°C and 33°C. On various days of their fifth nymphal stage, RNA was isolated from the bugs. Using *Ribosomal protein subunit 3* as the housekeeping gene control, real time quantitative polymerase chain reaction (qPCR) was performed using Bio-Rad iCycler iQ™ Real-Time PCR Detection System to obtain gene expression at the two different temperatures. The threshold cycle (*C*ₜ) value obtained for D0 20°C was used as the reference to obtain an expression ratio for each gene on various days of fifth nymphal stage.
III. **E75A expression is temperature dependent.**

*Dopa decarboxylase* (*Ddc*) is the gene involved in the expression of Dopa decarboxylase (DDC), an essential enzyme involved in the conversion of dopa to dopamine (Fig. 1) (Anderson, 2005). *Ddc* is an epidermal target of ecdysone signaling (Davis et al., 2007), and its transcription in the epidermis in other insects is under the control of the ecdysone response element (EcRE) (Chen et al., 2002). Thus, we sought to investigate the hormonal involvement in determining abdominal melanin pigmentation by studying the relative expression of ecdysteroid-induced transcription factor isoform E75A. Because its RNA transcription is directly induced by 20-hydroxyecdysone (20E), an ecdysteroid, E75A can give a direct estimate of ecdysone titers (Zhou et al., 1998). Figure 10 illustrates the preliminary data obtained for E75A relative expression in 20°C and 33°C fifth instar nymphs. There was a steady increase in the E75A relative expression at 20°C which peaked on D12 and dropped back down (Fig. 10). At 33°C, the expression of E75A started off higher than 20°C on D0 (Fig. 10). The increase from D0 to D1 was very high followed by a dramatic drop on D2 (Fig. 10). At 33°C, the expression was steady starting D2 with little fluctuation. The relative expression of *E75A* was found to be much higher at 20°C than 33°C (Fig. 10) during the most sensitive time period for the determination of abdominal pigmentation for both the temperatures (Fig. 3 and 4). Moreover, the difference in the relative expression of *E75A* was greater than that of the any of the other genes studied here (Fig. 10). Thus, *E75A* expression appears to be to be temperature sensitive, indicating that ecdysteroids may mediate the phenotypic plasticity underlying the abdominal pigmentation. The higher expression of *E75A* at 20°C during the sensitive period for ventral abdominal melanin pigmentation also indicates that ecdysone might promote melanism during this period.
Additionally, the dramatic rise and drop in the relative expression of *E75A* in D1 nymphs at 33°C suggests ecdysone-mediated inhibition of melanism. Previous studies in the tobacco hornworm, *Manduca sexta*, have shown that too high or too low expression ecdysone inhibits DDC activity, whereas intermediate hormonal levels promote DDC expression (Hiruma and Riddiford, 2009). However, further analysis is required to support this deduction. Since we were unable to obtain consistent data for the standardization of *E75A* relative expression, additional investigation is required to corroborate the preliminary data obtained in this study for the *E75A* expression profile.
Figure 10. Relative expression profile of E75A with SEM in fifth instar nymphs raised at 20°C and 33°C using real-time quantitative PCR. Milkweed bugs, Oncopeltus fasciatus, were reared at 20°C, 26.5°C and 33°C. On various days of their fifth nymphal stage, RNA was isolated from the bugs followed by cDNA synthesis as described in Materials and Methods. Using ribosomal protein subunit 3 as the housekeeping gene control, real time quantitative polymerase chain reaction (qPCR) was performed using Bio-Rad iCycler iQ™ Real-Time PCR Detection System to obtain E75A expression at two different temperatures. The threshold cycle (C_T) value obtained for D0 20°C was used as the reference to obtain the preliminary relative expression ratios along with their SEM on various days of fifth nymphal stage using comparative C_T method (Schmittgen and Livak, 2008).
IV. Higher abdominal pigmentation in *Oncopeltus fasciatus* does not confer higher desiccation tolerance.

One potential reason for this dramatic plasticity in abdominal pigmentation could be to provide desiccation tolerance to *O. fasciatus*. Alternatively, it may not have any adaptive significance. To determine whether or not ventral abdominal melanic pigmentation has an adaptive significance related to desiccation tolerance, we subjected *O. fasciatus* Day-0 (D0) adults from three different temperatures (20°C, 26.5°C and 33°C) to extremely low humidity level (5%) at 26.5°C and measured the number of days it took them to die. We found that the amount of melanic abdominal pigmentation does not have a significant effect on survival of adult male or female *O. fasciatus* at extremely low humidity conditions (ANOVA and Tukey Kramer HSD test showed no significant difference. Female ANOVA: p=0.8270, df = 2, F = 0.1904; Male ANOVA p = 0.3449, df = 2, F = 1.0816) (Fig. 11).
Figure 11. Effect of pigmentation on survival in low humidity conditions. The mean number of days survived by adult *Oncopeltus fasciatus* males and females starting Day-0 (D0) of their adult stage at 26.5ºC and 5% humidity. Error bars represent standard errors. Female one way ANOVA: p=0.8270, df = 2, F= 0.1904; Tukey-Kramer HSD analysis showed no significant difference between the average female values. Male one way ANOVA: p = 0.3449, df = 2, F= 1.0816; Tukey-Kramer HSD analysis showed no significant difference between the average male values. Wild-type (WT) milkweed bugs, *O. fasciatus*, were reared at 20ºC, 26.5ºC and 33ºC and transferred to 26.5ºC at 5% humidity level on first day (D0) of their adult stage. The number of days that each bug survived under this condition was recorded and processed using JMP Pro 9 and Microsoft Excel.
DISCUSSION

In order to understand how temperature affects the regulation of abdominal pigmentation of *Oncopeltus fasciatus*, we determined the temperature sensitive period and investigated the functions and expression profiles of abdominal –A (*abd-A*), *Abdominal-B* (*Abd-B*) and *Tyrosine Hydroxylase* (*TH*). *E75A* expression was also studied to determine hormonal involvement in melanism. *O. fasciatus* adults were also subjected to extreme humidity conditions (5%) to investigate the adaptive significance of abdominal pigmentation.

I. The temperature sensitive period for *Oncopeltus fasciatus* abdominal melanic pigmentation occurs during the fifth nymphal stage

In the present study, we found that lower temperature induces significantly darker pigmentation, while a higher temperature induces a significantly lighter pigmentation in *O. fasciatus* (Fig. 2). Our findings are consistent with a previous study by Novak where adult pigmentation was also found to be affected by temperature in a similar manner (Novak, 1955). Previous studies done on *Drosophila melanogaster* have also shown that a cold temperature induces a dark pigmentation in the posterior *D. melanogaster* abdomen, while a higher temperature has the opposite effect (Gibert et al., 2007).

In the current study, we found that the temperature sensitive period for the abdominal melanism occurs during the fifth instar. While the sensitive period is rather broad, the most sensitive period appears to fall around day 10-12 and day 3 for nymphs raised at 20°C and 33°C, respectively (Fig. 3 & 4). In arthropods, such as the grasshopper *Kosciuscola tristis*, pigment granule movement is affected by temperature, allowing them to change the level of
melanism depending on climate even as an adult (Key and Day, 1954). However, in other insects, adult pigmentation is fixed earlier in development through alterations in the expression of pigment synthesis enzymes and melanin precursors (Hiruma and Riddiford, 1985). Based on the findings of this study, the type of plasticity observed in *O. fasciatus* adult cuticular melanism is more similar to the latter, where a temperature sensitive mechanism determines the level of melanism before the adult cuticle is laid down.
II. The study of the genetic mechanism underlying the ventral abdominal pigmentation in *Oncopeltus fasciatus*

Pigmentation in insects is regulated by a two-step process where patterning genes control the positioning of pigmentation in space and time, and effector genes perform the actual biochemical synthesis of pigments (Wittkopp and Beldade, 2009). In the current study, we examined the phenotypes which resulted from the RNAi-induced post-transcriptional knock-down of posterior Hox genes, *abd-A* and *Abd-B*, and the effector gene, *TH*, via microinjection of double stranded RNA (dsRNA).

a. *abd-A* is required for melanin development in the ventral abdomen of *Oncopeltus fasciatus*.

The knock-down of *abd-A* resulted in an almost-melanin-less ventral abdomen (Fig. 5) with extensive melansim on the dorsal abdomen of *O. fasciatus* (Fig. 6). This suggests that *abd-A* acts as a promoter of dorsal melanic pigmentation and repressor of ventral abdominal melanic pigmentation. A fourth instar knock-down of *abd-A* performed in a previous study also reported missing pigmentation in anterior abdomen (A2-A8) in both the sexes (Aspiras et al., 2011), similar to the one noted in this study. The study of *abd-A* expression in posterior abdomen could elaborate on the extensive melanism observed in dorsal abdomen of *abd-A* knock-down adults. This will be an interesting aspect to investigate since *abd-A* hasn’t been directly linked to melanism patterning in *D. melanogaster* or other arthropods.

Previous studies have shown expression of *abd-A* to be strong and stable in posterior segment A1 to A7 of *O. fasciatus* embryos (Angelini et al., 2005). Similar studies done on *D. melanogaster* embryos have reported strong expression of *abd-A* in segments A1-A7.
(parasegments 7-13) (Karch et al., 1990). In *D. melanogaster*, *abd-A* along with *Ubx* directly repress *Distal-less (Dll)* to prevent the development of limbs in the abdomen (Gebelein et al., 2002; Vachon et al., 1992). Thus, understanding *abd-A* functions in anterior and posterior *O. fasciatus* abdomen and an investigation of *Ubx* in *O. fasciatus* could elaborate on the various roles in patterning and melanism played by the two genes.

The general segmentation and the shape of genitalia in both sexes were also affected by the knock-down of *abd-A* (Fig. 5), similar to what was reported previously (Aspiras et al., 2011). The lack of pigmentation noted in anterior A2-A8 segments of *abd-A* knock-down adults in this study is consistent with the region of *abd-A* expression in embryos (A1-A7) and the region of genitalia (A8-A11) in *O. fasciatus* (Angelini et al., 2005). Additionally, *abd-A* knock-down adults could not eclose properly. This may result from pleitropic effects of *abd-A* knock-down.

Relative expression profiling revealed that *abd-A* expression does not differ with temperature during the most sensitive time for the determination of abdominal melanic temperature (Fig. 9A). Nevertheless, the difference in the relative expression of *abd-A* at 20°C and 33°C during the earlier stage suggests that *abd-A* might contribute to some extent in regulating temperature sensitive pigmentation. Further studies with *abd-A* knock-down at different temperatures would shed more light on the interaction between *abd-A* and temperature.
b. **Abd-B represses the development of melanic patterns in the Oncopeltus fasciatus ventral abdomen.**

The knock-down of posterior Hox gene *Abd-B* resulted in the production of extra pigmentation in segment A6 that normally lacks pigmentation (Fig. 5 and 7). Previous works on *Drosophila melanogaster* have shown the involvement of *Abd-B* in the pigmentation pathway (Wittkopp and Beldade, 2009) where it represses several crucial pigmentation enzymes (Gibert et al., 2007). The results of this study indicate that *Abd-B* also plays a similar role in regulating the abdominal pigmentation since its knock-down resulted in extra melanism in A6 segment (Fig.5B). In addition to the anterior melanic pigmentation, the knock-down also affected the genitalia of both the sexes and general patterning of the abdomen, as reported previously (Aspiras et al., 2011). In fact, there was lack of pigmentation from A7-A10 segments, which normally contain melanic pigmentation (Fig. 5B), consistent with previous reports (Aspiras et al., 2011). A11 pigmentation was unaffected. Previous studies have shown that *Abd-B* is expressed at the highest level in the A10 and anterior A11 segments and more weakly anteriorly up to the A4 segment in *O. fasciatus* embryos (Angelini et al., 2005). Consistent with this observation, *Abd-B* knock-down adults had larger sized abdomen that lacked pigmentation from A7 – A10 and had extra melanism in A6 (Fig. 5). The difficulty to eclose and the change in general shape and size of the abdomen was most probably resulted from the homeotic transformation caused by the knock-down.

Previous studies on *Drosophila melanogaster* have shown that Abd-B influences TH, which is required for the production of all pigments, and Ebony, which is required for the production of the yellowish pigmentation, in a temperature dependent manner (Gibert et al.,
At a lower temperature, Abd-B strongly represses *ebony* but weakly represses *TH* expression, resulting in increased melanin production (Gibert et al., 2007). Additionally, Abd-B has been shown to induce melanin production through the repression of the transcription factor *bric-à-brac (bab)* and independently of *bab* (Kopp et al., 2000). Analysis of *ebony* and *bab* knock-down in *O. fasciatus*, and studying their interaction with Abd-B would be helpful in further understanding the temperature induced plasticity observed in the melanic patterns of the *O. fasciatus* ventral abdomen.

The extra melanism which is diminished from the RNAi mediated knock-down of *Abd-B* at 33°C (Fig. 7) suggests that posterior Hox gene *Abd-B* does not mediate the temperature induced phenotypic plasticity. A comparison of *Abd-B* expression at 33°C to 20°C in fifth instar nymphs, showed no notable difference in the relative expression of *Abd-B* during the most sensitive period for the determination of ventral abdominal melanic pigmentation (Fig. 9B). Based on these findings, *Abd-B* in *O. fasciatus*, unlike *D. melanogaster*, might not interact with temperature at all and repress genes other than *TH* for the repression of melanin production in *O. fasciatus* ventral abdomen. Further studies are required to corroborate these findings.

Fluctuations were observed in *Abd-B* expression during early fifth nymphal stage at 20°C and 33°C and late fifth nymphal stage at 20°C. Whether this was due to noise or an actual biologically relevant phenomenon needs further investigation as well. Study of *Abd-B* expression in relation to other melanism relevant genes like *bab* and *ebony* at different temperatures would be required to understand other players which could contribute to phenotypic plasticity observed in *O. fasciatus*. 

- 56 -
c. **TH is required for melanism in the entire Oncopeltus fasciatus body.**

Unlike the effect of Hox gene knockdowns where melanism is affected just locally in the abdomen (Fig. 5A), the knock-down of TH in *O. fasciatus* resulted in a complete lack of pigmentation in the abdomen (Fig. 5B), as well as the entire body (Fig. 5A). Tyrosine hydroxylase is an essential enzyme that converts Tyrosine to Dopa, which is required in pigmentation pathway of majority of insects for the production of melanin (Fig. 1) (Hiruma and Riddiford, 2009; Wittkopp and Beldade, 2009). Thus, the phenotype observed for TH knock-down establishes TH as an essential enzyme for the production of melanin in *O. fasciatus* as well.

The TH knock-down bugs also had issues eclosing although the general architecture of the body was unaffected. This could be because of the involvement of TH in the sclerotization similar to previous involvement of Tyrosine hydroxylase in cuticular sclerotization and pigmentation in *Tribolium castaneum* (Gorman and Arakane, 2010). Immediately after its synthesis, insect cuticle is soft and pale and needs maturation over a period of several hours to days to become sclerotized via cross-linking of cuticle proteins and dehydration (Anderson, 2005). TH knock-down likely prevented sclerotization and as a consequence, animals were unable to eclose.

In contrast to the previous studies done on *D. melanogaster* (Gibert et al., 2007), TH expression in *O. fasciatus* was found to be mostly temperature independent (Fig. 9C). Although the relative expression of TH was much lower at 33ºC than at 20ºC during the earliest portion of fifth nymphal stage, the relative expression was similar and very low during the sensitive periods for ventral abdominal pigmentation of *O. fasciatus* (Fig. 9C). Further studies on TH expression during final fourth nymphal period and early fifth nymphal
period could shed more light on the difference in relative expression of $TH$ observed during the early fifth nymphal stage at different temperatures.

The peak observed in relative expression of $TH$ at both 20°C and 33°C on the last day of fifth nymphal stage was puzzling especially since it was past the most temperature sensitive period. In black *Manduca sexta* larvae, premelanin granules are synthesized by epidermis and deposited into the newly forming cuticle during a molt (Hiruma and Riddiford, 1985). Enzymes like di-phenoloxidase (PO) are activated during the molt, and melanization occurs within the granules following the pathway described previously (Fig. 1) (Hiruma and Riddiford, 1985). Since TH would be required to produce dopa, a melanin precursor, the peak observed in relative expression of $TH$ in *O. fasciatus* at both 20°C and 33°C on the last day of fifth nymphal stage (Fig. 9C) could be because of the demand of melanin precursors needed during the molt. In this case, the temperature sensitive period would determine the quantity of enzymes and precursors demanded and they would be produced as the adult cuticle is being laid out. However, additional investigation would be required to understand the biological relevance of the peaks for relative expression of $TH$ at both the temperatures and verify this hypothesis.
III.  **E75A expression is temperature dependent.**

In order to investigate the hormonal involvement in determining abdominal melanic pigmentation, we studied the relative expression of ecdysteroid induced transcription factor isoform E75A. Because its RNA transcription is directly induced by 20-hydroxyecdysone (20E), an ecdysteroid, E75A can give a direct estimate of the circulating ecdysone titers (Zhou et al., 1998). DDC is an essential enzyme involved in the conversion of dopa to dopamine (Fig.1) (Anderson, 2005). Since Ddc is an epidermal target of ecdysone signaling (Davis et al., 2007) and its transcription in the epidermis in other insects is under the control of the ecdysone response element (EcRE) (Chen et al., 2002), we hypothesized an involvement of ecdysone (E) in ventral abdominal melanism of *O. fasciatus*.

A comparison of preliminary data obtained for E75A expression at 20°C and 33°C revealed a much higher relative expression of E75A at 20°C (Fig. 10) during the most temperature sensitive time period for the determination of abdominal pigmentation (Fig. 3 and 4). The difference in the relative expression of E75A was greater than any of the other genes studied here (Fig. 9 and 10).

*O. fasciatus* from early fifth nymphal stage at 33°C exhibited a higher relative expression of E75A compared that at 20°C. A dramatic drop was observed on D2 for the 33°C fifth instars (Fig. 10). The relative expression after D2 was low and steady for 33°C fifth instar nymphs. At 20°C, the relative expression of E75A increased steadily to a peak level on D12 and dropped back down (Fig. 10). Based on these preliminary data, E75A expression appears to be temperature sensitive, indicating that ecdysteroids might mediate the phenotypic plasticity underlying the abdominal pigmentation. The higher expression of E75A at 20°C during the sensitive period for ventral abdominal pigmentation also suggests
that ecdysone might be positively correlated to melanism during this period. Previous studies on the tobacco hornworm, *Manduca sexta*, have shown that too high or too low expression of ecdysone inhibits the activity DDC activity, whereas intermediate hormonal levels promote the expression of DDC (Hiruma and Riddiford, 2009). The dramatic increase and drop in *E75A* relative expression observed in fifth instar nymphs on D1 and D2 at 33°C could reflect extreme levels of ecdysone which inhibits melanism. A study involving the measurement of ecdysteroid titers at various temperatures and developmental stage of *O. fasciatus* would elucidate this further.

Based on the findings of this study, we propose two different types of melanin synthesis modules in *O. fasciatus* – a robust melanin synthesis module unaffected by temperature and a plastic melanin synthesis module, which is temperature sensitive (Fig. 12). Tyrosine Hydroxylase, being an upstream enzyme indispensable for both the plastic and robust pathways, is most probably unaffected by the temperature differences although further studies are required to establish this hypothesis. The robust melanism system most probably controls the robust melanism in *O. fasciatus* body that includes wings, thorax and legs. In contrast, the abdominal pigmentation, which is plastic, must be controlled by the temperature sensitive melanism pathway. Although *abd-A* and *Abd-B* are not directly involved in the temperature regulated plasticity, we hypothesize that they work in parallel with ecdysone. The ecdysone levels must mediate the temperature of the environment and thus control the amount of melanin synthesized for the ventral abdomen in *O. fasciatus*. However, repetition and validation of these preliminary data are required.

Ecdysteroids have been associated with the control of polyphenism in many insects (Nijhout, 1994; Nijhout, 2003). For example, the seasonal polyphenisms of eyespot size in
the butterfly *Bicyclus anynana* is mediated by different levels of ecdysteroids that are temperature-dependent (Oostra et al., 2011). Therefore, it would not be surprising to find direct involvement of ecdysone in the regulation of temperature mediated phenotypic plasticity observed in *O. fasciatus*. Future studies involving ecdysone titers, genes affected by *abd-A* and *Abd-B* and knock-down of *abd-A* and *Abd-B* at extreme temperatures would help us understand the complete pathway for epidermal melanization in *O. fasciatus*. This would not only help us further understand the mechanisms underlying phenotypic plasticity but also shed light on the hormonal involvement in *O. fasciatus* melanism.

**Figure 12.** Proposed pathway for cuticular melanization in *Oncopeltus fasciatus.*
IV. Higher abdominal pigmentation in *Oncopeltus fasciatus* does not confer higher desiccation tolerance.

Why would *O. fasciatus* have more melanic pigmentation at lower temperature and least abdominal melanic pigmentation at higher temperature? Does melanic pigmentation have any adaptive significance? Previous studies have associated melanism to thermoregulation (Majerus, 1998; True, 2003; Watt, 1968; Watt, 1969) or disease resistance (Wilson et al., 2001). Other studies have indicated the involvement of melanism in providing desiccation resistance to *Drosophila jambulina* (Parkash et al., 2009). Does desiccation resistance apply to *O. fasciatus* abdominal melanism as well?

In order to test the adaptive significance of abdominal melanism, we subjected D0 *O. fasciatus* adults from three different temperatures (20ºC, 26.5ºC and 33ºC) to extreme humidity condition (5%) and maintained them at 26.5ºC. We found that higher or lower amount of ventral melanic abdominal pigmentation does not have a significant effect on survival of both male and female *O. fasciatus* (Fig. 11). This suggests that there is no obvious adaptive significance in having higher or lower amounts of abdominal melanic pigmentation to confer desiccation tolerance. Moreover, the abdominal pigmentation is on the ventral side of the animal. Therefore, we hypothesize that pigmentation is unlikely to confer significant roles in thermoregulation of sexual selection. The lack of adaptive significance suggests that plasticity in this system is not under selection.

Since plasticity in *O. fasciatus* does not have obvious adaptive functions, it can be inferred that ventral abdominal pigmentation of *O. fasciatus* might be a useful incipient system to investigate the origins of polyphenisms. Nijhout suggests the origin of phenotypic plasticity to be gratis, as a by-product of the physics and chemistry of development (Nijhout,
2003). In case of *O. fasciatus*, the plasticity in abdominal melanism may simply be a by-product of a developmental system with no effects on fitness. Thus, we believe that this trait could be molded by natural selection by either eliminating it or exploiting it (Nijhout, 2003).

The abdominal pigmentation in *O. fasciatus* therefore provides a model system with an interesting opportunity for future studies on how evolution might act to shape the developmental systems underlying adaptive polyphenisms and developmental robustness.
REFERENCES


