From Metamorphosis to Puberty: The Role of Ventral Veins Lacking in Developmental Regulation

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From Metamorphosis to Puberty: The Role of *Ventral Veins Lacking* in Developmental Regulation

A Thesis Presented by
CeCe Cheng

Submitted in Partial Fulfillment of the Prerequisite for the Degree of Bachelor of Arts with Honors in Biological Sciences

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ABSTRACT

Metamorphosis is a drastic postembryonic developmental change that results in major morphological, physiological and behavioral alterations in an organism. Metamorphosis is a ubiquitous process, and some argue that there is a human equivalent – puberty. The hormonal basis of metamorphosis and puberty are very similar in that both processes are initiated and regulated by neurohormones of the brain. In vertebrates, POU domain transcription factors (TFs) have been linked to neuroendocrine changes associated with puberty. The primary purpose of this study was to understand the processes of metamorphosis and puberty by investigating a homolog of a vertebrate POU domain TF, ventral veins lacking (vvl), in the red flour beetle, Tribolium castaneum. RNA interference-mediated silencing of vvl expression was found to induce precocious metamorphosis. Furthermore, ectopic application of juvenile hormone (JH) or methoprene, a JH analog, prolonged the larval stage, but was unable to prevent metamorphosis. Semi-quantitative RT-PCR analysis showed that the expression of a downstream target of Juvenile Hormone signaling, kruppel homolog 1, was reduced in vvl knockdown larvae. This expression could be rescued by exogenous methoprene application, indicating that vvl might act upstream of JH signaling. Together with the inhibition of molting, these results indicate that vvl might act to repress neuroendocrine changes associated with metamorphosis. Thus, similar to the POU domain TFs in vertebrates, POU domain TFs in insects may play a critical role in regulating the timing of reproductive maturation.
INTRODUCTION

Metamorphosis is the physical transformation of an organism from one stage of its life cycle to another. Many organisms, including amphibians, echinoderms, marine vertebrates, and insects, undergo metamorphosis. Even mammals seem to undergo a variant of metamorphosis during puberty. However, not much is known about the underlying molecular mechanisms that drive the drastic morphological changes that occur during this process or its evolutionary origins. This study investigates a potentially conserved mechanism underlying metamorphosis and puberty across metazoans.

Insect development and metamorphosis

Insects are a class of living organisms within the phylum Arthropoda, characterized by chitinous exoskeletons, three tagma (including the head, thorax, and abdomen), jointed legs, and antennae. Insects are among the most diverse groups of animals on the planet, accounting for over a million known species, more than half of all living organisms (Chapman, 2006). In addition, certain insects also hold important roles in biological research where they are used as model organisms in studies of key developmental processes.

Postembryonic development in insects can be classified into three major categories: ametabolous, hemimetabolous, and holometabolous development. These terms reflect the degree of change that the body undergoes during an organism’s life cycle. The earliest insects were ametabolous – that is, juveniles would develop to the adult life stage without any major changes in form. Except for the lack of functional reproductive structure, immature Ametabola
look like miniature versions of adults (Truman and Riddiford, 2002). Insects that undergo incomplete metamorphosis, the Hemimetobola, hatch as nymphs that have an adult-like morphology but lack functional genitalia and wings. Examples of hemimetabolous insects include grasshoppers and cockroaches (Yang, 2001). Holometabolous insects undergo three morphologically distinct life stages: the larva, the pupa and the adult. The larvae of holometabolous insects bear no resemblance to the adults. During metamorphosis, holometabolous larvae transform into immobile prepupae and, subsequently, pupate. During this dramatic transformation, the larval tissues and organs begin to reorganize into adult structures. After the rearrangement is complete, the mature adult ecloses from the pupal cuticle. Most insects on Earth, including butterflies, flies, and beetles undergo this type of development, and this innovation is thought to have fueled the dramatic diversification of insects (Truman and Riddiford, 1999).

Over the years, several theories have been proposed to explain the evolution of complete metamorphosis in holometabolous insects. One hypothesis suggests that holometabolous larvae and hemimetabolous nymphs are equivalent to one another, and that the pupal stage arose independently as differences between the larva and adult increased (Hinton, 1914). In contrast, Berlese (1913) proposed that there is a clear distinction between the hemimetabolous nymph and the holometabolous larva. This hypothesis suggests that holometabolous larvae are merely free-living feeding embryos that arose from premature termination of embryogenesis (Truman and Riddiford, 1999). As the larvae of holometabolous insects became the major feeding stage, the number of nymphal stages were reduced to just a single pupal stage (Truman and Riddiford, 2002). This hypothesis suggests that the pronymph
stage, which lasts for 3-4 days after hatching in ametabolous insects and hours after hatching in hemimetabolous insects, corresponds to the active, feeding juvenile in holometabolous insects (Bernays, 1971).

Recent studies on the endocrinology of embryonic and postembryonic development of insects support the notion that the pronymph-to-nymph transition in ametabolous and hemimetabolous insects corresponds to the larva-to-pupa transition in holometabolous insects (Truman and Riddiford, 1999). Truman and Riddiford (1999) hypothesize that a shift in hormone secretion during embryogenesis may have led to the retention of embryonic characteristics in the hatchings of holometabolous insects. Thus, they propose that the hemimetabolous pronymph became the holometabolous larva, supporting Berlese’s theory. In order to understand how metamorphosis is regulated in insects, it is necessary to gain a better understanding of the endocrinology of insect development.

**Endocrine regulation underlying holometabolous insect development**

Hormones are chemical messengers crucial for metabolic and developmental processes. They are secreted into the bloodstream as part of the endocrine system and act as important regulators of cells, organs, and tissues in the body. In particular, two well-characterized classes of hormones regulate holometabolous insect development: ecdysteroids - controlled by prothoracicotropic hormone (PTTH) - and juvenile hormone (JH). It is the interaction between these two classes of hormones that has led to the evolution in timing and regulation of metamorphosis (Nijhout, 1994).
**Figure 1.** Current model of the ecdysteroid and JH secretion. (A) The prothoracicotropic hormone (PTTH) secreted from the corpora cardiaca in the brain provides the primary drive for ecdysone secretion. (B) Juvenile hormone (JH) is secreted from the corporal allatum, but not much else is known about what triggers its secretion.

**Ecdysteroid signaling in development**

Ecdysteroids are a major class of steroid hormone in insects that are responsible for coordinating developmental transitions such as larval molting – the process associated with the production of new cuticle and the shedding of old cuticle – and metamorphosis. In many insects, ecdysone is carried to target tissues by the hemolymph and is converted to a more active form, 20-hydroxyecdysone (20E) (Truman and Riddiford, 2002).

Previous studies have established that ecdysone regulates insect growth and development by binding to a nuclear heterodimeric receptor complex that consists of Ecdysone receptor (EcR) and Ultraspiracle (USP) (Evans, 1988; Tan and Palli, 2008). Once bound, the hormone receptor complex can then activate, repress, or de-repress downstream gene transcription (King-Jones and Thummel, 2005). Interestingly, the USP ortholog found in beetles (order Coleoptera) and hemimetabolous insects is very similar to the vertebrate Retinoid X
receptor (RXR), which is an important factor in development, reproduction, homeostasis and cell differentiation (Philip et al., 2012; Riddiford, 2008).

It is clear that the sensitivity to 20E requires the interaction of USP/RXR and EcR. In the common fruit fly, *Drosophila melanogaster*, it has been demonstrated that EcR is essential for larval molting and metamorphosis (Lam and Thummel, 2000). Although the *Drosophila* EcR gene encodes three different isoforms and one USP protein, two isoforms of each EcR and USP/RXR have been identified in the red flour beetle *Tribolium castaneum*. RNAi experiments in *Tribolium* by Tan and Palli (2008) showed that EcR but not USP isoforms play distinct roles during metamorphosis, with EcRA playing a dominant role in regulating ecdysone response genes. In fact, functional analysis of the Ecdysone receptors demonstrated that animals with EcRA knocked down arrest their development at the quiescent stage, while those with EcRB knocked down progress through larval development and pupate, although most pupae showed developmental defects.

*Prothoracicotropic Hormone control of ecdysteroid secretion*

In most insects, the production and release of ecdysone is regulated by a small, secreted peptide known as PTTH (McBrayer et al., 2007). PTTH was originally purified from *Bombyx mori* brain extracts as a substance that could stimulate ecdysone production in the prothoracic glands (Fig. 1A) (Kataoka and Suzuki, 1991). Understanding the regulation of PTTH production and release, and its interaction with ecdysone, is a key step to deciphering the mechanisms that regulate developmental timing of metamorphosis in insects.
PTTH has been proposed to play an essential role in regulating developmental timing and body size through the production and release of ecdysone. In fact, studies in *Manduca* and *Drosophila* have shown that PTTH secretion plays an important role in regulating the pulse of ecdysone at the end of the larval life to stop feeding and initiate metamorphosis (McBrayer et al., 2007). While the loss of PTTH does not prevent molting and metamorphosis, it does extend the larval stage by prolonging feeding, ultimately causing overgrowth.

Previous studies suggest that there are two main components to PTTH secretion: body size and photoperiod (McBrayer et al., 2007). Most importantly, larvae need to attain a “minimal viable weight” in order to survive metamorphosis. If larvae are starved and this weight is not reached, animals delay metamorphosis and eventually die without ever initiating pupation. Once the larvae reach a certain size, known as the “critical weight”, animals will always undergo metamorphosis even when faced with limited food sources (Nijhout, 2003). Only when this “critical weight” is reached and the appropriate photoperiod is present can PTTH be released (Truman, 1972; Truman et al., 1974).

*Juvenile Hormone mediation of insect development*

The processes of molting and metamorphosis are intricately connected by the activity of two hormones. Although it has been established that ecdysone is responsible for regulating molting, the nature of a molt is dependent upon another endocrine regulator, juvenile hormone (JH). JH is a sesquiterpenoid hormone that is secreted from a glandular structure in the brain known as the corpora allata (Fig. 1B) (Nijhout and Williams, 1974). The role of JH as a “status quo” hormone was first elucidated by Wigglesworth in the blood sucking bug, *Rhodnius*.
prolixus (Wigglesworth, 1934). It is known as a “status quo” hormone because it acts with other factors to maintain the same developmental stage after a molt and prevents the animal from undergoing metamorphosis. In hemimetabolous and holometabolous insects, JH is present at high levels during the nymphal or larval stages (instars) and modulates the activity of ecdysone so that another nymphal or larval stage follows a molt. Only when JH levels drop in the last instar can ecdysone initiate metamorphosis (Fig. 3). In addition, during metamorphosis, JH titers experience a distinct rise, helping to maintain the pupal stage instead of precociously becoming an adult. Previous studies have shown that the removal of the JH-producing corpora allata can induce precocious metamorphosis (Konopova and Jindra, 2007; Williams, 1961).

**Figure 2.** Hypothetical model of JH and Ecdysone levels throughout the life cycle of *T. castaneum.*
**Methoprene-tolerant (met) – the proposed JH receptor**

Recent studies have demonstrated that the basic helix-loop-helix (bHLH)- Per-Arnt-Sim (PAS) domain protein encoded by *methoprene-tolerant (met)* plays a key role in mediating sensitivity to JH in *Tribolium castaneum*. Ever since its discovery in mutant *D. melanogaster*, met has been thought to be a potential JH receptor involved in JH action (Zhang et al., 2011). Its critical role in the regulation of metamorphosis has been shown in the red flour beetle *Tribolium*, where the knockdown of the single met gene produces a precocious metamorphosis phenotype similar to that caused by loss of JH itself (Konopova and Jindra, 2007; Parthasarathy et al., 2008).

How Met transduces the hormonal signal is not well understood, but there must be some type of association between met and JH, which in turn modulates the EcR complex. Zhang et al. (2011) demonstrated that Met must heterodimerize with another bHLH transcription factor, steroid receptor co-activator (SRC), otherwise known as Taiman, to regulate the expression of JH response genes. Recently, it has been confirmed that Met will only bind to SRC when JH binds to its PAS-B domain, and that in the absence of the JH ligand, Met is found mainly as an inactive homodimer (Charles et al., 2011). These studies indicate that SRC plays an important role in JH action. In addition, SRC has also been shown to interact with EcR in the presence of 20E and promotes transcription of ecdysone-response genes. Although more research is needed to elucidate SRC-Met and SRC-EcR/USP action, two known downstream targets are Kruppel-homolog (Kr-H1) and Broad (Br).
Role of the BTB-domain protein Broad in metamorphosis its relation to the JH pathway

The ecdysone-induced gene *brood (br)* belongs to the bric-a-brac, tramtrack, broad-complex/Pox virus and zinc finger (BTB/POZ) domain transcription factor family of proteins (Zhou and Riddiford, 2002). Recent studies have shown that this transcriptional regulator plays an important role during metamorphosis (Konopova and Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008; Zhou and Riddiford, 2002). In fact, studies in *Drosophila melanogaster, Manduca sexta*, and *Tribolium* have demonstrated that Br is a key protein involved in specifying pupal development (Suzuki et al., 2008; Zhou et al., 1998; Zhou and Riddiford, 2002). When JH levels drop and 20E levels peak at the end of the last instar, Br becomes highly expressed allowing for entry into and progression through metamorphosis. During the prepupal period, the expression of Br is critical for directing various metamorphic modifications, such as remodeling of the CNS and the formation of the adult cuticle (Emery et al., 1994). In *Tribolium*, knockdown of Br expression results in animals that skip the pupal phase and exhibit a larva-adult intermediate phenotype (Konopova and Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008). Furthermore, *Drosophila* studies by Zhou and Riddiford (2002) have shown that misexpression of Br during the penultimate larval stage leads to the induction of precocious pupal-specific gene expression in the larva and misexpression of Br during late pupal stage leads to production of adults with pupal cuticles, demonstrating the important role of Br in mediating pupal fates (Zhou and Riddiford 2002).
Kruppel-homolog as a downstream target of JH signaling

Kruppel-homolog (Kr-H1) is an early JH-inducible transcription factor (TF) that has been demonstrated to mediate JH action during metamorphosis (Minakuchi et al., 2008; Zhou and Riddiford, 2002). Minakuchi et al. (2009) showed that kr-H1 is downstream of met but upstream of br in the JH signaling pathway. The interaction between JH, kr-H1, and br varies depending on the presence or absence of JH. Since kr-H1 is a JH response gene, it is expressed at high levels during the embryonic and larval stages but disappears during the pupal and adult stages. During the juvenile stage, kr-H1 suppresses br expression so that the larval characteristics are maintained during a larval-larval molt. However, in the absence of JH, kr-H1 expression down regulated, resulting in the induction of br expression. This action of kr-H1 seems to provide a link between met and br in the JH pathway. Although seemingly regulated by kr-H1, br expression is actually coordinated by the activity of both JH and ecdysone. As a primary ecdysone response gene and pupal specifier, br expression is activated in the pupal state when 20E binds with the EcR complex in the absence of JH. However, once br is expressed and the larvae becomes committed to the prepupal/pupal state, ectopic application of JH somewhat counter intuitively helps to maintain both kr-H1 and br expression, thus preventing progression to the adult form and maintaining the “status quo”.

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Figure 3. Current model of the JH and Ecdysone pathways in relation to metamorphosis.

Vertebrate puberty and insect metamorphosis

The hormonal bases of metamorphosis and puberty are similar in that both are initiated and regulated by neurohormones of the brain. One critical player in the initiation of puberty in mammals is gonadotropin-releasing hormone (GnRH). In normal development, pulses of GnRH are released from the hypothalamus and acts on the pituitary, where it regulates the synthesis and release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These gonadotropins are not necessary for life, but are essential for the release of testosterone and estrogen at the beginning of puberty as well as for reproduction. During puberty, the frequency of pulsatile GnRH release increases, and this increased frequency of GnRH release ultimately leads to endocrine changes associated with the onset of puberty.
(Mauras et al., 1996). It has been shown that the neuron-specific transcription factors are important in the developmental control of GnRH expression.

**Figure 4. Pattern of GnRH secretion as a female approaches puberty.**

**POU domain transcription factors**

In vertebrates, POU domain transcription factors have been linked to neuroendocrine changes associated with puberty. Structurally, POU proteins have a highly conserved POU homeodomain and have been shown to regulate gene expression by binding to high-affinity octamer sites (Herr and Cleary, 1995; Rosenfeld, 1991). Many known POU factors are expressed in cell or region-specific patterns within the developing central nervous system (CNS) suggesting a role in neural development. Of particular interest is that a few members of the POU family have been shown to contribute to the functioning of the neuroendocrine system and the early development of an organism (Andersen and Rosenfeld, 2001). For example, Pit-1
regulates the expression of Prolactin and growth hormone (Rosenfeld, 1991). In addition, the deletion of the gene encoding Brn-2, a member of the class 3 POU domain factors, results in the failure of activation of the corticotropin-releasing hormone gene after the neurons have already migrated to their final destination (Ramkumar and Adler, 1999). POU proteins seem to have such an important role in the developmental control of hypothalamic neuropeptide expression and it has been demonstrated that POU proteins are important in regulating the neural expression of GnRH.

One POU domain transcription factor in particular, SCIP/Oct-6/Tst-1 (henceforth referred to as Oct-6) has been shown to be physiologically involved in regulating the expression of GnRH. Previous research has demonstrated that overexpression of Oct-6 in certain neuronal cells repressed rat GnRH promoter activity in a dose-dependent fashion (Wierman et al., 1997). Its role as a repressor of GnRH release is interesting because it suggests that Oct-6 plays a key role in regulating the timing of mammalian sexual maturation. Because POU domain transcription factors have been found in both vertebrates and invertebrates, aspects of their functions may be a conserved across species. In order to better understand the processes of metamorphosis and puberty, we investigated a homolog of the POU domain TF, *ventral veins lacking* (*vvl*), in the holometabolous insect, *Tribolium castaneum*. 
Background on the POU domain Transcription factor Ventral Veins Lacking

**Vvl** is part of the POU family (class 3) of transcription factors found in organisms as divergent as nematode worms (*Caenorhabditis elegans*), insects (*Drosophila*), amphibians (*Xenopus*), and humans. Most of the information known about the Vvl protein comes from studies conducted in the common fruit fly *Drosophila*. In *Drosophila*, *vvl* is responsible for regulating tracheal development, a system of vessels running throughout the body of an insect to deliver oxygen. First cloned in 1995, *vvl* mutants displayed severe tracheal defects and defects in ventral midline glia migration in the CNS (Anderson et al., 1995). However, the function of *vvl* in more basal insects like *Tribolium* has yet to be established.
Tribolium castaneum as a model organism

Figure 5. Life stages of Tribolium castaneum.

[Tribolium castaneum image]

Tribolium castaneum, commonly known as the red flour beetle, is a powerful model organism used in developmental biology. It is a member of the most species-rich eukaryotic order, Coleoptera. As a major pest of stored grains and grain products, Tribolium has been distributed globally with human agriculture. Its ease of culture, relatively short generation time, sequenced genome, and use in reverse genetics studies via RNA interference (RNAi) make it an ideal model organism for genetic analyses.

The life cycle of Tribolium usually lasts 7-12 weeks, but adults can live for over 3 years. These beetles breed in grains and other dried foods, and females can lay up to 400 eggs within a two-month period. The incubation period for the embryos is relatively short; within 5-12 days, the eggs hatch into slender, cylindrical larvae. As Tribolium larvae grow and develop, they undergo 7-8 instars, or larval stages, separated by molts but without any major structural
changes. Fully grown larvae transform into pupae, and within approximately 4-5 days, the adults eclose.

Recently, *Tribolium* has become a prominent system for studies of evolution of development because its mode of development is more typical of insects than that of the classical model system *Drosophila*. While both insects undergo a holometabolous mode of development, *Tribolium* embryos exhibit more ancestral features and undergo a more primitive mode of development than *Drosophila* embryos (Klingler, 2004). In addition, that *Drosophila* is a very derived insect has also been observed at the molecular level, where genetic analyses have demonstrated that many *Tribolium* genes are more similar to vertebrate homologs, rather than those found in *Drosophila* (Richards et al. 2008).

Moreover, the use of *Tribolium* is advantageous over *Drosophila* because they demonstrate the classical developmental response to JH (Konopova and Jindra, 2007). It has been shown that *met* knockdown *Drosophila* mutants are able to develop without any major defects (Wilson et al., 2006). This lack of developmental phenotype is because the effect of JH on pre-adult *Drosophila* is relatively weak. In fact, *Drosophila* larvae will develop normally even with ectopic application of JH on their abdomen indicating that these animals have mostly lost the ability to sense JH. Since the presence of JH retains its functional effect in *Tribolium*, the use of this organism offers an ideal opportunity to examine the JH pathway and related factors in metamorphosis.
The mechanism of RNA interference

RNA interference (RNAi) is a gene-silencing phenomenon that occurs naturally in all animals. This process was first described in the nematode *Caenorhabditis elegans* in a Nobel prize-winning article by Fire and Mello (Fire et al., 1998). While RNAi is an endogenous process that plays essential roles in regulating gene expression, it can also be harnessed as a tool for the study of gene function. More specifically, in the process of RNAi, gene function is inhibited by using double-stranded RNA (dsRNA) to target complementary mRNA transcripts for destruction. After the dsRNA enters the cell, it is processed by an enzyme called Dicer, which cleaves the dsRNA into small 21-25 nucleotide fragments called short interfering RNA (siRNA). These siRNAs are incorporated into a protein complex known as the RNA-induced silencing complex (RISC), where the two strands are separated into single stranded RNA (Fortunato and Fraser, 2005). The single-stranded RNA in the RISC complex then binds to a complementary sequence of mRNA within the cell. Once bound, the RISC cleaves and degrades the mRNA, preventing translation from occurring. By deliberately introducing sequences of dsRNA that correspond to a specific gene of interest, it is possible to observe the physiological consequences of gene silencing.

The use of RNAi for functional studies has proved to be especially effective in *Tribolium* (Denell, 2008). While injection of dsRNA into *Drosophila* and *Tribolium* results in gene knockdown at that stage of development, RNAi is systemic only in *Tribolium* (Richards et al., 2008). Not only can RNAi be used effectively at any life stage of *Tribolium*, this technique also results in the knockdown of specific gene products in any tissue, developmental stage and offspring of dsRNA-injected females.
Figure 6. RNAi Interference (RNAi) mechanism. dsRNA injected into the cytoplasm of the cell is cleaved into siRNAs by an enzyme called Dicer. The siRNA-Dicer complex recruits additional components to form an RNA-induced Silencing Complex (RISC). The RISC complex unwind the siRNA into ssRNA, which then targets complementary mRNA for cleavage and degradation.

The Question

The objective of this study was to examine and characterize the role of vvl in the regulation of metamorphosis in Tribolium. Because of its homology to a vertebrate factor Brn-2 known to be involved in the neuroendocrine regulation of puberty, it seems possible for vvl to be a critical regulator of the neuroendocrine hormones involved in metamorphosis in Tribolium castaneum. This study aims to answer the question: is vvl involved in metamorphosis? And if so, how? There are many potential pathways to explain changes in developmental timing of metamorphosis, but the exact mechanism of action has yet to be elucidated. Could vvl be regulating JH synthesis or sensitivity? How does vvl knockdown differ from met knockdown? Is
vvl interacting with EcR complex? Understanding the interactions of vvl may provide mechanistic and evolutionary insights into the regulation of metamorphosis and puberty.
MATERIALS AND METHODS

The Organism

Wildtype *Tribolium castaneum* were obtained from Dr. Richard Beeman (USDA ARS Biological Research Unit, Grain Marketing & Production Research Center, Manhattan, Kansas). All beetles were raised on organic whole wheat flour fortified with 5% nutritional yeast at 29°C, 50% humidity.

RNA isolation

*T. castaneum* RNA from fifth instars, sixth instars, seventh instars, prepupae, and pupae was isolated by homogenizing the tissues in 500 μl of TRIzol, which inhibits RNase activity, dissolves cells components, but maintains the integrity of the RNA. Addition of 100 μl of chloroform followed by centrifugation at 11,500 rpm at 4°C for 15 min separated the solution into an aqueous (RNA) and organic (protein) phases. The RNA isolated in the aqueous layer was transferred to RNase free tubes and recovered by precipitation with 250 μl of isopropanol at room temperature for 15 min. The RNA was condensed to a pellet form by centrifugation at 11,500 rpm at 4°C for 10 min. After discarding the supernatant, the RNA pellet was washed with 500 μl 75% ethanol/DEPC water and subsequently centrifuged at 7,400 rpm for 5 min. The supernatant was removed and pellet air-dried before being resuspended in 13 μl of DEPC water. The RNA-DEPC water mix was incubated at 60°C for 5 min to dissolve the pellet.

In order to ensure the removal of all DNA from the RNA sample, the PROMEGA RQ1 RNase-Free DNase was used according to the manufacturer’s instructions (Promega). Upon
completion, RNA was precipitated with 20 μl of isopropanol and 10% volume (2 μl) of 3M sodium acetate (pH 5.2). All reactions were kept at -20°C for at least one hour. RNA purification was completed by centrifuging the samples at 14,000 rpm for 10 min at 4°C and rinsing the pellet with 75% ethanol. The centrifugation process was repeated and the pellet air-dried for 10-15 min. The resulting pellet was dissolved in DEPC water. Using the ThermoScientific NanoDrop 2000 spectrophotometer, the RNA concentration was determined for use in cDNA synthesis.

**cDNA synthesis**

In order to perform RT-PCR analysis, it was necessary to convert the purified *T. castaneum* single stranded RNA into a more stable copy DNA (cDNA). cDNA was synthesized from 1μg of RNA by using RNAse-free tube containing 1μl of Oligo dT primer, which binds to the poly A tail of the mRNA and allows for the priming of all mRNAs simultaneously. DEPC water was added to bring the total volume for each sample up to 12μl. The samples were mixed and briefly spun down and incubated at 70°C for 5 min. The process of reverse transcription occurred through further mixing the mRNA with 4μl of 5X reaction buffer, 1μl of nuclease inhibitor, 2μl of 10mM dNTP mix, and 1μl reverse transcriptase enzyme. All reactions were incubated at 42°C for 1 hr, followed by enzyme inactivation at 70°C for 5 min. Synthesized cDNA was stored at -20°C.
**Primer design**

The gene sequences were obtained using the NCBI Basic Local Alignment Search Tool (BLAST) network service (www.ncbi.nlm.nih.gov) and Beetlebase (www.beetlebase.org). Using Primer3 (http://frodo.wi.mit.edu/primer3/) primers were designed to amplify particular regions of interest in the synthesized cDNA (Table 1).

### Table 1. Forward (FW) and reverse (RV) primers designed with Primer3 (http://frodo.wi.mit.edu/primer3/) used for dsRNA synthesis, expression profiling and knockdown verification.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’ – 3’)</th>
<th>bp</th>
<th>Cycle</th>
<th>Exp/Vf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br Core</td>
<td>TCGGCAACAACAAA CATCGGTTCGCTCT</td>
<td>251</td>
<td>30</td>
<td>Exp</td>
</tr>
<tr>
<td>E75</td>
<td>CGGTCCCTCAATGGAAAA GTCGGCAGTATTGACATC</td>
<td>473</td>
<td>34</td>
<td>Exp</td>
</tr>
<tr>
<td>Ftz-F1</td>
<td>ATTTGTGGAACCTGTGG GGATTTCTTGCTTGATG</td>
<td>804</td>
<td>40</td>
<td>Exp</td>
</tr>
<tr>
<td>HR3</td>
<td>CCGTGCAAAGTATGTGG GTCGGCAGTATTGACATC</td>
<td>220</td>
<td>34</td>
<td>Exp</td>
</tr>
<tr>
<td>HR4</td>
<td>CCCAATGCTGCTCAACT CGCTGGAAATACTGGATGG</td>
<td>650</td>
<td>38</td>
<td>Exp</td>
</tr>
<tr>
<td>KrH</td>
<td>TGAATAACACCAACCAAGTGCTG CTGGATGAGCAGGAGGATT</td>
<td>989</td>
<td>34</td>
<td>Exp</td>
</tr>
<tr>
<td>Met</td>
<td>GAGCAGTTGGTGTTTTTC</td>
<td>774</td>
<td>35</td>
<td>Exp/Vf</td>
</tr>
<tr>
<td>Met RNAi</td>
<td>GAAGCTTTCAAGAGAGGAATATG TTTCAACAGTTCCCTGGTCG</td>
<td>295</td>
<td>N/A</td>
<td>Vf</td>
</tr>
<tr>
<td>RP49</td>
<td>TGACGGTTATGGCAGAGGCAATG</td>
<td>136</td>
<td>28 for pp cDNA; 34 for D4 5&lt;sup&gt;th&lt;/sup&gt;*</td>
<td>Exp/Vf</td>
</tr>
<tr>
<td>Vvl INSITU</td>
<td>CACCATCACCAACCCACCA CATCGGTTCGCTTGATTACA</td>
<td>917</td>
<td>34</td>
<td>Exp/Vf</td>
</tr>
<tr>
<td>Vvl RNAi</td>
<td>GTCTCTCGGCTTTCATTACA GTCCGCTTGCGTAAATCC</td>
<td>267</td>
<td>N/A</td>
<td>Vf</td>
</tr>
</tbody>
</table>
Semi-quantitative RT-PCR

In order to perform semi-quantitative RT-PCR, fifth instar larvae and early prepupae were collected. Fifth instar larvae were collected four days post injection with *amp*’, *vvl* or *met* dsRNA and ectopic treatment with acetone, JH III, or methoprene. Five animals for each treatment (*amp*’ and acetone, *amp*’ and JH III, *amp*’ and methoprene, *vvl* and acetone, *vvl* and JH III, *vvl* and methoprene, *met* and acetone, *met* and JH III, and *met* and methoprene) were pooled in Trizol and prepared for total RNA isolation and cDNA synthesis as previously described. Last instars injected with *amp*’, *vvl*, and *met* dsRNA were collected as they prepupated. Five prepupae for each sample pooled and prepared for total RNA isolation and cDNA synthesis.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Using the GoTaq® PCR Core System I kit (Promega) and the primer pairs listed above (Table 1), RT-PCR reactions were set up with 1X Green GoTaq® Flexi Buffer, sterile water, dNTPs (0.2mM), MgCl₂ (1.5mM), GoTaq® DNA Polymerase (1.25μM), forward primer (0.2μM), reverse primer (0.2μM), and template cDNA (0.5μL). Each reaction was subjected to an optimal level of PCR amplification in the thermal cycler (BioRad®) as follows: Initial hold for 2 min at 94°C to melt template cDNA followed by X number of cycles of the following thermal conditions - 30s at 94°C, 30s at 55°C, 1 min at 72°C. The final extension was completed at 72°C for 5 min before being cooled to 4°C.

Successful amplification of the genes was determined by gel electrophoresis using 2.0% (25/vol) agarose gel in 1X TBE buffer (0.1 M Tris, 0.09 M Boric Acid, and 1mM EDTA) with
SybrSafe™ stain (Invitrogen) used according to manufacturer specifications. A DNA standards ladder was used to locate the DNA under UV light after the DNA was subjected to electrophoretic migration at 100V for 20 min.

**ssRNA and dsRNA synthesis**

![Diagram of TOPO® TA cloning vector](image)

**Figure 7. Insertion of the amplified cDNA product into the TOPO® TA cloning vector**

Using the MinElute Gel Extraction Kit (Qiagen), the amplified cDNA product was extracted and purified. The product was subsequently cloned into the TOPO® vector (Invitrogen; Fig 7). The TOPO® reaction was set up in a 2:3 DNA product to reaction volume ratio using salt solution (0.2M NaCl, 10mM MgCl₂) and TOPO® vector (8.3mM Tris-HCl pH 7.4, 0.167mM EDTA, 1.67ng/μL plasmid in 50% glycerol, 0.0167% TritonX-100, 0.33mM DTT,
16.67μg/μL BSA, 5μM phenol red). The reaction was incubated at room temperature for 20 min and immediately used for transformation.

The newly integrated TOPO® vector was added to One Shot Chemically competent Top10 E.coli (Invitrogen) in a 2:25 mixture and incubated on ice for 20 min. The cells were heat shocked for exactly 30 seconds at 42°C and returned to ice. The transformed E. coli cells were subsequently incubated in Super Optimal broth with catabolite repression (S.O.C.) medium at 37°C for one hour while shaken at 250rpm. Transformants were plated onto Luria Broth (LB) solid medium agar plates containing 0.1 mg/ml ampicillin and incubated at 37°C overnight. A few colonies that were confirmed to contain the gene of interest were individually cultured overnight at 37°C with shaking (250rpm) in 25ml of LB broth with ampicillin (100mg/ml) for DNA plasmid amplification.

Plasmid DNA was extracted from transformed E. coli Top10™ cells using the QIAprep Spin Miniprep Kit (Qiagen) as follows: Cells were first pelleted by centrifugation for 1 min at 14,000 rpm. Cell pellets were resuspended thoroughly in suspension buffer (50mM Tris –Cl, pH 8.0, 10mM EDTA, 100μg/ml RNase A) and lysed thoroughly with lysis buffer (200mM NaOH, 1% SDSw/v) to optimize the release and denaturation of plasmid DNA. Plasmid DNA was renatured upon addition of a neutralization buffer (3.0M Potassium acetate, pH 5.5) and isolated from the precipitated chromosomal DNA through centrifugation at 14,000rpm for 10 min. The supernatant, which contained the plasmid DNA, was decanted into a QIAtrep spin column and spun for 1 min at 14,000rpm to bind the DNA. The column was washed with Buffer PE and centrifuged twice, followed by elution of plasmid DNA from the spin column with deionized water.
Sequencing the purified plasmid DNA using the ABI PRISM® 3100 Genetic Analyzer confirmed the identity of cloned gene and the accuracy of the insertion into the TOPO vector. The reactions were prepared with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Upon confirmation of the plasmid identity by sequencing, the plasmid DNA was linearized by restriction digestion. Using the Spe1 and Not1 restriction enzymes (10,000 units/ml in 10mM Tris-HCl, 250mM NaCl, 1mM Dithiothreitol, 0.1mM EDTA, 200μg/ml BSA, 50% Glycerol, 0.15% Triton X-100), two separate restriction digests were set up to cut the plasmid DNA on either side of the insert in the TOPO® TA vector. The Spe1 digestion reaction contained the Spe1 enzyme, reaction buffer (50mM NaCl, 10mM Tris-HCl, 10mM MgCl2, 1mM dithiothreitol, pH 7.9), plasmid DNA, and sterile water. Not1 digests consisted of Not1 enzyme, reaction buffer (50mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate, 1mM dithiothreitol, pH 7.9), plasmid DNA, and sterile water. The restriction digests were incubated at 37°C overnight and plasmid linearization was confirmed by agarose gel electrophoresis as described above. The restriction reaction was completed with the addition of phenol-chloroform (Tris-saturated, pH 8.0) and the linearized DNA was precipitated in isopropyl alcohol and sodium acetate (pH 5.2) at -20°C overnight. The pellet was then rinsed in 75% ethanol before being dissolved in DEPC water.

To begin ssRNA synthesis, the concentration of digested DNA was obtained using the NanoDrop1000 spectrophotometer (Thermo Fisher Scientific) to determine the amount of plasmid DNA needed to make 1μg of RNA. ssRNA was synthesized using Ambion® MEGAscript™ T3 and T7 kits for Not1 and Spe1, respectively, according to the manufacturer’s instructions.
The reactions were incubated at 37°C overnight and terminated with the addition of ammonium acetate (Ambion). The ssRNA was extracted sequentially with phenol-chloroform (pH 4.0) and chloroform and precipitated with isopropyl alcohol. The ssRNAs were kept at -20°C overnight. After the overnight precipitation, ssRNAs was resuspended in DEPC water. The complementary ssRNA were then combined and annealed to form a 2μg/μl dsRNA solution using the thermal cycler (BioRad) with the following conditions: 85°C for 3 min, 20 min ramp down to 55°C, 55°C for 10 min, 10 min ramp down to 40°C, hold at 40°C for 20 min, 5 min ramp down to 30°C, hold at 30°C for 10 min and hold at 4°C (Hughes and Kaufman, 2000). The final annealed product was analyzed via gel electrophoresis as described above and stored at -80°C until further use.

**dsRNA Injection and treatment**

To characterize the effects of vvl on the neuroendocrine network during metamorphosis, *Tribolium* larvae of the 5th and final instars, as well as early prepupae, were collected for dsRNA injection. Using a pulled 10μl glass capillary needle connected to a syringe, dsRNA was manually injected into each animal. Approximately 1μg (0.5μl) of dsRNA was injected into animals at seventh instar stage and older, while for younger animals, dsRNA was injected until the abdomen began to stretch. Controls were injected with the same volume of bacterial *ampicillin-resistance (amp)* dsRNA. Animals were also injected with *methoprene-tolerant (met)* dsRNA as a comparison to vvl knockdown animals.

Within each group, knockdown animals were also topically treated with 0.5μl of JHIII (25mg/ml) and methoprene (30mM) – a JH analog – dissolved in acetone. The same amount of
acetone was applied onto control larvae. All solutions were applied to the dorsal side of the animals immediately following injection with dsRNA to mimic a constant level of JHIII. *Tribolium* were maintained at 29°C and 50% humidity. The animals were examined every other day and characterized in comparison to *amp* dsRNA-injected or *amp* dsRNA injected animals treated with acetone.
RESULTS

Structure of Vvl in *Tribolium castaneum* and homologs

The *Tribolium castaneum* Vvl protein sequence was identified in the *Tribolium* Genome Base (http://www.bioinformatics.ksu.edu/BeetleBase/) and blasted in Geneious (http://www.geneious.com/) to find its homologs in other invertebrates and vertebrates. The POU domain in the Vvl core region shares high amino acid sequence conservation with POU domain proteins such as Vvl in *Drosophila melanogaster*, Brn-1, Brn-2, Brn-4, and Oct-6 in *Mus musculus*, and Brn-1, Brn-2, Brn-4, and Oct-6 *Homo sapiens* (Fig. 8). The *Drosophila* Vvl protein displayed the most similar amino acid sequence to *Tribolium* Vvl (64% sequence identity). Although the other protein sequences were not as conserved outside of the POU homeodomain, it is interesting to note that Brn-4 in *M. musculus* and *H. sapiens* displays similar regions upstream of the POU domain. Moreover, the Oct-6 protein in *H. sapiens* is the only other aligned vertebrate POU protein that exhibits two similar amino acid regions downstream of the POU homeodomain.
Figure 8. Alignment of *Tribolium vvl* with homologous protein sequences in *Drosophila*, *Homo sapiens*, and *mus musculus*. 
Expression of vvl in wild type Tribolium

An expression profile for vvl was determined via semi-quantitative RT-PCR in the sixth and seventh larval instars, pre-pupae, and pupae of wild type Tribolium (Fig. 9). The expression profile was based on mRNA extracted from whole body tissues, excluding the gut, of animals from each stage. Vvl was detected throughout the various stages of development (Fig. 9). It appears that vvl expression cycles throughout the different developmental phases, with its expression being the highest on Day 0 or Day 1 of each stage. However, it is possible that vvl expression is merely a reflection of varying levels of cDNA in each sample, as indicated by the variation in the brightness of rp49 expression throughout the expression profile (Fig 9).

Additional studies are needed to confirm the expression of vvl.

Figure 9. Expression profile of vvl during the late larval and pupal stages of T. castaneum. Expression profile determined by RT-PCR of vvl from whole body sixth and seventh instars, prepupae, and pupae. Cycle number for vvl was 34. Ribosomal protein 49 (rp49) was used as the control. Cycle number for rp49 was 34. D represents day.
Functional analysis of vvl by RNA interference

To investigate the biological functions of vvl during Tribolium development, and in particular, metamorphosis, vvl dsRNA was injected into Day 0 fifth instar Tribolium larvae. All animals injected with vvl dsRNA underwent precocious metamorphosis and entered the quiescent stage without molting (Fig. 10). Control larvae injected with amp’ dsRNA at the beginning of the fifth instar molted at least two more times before metamorphosis, typically at the end of the seventh instar stage. These amp’ dsRNA-injected larvae took approximately 12 days after injection to enter the quiescent stage; in contrast, the timing of metamorphosis was shifted about four days earlier when animals were injected with 1 μg of vvl dsRNA (Table 2). Since none of the animals injected with vvl dsRNA could molt to subsequent instars but eventually began to undergo metamorphosis (Table 3), the size of the amp’ dsRNA-injected prepupae was much larger than vvl dsRNA-injected prepupae (Fig 11A). The larvae injected with vvl dsRNA arrested at the prepupal stage, but the pupal structures were evident under the larval cuticle: gin traps were visible on the sides of the body, and compound eyes developed and matured much further than the extent of eye development seen in normal prepupae (Fig. 11B-I). Unlike amp’ dsRNA-injected animals, vvl dsRNA-injected animals never developed into adults.

The precocious metamorphosis seen in vvl dsRNA injected larvae suggested the possibility that JH signaling might be affected. To determine and compare how larvae respond to met knockdown, met dsRNA was injected into day 0 fifth instar larvae. Compared to the lack of a larval-larval molt seen in vvl dsRNA injected animals, most larvae injected with met dsRNA molted once before entering metamorphosis (Table 3). While the met dsRNA-injected animals
underwent precocious metamorphosis, unlike vvl dsRNA-injected animals, they were not arrested in the prepupal stage. Nearly 67% of met injected animals (n=21) began to develop adult tissues in the head and thoracic regions under the old larval cuticle and eventually eclosed as adults, similar to those reported by Parthasaraty et al (2008) (Table 3; Fig. 10; Fig. 11J-L).

![Figure 10. Timing of metamorphosis in dsRNA injected 5th instars. amp' RNAi, vvl RNAi and met RNAi indicate animals injected with amp' dsRNA, vvl dsRNA and met dsRNA, respectively. The time to the onset of prepupal period was recorded. All animals were maintained at 29°C and 50% humidity.](image)

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>N</th>
<th>Time (days) to metamorphosis Mean±SE</th>
<th>Tukey Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>amp'</td>
<td>13</td>
<td>15.2±0.785</td>
<td>A</td>
</tr>
<tr>
<td>vvl</td>
<td>16</td>
<td>11.2±0.708</td>
<td>B</td>
</tr>
<tr>
<td>met</td>
<td>21</td>
<td>11.0±0.618</td>
<td>B</td>
</tr>
</tbody>
</table>
Figure 11. Phenotypic effect of dsRNA injections in fifth instar larvae. (A) Size comparison of \textit{amp'}, \textit{vvl} and \textit{met} dsRNA-injected prepupae. (B) \textit{amp'} dsRNA-injected prepupal eyes. (C) \textit{amp'} dsRNA injected normal adult (D) \textit{amp'} dsRNA-injected pupa (E) \textit{amp'} dsRNA-injected pupal eyes (F) \textit{amp'} dsRNA-injected pupal gin traps (G) \textit{vvl} dsRNA-injected prepupa (H) \textit{vvl} dsRNA-injected prepupal eyes (I) \textit{vvl} dsRNA-injected prepupal gin traps (J) \textit{met} dsRNA-injected prepupa (K) \textit{met} dsRNA-injected prepupal eyes (L) \textit{met} dsRNA-injected eclosed adult Scale bars indicate 0.5 mm
Table 3. Phenotypic effects of dsRNA injection into fifth instar larvae.

<table>
<thead>
<tr>
<th>dsRNA injected</th>
<th>N</th>
<th>Metamorphosis after...</th>
<th>Maximum stage obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 molt</td>
<td>1 molt</td>
</tr>
<tr>
<td>amp'</td>
<td>16</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>vvl</td>
<td>15</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>met</td>
<td>21</td>
<td>3</td>
<td>18</td>
</tr>
</tbody>
</table>
Vvl knockdown verification

Semi-qualitative RT-PCR was used to verify knockdowns of vvl expression in early prepupae after last instar injection with 0.5μg of dsRNA (Fig. 12). As a control, amp’ dsRNA was also injected, and met dsRNA-injected larvae were used as comparisons. On the day of prepupation, semi-quantitative RT-PCR was performed to determine if the mRNA levels of the targeted genes were knocked down. Vvl expression levels were lower in animals injected with vvl dsRNA than in the control larvae injected with amp’ dsRNA and larvae injected with met dsRNA (Fig. 12). In addition, met expression level was lower in animals injected with met dsRNA than in those injected with amp’ dsRNA or vvl dsRNA. These results demonstrate that the vvl and met expression was effectively silenced.

Figure 12. Knockdown verification of vvl and met in early prepupae. Expression profiles for vvl, met, and rp49 (control) in amp’, vvl, and met dsRNA-injected animals. Cycle numbers for vvl, met and rp49 were 34, 35, and 28, respectively.
Effect of methoprene and JH application in Tribolium

The precocious metamorphosis observed in vvl dsRNA-injected Tribolium suggests that JH signaling might somehow be altered. However, nothing is known about whether Vvl interacts with the JH pathway. In order to determine if vvl is involved in the synthesis or reception of JH, we ectopically applied JH and a juvenile hormone analog, methoprene, to Day 0 fifth instar larvae injected with vvl dsRNA. Acetone treatments were used as controls and amp′ and met dsRNA-injected larvae were also treated similarly.

Application of 30mM of the JH analog methoprene to Day 0 fifth instar larvae injected with amp′ dsRNA caused supernumerary molts (extra larval molts after the eighth instar) in seven out of 14 larvae (Table 4). Five of the methoprene treated control animals never progressed beyond the larval stage, but nine were able to undergo metamorphosis; however, most larvae that underwent metamorphosis arrested their development and died as either prepupae or pupae. amp′ dsRNA-injected animals treated with JHIII all progressed normally through development and eclosed as adults. However, their development was delayed compared to those treated with acetone (Fig. 13A; Table 5). This difference is most likely due to the fact that the JH used was not as strong (or concentrated) as methoprene. All controls injected with amp′ dsRNA and treated with acetone molted into pupae and formed normal adults (Table 4; Fig. 14F).

In contrast, larvae injected with vvl dsRNA and treated with methoprene were still unable to molt, and most died as prepupae (Table 4). These animals developed pupal-like characteristics similar to control vvl dsRNA-injected animals treated with acetone (Fig. 14A-D; G-J). Despite this similarity, ectopic application of methoprene drastically delayed the timing of
metamorphosis (Fig. 13B). While about 80% of vvl dsRNA-injected larvae treated with acetone underwent prepupal formation within nine days, it took 15 days for the same proportion of methoprene treated animals to reach the prepupal stage. Additionally, ectopic application of JH also delayed metamorphosis, although not as dramatically as methoprene. The results show that vvl knockdown animals are able to sense JH/methoprene yet cannot be entirely rescued by JH/methoprene application.

As a comparison, larvae were also injected with met dsRNA and treated with acetone, JH, or methoprene. For all three treatments, the majority of met dsRNA injected animals underwent metamorphosis and developed into prepupae or eclosed as an adult (Table 4; Fig. 14F&L). In agreement with previous studies suggesting that Met is a receptor for JH, there was no significant difference between the timing of metamorphosis in larvae treated with acetone or JH/methoprene (Fig. 13C). This finding indicates that met knockdown animals are insensitive to JH.
Table 5. Effects of ectopic application of JH and methoprene on developmental timing in larvae injected with dsRNA as fifth instars.

<table>
<thead>
<tr>
<th>dsRNA injected</th>
<th>N</th>
<th>Conc. of JH and methoprene</th>
<th>Metamorphosis after...</th>
<th>Maximum Stage Obtained</th>
<th>Prepupa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 1 2 3 4</td>
<td>Larva</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>molt molt molts molts</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 1 2 3 4</td>
<td>No complex eye development With complex eye development Adult cuticle formation Pupa Adult</td>
<td></td>
</tr>
<tr>
<td>amp' + acetone</td>
<td>19</td>
<td>N/A</td>
<td>0 6 11 2 0</td>
<td>3 0 2 0 1 13</td>
<td></td>
</tr>
<tr>
<td>amp' + JH</td>
<td>7</td>
<td>25ng/μl</td>
<td>0 0 7 0 0</td>
<td>1 0 0 0 0 6</td>
<td></td>
</tr>
<tr>
<td>amp' + methoprene</td>
<td>14</td>
<td>30mM</td>
<td>0 2 5 5 2</td>
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</tr>
<tr>
<td>vvl + acetone</td>
<td>20</td>
<td>N/A</td>
<td>20 0 0 0 0</td>
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<tr>
<td>vvl + JH</td>
<td>11</td>
<td>25ng/μl</td>
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<td>vvl + methoprene</td>
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<td>30mM</td>
<td>16 0 0 0 0</td>
<td>6 3 7 0 0 0</td>
<td></td>
</tr>
<tr>
<td>met + acetone</td>
<td>25</td>
<td>N/A</td>
<td>10 15 0 0 0</td>
<td>0 1 8 11 4 1</td>
<td></td>
</tr>
<tr>
<td>met + JH</td>
<td>17</td>
<td>25ng/μl</td>
<td>10 7 0 0 0</td>
<td>1 0 5 11 0 0</td>
<td></td>
</tr>
<tr>
<td>met + methoprene</td>
<td>16</td>
<td>30mM</td>
<td>11 5 0 0 0</td>
<td>1 1 4 8 2 0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 13. Timing of metamorphosis in dsRNA injected fifth instars treated with acetone, JH, and methoprene. (A) Animals injected with *amp* dsRNA and treated with acetone, JH III, or methoprene. (B) Animals injected with *vvl* dsRNA and treated with acetone, JH III, or methoprene. (C) Animals injected with *met* dsRNA and treated with acetone, JH III, or methoprene. The time to the onset of prepupal period was recorded for all treatments. All animals were maintained at 29°C and 50% humidity.
Table 5. Mean ± SE for the timing (days) to metamorphosis in *amp’*, *vvl*, and *met* dsRNA injected *T. castaneum* treated with acetone, JH, or methoprene. ANOVA, df = 2, α = 0.05, Tukey HSD test. Tukey levels not connected by the same letter are significantly different.

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>N</th>
<th>Time (days) to metamorphosis Mean±SE</th>
<th>Tukey Level</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>amp’</em> + acetone</td>
<td>17</td>
<td>12.0±1.03</td>
<td>A</td>
</tr>
<tr>
<td><em>amp’</em> + JH</td>
<td>16</td>
<td>12.1±1.06</td>
<td>A</td>
</tr>
<tr>
<td><em>amp’</em> + methoprene</td>
<td>10</td>
<td>19.4±1.34</td>
<td>B</td>
</tr>
<tr>
<td><em>vvl</em> + acetone</td>
<td>19</td>
<td>9.37±0.577</td>
<td>A</td>
</tr>
<tr>
<td><em>vvl</em> + JH</td>
<td>19</td>
<td>9.89±0.577</td>
<td>A</td>
</tr>
<tr>
<td><em>vvl</em> + methoprene</td>
<td>11</td>
<td>14.9±0.759</td>
<td>B</td>
</tr>
<tr>
<td><em>met</em> + acetone</td>
<td>25</td>
<td>10.6±0.618</td>
<td>A</td>
</tr>
<tr>
<td><em>met</em> + JH</td>
<td>17</td>
<td>10.9±0.749</td>
<td>A</td>
</tr>
<tr>
<td><em>met</em> + methoprene</td>
<td>15</td>
<td>11.1±0.798</td>
<td>A</td>
</tr>
</tbody>
</table>
Figure 14. Phenotypic effect of dsRNA injection with methoprene and acetone treatments. (A-F) Acetone-treated animals. (A) Size comparison of amp, vvl and met dsRNA injected animals treated with acetone. (B) vvl dsRNA-injected prepupa (C) vvl dsRNA-injected prepupal gin traps (D) vvl dsRNA-injected prepupal complex eyes (E) amp’ dsRNA-injected adult (F) met dsRNA-injected eclosed adult. (G-L) Methoprene-treated animals (G) Size comparison of amp, vvl and met dsRNA injected animals treated with methoprene. (H) vvl dsRNA-injected prepupa. (I) vvl dsRNA-injected prepupal gin traps (J) vvl dsRNA-injected prepupal complex eyes (K) amp’ dsRNA-injected arrested pupa (L) met dsRNA-injected eclosed adult. All animals (G-L) were treated with methoprene. Scale bars represent 0.5mm.
Expression of Kr-h1 in normal and JH treated larvae

To determine whether vvl was affecting downstream targets of the JH pathway, semi-quantitative RT-PCR analysis was performed on Day 4 fifth instar Tribolium larvae injected with vvl dsRNA and treated with either acetone (control) or methoprene on Day 0 of the fifth instar stage (Fig. 15). *amp’* dsRNA-injected animals treated similarly were used as a comparison. An apparent decrease in kr-h1 levels was observed in control vvl knockdown animals treated with acetone (Fig. 15), while kr-h1 expression was normal in *amp’* dsRNA-injected animals treated with acetone. However, when larvae were treated with 30mM of methoprene immediately after injection of vvl dsRNA on Day 0 of the fifth instar, kr-h1 expression was elevated to a level similar to that seen in the *amp’* dsRNA-injected animals treated with acetone. RT-PCR of vvl expression verified the successful knockdown of the gene of interest and rp49 expression was constant throughout all treatments.
Figure 15. The expression levels of the juvenile hormone response gene, *kr-h1* in dsRNA injected fifth instars. Larvae in the first day of the fifth instar were injected with *amp′*, *vvl*, or *met* dsRNA, and total RNA was extracted from whole body homogenates on day 4 of the fifth instar. The expression levels were determined by RT-PCR and *rp49* expression was used as a control for loading. Cycle numbers for *kr-h1*, *vvl*, and *rp49* were 34 for all genes.
Effect of vvl RNAi on the expression of 20E response genes

Vvl dsRNA-injected larvae exhibit a unique phenotype in that they were unable to molt before metamorphosis. In order to determine if vvl is interacting with the ecdysone response pathway, we investigated the expression of 20E-response genes in prepupae injected with vvl dsRNA as Day 0 final instar larvae using semi-quantitative RT-PCR. The expression levels of the ecdysone inducible early genes, br and E75, the ecdysone-inducible delayed-early genes, hormone receptor 3 and 4 (hR3, hR4), and a nuclear receptor known to be involved in ecdysone signal transduction, FTZ-F1, were investigated. The relative levels of these genes were compared with levels in animals injected with amp′ dsRNA and met dsRNA. The br mRNA levels in vvl knockdown prepupae were similar to those found in amp′ knockdown prepupae. In addition, the expression of all the downstream genes, E75, hr3, hr4, and ftz-f1 during the prepupal stage was not affected by the vvl knockdown (Fig. 16). These data suggest that vvl is not downstream of 20E in the ecdysone pathway during the prepupal period even though vvl dsRNA injected animals have extremely limited molting capabilities.
Figure 16. The expression levels of 20E-regulated genes in prepupae injected with \textit{amp'}, \textit{vvl}, and \textit{met} dsRNA as day 0 seventh instar larvae. Larvae in the first day of the final instar were injected with \textit{amp'}, \textit{vvl} or \textit{met} dsRNA, and total RNA was extracted from whole body homogenates on the day of prepupa formation. The mRNA levels were determined by semi-quantitative RT-PCR and were compared to the \textit{Tribolium castaneum} \textit{rp49} expression. Cycle numbers for \textit{br}, \textit{E75}, \textit{HR3}, \textit{HR4}, \textit{Ftz-F1} and \textit{rp49} were 30, 34, 34, 38, 40, and 28, respectively.
DISCUSSION

In this study, we determined the knockdown effects of *vvl* in *Tribolium* and elucidated a possible mechanism by which it interacts with JH and ecdysone, the two major hormones involved in metamorphosis. Use of dsRNA-mediated expression knockdown revealed that *vvl* plays a major role in regulating the developmental timing of metamorphosis, and that suppression of *vvl* results in precocious metamorphosis. Although similar findings have been described by Konopova and Jindra (2007) regarding *met*, it is apparent that *vvl* plays a different role in regulating metamorphosis.

Role of *vvl* during metamorphosis – A JH perspective

In fifth instar animals injected with *vvl* dsRNA, the larvae undergo premature metamorphosis without molting (Fig. 10). Since significant growth normally occurs between larval-larval molts before metamorphosis at the end of the seventh or eighth instar, these animals developed into miniature prepupae (Table 2; Fig. 11). The precocious metamorphosis observed in *vvl* dsRNA-injected larvae indicates that these larvae commit early to become a pupa, possibly by disrupting JH signaling. Consistent with this interpretation, we determined that knockdown of *vvl* in the fifth instars causes a down-regulation of the expression of *kr-h1*, a JH-response gene, known to be transcriptionally active during the larval stage but absent in the pupal stage (Minakuchi et al., 2009).

In addition, we found that *vvl* knockdown animals were not completely resistant to JH/methoprene; ectopic application of JH or methoprene delayed the timing of prepupal
development, but was unable to maintain the juvenile stage forever (Fig. 13A&B; Table 3). The delay in metamorphosis observed in these JH-treated vvl knockdown larvae suggests that vvl does not affect the sensitivity to JH. In contrast, JH had little effect on the final phenotype of vvl injected larvae since many still developed pupal characteristics such as complex eyes and ginv traps (Fig. 14). This suggests that beyond a certain point, the larval tissues are irreversibly committed to a prepupal/pupal fate and are no longer sensitive to JH. Similarly, ectopic application of methoprene, a JH analog, was able to upregulate kr-h1 expression in vvl knockdown animals (Fig. 15). Since kr-h1 is a direct downstream target of JH (Minakuchi et al., 2009), the down-regulation of kr-h1 observed in vvl knockdown animals and the rescue of its expression with ectopically applied methoprene indicate that Vvl knockdown causes a decrease in JH biosynthesis or action upstream of kr-h1. Thus, vvl appears to act either upstream of or in parallel to JH to mediate developmental timing.

Role of vvl during metamorphosis – An ecdysone perspective

Another important finding regarding the mechanism by which vvl controls metamorphosis came from differences in molting patterns between vvl knockdown animals and met knockdown animals. All larvae injected with vvl dsRNA underwent precocious metamorphosis without molting, while met dsRNA-injected animals almost always molted before metamorphosis (Table 2). This evidence suggests that vvl suppression has a strong inhibitory effect on the ecdysone-signaling pathway. However, both met RNAi and vvl RNAi animals undergo metamorphosis at the same time (Fig. 10), suggesting that a minimum length of time is needed for larvae to commit to pupal development and initiate metamorphosis.
Through RT-PCR analysis of downstream targets of the ecdysone pathway during the prepupal period, we found that the mRNA expressions of downstream ecdysone signaling factors, such as br, E75, hr3, hr4, and ftz-f1, do not show any changes when Vvl expression is silenced (Fig. 16). These findings suggest that the secretion or synthesis of ecdysteroids may be disrupted in vvl knockdown animals prior to entry into metamorphosis such that molting is inhibited.

Ecdysone synthesis and release from the prothoracic glands is regulated by multiple factors. For most arthropods, the timing of molts and metamorphosis is coordinated by a rise in the titers of 20E in response to PTTH action on the prothoracic glands (Henrich et al., 1999). PTTH mutant flies have delayed onset of metamorphosis (McBrayer et al., 2007). However, there are other factors that influence the synthesis and release of ecdysteroids from the prothoracic gland. Recent studies have also implicated the insulin-signaling pathway in the regulation of the timing of metamorphosis. In the silkworm Bombix mori, insulin and PTTH act together to mediate ecdysteroidogenesis via the P13K/Akt signaling pathway, whereas PTTH alone acts along the ERK signaling pathway to influence ecdysteroidogenesis (Gu et al., 2009; Gu et al., 2011). Since no study has demonstrated whether insulin and PTTH are sufficient to explain the fluctuations of ecdysteroid titers, there may be additional factors that regulate the timing and production of ecdysteroids.

Ecdysis was delayed in vvl dsRNA-injected animals, but not completely blocked since these animals were still able to undergo prepupal development, which requires an increase in 20E titers (Riddiford, 1994). The ability of vvl dsRNA-injected animals to begin metamorphosis
suggests that some 20E is still released. Thus, Vvl might be involved in regulating one of the many factors involved in ecdysone synthesis and release.

Figure 17. Proposed mechanism of Vvl action and homology with GnRH pathway.

Possible conservation of control of the reproductive maturation in insects and vertebrates

It has been suggested that puberty in humans and other mammals is another form of metamorphosis (Gilbert, 2010). Although seemingly different, both processes are marked by dramatic changes in physiology. Puberty in humans is regulated by endocrine changes in response to increased pulsatile release of gonadotropin releasing hormone, GnRH, from the hypothalamus. Although it is not entirely clear what factors are responsible for the initiation of puberty, this process is regulated at least in part by the release of Kisspeptin (Gottsch et al., 2004). An integrator of environmental conditions and an unknown developmental timer have
also been suggested to play roles in regulating the timing of puberty (Gajdos et al., 2010; Sisk and Foster, 2004).

Previous studies in rats have shown that GnRH mRNA levels are present long before puberty, and that any pulsatile changes in mRNA abundance occurring at the time of puberty are most likely due to post-transcriptional modifications, rather than changes in the primary transcription rate (Gore and Roberts, 1997). In effect, puberty can be viewed as a reactivation of GnRH secretion. Research has suggested that one protein playing a key role in modulating GnRH promoter activity is the POU domain transcription factor Oct-6 (Wierman et al., 1997). According to Wierman et al. (1997), Oct-6 represses GnRH activity, delaying the onset of puberty. In the absence of Oct-6, GnRH is released causing pubertal development similar to how the absence of vvl initiates metamorphosis in insects. Furthermore, our protein alignment indicates that in addition to sharing a homeodomain with vertebrate Oct-6, Tribolium vvl shares two additional amino acid sites near the 3’-carboxyl end not observed in other POU domain transcription factors such as Brn-1, Brn-2, or Brn-4 (Fig. 8). Whether or not these residues are important remains to be seen.

Recently, new GnRH-like peptide sequences have been identified in various invertebrates, ranging from tunicates to annelids (Roch et al., 2011). Some examples of invertebrate neuropeptides homologous to the GnRH family include coronasin and adipokinetic hormone (AKH). A few studies have shown a potential link between these neuropeptides and JH synthesis. For example, studies in adult crickets (Gryllus bimaculatus and Acheta domesticus) have shown that AKH can inhibit JH III synthesis in vitro (Woodring and Hoffmann, 1997). It is possible that vvl interacts with a GnRH insect homolog, referred to here as factor X, to regulate
the JH pathway as well as ecdysone secretion (Fig. 17). Future studies are needed to determine the downstream targets of vv. A potentially conserved role of POU domain transcription factors during puberty and metamorphosis suggests an intriguing possibility that the regulation of the timing of reproductive maturation may be conserved throughout the Metazoa.
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