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Investigating the Role of ventral veins lacking in the Endocrine Regulation of Metamorphic Timing

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Investigating the Role of *ventral veins lacking* in the Endocrine Regulation of Metamorphic Timing

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ABSTRACT

Metamorphosis and puberty are characterized by dramatic morphological and behavioral changes, and their regulation and evolution continues to be a puzzling scientific enigma. In insects, the timing of metamorphosis is regulated by the interaction between juvenile hormone (JH), prothoracicotropic hormone (PTTH) and ecdysteroids. However, the transcriptional control of these neuroendocrine regulators remains unknown. In vertebrates, POU domain transcription factors have been linked to endocrine changes associated with puberty. POU factors play key roles in regulating the timing of puberty, having been associated with the production of gonadotropin releasing hormone, which regulates the onset of puberty. Since POU factors are highly conserved transcription factors found across all metazoans, the role of one of the POU transcription factors, Ventral vein lacking (Vvl), was examined during development in the postembryonic stages. Previous study has shown that silencing *vvl* expression using RNA interference (RNAi) results in the induction of precocious metamorphosis in *Tribolium castaneum* (Cheng, 2013). Here, I demonstrate that this effect is mediated by a reduction in the expression of the JH-inducible gene *krüppel homolog 1* (*kr-h1*), as well as a decrease in the expression of the JH biosynthesis enzyme coding gene *JH acid methyltransferase 3* (*jhamt3*). Furthermore, the expression of *kr-h1* could be rescued by topical application of the JH analog methoprene. Interestingly, in addition to inducing precocious metamorphosis, molting was also inhibited in *vvl* RNAi-induced animals. I show that ecdysone biosynthesis is reduced in *vvl*-knockdown animals. Thus, Vvl influences both JH and ecdysone signaling and biosynthesis, potentially acting as an integrator of both hormonal pathways to regulate the metamorphic onset. In addition, I also provide preliminary findings on the relationship between *vvl* expression and nutritional status. Taken together, our study suggests that Vvl may serve as a key transcriptional regulator of major developmental endocrine events in both insects and vertebrates.

INTRODUCTION

In nature, animals experience complex and dramatic changes in their embryonic forms as their cells undergo countless rounds of proliferation and differentiation. However, many animals also continue to undergo major transformations in their postembryonic stages through a process known as metamorphosis. Metamorphosis is a process in which an organism undergoes a series of drastic morphological and physiological changes and is observed in insects, amphibians, and even mammals. For example, caterpillars undergo dramatic physiological and structural changes in morphology to become butterflies. Similarly, mammals also undergo drastic developmental changes to reach reproductive maturity. In both of these cases, the hormones involved are known, but the regulation of the timing of these hormonal events remains a mystery.

Regulation of endocrinology in insect development

In the field of developmental biology, the regulation of body size and allometry in animals continues to be a perplexing scientific enigma. The final size that an organism grows to is influenced by both genetic and environmental factors that function through complex molecular and physiological mechanisms that have yet to be fully understood. In most animals, these major developmental changes are coordinated by the specific timing and release of hormones. Similar to how humans undergo puberty at a certain stage in their lives, other organisms also metamorphose at a certain point in their development, and these events are typically controlled and regulated by a series of

different hormones and signals. In insects, growth and metamorphosis is punctuated by the process of molting, which is regulated by the interaction of three hormones: prothoracicotropic hormone (PTTH), juvenile hormone (JH), and ecdysteroids (Wigglesworth, 1970; Nijhout, 1981; Konopova and Jindra, 2007). We know that, in most and probably all insects, the brain controls the biosynthesis and secretion of ecdysteroids by the action of PTTH on the prothoracic glands (PG). To initiate a molt, the brain releases PTTH, which causes the synthesis and secretion of ecdysteroids from the PGs. At the target site, ecdysteroids are converted to 20-hydroxyecdysone (20E), which is the primary ecdysteroid involved in molting and triggers the detachment of the epidermis from the old cuticle and the subsequent secretion of the new cuticle. The nature of the molt – whether it is a larval-larval molt or larval-pupal molt – depends on the presence or absence of JH, respectively (Nijhout, 1975). Thus, the roles of PTTH, JH and ecdysteroids and how they interact in controlling the molting and metamorphosis of insects have been the focus of numerous investigations.

In the final larval instar of *Manduca* larvae, JH initially prevents the brain from synthesizing and releasing PTTH (Nijhout and Williams, 1974b). Midway through this instar, upon reaching a particular size known as the critical weight, JH levels drop, and the larval brain becomes competent to release PTTH (Nijhout and Williams, 1974b). Reaching the critical weight thus sets in motion a series of physiological events involving signaling of the brain to release PTTH, which activates the prothoracic glands to secrete ecdysone, triggering the onset of metamorphosis (Nijhout and Williams, 1974a; Truman and Riddiford, 1974).

The roles of critical weight and threshold size on the onset of metamorphosis

Critical weight

In the past four decades, countless studies have been conducted to find the underlying physiological mechanisms that regulate size control and critical weight. In many insects, starvation during the final instar results in a delay in metamorphosis. The critical weight is defined as the point where starvation no longer delays the initiation of metamorphosis (Nijhout). The critical weight is determined by both genetic and environmental factors, and the body size in *Manduca sexta* has been shown to evolve through changes in critical weight (Davidowitz et al., 2003). Though it is evident that genetics play a major role in controlling growth and the initiation of metamorphosis, recent scientific approaches to the regulation of growth and size have largely focused on the interaction between genetic and environmental effects. Environmental conditions, such as nutrition, quality of the diet and exposure to the elements, have been shown to have a significant role in the regulation of growth. In *Drosophila*, the critical weight is hypothesized to be controlled levels of insulin sensed by the PG. Thus, interrupting insulin signaling in the PGs can impede the point of critical weight, hindering the initiation of metamorphosis, or induce precocious onset of metamorphosis (Caldwell et al., 2005; Mirth et al., 2005).

In *Manduca sexta*, the activity of a larval insect's corpora allata (CA), or site of JH production in early stages of larval development, depends on the weight that the individual had attained (Nijhout and Williams, 1974a). In this species, the critical weight marks the attainment of a particular threshold body size and leads to the removal of JH from the hemolymph. If larvae are starved below the critical weight, JH is prevented

from being cleared, interrupting the onset of PTTH secretion. The mechanism by which a larva assesses its body size and its critical weight are not known, but the critical weight appears to be a function of the initial weight of the instar (Nijhout, 1981). Nijhout (1975) suggested the presence of an allometric size-monitoring system mechanism that keeps track of the overall size in relation to some other part or to body as a whole. A recent study that used *Manduca sexta* as its model system has shown that oxygen levels in the larva indicate critical weight achievement, thus leading to the cessation of JH secretion (Callier and Nijhout, 2011). Experiments have revealed that larvae exposed to hypoxic conditions form smaller-sized pupae because the critical weight is shifted to a lower size. Insects rely on diffusion of oxygen for respiration. Oxygen is delivered to various glands and tissues by tracheal tubes. As larvae grow, the concentration of oxygen delivered to the inner portions of the animal drops. The hypothesis then is that there is an unidentified oxygen sensor that detects oxygen levels in the growing larva and that the larva uses oxygen levels as a proxy for body size (Callier and Nijhout, 2011).

Threshold size

The decision for a larva to initiate metamorphosis is actually influenced by an even earlier checkpoint known as the threshold size. In most insects, the final larval instar exhibits a distinct morphology that differs from the rest of the larval instar. This commitment to enter the final (penultimate) instar is made prior to the final instar. From previous studies, we know that nutrition is a key in the regulating the initiation of metamorphosis, most likely having to do with the determination of the threshold size,

which determines the number of molts preceding metamorphosis. Whether or not the larva enters the final instar in turn is determined by the threshold size. Starvation during the penultimate (the instar before the last) instar result in the formation of supernumerary penultimate instar (i.e. it repeats the penultimate instar) (Nijhout, 1975). Thus, the threshold size determines the number of molts the larvae will go through before pupation. However, what determines the threshold size currently remains unknown, though the decision is probably made near the end of the instar prior to the dramatic plunge in the levels of JH.

In the final larval instar, an organism's brain cells are sensitive to levels of JH, which delays PTTH secretion (Nijhout, 1975). In earlier larval instars, molting occurs in the presence of high JH, which means PTTH secretion from the brain occurs independently of JH. In the final instar, the brain becomes independent of the presence of JH. Therefore, a subtle switch occurs in the CNS at some point during the penultimate instar that alters the brain's responsiveness to JH. The attainment of the threshold size is probably triggered by this switch; however, the molecular nature of this switch remains elusive.

Juvenile hormone and prothoracicotropic hormone

Though the role of the JH titer decline in metamorphosis is well understood, the mechanism controlling the timing of the pulse of PTTH that initiates metamorphosis is still a mystery. Nijhout and Williams (1974b) found that the removal of the CA allowed for earlier release of PTTH for initiation of gut purge, or when the organism purges its gut contents near the end of the larval period. In fact, the CA stops releasing JH during

the final larval instar in most species of insects (Patel and Madhavan, 1969; Nijhout and Williams, 1974b). However, what induces the brain to secrete or not to secrete PTTH, or what signals the CA to stop secreting JH at the appropriate time, still remains unclear. The only definitive study on the intrinsic mechanism for controlling PTTH secretion was carried out by Wigglesworth (1934) on *Rhodnius prolixus*. In this insect, the brain is stimulated to secrete PTTH by nerve impulses from the ventral nerve cord. Wigglesworth (1970) cites persuasive evidence that the CA do not take note of the number of instars and stop JH release at a predetermined stage, but are controlled by complex internal factors which are yet to be clarified.

Halloween genes and their role in the regulation of ecdysone biosynthesis

In the process of metamorphosis, ecdysteroids play a major role as timing regulators. As aforementioned in this paper, during the final instar, these hormones activate the initiation of the entrance into metamorphosis as soon as JH is emptied from the system. Ecdysteroids are produced in the prothoracic gland (PG) and are produced from cholesterol. Inside the PG, ecdysone synthesis is regulated by several Halloween genes, and the genes in this family include *phantom* (*phm*), *spook* (*spo*), *disembodied* (*dib*), *shade* (*shd*) and *shadow* (*sad*) (Gilbert et al., 2004). The Halloween genes encode for P450 enzymes necessary for catalyzing a series of reactions that ultimately convert cholesterol into E, which is then converted to 20E in the peripheral tissues by *shd* (Petryk et al., 2003). Previous studies have shown that disrupting the expression of Halloween genes lead to decreased levels of ecdysteroids in *Drosophila* (Rewitz et al., 2007). The expressions of the Halloween genes are controlled by both PTTH and

insulin signaling and correlate with the ecdysteroid titers. Thus, the regulation of the timing of metamorphosis involves the intricate interaction of factors that are involved in the production and expression of ecdysteroids and JH titers. These factors are monitored by both the physical size and physiological state of the developing organism. However, the molecular nature of the processes that combine the various organismal and environmental contexts to influence the interplay between JH and ecdysteroid regulators remains unclear.

The significance of Vvl, a POU transcription factor

Though it is evident that the regulation of growth and the initiation of metamorphosis size are regulated by a complex interplay between hormones and environmental inputs, the transcriptional regulation of hormonal changes are not well understood. In vertebrates, POU (Pit-Oct-Unc) domain transcription factors have been linked to endocrine changes associated with puberty and are of particular interest because they play significant roles in the development and regulation of the vertebrate neuroendocrine centers. They also play key roles in regulating the timing of puberty, having been associated with the production of gonadotropin releasing hormone, which regulates the onset of puberty (Wierman et al., 1997). Many known POU factors are expressed in region-specific areas within the developing central nervous system (CNS), and POU proteins also appear to regulate changes in neuroendocrine signal expressions linked to puberty. POU factors are also expressed in the CNS of insects; however, it is not fully understood whether or not they actually monitor neuroendocrine regulation during metamorphosis.

Initially, *ventral veins lacking* (*vvl*) was studied in *Drosophila* as a gene responsible for regulating development of the trachea, a structure that serves to deliver oxygen (Anderson et al., 1995). Vvl, a POU transcription factor, was also found to be crucial in directing early cellular migration in the CNS. Because POU factors are highly conserved across metazoans, studying them will give us a better understanding of how sexual maturation is regulated across species. From previous experimental data, we believe that the POU transcription factor Vvl may play an important role in regulating metamorphosis (Cheng, 2013). The function of Vvl was analyzed in the flour beetle *Tribolium* using RNA interference (RNAi). Silencing Vvl expression resulted in the induction of precocious metamorphosis in *Tribolium.* The study also reported a preliminary finding that suggested a reduction in the expression of the JH-inducible gene *krüppel homolog 1* (*kr-h1*). However, topical application of JH on individuals lacking Vvl delayed the onset of metamorphosis and rescued the normal expression of *kr-h1*, indicating that JH levels are reduced in *vvl* RNAi animals. In addition, these *vvl*-knockdown animals failed to molt, suggesting that ecdysteroid regulation might be affected. The findings in this study strongly suggest that Vvl acts as an activator of both JH biosynthesis and ecdysone signaling to influence molting and the timing of metamorphosis. Vvl seems to integrate both JH and ecdysone signaling, meaning understanding how its expression is regulated and its relationship with body size may reveal interesting implications how development is initiated, thus linking the central nervous system with hormonal centers.

Oenocytes as a site of *vvl* expression

Oenocytes are a specialized group of cells that are necessary for multiple important metabolic processes, such as lipid generation and developmental signaling (Chapman, 1998). Oenocyte development is categorized into two major steps: cell specification and cell maturation. At the time of cell specification, oenocytes also express several other transcription factors, such as Spalt and HNF4 (two important proteins in lipid processing), and Vvl (Inbal et al., 2003; Burns et al., 2012). Burns et al. (2012) showed that, similar to *Drosophila*, the oenocytes of *Tribolium* larvae are localized in the abdominal region. Previous studies have also shown that oenocytes are able to generate ecdysone from cholesterol in another beetle, *Tenebrio molitor* (Romer et al., 1974; Delbecque et al., 1990); thus, investigating Vvl localization at the oenocytes, may elucidate its role in ecdysone biosynthesis.

Objectives and hypothesis

The purpose of this study is to further understand the role of *vvl* in endocrine regulation. Thus, this study: (1) investigates *vvl* and its possible role in controlling the initiation of metamorphosis in *Tribolium* through the analysis of JH and ecdysteroid biosynthesis genes; (2) examines the tissue-specific expression profiles of *vvl* in *Manduca*; (3) analyzes how Vvl interacts with other known regulators of JH and ecdysteroids biosynthesis; and (4) evaluates the expression of *vvl at* pre- and post-critical weights in *Manduca* using quantitative PCR. I propose that Vvl might regulate the timing of metamorphosis by controlling both JH and ecdysone biosynthesis, thus linking the central nervous system with hormonal centers. I also hypothesize that *vvl*

may play a role in critical weight attainment. Examining *vvl* from a genetic perspective may provide further insight on the evolution of the functions of this gene in growth and development.

Gene silencing using RNA interference (RNAi)

In *Tribolium*, using RNAi for studying growth and development is a very powerful tool in this particular model system (Denell, 2008). RNAi is a method used to inhibit gene function and expression by introducing double-stranded RNA (dsRNA) into an organism, resulting in specific silencing of the targeted gene (Fire et al., 1998). Thus, by targeting a specific gene and silencing it with the use of dsRNA, protein products are not translated.

When the foreign dsRNA enters the organism, an enzyme called Dicer cleaves the dsRNA into small nucleotide fragments known as short interfering RNA (siRNA), which then are integrated with additional components to form a complex called the RNA-induced silencing complex (RISC). The RISC then unwinds the siRNA into single-stranded RNA, which then targets complementary mRNA for cleavage and degradation (Fortunato and Fraser, 2005). The single-stranded RNA in the RISC complex then binds to a complementary sequence of mRNA within the host cell, and the RISC proceeds to cut and degrade the mRNA, preventing the process of translation. With the obstruction of genetic expression, the production of certain genetic products is also hindered, leading to altered development. Thus, to examine gene function of any individual gene, we start off with the sequence of a gene of interest, knock down the expression levels of

that gene, and study and observe the resulting phenotypic effects (Fortunato and Fraser, 2005).

Tribolium castaneum and *Manduca sexta* as model organisms

Both *Tribolium* and *Manduca*, unlike *Drosophila*, depend on JH as a key regulator of metamorphosis. *Tribolium castaneum,* or the red flour beetle, is an ideal model organism in developmental biology. It is easy to maintain, has a rapid generation time, an already sequenced genome, and is susceptible to RNA interference (RNAi), making it an excellent model organism for genetic studies. As *Tribolium* larvae grow and develop, they undergo 7-8 instars, or larval stages, separated by periods of molts. Fully grown larvae transform into pupae, and within approximately 4-5 days, emerge into their adult forms.

Tribolium have become a major system for evolutionary developmental biology because its developmental process is more characteristic of a broader range of insects than that of *Drosophila.* While both *Tribolium* and *Drosophila* undergo complete metamorphosis, *Tribolium* embryos undergo a more primitive form of development than those of *Drosophila*, making it a suitable candidate for studies involving evolution and genetic conservation (Klingler, 2004). Moreover, using *Tribolium* is advantageous over using *Drosophila* in experiments pertaining to metamorphosis because they respond predictably to JH (Konopova and Jindra, 2007). Whereas *Drosophila* larvae will pupariate even when JH is ectopically applied, the presence of JH induces supernumerary molts in *Tribolium* larvae. Thus, using this organism presents an ideal

opportunity to investigate the JH pathway and related factors involved in the process of metamorphosis.

Manduca also serves as a model system for the experiments presented in this study because it offers several advantages over *Tribolium*. With its large body size, the organism is the ideal candidate for examining tissue specific genetic expression. It also has well characterized physiological regulation and a very standard pattern of development with a fixed number of molts. Because their critical and threshold weights can be altered with environmental (such as dietary and oxygen level) manipulation, *Manduca* are the ideal system to study the complexity of these two size assessment events and their respective roles in the initiation of metamorphosis. Like *Tribolium, Manduca* also have a sequenced genome, which allows us to find genes easily. The combination of these two species will allow us to best address the experimental questions presented in this study.

MATERIALS AND METHODS

Animal husbandry

Wildtype GA1 strain *Tribolium castaneum* beetles were raised on organic whole wheat flour supplemented with 5% nutritional yeast at 29°C and 50% humidity. *Manduca sexta* larvae were raised on artificial diet at 26.5°C.

Isolating RNA and synthesizing complementary DNA (cDNA)

RNA from various larval instars of *Tribolium* or *Manduca* was isolated by homogenizing the tissues in 500µL TRIzol. Subsequently, 100 µL of chloroform was added, and the product was centrifuged in 4°C at 11,500 rpm for 15 min. The top aqueous layer, which contains the RNA, was collected and precipitated by the addition of 250 µL isopropanol at room temperature for 15 min. The RNA was then pelleted by another round of centrifugation in 4°C at 11,500 rpm for 10 min, and the resulting supernatant was removed. The remaining pellet containing the RNA was washed with 500 µL 80% ethanol/DEPC water, followed by centrifugation at a slower rate of 7,400 rpm for 5 min. The supernatant was discarded, and pellet was allowed to dry before being resuspended in 13 µL DEPC water. The product was then incubated at 60°C for 5 min to dissolve the pellet. Promega RQ1 RNase-Free DNase was used to remove traces of DNA from the RNA sample, followed by the RNA being precipitated with 20 µL isopropanol and 10% volume 3M sodium acetate (pH 5.2). The mixture was kept at -20°C for at least one hour, and to isolate the RNA, samples were centrifuged in 4°C at 14,000 rpm for 10 min. Pellets were then washed with 75% ethanol and the previous

step of centrifugation was repeated, after which the pellets were dried until they appeared transparent. After drying, the RNA pellet was dissolved in 10 µL DEPC water, and its concentration was measured using the *NanoDrop 2000* spectrophotometer in preparation for its use in complementary DNA (cDNA) synthesis.

cDNA was synthesized from 1 µg of RNA by the process of reverse transcription. One µL Oligo dT primer was added to 1 µg RNA, with enough DEPC water added to bring the total volume for each sample to 12 µL, and the mix was then incubated at 65°C for 5 min. Reverse transcription was initiated by mixing the following with the 12 µL RNA: 4 µL of 5X reaction buffer, 2 µL of 10mM dNTP mix, 1 µL of nuclease inhibitor and 1 µL reverse transcriptase enzyme. The mixture was then incubated at 42°C for one hour, followed by inactivating the enzyme by heating at 70°C for 5 minutes. The resulting cDNA was then stored at -20°C until it was ready for either double-stranded RNA synthesis or quantitative polymerase chain reaction (qPCR) analysis.

Polymerase chain reaction (PCR) and gel electrophoresis

To amplify synthesized cDNA, 0.5 µL cDNA was combined with 0.5 µL forward primer and 0.5µL reverse primer, along with 23.5 µL of the following mix: 5 µL Taq PCR buffer, 15.875 µL distilled water, 0.5µL dNTPs, 2 µL $MgCl_2$, 0.125 µL Taq Polymerase. The resulting solution was then amplified by PCR in a thermal cycler using: an initial hold for 2 minutes at 94°C, followed by 40-50 cycles of the following thermal conditions – 94°C for 20 sec, 55°C for 30 sec and 72°C for 1 min. The final step involved keeping the reaction at 72°C for 5 min before being lowered and kept at 4°C.

To ensure that correct amplification of the gene of interest, PCR products were mixed with SYBR Safe stain and loaded into a 1.5% agarose gel in 1X TBE buffer. Gel electrophoresis was then initiated at 100V for 20 min and the DNA bands were visualized under UV light.

Cloning using TOPO© TA and synthesizing double-stranded RNA (dsRNA)

The amplified cDNA product was extracted from the gel (previously described in "Materials and Methods" in "Polymerase chain reaction (PCR) and gel electrophoresis") and purified using the MinElute Gel Extraction Kit (Qiagen). The PCR product was then cloned into the TOPO© cloning vector and transformed using this product. Plasmid DNA was extracted from transformed *E. coli* cells using the QIAprep Spin Miniprep Kit (Qiagen). Restriction digestion using *Spe1* and *Not1* linearized the plasmid DNA, which was then used for single-stranded RNA (ssRNA) synthesis.

To prepare for ssRNA synthesis, DNA concentration was first obtained using the *NanoDrop2000* to determine the amount of linearized plasmid DNA. ssRNA was then synthesized from 1 µg of plasmid DNA using MEGAscript T3 and T7 kits according to the manufacturer's instructions. Both reactions were kept at 37°C overnight and stopped by adding 30 µL ammonium acetate. ssRNAs were then isolated by adding 250 µL phenol-chloroform (pH 4.0), followed by chloroform and was finally precipitated with isopropanol. The ssRNAs were kept at -20°C overnight to allow precipitation and then resuspended in DEPC water. The two complementary ssRNA were then annealed to produce a 2 µg/µL dsRNA solution with the following setting: 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. The final annealed product was examined using gel electrophoresis to verify that annealing occurred properly and then stored at -80°C until future use.

Injecting double-stranded RNA (dsRNA) into *Tribolium* and treatment of 20E

To investigate the role of *vvl* on the different aspects of metamorphosis initiation, day 0 fifth instar *Tribolium* larvae were injected with double-stranded RNA (dsRNA). Animals were prepared for injection by first anesthetizing them on ice. A 10 µL glass capillary needle was connected to a syringe, and 0.5-1 µL dsRNA was injected into each specimen. dsRNA was injected on the dorsal side in between the segmental ridges, midway from head to tail end. Controls were injected with the same amount of bacterial *ampicillin-resistance (amp^r)* dsRNA.

To see if applying 20E could rescue *hr3* expression, day 0 fifth instar larvae were injected with *vvl* dsRNA as previously described earlier in this section, followed by the injection of either 0.15 µg of 20E or water after two days.

Quantitative RT-PCR

To better understanding of the developmental expression profile of *vvl* in *Tribolium*, RNA was isolated from the whole body of larvae that were in the final two instars (sixth and seventh), as well as from prepupae. Three biological replicates were generated. To investigate how genetic knockdown of *vvl* affects JH biosynthesis and signaling, the expressions of JH-target gene *kr-h1*, the JH-receptor *Met* and the JH-biosynthesis gene *jhamt3* were measured. Day 0 fifth instar larvae were injected with *vvl*

dsRNA or *amp*^{r} dsRNA, and their RNA was isolated and cDNA was synthesized as discussed previously in the “Methods and Materials” in “Isolating RNA and synthesizing cDNA”. Thus, to observe the expression of *vvl* in *Tribolium* larvae, gene expression analysis was conducted on the fat body, the gut, the epidermis and the central nervous system (CNS) pooled from twenty day 0 seventh instar animals.

The effect of *vvl*-knockdown on ecdysone signaling was analyzed by examining the expression of two ecdysteroid-inducible genes, *E75* and *hr3.* How removal of *vvl* influences ecdysone biosynthesis was also examined through analyzing, in *vvl*-knockdowns, the expression of five ecdysone biosynthesis genes: *spo*, *phm*, *dib*, *sad* and *shd*. Day 0 fifth instar larvae were injected with either *vvl* or *amp*^{r} dsRNA and were dissected and assayed four days later for ecdysone biosynthesis gene expression using RT-PCR.

To determine the localization of *vvl* (either in the prothoracic glands or the oenocytes), and how it may influence ecdysone biosynthesis, larvae were split into the anterior (containing the thorax) and posterior (containing the abdomen) halves. Biological triplicates for each treatment were prepared. To determine if *spo* expression in the oenoctyes of the *vvl*- knockdown animals was decreased, day 0 fifth instar KT817 larvae were injected with either *vvl* or *amp*^{r} dsRNA, and oenocytes and fat body collected from fifteen biological samples were separately isolated on day 4. SsoAdvanced SYBR Green Supermix (Bio-rad) was used for qRT-PCR analyses. All qPCR analyses were performed in triplicates.

Manduca body dissections

To examine the tissue-specific expression of *vvl* in *Manduca sexta*, larval specimens were raised until the fourth instar and dissected for isolated tissue samples. Instars (fourth or fifth) and day numbers (0-5) were tracked, and larvae were dissected for various tissues: central nervous system and corpora allata complex (CNS/CA), prothoracic glands (PG), epidermis and fat body. For the fourth instar animals, 10 samples of PG and CNS/CA and three samples of fat body and epidermis were collected. For the fifth instar animals, five samples of PG and CNS/CA and three samples of fat body and epidermis were collected. Samples were then prepared for RT-PCR to analyze localized genetic expression of *vvl*. To analyze the link between critical weight and expression of *vvl* and the timing of metamorphosis in *Manduca,* larvae were fed either a normal diet or a non-nutritive starvation diet, and after isolating the CNS+CA complex, qPCR was used to gauge levels of genetic expression, paying close attention to the critical weight.

RESULTS

vvl expression in *Tribolium*

In order to examine the expression profile of *vvl* in the late larval and prepupal stages of *Tribolium castaneum*, mRNA was harvested from whole body samples of larvae in the sixth and seventh larval instars, and prepupae, converted into cDNA and amplified and analyzed for *vvl* expression using quantitative PCR (qPCR) (Fig. 1A). *vvl* expression was the highest on day 0 of the sixth instar, but then dropped to a low level by day 3. Expression then rose again to a high level, coinciding with the molt into the final instar. During the seventh instar, *vvl* expression gradually declined until entry into the prepupal stage, which was marked by *vvl* expression intensifying again to a higher level. This seemingly erratic rise and fall observed in *vvl* expression is actually similar to the oscillating levels observed in ecdysteroids and JH expression during the penultimate and final instars.

To determine expression of *vvl* in specific tissues of *Tribolium*, its expression was analyzed in the central nervous system (CNS)/corpora allata (CA) complex, epidermis, fat body and gut of day 0 seventh instar larvae. Highest expression was observed in the CNS/CA complex and also in the epidermis, with low expression found in the fat body and gut (Fig. 1B). This is consistent with the idea of *vvl* being a regulator of JH biosynthesis, because JH is produced in the CA and *vvl* might act directly in the CA or indirectly via the CNS.

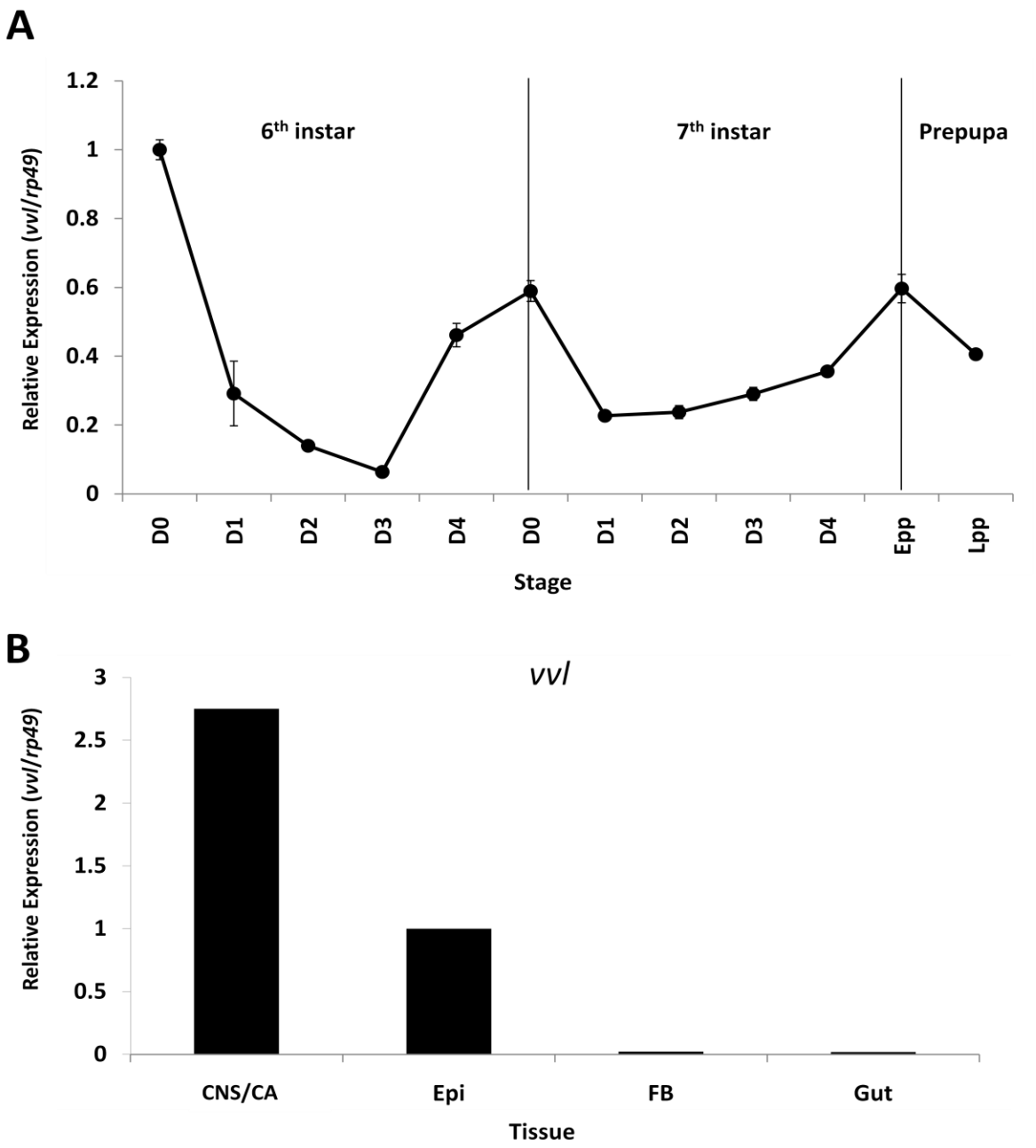

Figure 1. Expression profile of *vvl* in *Tribolium* larvae. A) Expression profile of *vvl* in the whole body of *Tribolium* during the late larval and prepupal stages. Expression determined by qPCR of *vvl* from whole body sixth and seventh instars and prepupae. *Ribosomal protein 49 (rp49)* was used as the standardizing control. For all treatments, each sample consisted of RNA pooled together from five sixth instars, three seventh instars and three prepupae. Three biological replicates were used per treatment, and each sample was run in triplicates. B) Expression of *vvl* in seventh instar day 0 *Tribolium* larvae. Expression of *vvl* in the CNS and CA, epidermis, fat body and gut of day 0 seventh instar larvae. mRNA was isolated from tissues that was pooled from 20 specimen.

vvl-knockdown results in reduction of *jhamt3* expression

Early onset metamorphosis observed in the *vvl* dsRNA-injected animals suggested that the JH pathway is interrupted in the *vvl*-knockdown animals. To determine whether JH biosynthesis or JH sensitivity was influenced, the expression of *Met*, which produces the JH receptor Met, and the expression of *jhamt3*, a JH biosynthesis gene, was analyzed. JHAMT3, an enzyme that produces JH from JH acid, is important for JH biosynthesis, evidenced by how silencing its expression in *Tribolium* results in precocious metamorphosis. Thus, I investigated whether the knockdown of *vvl* affects the expression of *jhamt3*. Fifth instar day 0 *Tribolium* larvae were injected with either *vvl* or *amp*^{r} dsRNA, which were then prepared for qPCR to analyze for expression of *met* or *jhamt3* on day 4. Though the expression of *Met* was not affected by *vvl*-knockdown (Fig. 2A), the expression of *jhamt3* was significantly reduced in larvae that were treated with *vvl* dsRNA (Fig. 2B). Thus, *vvl* appears to play an important role in JH biosynthesis, and not JH sensitivity, by regulating *jhamt3* expression.

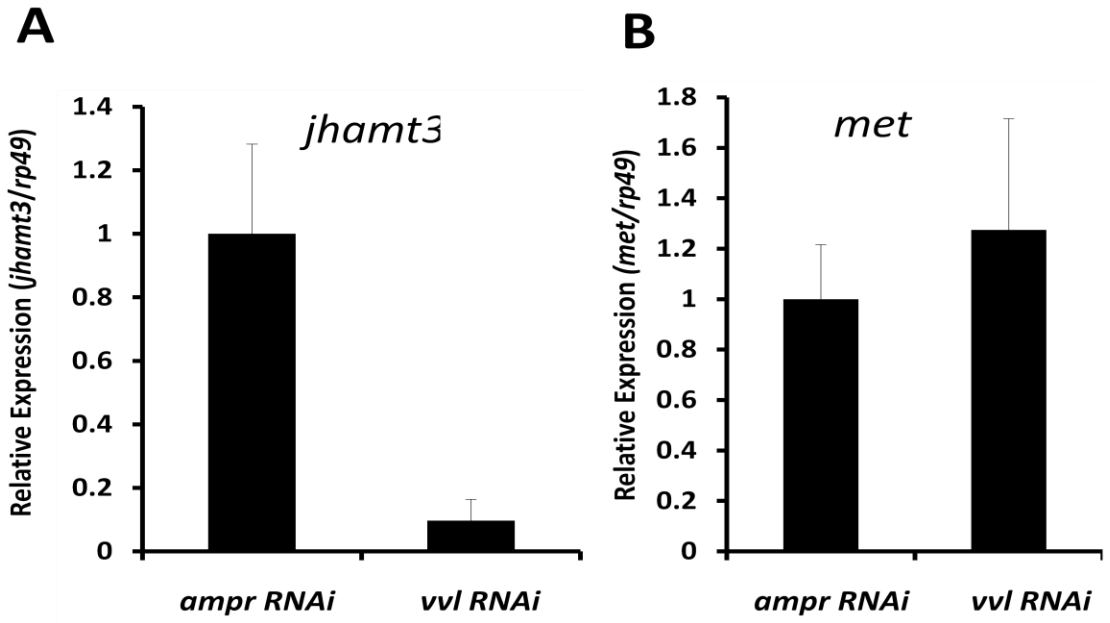

Figure. 2. *jhamt3* (a JH biosynthesis gene) expression is decreased in larvae injected with *vvl* dsRNA. (A) Effect of *vvl*-knockdown on *jhamt3* expression ($p < 0.05$). (B) Effect of *vvl*-knockdown on *met* expression ($p = 0.60$; Student's t-test). Three biological replicates were used in each treatment, with each sample being run in triplicates and *ampr* dsRNA-injected specimens as the negative control. For (A) and (B), each sample consisted of RNA pooled from five fifth instar larvae. Data are represented as mean +/- SEM.

vvl-knockdown results in reduction of *kr-h1* expression

To further examine whether *vvl* influences the expression of a target gene downstream of the JH pathway, quantitative PCR was conducted on *Tribolium* larvae on day 4 of the fifth instar that were previously injected with *vvl* dsRNA and treated with either 15 µg of the JH analog methoprene or acetone four days prior to RNA isolation. Another group of animals were treated in a similar manner, except they were injected with *amp*^{r} dsRNA, and were used as a comparison. As seen in Figure 3A, expression of *kr-h1* was decreased in the *vvl*-knockdown larvae in comparison to the *amp*^{r} dsRNA-injected larvae. However, in larvae treated with ectopically applied methoprene following injection of *vvl* dsRNA on day 0 of the fifth instar, *kr-h1* expression recovered a normal level, when compared to that of the *amp*^{r} dsRNA-injected animals treated with methoprene (Fig. 3B). This suggests that *vvl*-knockdown leads to decreased *kr-h1* expression, which can be rescued with the treatment of methoprene, further supporting that JH biosynthesis, rather than reception, is affected by the presence and/or absence of Vvl.

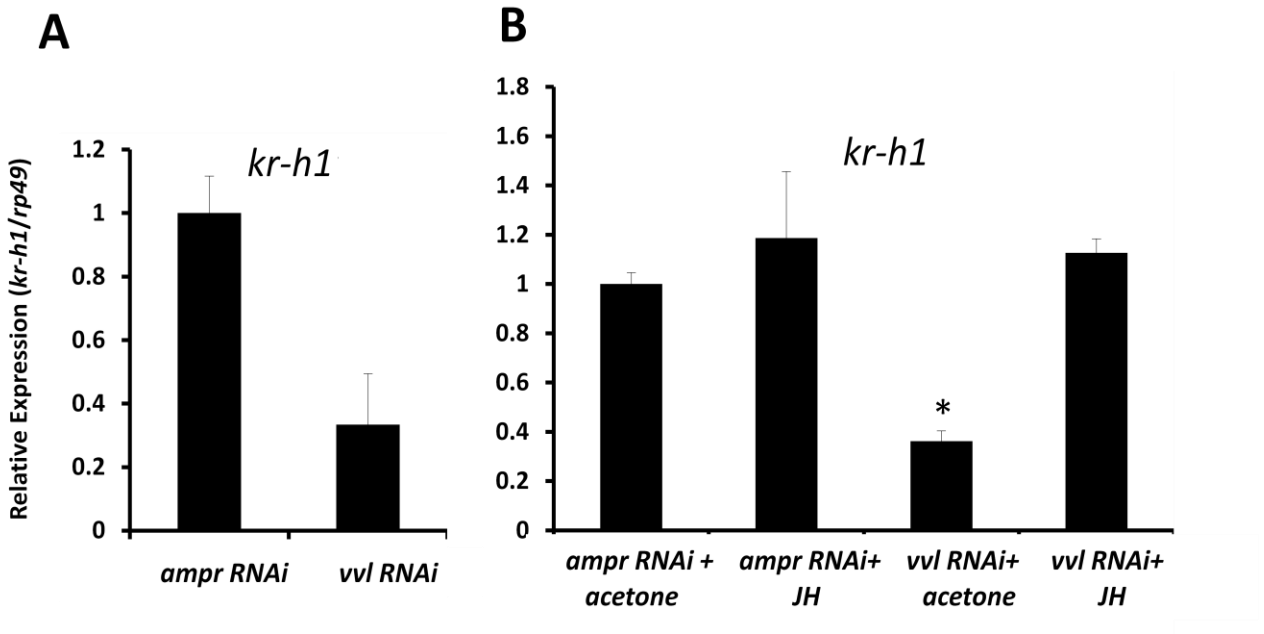

Figure 3. *kr-h1* (a JH-target gene) expression is decreased in larvae injected with *vvl* dsRNA, but expression in *vvl*-knockdown larvae is rescued with JH application. (A) Effect of *vvl*-knockdown on *kr-h1* expression ($p < 0.05$; Student's t-test). (B) Effect of ectopic methoprene application on the *kr-h1* expression in *vvl* dsRNA-injected animals. *vvl*-knockdown larvae and *amp*^{r}–knockdown animals were treated with either acetone (control) or methoprene (15 µg) on day 0 of the fifth instar. * indicates significantly lowered level relative to other treatments (ANOVA with Tukey HSD-test). Four biological replicates were used per treatment, and each sample was run in triplicates. Data are represented as mean +/- SEM.

vvl-knockdown larvae exhibit decreased ecdysone-response gene expression

Because *vvl*-knockdown larvae fail to molt, ecdysteroid signaling pathway, an essential part of the molting process, was analyzed in the *vvl* dsRNA-injected animals. To determine whether Vvl influences ecdysteroid signaling, I looked at the expression of two ecdysone-response genes associated with molting, *E75* and *hr3*, in both *vvl*-knockdown and *amp*^{r}-knockdown larvae that were treated four days before qPCR analysis, right before the control larvae entered the sixth instar. Though the expression level of *E75*, the ecdysone inducible early gene, did slightly decrease in the animals injected with *vvl* dsRNA, the difference was not enough to be significant (Fig. 4A). On the other hand, the expression of *hr3*, an ecdysone-inducible gene, was significantly reduced in animals injected with *vvl* dsRNA (Fig. 4B). To see whether application of 20E can rescue the expression of *hr3* when *vvl* is silenced, day 0 fifth instar larvae were injected with *vvl* dsRNA, followed by injection of either 0.15 µg of 20E or water two days later, and dissected six hours later. Figure 4C shows that larvae injected with 20E had significantly higher levels of *hr3* expression, suggesting that introducing 20E into the system can rescue the expression of *hr3* in *vvl*-knockdown organisms. These results strongly suggest that, in the *vvl*-knockdown animals, ecdysteroid signaling is disrupted and that ecdysteroid biosynthesis rather than sensitivity is affected. Thus, Vvl may have a dual role as both an activator of JH signaling and as a regulator of molting through its influence on ecdysteroid signaling.

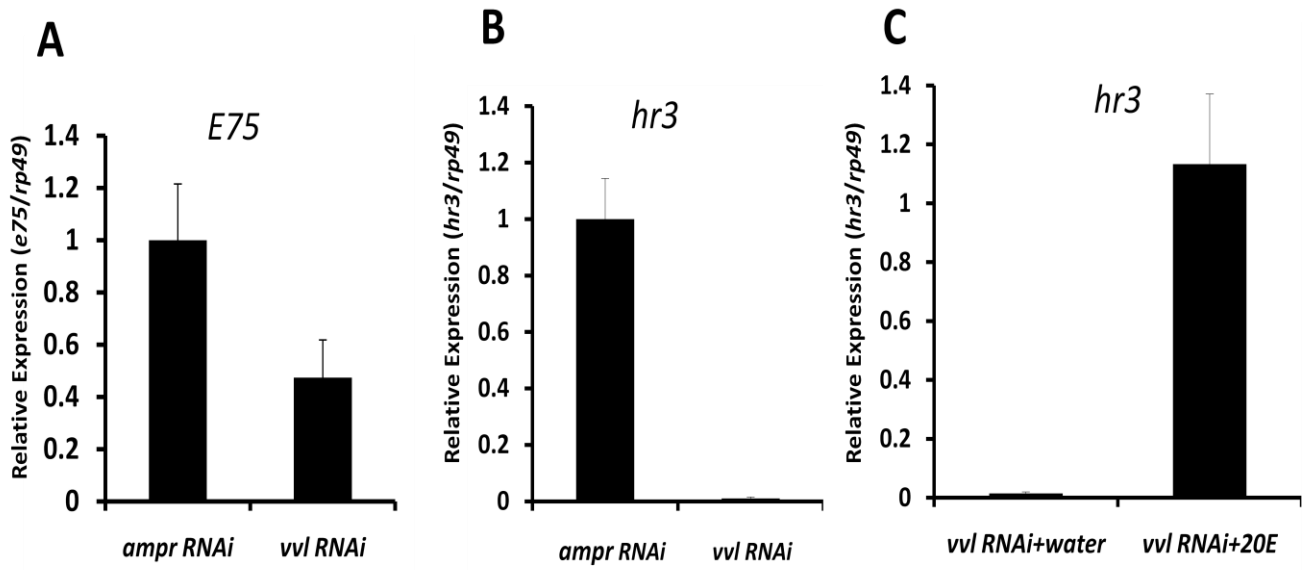

Figure. 4. Effect of *vvl*-knockdown on the expression of ecdysone-regulated genes. (A) Effect of *vvl*-knockdown on *E75* expression (p = 0.09; Student's t-test). (B) Effect of *vvl*-knockdown on *hr3* expression (p <0.005). (C) Effect of 20E application in *hr3* expression in *vvl* dsRNA-injected animals (p = <0.001; Student's t-test). Three biological replicates were used in each treatment, with each sample being run in triplicates and *amp*^{r} dsRNA-injected specimens as the control. For (A) and (B), each sample consisted of RNA pooled from five larvae and in (C), each biological replicate consisted of RNA pooled from three larvae. Data are represented as mean +/- SEM.

***vvl*-knockdown influences the expression of certain Halloween genes**

A past study used a GFP enhancer trap line that expresses GFP under the control of the *vvl* enhancer, and found that, during the early larval stages, oenocytes of *Tribolium* larvae exhibit expression of GFP (Burns et al., 2012). Based on this find, we observed late sixth instar larvae and found GFP expression in localized structures of the abdominal area that were identified as oenocytes (Fig. 5A, 5B).

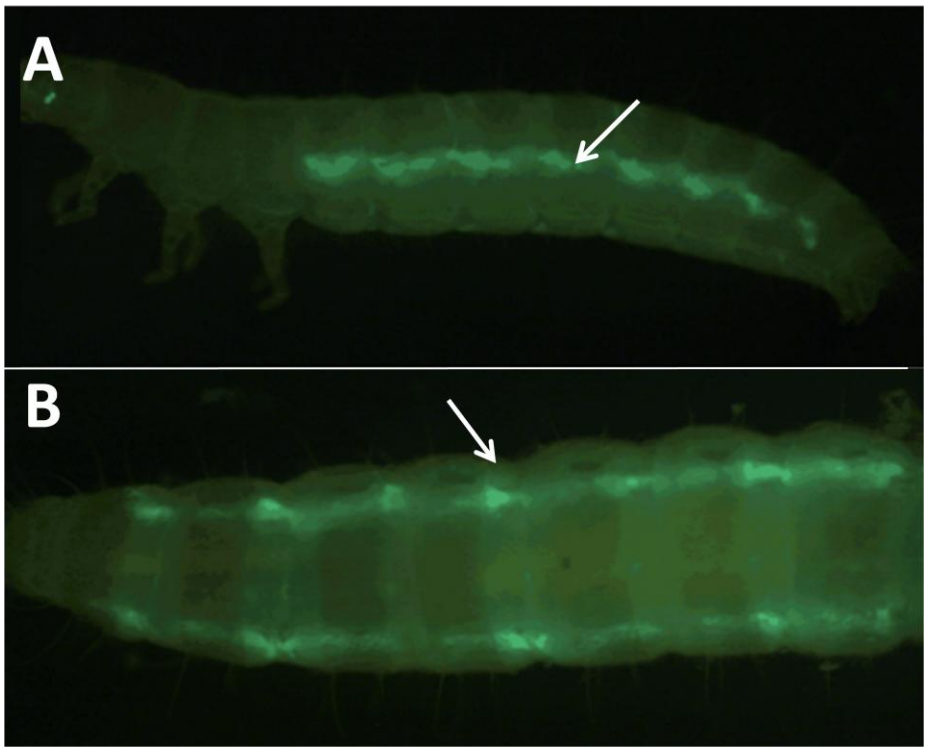

Figure 5. GFP image showing *vvl* expression in a late sixth instar larval whole body. (A-B) Vvl-GFP expression in oenocytes of sixth instar KT817 enhancer trap line from (A) lateral and (B) dorsal view. White arrows indicate location of oenocytes.

Because oenocytes have been found to be possible sites of ecdysone synthesis in another species of beetle, we hypothesized that *vvl* may be linked to ecdysone biosynthesis in the oenocytes. To pinpoint where ecdysone biosynthesis was being altered, we first deduced that the two candidates were either the prothoracic glands or the oenocytes. *Tribolium* larvae were thus injected with either *vvl* or *amp*^{r} dsRNA, and cut in half into posterior and anterior sections, which were then analyzed for ecdysone biosynthesis gene expression using quantitative PCR. In this manner, expression of several ecdysone biosynthesis "Halloween" genes (*phm*, *spo*, *sad*, *shd*, *dib*) in the knockdown animals were examined. Results showed that *phm* expression was lower in the anterior sections of *vvl*-knockdown animals compared to *amp*^{r}-knockdown animals, while the posterior expression of *phm* in the *vvl*-knockdowns remained unchanged compared to the *amp*^{r}-knockdowns (Fig. 6A,6B).

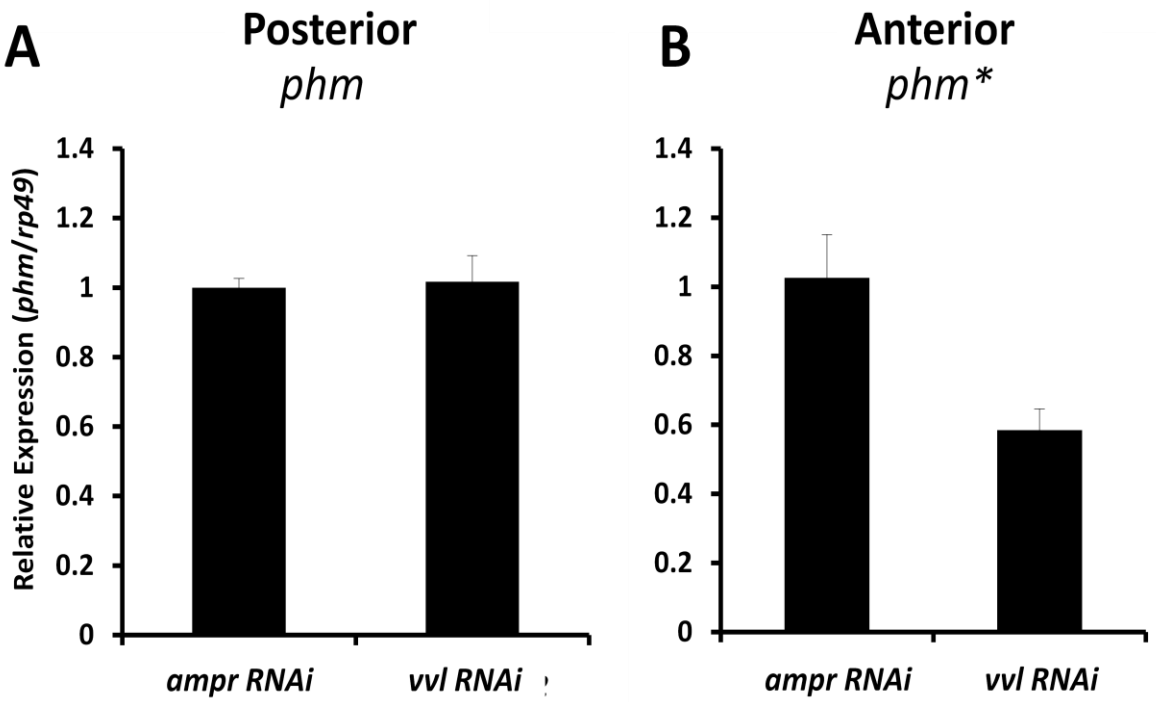

Figure 6. *phm* expression in *vvl*-knockdown larvae was reduced in the anterior. (A-B) *phm* expression in *amp*^{r} and *vvl* dsRNA-injected instar larvae on day 4 in (A) the posterior, containing the abdomen ($p = 0.85$), and (B) the anterior, containing the head and thorax ($p < 0.05$). Three biological replicates were used per treatment, and each sample was run in triplicates. Each biological sample consisted of pooled RNA from five larvae. Data are represented as mean +/- SEM.

In the *spo* expression, however, we saw the reverse. Expression of *spo* was decreased significantly in the posterior section of the *vvl*-knockdown larvae (Fig. 7A). Interestingly, the expression of *spo* in the anterior section of the *vvl*-knockdown specimen did not differ significantly from that of the *amp*^{r}-knockdown animals (Fig. 7B). To further investigate if *spo* expression was reduced in the oenoctyes of the animals injected with *vvl* dsRNA, either *vvl* or *amp*^{r} dsRNA was injected into 0 fifth instar animals and on day 4 the oenocytes with their associated tissues were collected. In the oenocytes of the *vvl*-knockdown larvae, we saw that *spo* expression was reduced compared to those of the *amp*^{r}-knockdown larvae (Fig. 7C). Fat body from the dsRNA-injected larvae was also collected, which showed that *spo* expression was slightly increased in the *vvl*-knockdown animals compared to the controls.

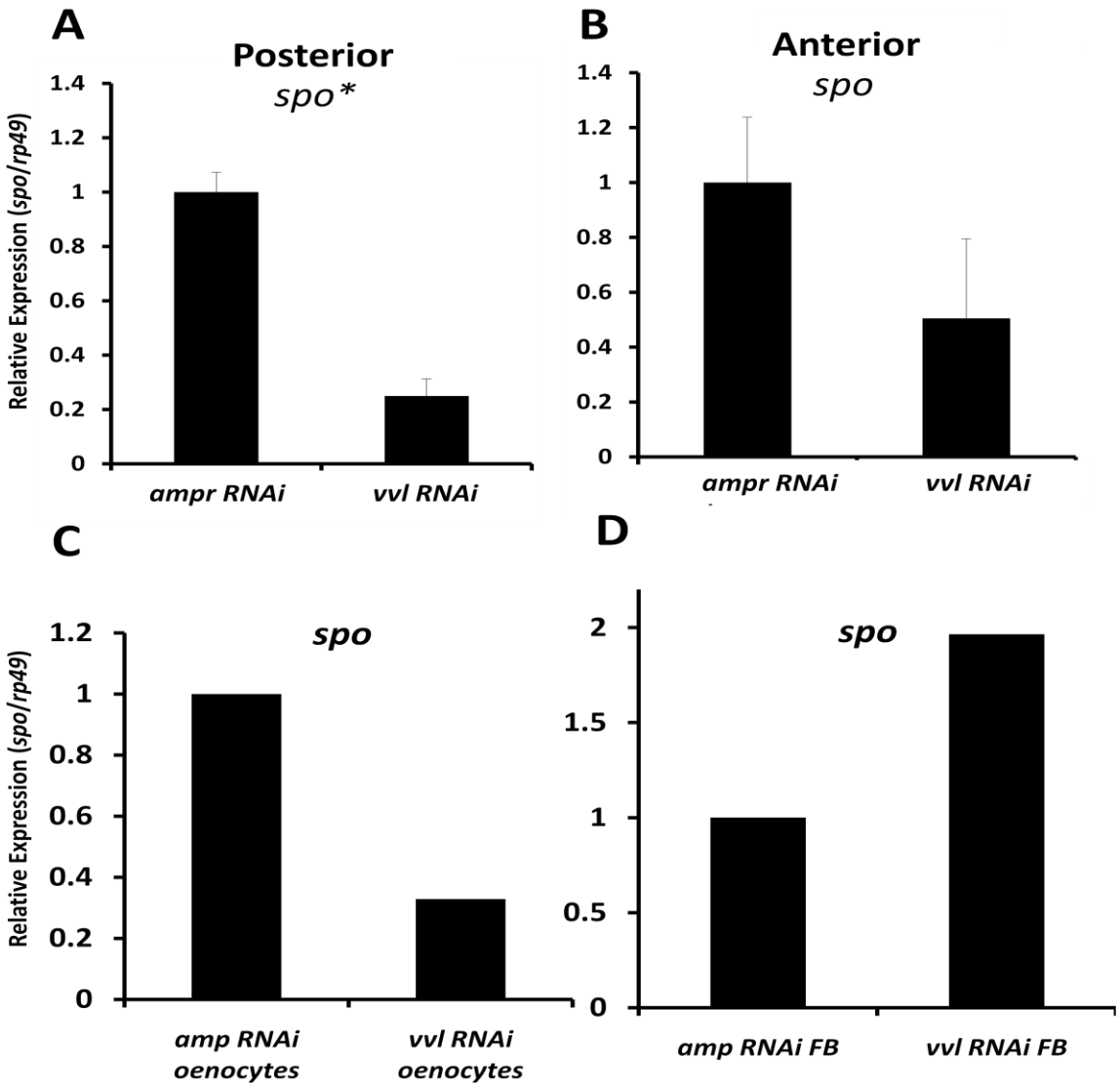

Figure 7. *spo* expression in *vvl*-knockdown larvae was reduced in the posterior. (A-B) *spo* expression in *amp*^{r} instar larvae on day 4 in (A) the posterior, containing the abdomen ($p < 0.05$), and (B) the anterior, containing the head and thorax ($p = 0.27$). Three biological replicates were used per treatment, and each sample was run in triplicates. Each biological sample consisted of pooled RNA from five larvae. (C) Expression of *spo* in oenocytes of day 4 fifth instar KT817 larvae with *amp*^{r} and *vvl* knockdowns. Oenocytes pooled from 15 larvae per treatment. (D) Expression of *spo* in fat body of day 4 fifth instar KT817 larvae with *amp*^{r} and *vvl* knockdowns. Fat body pooled from 15 larvae per treatment. Data are represented as mean +/- SEM.

For the Halloween genes *sad, shd* and *dib*, there was no difference between the expression of *vvl* and *amp*^{r} dsRNA-injected larvae in either the anterior and posterior sections (Fig. 8A-F). All together, our results suggest that Vvl may control the synthesis of ecdysone in different areas by selectively influencing the expression of certain ecdysone biosynthesis genes.

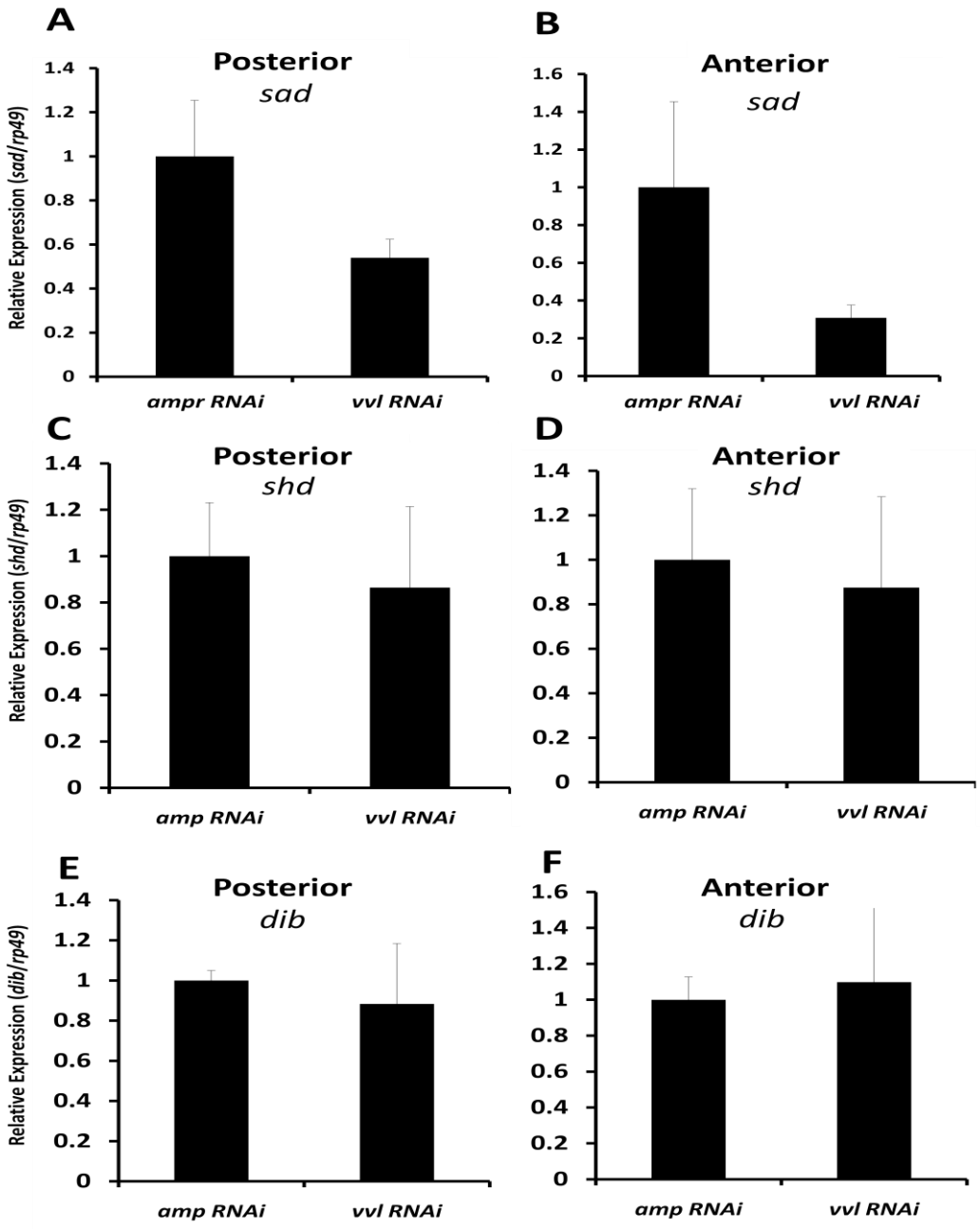

Figure 8. *sad*, *shd* and *dis* expression in *vvl*-knockdown larvae in the anterior and posterior portions. (A-F) *sad*, *shd* and *dis* expression in *amp*^{r} and *vvl*-knockdown fifth instar larvae on day 4 in (A,C,E) the posterior, containing the abdomen (respective p-values=0.18; 0.75; 0.74), and (B,D,F) the anterior, containing the head and thorax (respective p-values=0.20; 0.80; 0.71). Three biological replicates were used per treatment, and each sample was run in triplicates. Each biological sample consisted of pooled RNA from five larvae. Data are represented as mean +/- SEM.

Effect of starvation on *vvl* expression in *Manduca*

To determine the expression of *vvl* in *Manduca*, *vvl* expression was analyzed in several tissues of penultimate (fourth) and final (fifth) instar larvae. Larvae were raised until they reached the fourth instar and were dissected daily to isolate total RNA from the central nervous system (CNS)/corpora allata (CA) complex and prothoracic glands (PG). In the CNS/CA, minimal expression of *vvl* was observed during the fourth instar, but higher levels were seen in the fifth instar (Fig. 9A). This suggests that there is a distinct change in the expression of *vvl* in the CNS during the final instar. In addition, the expression of *vvl* was also low on day 3 of the fifth instar, which coincides with the timing of critical weight attainment. Thus, a decline in the *vvl* expression might be required for the signal to release PTTH which marks the attainment of critical weight. In the PG, high expression of *vvl* was seen on day 0 of both the fourth and fifth instars (Fig. 10).

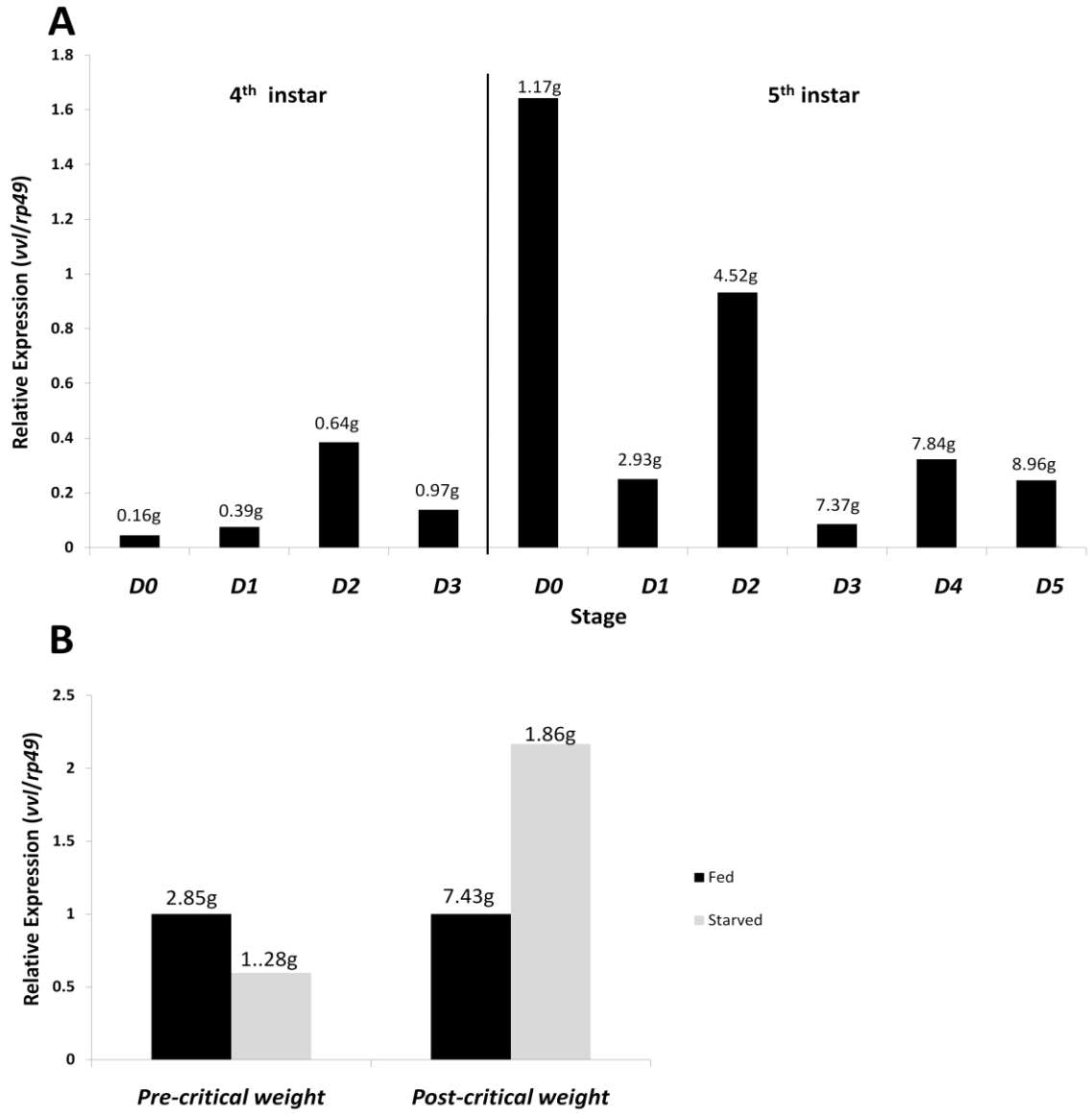

Figure 9. Expression of *vvl* in CNS/CA tissue samples in *Manduca* larvae at various days of the fourth and fifth instars. A) *vvl* expression at various stages of the fourth and fifth instar. Ten animals were dissected from the fourth instar and five samples from the fifth instar. Specimens were fed on normal diet and central nervous systems were collected when animals reached different stages of the fourth and fifth instars. B) *vvl* expression in CNS/CA tissue samples of fed or starved animals at various stages of the fifth instar. CNS/CA tissue was isolated from dissected animals at the fifth instar on day 0, day 1 (pre-critical weight: fed average weight = 2.85g, starved average weight =1.28g) and day 3/4 (post-critical weight: fed average weight =7.43g, starved average weight =1.86g). Animals to be dissected on day 1 and day 3/4 were either continued to be fed on a normal diet or starved with non-nutritional diet. Starved animals weighed much less than critical weight on both days. *vvl* expression was quantified using qPCR, with *rp49* used as the standardizing control. Average weights are indicated above each bar.

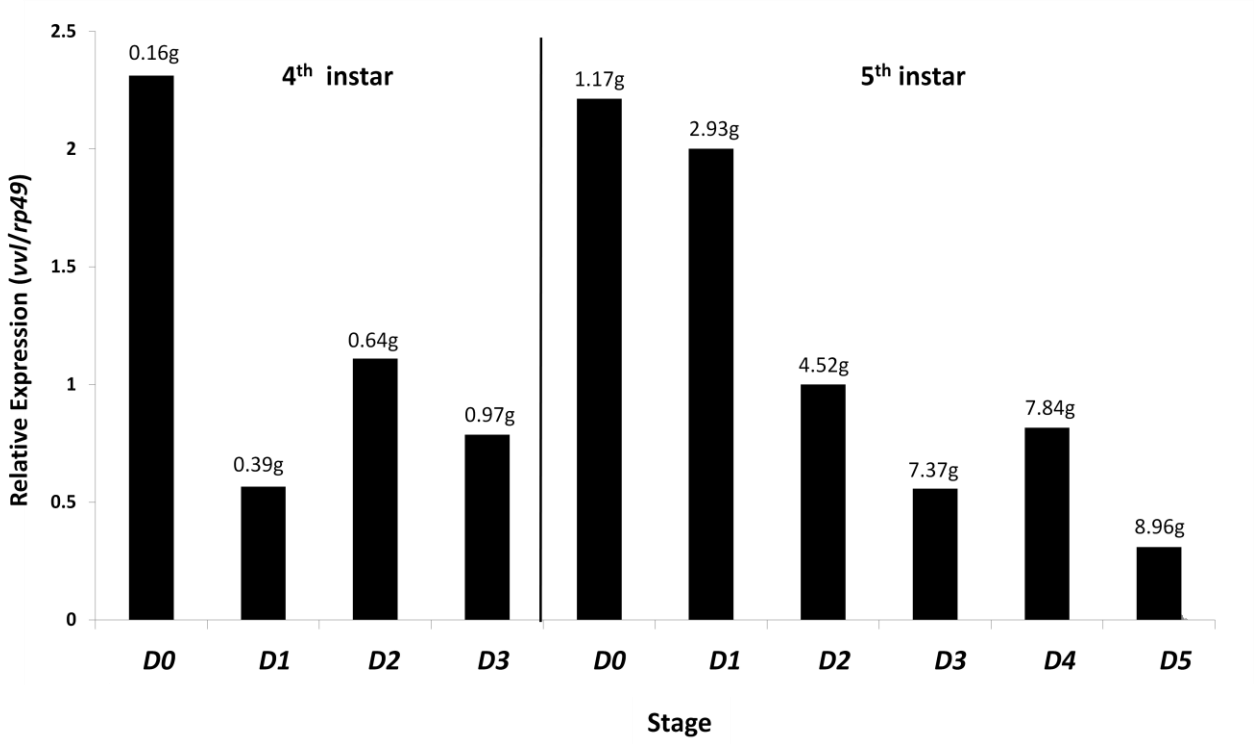

Figure 10. Expression of *vvl* in PG tissue samples in *Manduca* larvae at various days of the fourth and fifth instars. Ten animals were dissected from the fourth instar and five samples from the fifth instar. Specimens were fed on normal diet and the prothoracic glands were collected when animals reached different stages of the fourth and fifth instars. *vvl* expression was quantified using qPCR, with *rp49* used as the standardizing control. Average weights are indicated above each bar.

To further investigate the possibility of the role of *vvl* in the timing of metamorphosis and its relationship to nutritional intake, the effect of inadequate nutrition on the expression of *vvl* in *Manduca* larvae was analyzed. Larval specimens were fed on a normal diet until they reached the fifth instar, and upon reaching this cornerstone, the animals were separated into two different feeding groups: larvae were continued to be fed on a normal diet or larvae were fed starvation diet for two days. All fifth instar animals were then dissected on day 1 for pre-critical weight, and on day 3-4 for post-critical weight, in which the CNS/CA complex was collected for quantitative analysis using qPCR. Expression of *vvl* was high on day 0 (not shown), and I saw that during the pre-critical weight, the starved animals have less *vvl* expression compared to the fed animals (Fig. 9B). However, once the larvae passed the critical weight, thus reaching the post-critical weight, the starved animals had than 2-fold higher *vvl* expression compared to the fed animals.

Taken together, the results of these experiments strongly suggest that *vvl* plays an important role in regulating the timing of metamorphosis by controlling both JH and ecdysone biosynthesis.

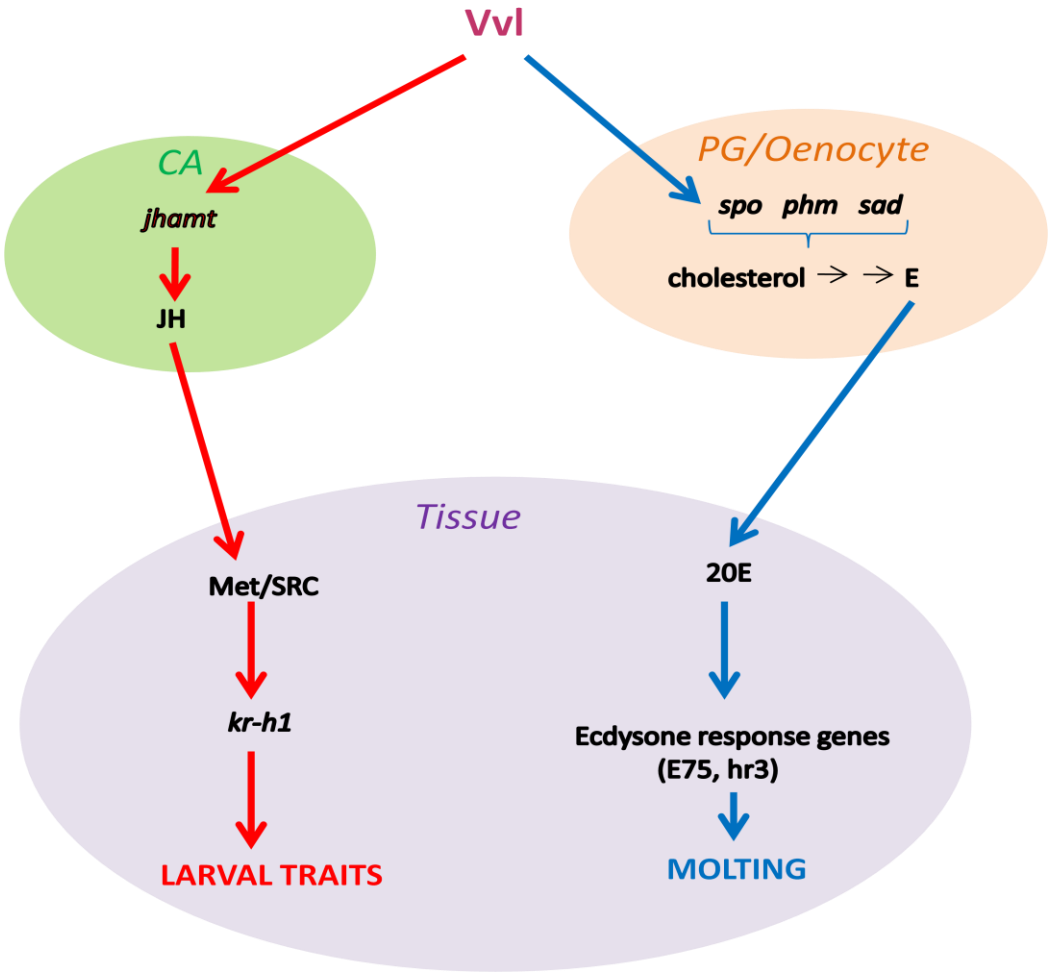

Figure 11. Theoretical mechanism of Vvl action in determining initiation of metamorphosis. Vvl influences JH production by regulating *jhamt* expression in the corpora allata (CA), thus determining the timing of metamorphosis. Vvl also plays a role in the ecdysteroid biosynthesis, as well as the molting pathway through the Halloween genes in the prothoracic glands (PG) and oencytes.

DISCUSSION

We have previously seen that the whole body expression of *vvl* correlates with the levels of juvenile hormone (JH) and ecdysone (Cheng, 2013). Thus, we investigated the effects of *vvl*-knockdown on various JH and ecdysone genes in *Tribolium castaneum* larvae. In addition, we observed *vvl* expression in localized tissue areas of *Manduca sexta* during its last two larval instars, which contain crucial size-assessing points that specify the timing of the initiation of metamorphosis. The results of this study present new and exciting revelations on the complex and multifaceted role of *vvl* in the timing of the initiation of metamorphosis in *Tribolium* and *Manduca*, supporting the notion that the gene may be the link that bridges the gap between the two hormonal pathways important to metamorphosis initiation (Fig. 11). By knocking down *vvl* expression in larval *Tribolium*, we identified a potential mechanism by which *vvl* regulates the physiological aspects of early insect development. I found that Vvl regulates JH biosynthesis, and that *vv*-knockdown affects the expression of an ecdysone-response gene, as well as a series of genes involved in the biosynthesis of ecdysone.

Juvenile hormone

The role of Vvl in JH biosynthesis

In invertebrates, the role of JH is to maintain the organism in its larval stage so that it does not pupate in an untimely fashion. Thus, production of this hormone and its regulation is essential during the process of metamorphosis. As aforementioned, JH

determine the nature of the molt and acts as a *status quo* hormone (Riddiford, 1996). In the fifth larval instar, *Manduca* ready themselves for pupation by initiating JH levels to drop, thus signaling that the animals are ready to metamorphose (Nijhout and Williams, 1974b). As a whole, JH production is monitored by JH acid methyltransferase 3 (*jhamt3*), a gene involved in JH biosynthesis. Our results show that the expression of *jhamt3* expression in *Tribolium* is dramatically decreased in *vvl*-knockdown larvae, indicating that *vvl* might play a direct role in the production of JH. A recent study also found that *vvl* was expressed in the corpora allata (CA) of *Drosophila* embryos, implying that there may be a direct relationship between Vvl and JHAMT in the CA (Sanchez-Higueras et al., 2013).

To better study the expression of *vvl* and the areas in which it is heavily expressed, we investigated the gene of interest in another model system, the *Manduca*. Because of its larger size, *Manduca* are excellent organisms to dissect and analyze localized areas of tissue-specific expression of *vvl*. We saw that *vvl* expression in whole body samples of *Tribolium* larvae was high at the start of the sixth and seventh instars, and that the CNS/CA complex of day 0 seventh instar larvae had a very high expression of *vvl*. Similarly, CNS of day 0 of the fifth instar *Manduca* larvae also expressed high levels of *vvl* expression (Fig. 9). However, *Manduca* had lower levels of *vvl* in the CNS the second–to-last instar before pupation. The difference in *vvl* expression between the penultimate and final instars may be due to several reasons. It is possible that during the final instar, *vvl* may be activated in the CNS in preparation for metamorphosis, especially since Vvl has been shown to regulate neuronal development in *Drosophila*. In addition, if *vvl* is indeed a dual regulator of JH and ecdysone as this study suggest,

there is a possibility that *vvl* may also have multiple other neuroendocrine functions in the CNS besides monitoring the biosynthesis of these two important developmental hormones. For example, in *Bombyx*, a homolog of Vvl, POUM2, has been shown to regulate the expression of Diapause hormone and pheromone biosynthesis-activating neuropeptide (Zhang et al., 2004).

In addition, the difference between fourth and fifth instars observed in CNS of day 0 *Manduca* may be due to the fact that the brain changes in its response to JH during different instars. In the fourth instar, the release of PTTH from the brain remains insensitive to JH, but in the final instar, it becomes sensitive to the hormone. Since we found that Vvl regulates ecdysteroid biosynthesis gene expression, *vvl* may underlie the change in the brain's responsiveness to PTTH secretion that is found in the final instar brain. Future studies would benefit from an examination of *ptth* expression in the brain of *vvl*-knockdown larvae.

Nutritional effects on Vvl at critical weight

In insects such as *Manduca*, the critical weight, or a specific size that an organism needs to obtain for it to metamorphose without developmental delay under starvation conditions, plays an important role in determining the timing of metamorphosis. Nutrition plays an important role in the regulation of the neuroendocrine system, and thus, the onset of metamorphosis. In *Manduca*, the critical weight is associated JH clearance, which then initiates the release of PTTH secretion and signaling the initiation of metamorphosis (Nijhout and Williams, 1974b). The decrease in JH levels following the attainment of the critical weight sanctions the release of PTTH,

resulting in the PGs to start up the synthesis of ecdysone (Nijhout and Williams, 1974a, b). Thus, if larvae are starved before they reach the critical weight, JH clearance is delayed, therefore disrupting when an organism pupates and delaying metamorphosis. To further understand and establish the potential relationship between *vvl* and critical weight, the effect of starvation on pre-critical and post-critical weights was compared. The increased expression of *vvl* observed in the day 3/ 4 starved animals compared to the fed post-critical animals suggests that JH production remains high in starved animals, possibly contributing to the delay of metamorphic entry. These preliminary findings suggest that there is a potential difference in responsiveness to *vvl* between the fourth and fifth instar depending on the CA or the CNS. This suggests that *vvl*, which plays a crucial role in JH biosynthesis, may also be involved in how and when critical weight is reached. It would be interesting for future studies to isolate only the CA and to see whether the expression in these different localized tissue samples is dramatically altered.

The implications of these findings are fascinating and truly exciting. As mentioned before, the critical weight has been suggested to be determined by an unidentified oxygen sensor (Callier and Nijhout, 2011). In arthropods, endocrine glands and trachea were previously believed to be two unrelated and dissociated systems that independently evolved due to various adaptive pressures (Grillo et al., 2014). However, recent studies show that the embryonic primordia of the CA and the prothoracic gland are in fact homologous to the primordial of tracheal cells (Sanchez-Higueras et al., 2014). Sanchez-Higueras et al. (2014) show that in *Drosophila melanogaster*, the CA, prothoracic gland and tracheal primordia may have originated at the same location in

regions of the head and trunk, having homologous origin and the ability to turn into each other. During development, all of these structures are derived from cells that express *vvl*. Because *vvl* expression also influences the establishment of the endocrine and tracheal primordia, the gene may also be involved in the oxygen-sensing mechanism that regulates insect development. My findings that Vvl regulates endocrine regulators suggest that *vvl* may link together aspects of the endocrine system with the physiological mechanism of respiratory tracheal development in insects, and potentially other invertebrates. Thus, oxygen concentration may affect ecdysone and JH levels via Vvl. Additional studies will be necessary, however, to provide further evidence of this possible link.

Ecdysone

The role of Vvl in ecdysone biosynthesis

Previous results have shown that *vvl*-knockdown larvae not only experience early onset metamorphosis, but also fail to molt (Cheng, 2013). This led us to believe that Vvl may have an activating role on the ecdysteroid signaling pathway. A recent study analyzed the ecdysteroid titers of *Tribolium* injected with *vvl* dsRNA, and found that the *vvl*-knockdown animals had overall lower ecdysteroid titers compared to the control animals (Cheng et al., in press). This finding supplies further evidence that the regulation and expression of *vvl* influences the production and regulation of ecdysteroids.

From the results of this study, we also found that the expression of *hr3,* the ecdysone response gene, was significantly reduced in *Tribolium* injected with *vvl*

dsRNA, but treatments of 20E could rescue normal expression of *hr3*, giving substantial evidence that *vvl* plays a role in the regulation of ecdysone production, rather than response (Fig. 2B,C). We also saw that, even though exogenous 20E treatments rescued the expression of *hr3,* it failed to rescue the ability of the larvae to molt (not shown), implying that Vvl might influence a more downstream aspect of the molting process rather than the early ecdysone biosynthesis signaling pathway. In insects, 20E controls the expression of *E75* and *hr3* (Segraves and Hogness, 1990; Palli et al., 1992; Jindra et al., 1994). The difference in expression observed in the two ecdysone response genes *hr3* and *E75* may be due to the fact that rise in *E75* comes prior to the rise in *hr3*, because *E75* is an “early” gene and *hr3* is an “early-late” gene in the biosynthesis of 20E (Christiaens et al., 2010). In this pathway, “early” genes are responsible for upregulating “early-late” gene, and there is a time lapse between the expressions of these two genes. Because *hr3* is downstream of *E75*, it could be more sensitive to the *vvl*-knockdown.

Localized expression of Vvl implies various sites of ecdysone production

We analyzed the effect of *vvl*-knockdown on the expression of various Halloween genes to better understand the role of *vvl* in ecdysone biosynthesis, and saw that the expression of *phm* and *spo*, two genes involved in ecdysone biosynthesis, in *vvl*-knockdown animals were different depending on the sections of the body. Whereas *phm* expression was only lowered in the anterior section, *spo* expression was only lowered in the posterior section (Fig. 6, Fig. 7). Furthermore, I found evidence that *spo* expression in the oenocytes was one target of Vvl. Based on previous studies observing

oenocytes in other insect species, such as *Tenebrio molitor*, we know that these sites synthesize ecdysone from cholesterol (Romer et al., 1974). Thus, Vvl might play a role in the oenocytes in addition to the prothoracic glands to regulate the process of ecdysone biosynthesis.

Due to the disparity observed between *spo* and *phm* expression in the posterior vs. anterior when *vvl* is knocked down, we are inclined to believe that Vvl plays different roles in controlling ecdysteroid production depending on the area of its expression, and that having multiple sites and degrees of ecdysone biosynthesis may be related to how the organism responds to signals from the oenocytes or signals from the central nervous system. In various species of arthropods, the synthesis of ecdysone is found in ovaries and the epidermis, with several ecdysteroids having also been found to play a role in spermatogenesis (Hagedorn et al., 1975; Zhu et al., 1991; Bellé, 2005). Thus, the possibility of there being area specific regulation of endocrine factors should not come as a surprise.

Though it still remains unclear exactly how the ecdysone biosynthesis genes are regulated in different areas of expression, we propose that multiple areas of *vvl*-controlled ecdysone regulation may be beneficial to insects undergoing molts because the spread of ecdysoneis faster if it is released from several sites instead of one single location. Such regulation might facilitate rapid, massive and more efficient ecdysone production and release, allowing for synchronized molting of different segments. Moreover, this study presents novel data in which ecdysone biosynthesis genes were found in the oenocytes, which could mean that there is a possible homology between PG and oenocytes. Since in *Drosophila*, both oenocytes and prothoracic glands express

vvl during their development (Sanchez-Higueras et al., 2014), these observations suggest that the PGs and oenocytes likely share a common developmental and evolutionary origin.

The overall role of Vvl in the regulation of the initiation of metamorphosis

Members of the POU family are no strangers when it comes to early development. In vertebrates and invertebrates, they act as major players in cell type-specific gene expression and cell determination in both embryos and larvae (Fukuta et al., 1993; Ryan and Rosenfeld, 1997; Zhang and Xu, 2009). In the moth species *Bombyx mori, BmWCP4,* a protein gene primarily studied as a factor involved in wing development, plays a crucial role in pupal cuticle formation during the transition from the larval stage into the pupal stage. A study found that the expression of this gene increased in response to 20E, but the heightened expression was also found to be inhibited by the presence of JH (Deng et al., 2011). This phenomenon was explained to be due to the fact that JH hindered the expression of BmPOUM2, a POU homolog of Vvl found in *Bombyx*, which is the reverse of what we see in our study. This discrepancy may have been due to the differences in species used in each respective study, but another reason may be because prepupal specimens were used in the other study whereas this study looked at primarily the larval stages. This suggests that *vvl* may have stage-specific roles whose mechanistic pathways may differ from species to species.

Looking at all of the qualitative and quantitative results from this study, we see that there is a subtle similarity between the transcriptional regulation of neuroendocrine

factors involved in metamorphosis observed in invertebrates and puberty observed in vertebrates, which are two very crucial turning points in organismal growth and postembryonic development. Though it is highly unlikely that these two developmental processes share a common evolutionary origin, the fact that POU transcription factors are evolutionary conserved across several metazoan taxa suggests that the transcriptional regulation of these developmental processes may be very similar whereas the specific neuroendocrine regulators they target may be distinct. Because significant *vvl* expression is observed in the trachea, corpora allata and prothoracic glands during early development in *Drosophila*, recent studies have claimed these structures may had a common origin in an arthropod ancestor (Grillo and Casanova, 2014; Sanchez-Higueras et al., 2014). Together with the possibility that *vvl* expression may be tied to the critical weight, which has been linked to oxygen levels, the link between trachea and the neuroendocrine glands suggest that *vvl* may have played an ancient neuroendocrine function that was conserved throughout millions of years of evolution.

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