Ventral veins lacking regulates molting and oogenesis in Oncopeltus fasciatus

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Ventral veins lacking regulates molting and oogenesis in *Oncopeltus fasciatus*

A thesis by
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ABSTRACT

Recent studies have shown that in holometabolous insects, Ventral veins lacking (Vvl), a POU domain transcription factor, regulates ecdysteroid biosynthesis to influence molting in the red flour beetles, Tribolium and the fruit fly, Drosophila. To determine the degree of conservation in the transcriptional control of the endocrine system across more ancestral insect species, the expression of vvl was silenced in the hemimetabolous insect, Oncopeltus fasciatus. When vvl was silenced, Oncopeltus nymphs failed to molt and eventually died as nymphs. vvl RNAi led to reduced expression of the ecdysone response gene, HR3. Injection of active ecdysone, 20E, into vvl RNAi nymphs rescued the HR3 expression and allowed nymphs to initiate molting. vvl knockdown also led to subsequent reduction in the levels of the ecdysone biosynthesis gene, disembodied and shade in the anterior portion of the body. Furthermore, insect ovarian maturation and oogenesis has also been shown to be under the control of developmental hormones, ecdysone and juvenile hormone (JH). To investigate whether Vvl is involved in the transcriptional regulation of hormone biosynthesis during ovarian maturation and oogenesis vvl was silenced in adult females. vvl knockdown inhibited oogenesis and reduced the ovarian size. Preliminary results showed that simultaneous injection of 20E and ectopic application of the JH analogue, methoprene, could increase ovarian size and lead to the production of a few eggs. My results show that the transcriptional regulation of ecdysteroid biosynthesis during nymphal development is conserved between hemimetabolous and holometabolous insects. vvl also appears to affect hormone biosynthesis throughout the insect’s life cycle. Altogether, this study suggests that the endocrine role of Vvl in hormonal biosynthesis likely has ancient origins.
INTRODUCTION

Endocrine regulation of insect development has been well studied in many organisms, however the transcriptional regulation of the endocrine hormones remains poorly understood. Understanding the transcriptional regulation of endocrine events has the potential to illuminate us further on the origins of major developmental and physiological processes. In this work, I try to understand the evolutionary origins of endocrine functions and structures in insects by studying the role of a transcription factor, Ventral veins lacking (Vvl), during the hormonally regulated nymphal molts and adult oogenesis of the milkweed bug, *Oncopeltus fasciatus*.

Growth regulation in insects

In both mammals and invertebrates, juvenile growth and maturation have been shown to be under the control of steroid hormones, fat-soluble biomolecules that can pass through the cell membrane into the cell cytoplasm (Yamanaka et al., 2013). In insects, growth occurs by molting, the shedding of the exoskeleton to accommodate growing body size, and an important category of steroid hormones, known as ecdysteroids, regulate the molting process. This thesis focuses on the transcriptional regulation of the biosynthesis of ecdysteroids. In addition, two other factors have been identified as key regulators of growth and maturation: juvenile hormone (JH), a sesquiterprenoid hormone secreted from the corpora allata, and the neuropeptide prothoracicotopic hormone (PTTH) that is secreted from the brain (Yamanaka et al., 2013).

Ecdysteroids initiate molting and metamorphosis in insects (Yamanaka et al., 2013). JH is a status quo hormone that prevents progression from one life history stage to the next (Riddiford, 1996). During the larval stage, JH helps avoid precocious metamorphosis by allowing ecdysone to initiate larval-larval molts, enabling the animal to attain an appropriate
adult body size. The absence of JH in the last larval instar allows active ecdysone, 20-hydroxyecdysone (20E) to promote entry into the pupation phase. In holometabolous insects, exogenous JH at the last larval instar delays metamorphosis further and exogenous JH at the pupal phase causes ecdysone to initiate molting into a second pupal phase (Riddiford, 2012).

**Ecdysteroidogenesis by P450 enzymes**

The timing of molting or metamorphosis is principally controlled by ecdysteroidogenesis, the biosynthesis of ecdysteroids. The site of ecdysteroidogenesis is the prothoracic gland where a group of P450 enzymes, transcribed by a set of genes known as the Halloween genes, converts dietary cholesterol to ecdysone in a series of hydroxylation steps. The members of the Halloween genes were identified first in *Drosophila melanogaster* as a group of genes that cause embryonic lethality when mutated. These genes include *disembodied (dib)*, *shadow (sad)*, *shade (shd)*, *spook (spo)* and *phantom (phm)*, which encode different proteins in the cytochrome P450 superfamily (Gilbert, 2004).

The conversion of cholesterol to ecdysone by the P450 enzymes is summarized in Figure 1. The initial step in the conversion of cholesterol to ecdysone involves the production of 7-dehydrocholesterol (7dC) mediated by a 7,8-dehydrogenase. The rate-limiting step in ecdysone production however is thought to be the production of ketodiol from 7dC which occurs by a series of “Black box” oxidative steps. Elucidating the mechanism of conversion in the black box is made difficult because of the instability of the intermediates between 7dC and ketodiol. However, this Black box is hypothesized to be transcriptionally regulated by PTTH (Warren et al. 2009) and mediated by the P450 enzyme coded by the Halloween gene, *spo* and its paralogs, *spookier (spok)* and *spookiest (spot)*. In the fruit fly *Drosophila*, two paralogs *spo* and *spok* have
been found. Ono et al. (2006) have shown that developmentally arrested spo and spok RNAi knockdown Drosophila larvae can be rescued by feeding ecdysone, 20-hydroxyecdysone or ketodiol but not 7dC.

After the Black box, the conversion of ketodiol to 2-deoxyecdysone is mediated by the enzymes encoded by the genes phantom (phm) and disembodied (dib), respectively. The conversion of 2-deoxyecdysone to ecdysone is mediated by the enzyme encoded by shadow (sad) (Chavez et al., 1999; Niwa et al., 2004; Gilbert, 2004). Niwa et al. (2004) characterized Phm as a 25-hydroxylase that converts ketodiol to ketotriol in Drosophila and Bombyx mori. Gilbert (2004) further characterized Dib and Sad as the mitochondrial P450 enzymes, 22-hydroxylase and 2-hydroxylase, respectively. 22-hydroxylase encoded by dib is responsible for the conversion of ketotriol to 2-deoxyecdysone, which is then converted to ecdysone by the 2-hydroxylase encoded by sad. Both Dib and Sad colocalize with mitochondrial markers and have charged residues at the N-terminal typical of proteins targeted to mitochondrial import (Gilbert, 2004).

The last step is the conversion of ecdysone into the active 20-hydroxyecdysone which generally occurs in peripheral tissues once ecdysone has been released into the hemolymph. This conversion occurs via the 20-monoxygenase enzyme encoded by shd. Shd, like Dib and Sad, has a charged segment at its N-terminus characteristic of mitochondrial localization but also has a hydrophobic region that targets it to the endoplasmic reticulum (Gilbert, 2004) In Drosophila, shd is expressed in the epidermis, midgut, Malphigian tubules and fat body but not the prothoracic ring glands. However, the site of expression for shd does not appear to be important for function as misexpression of shd in embryonic mesoderm instead of the epidermis, can still rescue embryonic lethality (Petryk et al. 2003).
Figure 1. The conversion of cholesterol to 20-hydroxyecdysone as mediated by the Halloween genes. Figure adapted from Niwa and Niwa, 2014.
**JH biosynthesis**

JH biosynthesis occurs in the corpora allata (CA), another important insect endocrine gland usually found near the PG. Enzymes involved in the JH biosynthesis pathway are all expressed in the CA, and Kinjoh et al. (2007) showed that the transcript levels of JH biosynthetic enzymes in the *Bombyx mori* CA are positively correlated with the production of JH. The JH biosynthetic pathway involves the conversion of Acetyl CoA into farnesoic acid in several steps, and the final step involves the conversion of farnesoic acid to JH (Nijhout et al., 2013). In *Drosophila* and *Bombyx mori*, JH acid methyl transferases (JHAMT) have been shown to be important in this last step of JH biosynthesis. The transcript levels of JHAMT drops in the CA right before pupal formation, following the pattern of a drop in JH titer (Niwa et al., 2008). In *Tribolium castaneum*, three methyltransferase genes were identified that corresponded to the JHAMT gene in *Drosophila* and *Bombyx*. Of the three paralogs in *Tribolium*, *jhamt3* was present starting from the embryonic stages to the final larval instar with a drop occurring right before pupation, a transcript profile that matches the profiles of JHAMT in *Drosophila* and *Bombyx mori* (Minakuchi et al., 2008).

**Factors regulating prothoracic gland development and ecdysteroidogenesis**

The development of the prothoracic glands (PG) during embryogenesis and also subsequent ecdysteroidogenesis in the PG is regulated by many different environmental and physiological factors. One important factor is the secretion of PTTH, which is synthesized in the brain. PTTH targets the PG via the receptor tyrosine kinase, Torso, and a MAPK signaling pathway (Yamanaka et al, 2013). In *Drosophila*, it has been shown that weight gain and photoperiod is important in determining PTTH release. The absence of PTTH delays larval
molting or eclosion, prolonging larval development and feeding time, although it does not arrest molting (McBrayer et al., 2007). A recombinant PTTH also has been shown in vitro to directly initiate the transcription of phm, dib and spo in the PGs of Bombyx mori (Yamanaka et al. 2007). Furthermore, in Drosophila, PG growth is also controlled via the nutritional-dependent pathways, notably the insulin/TOR signaling pathway (Mirth et al. 2005; Layalle et al, 2008).

In insects, transcription factors have also been shown to control PG formation and ecdysteroidogenesis. The transcription factors, Sex comb reduced (Scr), Signal Transducers and Activator of Transcription (STAT), Ventral veins lacking (Vvl) and Snail are involved in the specification of the PG during Drosophila embryogenesis. Vvl along with other transcription factors like the bFtz-f1, Ultraspireacle (USP), Broad (Br) and DHR3 and Knirps (Kni) have been shown to affect the transcript levels of the ecdysone biosynthesis genes in Drosophila melanogaster (Niwa and Niwa, 2015).

**Functions of the transcription factor Vvl**

Vvl, the transcription factor being studied in this paper, is a POU domain transcription factor. The POU domain is a highly conserved transcription domain, and a vvl human homologue POU3F2 is associated with hypogonadotrophic hypogonadism and adrenal insufficiency (Niwa and Niwa, 2015). Vvl was initially identified by Anderson et al. (1995) as a transcription factor important in the differentiation of the trachea and the midline glia in Drosophila embryos. In these embryos, Vvl is expressed in the tracheal system, the middle pair of the midline glia and select CNS neurons. Vvl has also been shown to work downstream of the Hox gene labial to control neuronal patterning in the tritocerebrum of Drosophila embryos (Meier et al., 2006). The role of vvl in ecdysteroidogenesis through the transcriptional regulation of ecdysone biosynthesis
genes was elucidated through PG-specific knockdown and systemic knockdown studies in *Drosophila melanogaster* and *Tribolium castaneum*, respectively.

Danielsen et al. (2014) found that cis-regulatory elements responsible for the expression of the ecdysone biosynthesis genes *phm*, *spok* and *dib* contain *vvl* binding sites in *Drosophila melanogaster*. RNAi knockdown of *vvl* in *Drosophila* PG leads to reduced transcript levels of *phm*, *spo*, *dib* and *sad*, and arrests larval molting. Knockdown of *vvl* further causes a reduced PG size and reduction in the transcript level of the PTTH receptor *torso* and the TOR downstream positive growth regulator *S6 kinase* (*S6K*). Therefore, *vvl* likely indirectly affects PG size and ecdysteroidogenesis via regulatory action on the PTTH and insulin/TOR signaling pathways (Danielsen et al., 2014). In *Tribolium castaneum*, *vvl* RNAi causes the reduction in the transcript levels of the ecdysone biosynthesis genes *phm* and *spo* and also the JH biosynthesis gene *JH acid methyltransferase 3* (*jhamt3*) (Cheng et al., 2014). The varied effects of *vvl* knockdown on both ecdysone and JH suggest that *vvl* might be an important master transcriptional regulator of hormone biosynthesis in insects.

**Sites of *vvl* expression**

In *Drosophila*, *vvl* is expressed in the PG in the larval stages and the PG primordia of the embryos (Danielsen et al., 2014). In *Tribolium* embryos, the expression of *vvl* occurs in the prothoracic region, which likely develops into PGs and also abdominal structures known as oenocytes (Cheng et al., 2014). A few studies have proposed that in oenocytes may the site of ecdysone biosynthesis (Dorn and Romer, 1976). Whereas the role of PGs in hormone biosynthesis is generally accepted, the role of oenocytes in hormone biosynthesis is yet to be definitively established.
The oenocytes were initially identified as a group of cells important in lipid production and other metabolic processes. Several transcription factors important for lipid processing and metabolism are expressed in the oenocyte (Burns et al., 2012; Gutierrez et al., 2007). The notion that oenocytes may be sites of ecdysone production was first shown in Tenebrio molitor, a beetle species (Romer et al., 1974). vvl expression is found in Tribolium oenocytes (Cheng et al., 2014). Furthermore, in response to vvl RNAi, the expression of the biosynthesis gene spo decreases in the posterior half of the body, where the oenocytes are located. Dorn and Romer (1976) also suggest that the oenocytes may be a site of ecdysteroid production in other species, including Oncopeltus fasciatus, the model organism used in this study.

**Mechanism of ecdysone response in insects**

The ecdysone signaling is mediated by the formation of a heterodimeric complex through the binding of 20E to the nuclear receptors, ecdysone receptor (EcR) and Ultra spiracle (Usp) (Tan and Palli, 2008). Usp is a homologue of the vertebrate retinoid X receptor (RXR), a nuclear receptor of the steroid/thyroid hormone superfamily (Dawson and Xia, 2012). In Drosophila, the EcR and Usp/RXR complex with 20E is important for maternal fertility, embryonic morphogenesis and also metamorphosis. Usp has no specific activation function of its own but rather has an allosteric affect on EcR, which in turn cannot activate transcription on its own (King-Jones and Thummel, 2005). In Tribolium, EcR and Usp have been implicated in embryonic development and larval development as EcR and Usp knockdown in this species leads to embryonic lethality and arrested larval development (Tan and Palli, 2008). The function of EcR and the Usp/RXR complex as mediators of ecdysone signaling is conserved across many different species (Cruz et al., 2006; Hayward et al., 2003, Riddiford et al., 2003).
Upon binding 20E, the EcR/Usp heterodimeric complex induces the primary response genes, which code for transcription factors that activate secondary response genes (Jones and Thummel, 2005). In particular, the secondary response gene hormone receptor 3 (HR3) functions as a switch that helps define larval to prepupal transition by acting as a transcriptional activator of bFtz-f1 (Lam et al., 1997; King-Jones and Thummel, 2005).

**Function of developmental hormones in oogenesis and ovarian maturation**

In addition to molting and metamorphosis, ecdysteroids and JH both have roles in the reproductive maturation of adult insects. In most insects, except dipterans, JH predominantly regulates the synthesis of the female specific egg yolk protein Vitellogenin (Vg) in the adult fat body. In dipterans, however, vitellogenesis appears to be predominantly regulated by ecdysteroids (Belles et al., 2015).

In contrast, oocyte growth and ovarian maturation depends on ecdysteroids in beetles and *Bombyx mori*, whereas in *Drosophila* and *Aedes* aegypti, JH and ecdysteroids both appear to play critical roles (Parthasarathy et al. 2010; Belles et al. 2015). Additionally in *Drosophila*, ecdysteroids promotes lipid accumulation in oocytes and also regulates lipid metabolism in the germline via the ecdysone response gene, *EcR* (Sieber and Spradling, 2015). These studies speak to the varied roles that JH and ecdysteroids play in vitellogenesis and oogenesis across different insect species.

**Life cycles of insects**

The process of juvenile maturation in insects can be classified into three different forms of development: ametabolous, holometabolous and hemimetabolous. The most primitive form of
insect development is the ametabolous development where the juveniles look like the adults. Maturation into the adult stage occurs without much drastic physiological or morphological changes other than the formation of reproductive structures. An example of ametabolous insects is the silverfish. The second form of development is the hemimetabolous mode of development in which the nymphs resemble their adult form, but lack fully developed wings and functional gonads. Hemimetabolous development is also known as incomplete metamorphosis. The model organism used in this study, Oncopeltus fasciatus or milkweed bugs, are examples of hemimetabolous insects. Holometabolous insects, such as fruit flies, beetles, tobacco hornworms and silkworms, undergo larval molts, form a pupa and emerge as an adult that has undergone drastic physiological and morphological changes. This mode of development is the most derived form of insects maturation and its evolution appears to have been a key innovation that gave rise to the dramatic diversity of insect species (Truman and Riddiford, 2002).

**The evolutionary origin of insect endocrine glands**

In *Drosophila*, the prothoracic glands, corpora allata and trachea are serially homologous structures that can be transformed into each other by the misexpression of Hox genes. During development, the primordia of the three structures invaginate in a similar fashion. The development of the tracheal primordia is transcriptionally regulated by both *vvl* and *tracheless* (*trh*). In contrast, the corpora allata and prothoracic gland primordia only express *vvl* and migrate to later form the ring gland, an endocrine gland specific to *Drosophila*. (Sanchez-Higueras et al., 2014). Based on these findings, Grillo et al., (2015) proposed that endocrine functions might have originated within the respiratory structures of ancestral arthropods.
Furthermore, the role of Vvl in regulating the formation of trachea, CA and prothoracic glands implicates it as a transcription factor whose functions are shared between endocrine and respiratory primordia. The conservation of the endocrine and tracheal expression of vvl across the hemimetabolous and holometabolous boundary might imply that endocrine and respiratory structures share a common evolutionary origin.

To demonstrate that endocrine regulation and tracheal development were coupled in an ancestral arthropod requires an understanding of when Vvl first arose as a key regulator of hormonal biosynthesis. Because our current knowledge of the endocrine role of Vvl is confined to holometabolous insects, the objective of this study is to establish Vvl as a master regulator of ecdysone biosynthesis in a hemimetabolous insect.

**Oncopeltus fasciatus as a model organism for studying development**

*Oncopeltus fasciatus* are part of the taxonomic order Hemiptera and eat seeds of milkweed plants in the wild. In the lab, they can be reared on water and seeds of various plants including watermelon, pumpkin, squash and sunflower (Chaplin, 1980). *Oncopeltus* have five nymphal stages, and the growth and size of insects is temperature- and food-dependent. Higher temperatures, longer photoperiods and lower population densities increase reproduction and mortality (Dingle, 1968). Adults have two sets of fully formed wing with hind- and fore- wings, and studies have established them as migratory animals (Palmer and Dingle, 1989).

Adult females can lay up to 2,000 eggs in their lifespan and lays eggs in clusters of approximately 30 eggs. Oviposition occurs approximately five days after mating. Eggs can hatch within 4-10 days, and higher temperatures can reduce the time eggs take to hatch. An adult male may fertilize a female up to 30 minutes for egg laying, and a female can store sperm after mating.
for up to five weeks (Feir, 1974). *Oncopeltus fasciatus* have a sequenced genome, which makes identification of gene homologs easier. RNA interference (RNAi) in *Oncopeltus* using injection of dsRNA of the target knockdown gene is a well-established methodology in the literature and causes a systemic knockdown of gene transcription (Hughes and Kaufman, 2000). Additionally, RNAi knockdown of specific gene products is effective at various stages of *Oncopeltus* life cycle, including the nymphal stage and the adult stage, when it affects the offspring of injected females (Liu and Kaufman, 2004).

**RNA interference (RNAi)**

RNAi is a gene silencing mechanism that is naturally present in animals and was first described as a gene silencing mechanism in the lab using the nematode *Caenorhabditis elegans* (Fire et al, 1998). An enzyme called Dicer digests double stranded RNA (dsRNA) in the body. Dicer recognizes dsRNA as foreign compounds and cleaves it into short interfering RNA (siRNA), which are fragments 21-25 nucleotides long. The siRNA is recognized and bound by the RNA induced silencing complex (RISC) and is cleaved into two single-strand RNA (ssRNA). One ssRNA, called the passenger strand is destined for cleavage, while the other is the guide strand that stays bound to RISC and recognizes other single stranded RNA transcripts with complementary nucleotide base sequences. The cleavage is carried out by the Argonaute enzyme that is incorporated within RISC (Miyoshi et al., 2005). This natural mechanism is exploited in the lab as a gene silencing tool by the application of the dsRNA of a target gene and studying its loss of function effects.
In this study, I first describe the expression of vvl in *Oncopeltus* embryos. I then describe the effect of vvl knockdown on nymphal molts and the expression of ecdysone biosynthesis genes to determine the conservation of molting function between both hemi- and holometabolous insects. I finally demonstrate a novel role of Vvl in egg production. Thus, the origin of the endocrine role of Vvl likely dates back at least to the common ancestor of hemipterans and the lineage leading to the holometabolous insects.
MATERIALS AND METHODS

Animal husbandry

Wildtype *Oncopeltus* was obtained from Carolina Biologicals and raised on organic sunflower seeds. The adults were paired and kept separately with a cotton ball for the females to lay eggs.

RNA isolation and dsRNA synthesis

*Oncopeltus* RNA from 3rd and 4th instar nymphs was isolated by homogenizing the tissues in TRIzol (Life Technologies, Carlsbad, CA). The RNA sample was treated with DNase and 1µg RNA was converted to complementary DNA (cDNA) using reverse transcriptase in the cDNA synthesis kit as per the manufacturer's instructions (Thermo Scientific, Waltham, MA). The gene sequence of *vvl* was identified in the *Oncopeltus* genome (Vargas Jentzsch *et al*. 2015). Primers were designed to amplify genes of interest in the synthesized cDNA using polymerase chain reaction (PCR) with Taq Polymerase in a thermal cycler (Table 1). The PCR mix with cDNA and *vvl* primers were held for 2 minutes at 94°C, followed by 40 cycles of the following: 94°C for 20 sec, 55°C for 30 sec and 72°C for 1 min. In the final step the reaction was kept at 72°C for 5 min and held at 4°C.

The PCR product was purified after being run on a 1.5% agarose gel for 20 min at 100V using MinElute Gel Extraction Kit (Qiagen) as per the manufacturer's instructions. Purified product was cloned into the TOPO-TA cloning vector (Life Technologies, Carlsbad, CA), which was then used to transform *E. coli* cells. Plasmid DNA was extracted from transformed cells using the QIAprep Spin Miniprep Kit as per the manufacturer's instructions (Qiagen). The plasmid DNA was linearized using *SpeI* and *NotI* restriction enzymes (New England Biolabs,
Ipswich, MA) and then used for single-stranded RNA (ssRNA) synthesis using the MEGAscript T3 and T7 kits (Life Technologies, Carlsbad, CA). ssRNA synthesis was carried out according to manufacturer's instructions. The complementary ssRNA were combined and annealed to form a 2µg/µl dsRNA solution in a thermal cycler using standard annealing protocol (Hughes and Kaufman, 2000). The final annealed product was analyzed via gel electrophoresis to ensure proper annealing.

**dsRNA injection and rescue treatment**

To characterize the role of vvl in nymphal development day zero fourth and fifth instar Oncopeltus nymphs were injected with 0.5 µg vvl dsRNA and for controls with 0.5µg amp' dsRNA (0.25 µL of 2 µg/µL dsRNA). To understand the role of vvl in oogenesis day zero adult females were injected with 1µg vvl dsRNA and for controls with 1µg amp' dsRNA (0.5 µL of 2 µg/µL dsRNA).

Rescue treatments in nymphs involved injecting RNAi nymphs on day two with 0.25 µL of 5 µg/µL 20E or 0.25 µL water as a control for the rescue injection. The volume of liquid injected during rescue was kept consistent with volume of RNAi injection. Rescue treatments in adults were a simultaneous treatment with 20E and JH analogue, methoprene, nine days after mating. Females were injected with 1 µL of 5 µg/µL 20E and 1 µL of 10 µg/µL methoprene was ectopically applied on the ventral side of the female head. Animals were anesthetized on ice before injections and injections were carried out using a 10 µL glass capillary needle connected to a syringe.
**Knockdown verification with semi-quantitative RT-PCR**

To verify that *vvl* dsRNA injection caused knockdown of *vvl* expression day zero fifth *Oncopeltus* nymphs were injected with dsRNA with the same volume and concentration of *vvl* and control *amp* as specified in ‘dsRNA injection and rescue treatment’. Three whole bodies per sample of the fifths were collected in TRIzol on day four. RNA isolation and cDNA synthesis was carried out using 1 µg RNA as specified earlier in ‘RNA isolation and dsRNA synthesis’. PCR reactions were set up with the cDNA of *vvl* and *amp* injected nymphs to check the expression of *vvl* and the positive control *RPS3*. To compare the relative abundance of *vvl* and *RPS3* in the samples PCR with *vvl* primers was carried out with 30, 35 and 40 cycles and with *RPS3* primers was carried out with 25 and 30 cycles. The products were run on a 1.5% agarose gel at 100V for 20 min and the relative intensities of the bands were compared.

**Quantitative RT-PCR**

To look at the expression of *HR3* upon rescue of *vvl* RNAi nymphs with 20E, RNA was isolated from the whole body of day three fourth instar nymphs that underwent *vvl* or *amp* RNAi on day zero and rescued with 20E or water on day two. Three biological replicates were collected with two animals per sample replicate. To look at the effect of *vvl* RNAi on the expression of ecdysone biosynthesis genes, *phm*, *dib* and *shd*, total RNA was isolated separately from the anterior half and posterior half of day three fifth instar nymphs that were injected with *vvl* or *amp* dsRNA on day zero. Three biological replicates were collected with three animal halves per sample replicate. RNA isolation in TRIzol and RNA conversion to cDNA with 1 µg RNA was carried out as described above in ‘RNA isolation and dsRNA synthesis’.
Of the total 20 µL cDNA generated using the Thermo Scientific cDNA synthesis kit 0.5 µL was used for qRT-PCR analyses using the SsoAdvanced SYBR Green Supermix (Bio-rad). Primer sequences for HR3, phm, dib and shd were made from the genes identified in the Oncopeltus genome (Vargas Jentzsch et al. 2015; Table 1). A 20 µL total liquid mixture comprising of Supermix, forward and reverse primer and cDNA was loaded onto a 96 well-plate. Every sample from all biological replicates was loaded in triplicates and the average of the triplicate output was used to analyze gene expression. qRT-PCR analysis was done using the standard curve method. Gene expression from all samples was normalized to the corresponding RPS3 expression to control for variability in loading.

**vvv probe preparation and in situ hybridization**

Purified linearized plasmid was obtained as described. vvl sense and anti-sense Digoxigenin (DIG) labeled probes were synthesized from the linearized plasmid using MAXIscript kit (Life Technologies, Carlsbad, CA) and DIG RNA labeling mix (Roche Molecular Biochemicals, Indianapolis, IN). The probes were hydrolyzed for 20 min and suspended in hybe buffer and stored at −80 °C until use. Prior to use, the probe was thawed and boiled for 3–5 min.

For preparation of the embryos, day two, three and four embryos were dechorionated by boiling for 3 min and kept on ice. Egg chorions were cracked by shaking in heptane/methanol, followed by methanol. Individual embryos were dissected out of their egg shell and fixed in 4% formaldehyde in PBS overnight at 4°C. In situ hybridization protocol obtained from Yen Ta Chen and Dr. Kristen Panfilio was followed. Embryos were dehydrated through 5 min treatment in 50%, 25% methanol in PBST and washed several times in PBST. A 30 min proteinase K
(4 µg/µL) digest followed. Embryos were washed several times in PBST before being treated with 2 µg/µL glycine for 5 min. Embryos were again washed several times in PBST and postfixed in 5% formaldehyde for 25 min at room temperature. After several PBST washes embryos were gradually transferred to hybe buffer by rinsing with 50%, 75% and finally 100% hybe buffer. After a 30-min incubation in hybe buffer at 70 °C followed by a 30 min incubation at 60°C, the probe was added.

Embryos were incubated with the probe for 14–48 h in at 60°C, then the probe was removed, and washed in hybridization wash buffer twice and incubated in hybridization wash buffer for 20 minutes, four times at 60°C. The embryos were then rinsed at room temperature for 5 minutes in 5X SSC/50% Formamide/0.1%Tween20 and another 5 minutes in 2X SSC/50% Formamide/0.1%Tween20. After, the embryos were washed in PBST 6 times, with the last 4 times lasting 20 minutes each. Then embryos were blocked for 1 hr at room temperature in blocking buffer (Tris pH 7.4 with 2 mg/ml bovine serum albumin and 5% normal goat serum). Embryos were incubated overnight at 4 °C with anti-DIG-alkaline phosphatase (AP) conjugate (Roche Applied Science, Indianapolis, IN) at a concentration of 1:2000. After two washes in AP buffer and a 30 minute incubation in AP buffer at room temperature, the color reaction was performed using NBT/BCIP as the substrate. The color reaction was stopped by rinses in PBST and embryos were mounted on slides in glycerol for imaging.
Table 1. Forward (FW) and reverse (RV) primer sequences for dsRNA synthesis, expression profiling, in situ hybridization and knockdown verification.

<table>
<thead>
<tr>
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<th>Primer 5’ → 3’</th>
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<tr>
<td>dsRNA and in situ hybridization</td>
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<tr>
<td>Vvl</td>
<td>FW GTGGAGTCGGTGATGATGAA</td>
</tr>
<tr>
<td></td>
<td>RV A GCCAGTAGATGAGGTAAGG</td>
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<tr>
<td>Knockdown verification</td>
<td></td>
</tr>
<tr>
<td>Vvl</td>
<td>FW CCTCACGGCAAGTCAGTAGT</td>
</tr>
<tr>
<td></td>
<td>RV TTGGTGTTGGCCTAACAT</td>
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<td>Quantitative RT-PCR</td>
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</tr>
<tr>
<td>HR3</td>
<td>FW ACGAGGAGCCACCAACCACA</td>
</tr>
<tr>
<td></td>
<td>RV TCACCACTGACGACTGAGACC</td>
</tr>
<tr>
<td>Phm</td>
<td>FW TACATCGCCCTCAATCCTCA</td>
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<tr>
<td></td>
<td>RV GCAGCCTCCGTGAAAAGAAC</td>
</tr>
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<tr>
<td></td>
<td>RV CTTTCACCATTGAGCCAAC</td>
</tr>
<tr>
<td>RPS3</td>
<td>FW TTGATACCAAACCCCTT</td>
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<td></td>
<td>RV CAACCCCATAACACTTGACCT</td>
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RESULTS

Knockdown verification in vvl RNAi animals

To verify that the vvl dsRNA injection results in knockdown of vvl expression, 0.5 µg of either vvl double-stranded RNA (dsRNA) or amp' dsRNA was injected into day zero fifth instar nymphs. The nymphs were collected on day four to compare their expressions of vvl mRNA transcripts using semi-quantitative RT-PCR. In comparison to amp' knockdown animals, the expression of vvl in vvl knockdown animals was reduced (Figure 2), indicating successful knockdown of vvl.

![Figure 2. Knockdown verification for vvl RNA interference. vvl and RPS3 expression in day 4 fifth instar amp' and vvl dsRNA-injected nymphs. The PCR cycle numbers for the expression of RPS3 was 25 and 30 cycles and for the expression of vvl was 30, 35 and 40 cycles.](image)
**vvl knockdown in fourth and fifth instar nymphs arrests molting**

To determine the effects of vvl knockdown, fourth and fifth instar nymphs were injected with 0.5 µg of either vvl or control amp dsRNA and observed until death or attainment of adulthood. Of the 13 day zero fourth instars injected with vvl dsRNA, none molted into the next instar and of the 17 day zero fifth instars injected, only one molted to the adult. (Figure 3). The vvl knockdown nymphs that failed to molt stayed alive for approximately one month for both fourths and fifths. In contrast, 13 our of 14 amp dsRNA-injected control day zero fourth nymphs molted into the fifth instar, and of these, 11 successfully molted into the adult stage. The control treatment of the fifths injected with amp on day zero all reach adulthood. These results show that vvl knockdown causes molting defects in *Oncopeltus* and suggests the involvement of vvl in ecdysteroid signaling.

**Figure 3. vvl RNAi leads to arrested molting in 4th and 5th instar nymphs.** For vvl knockdown in day zero fourths, n = 13 and in day zero fifths, n = 17. For control amp knockdown in day zero fourths, n = 14 and in day zero fifths, n = 11.
Injection of 20-hydroxyecdysone in vvl RNAi nymphs rescues molting phenotype and HR3 expression

To determine whether the vvl knockdown impacts ecdysteroid production or ecdysteroid reception, day zero fourth nymphs were injected with 0.5 µg vvl dsRNA and rescued with 1.25 µg 20-hydroxyecdysone (20E) on day two. Control nymphs were injected with 0.5 µg amp<sup>r</sup> dsRNA on day zero and injected with water two days later. 20-E was able to rescue the molting phenotype in nine out of 12 nymphs (75%), whereas nymphs injected with water did not molt (Figure 4). All nine nymphs initiated molting but only one successfully completed eclosion. Most died during the molt, with the fifth instar partially eclosing out of the old cuticle. Molting initiation suggests that the ecdysone response mechanism is still effective during vvl knockdown.
Figure 4. 20-hydroxyecdysone rescues molting phenotype in vvl RNAi nymphs. Percentage of nymphs initiating molt are shown. Day zero fourth nymphs were injected with vvl dsRNA and then subsequently injected with water or 20E on day two. Inset shows a ‘half-molted’ Oncopeltus nymph that initiated molting into the fifth instar but died during molt. For day zero fourth vvl RNAi rescued with 20E, n=12 and for rescue with water, n=7.

To corroborate the phenotypic results, the relative expression of HR3, an ecdysone response gene, was compared between day zero fourth instar vvl RNAi knockdown animals injected with either 0.25 µL water or 0.25 µL of 5ul/ug 20E (1.25 µg) two days later. For controls, day zero fourth instar nymphs were injected with 0.5 ug ampr dsRNA and rescued with either water or 20E. HR3 expression was significantly increased in vvl RNAi nymphs rescued with 20E (p<0.01) compared to vvl RNAi nymphs injected with water. The ampr’ knockdown nymphs injected with 20E also showed an increase in HR3 expression, which is expected due to the extra 20E available to the nymph. However, this increase was not significant (p=0.27; Figure
These results demonstrate that the ecdysteroid signaling can be rescued with injection of 20E, indicating that the vvl knockdown likely impacts ecdysteroid biosynthesis.

Figure 5. *HR3* expression is rescued in *vvl* RNAi nymphs by 20-hydroxyecdysone. Effect of *vvl* RNAi and 20E rescue on the expression of *HR3*. Comparison is made between *vvl* RNAi rescue by water and 20E (p<0.01) and *ampr* RNAi rescue by water and 20E (p=0.27). Experiment was run in three biological replicates with two whole body nymphs per treatment in a replicate. Expression of *HR3* is relative to the expression of the control gene, *RPS3*. Data are represented as mean expression +/- SEM.
**vvl knockdown reduces the levels of ecdysone biosynthesis genes disembodied and shade**

To test the possible role of vvl in ecdysteroid biosynthesis, the expression of three ecdysteroid biosynthesis genes, phm, dib and shd, were analyzed in day three fifth instar nymphs injected with 0.5 µg of vvl or amp dsRNA. To distinguish between different sites of ecdysteroidogenesis, each nymph was separated into its anterior (thorax and head segments) and posterior region (abdomen) for analysis. The anterior region contains the prothoracic glands, the key site of ecdysone production during juvenile development and the site of phm and dib expression. The posterior region contains developing gonads and possibly cells parallel to the oenocytes in Tribolium and Drosophila (Dorn and Romer, 1976). Another important component is the fat body, the site of shade production, which is present in both the anterior and posterior regions of the nymph.

There is a significant decrease in the expression of dib and in the anterior region of the vvl RNAi nymph but not the posterior region (p<0.01 and p<0.05, respectively; Figure. 6). This suggests that vvl affects ecdysteroid production by initiating the transcription of the biosynthesis genes, dib and shd. The expression of shd, however, differ by less than 2-fold, so the difference is not profound.
Figure 6. *vvl* RNAi reduces the transcript levels of *disembodied* and *shade* in the anterior portion of *Oncopeltus* fifth instar nymphs. Effect of *vvl* RNAi on the transcript levels of (A) *phantom* (B) *disembodied* (C) *shade* in the anterior and posterior region of day zero fifth instar nymphs. All expression values are relative to the control gene RPS3. *p* value was =0.14 and =0.27 for *phm* in the anterior and posterior region, respectively. *p* value was <0.01 and =0.39 for *dib* in the anterior and posterior region, respectively. *p* value was <0.05 and =0.26 for *shd* in the anterior and posterior region, respectively. Comparison of the *vvl* RNAi rescue experiments were made to control animals being rescues after *amp* RNAi. Experiment was run in three biological replicates with three nymph halves per treatment replicate. Data are represented as mean expression +/- SEM.
**vvl knockdown in adult female arrests oogenesis and shows reduced ovarian size**

To understand the role of vvl in *Oncopeltus* reproductive processes, 0.5 µg of vvl dsRNA was injected into day zero adult females (n=3) that were then paired with untreated males throughout the remainder of their life. vvl RNAi adults stopped laying eggs. In contrast, day zero adults (n=4) that received the control injections of amp' dsRNA laid between 170 to 450 eggs until death (p<0.05; Figure 7). Ovaries were dissected approximately two weeks after injection of vvl or amp' dsRNA into day zero adults. The vvl RNAi ovary had no mature oocytes and a smaller ovarian size in comparison to the amp' RNAi control ovary that had a larger ovarian structure and many mature oocytes (Figure 8A-B).

To determine whether the observed inability to lay eggs was due to deficiencies in hormonal biosynthesis, a rescue experiment in vvl RNAi adults with ectopic 20E and methoprene was carried out. Nine days after first mating vvl RNAi females (n=2) were rescued with the simultaneous injection of 5 µg of 20E and topical application of 10 µg of the JH analogue, methoprene. The two females only produced a few eggs; one laid three while the other laid five. Even though the number of eggs laid by the rescued females was low, the rescue of oogenesis was also supported by increased ovarian size and egg deposition within ovaries of the rescued female, dissected approximately two weeks post rescue treatments (Figure 8C). The ovary of the rescued female showed an intermediate ovarian size and also some semblance of oogenesis although this seemed to be limited relative to the control amp' dsRNA-injected adults. These results suggest that vvl is involved in regulating *Oncopeltus* egg laying and ovarian growth possibly via regulation of the action of the developmental hormones, JH or ecdysteroids, although we cannot rule out the involvement of other factors.
Figure 7. vvl RNAi adults fail to produce eggs. Shown in the graph is the number of eggs laid by day zero females injected with vvl (n=3) or amp′ (n=4) dsRNA. p-value is <0.05. Data are represented as mean number of eggs laid +/- SEM.
Figure 8. Ovary from *vvl* RNAi female was smaller in size and had no mature oocytes, but the female rescued with JH and 20E had slightly larger ovaries and some semblance of oogenesis. (A-A’) Ovary from *amp*’ RNAi female (B-B’). Ovary from *vvl* RNAi female (C-C’). Ovary from *vvl* RNAi female rescued after nine days with JH and 20E. Ovaries were collected from *vvl* RNAi female 15 days after injection into day zero female and from *amp*’ RNAi female 21 days after injection into day zero female. Ovary from JH and 20E rescued females was collected 18 days after rescue treatment of day zero *vvl* RNAi female. A, B and C were taken at the same magnification. A’, B’ and C’ were taken at the same magnification. Images were taken using Leica Stereo Microscope, Model M165FC.
**vvl is expressed at different sites of *Oncopeltus* embryo**

Whole mount *in situ* hybridization of *Oncopeltus* embryos shows the expression of *vvl* in the prothoracic glands, brain region, central nervous system and in putative tracheal pits and oenocytes. *vvl* was expressed in the basal labial segment in the elongated germ band which could be the developing prothoracic glands (Figure 9A-A’). In the early retracting germ band stage *vvl* is expressed in the brain region, which is possibly the tritocerebrum, the prothoracic region, legs and antennal segments and the tracheal pits (Figure 9B-H). In the retracted germ band, *vvl* is expressed in the putative prothoracic glands, tracheal pits and the putative oenocytes (Figure 9J-M).
Figure 9. *In situ hybridization of vvl expression in Oncopeltus embryos.* vvl transcript expression in (A-A’) elongated germ band stage, (B-H) early retracting germ band stage and (J-M) retracted germ band stage. (I-I’) Control with sense strand. L shows the abdomen of a retracted germ band phase with vvl expression in the circular oenocytes and tracheal pits located side by side. White arrowheads specify vvl expression in putative prothoracic glands, grey arrows in the prothoracic region, white arrows in the legs and antennal segments, black arrow in an unidentified group of cells and black arrowhead in the tracheal pits. A, B, C and I were taken under the same magnification. A’, B’, D were taken under the same magnification.
DISCUSSION

In this study, I sought to determine whether the endocrine role of *ventral veins lacking* (vvl) is conserved across hemimetabolous and holometabolous insect. I also attempted to elucidate the endocrine roles of vvl across the different life history stages. The importance of vvl in insect endocrine processes would support an ancient origin for the endocrine role of Vvl in the prothoracic gland and corpora allata.

Knockdown of vvl expression using dsRNA-mediated RNAi led to the inhibition of nymphal molts and egg production in adult female. The molting phenotype in vvl knockdown nymphs could be rescued by ectopic injection of active ecdysone, 20-hydroxyecdysone (20E), indicating that Vvl affects the biosynthesis of ecdysone rather than ecdysone response. Furthermore, I found reduced transcript levels of the ecdysone biosynthesis gene, *disembodied* (dib), in response to vvl knockdown. The conserved role of vvl in ecdysone biosynthesis in *Oncopeltus* shows that its endocrine function likely has ancient origins.

Mechanism of vvl action

Cheng et al. (2014) and Danielsen et al. (2014) established the role of vvl in initiating the transcription of ecdysone biosynthesis genes in the holometabolous insects *Tribolium castaneum* and *Drosophila melanogaster*, respectively. In *Tribolium*, whole body knockdown of vvl has been shown to affect the transcription of the ecdysone biosynthesis genes, *phm* in the anterior region and *spo* in the posterior region of larvae. In *Drosophila*, PG-specific vvl knockout leads to the reduced transcription of *phm, spok, sro, dib* and *sad* in the larval prothoracic glands. My results show that in the fifth, or final, instar of *Oncopeltus*, vvl also modulates ecdysteroid production by regulating the transcription of one of the P450 enzyme coding genes, *dib.*
In addition, knockdown of \textit{vvl} led to modest reduction in the expression of \textit{shd} in the anterior region of the nymph. Shd is the very last P450 enzyme that acts during ecdysteroidogenesis activating ecdysone to 20E and is expressed in the target tissues of ecdysone but not the prothoracic gland in \textit{Drosophila} and other holometabolous insects (Petryk et al, 2003; Rewitz et al., 2006). The effect of \textit{vvl} on \textit{shd} in \textit{Oncopeltus} is different from the effect of \textit{vvl} on \textit{shd} in \textit{Tribolium} because knockdown of \textit{vvl} in \textit{Tribolium} showed no significant change in \textit{shd} expression.

My results of \textit{vvl} knockdown could indicate the slight difference in the way that \textit{vvl} regulates ecdysteroidogenesis across insect species. It is possible that \textit{shd} is expressed in the prothoracic glands in \textit{Oncopeltus}. However, it is also possible that the RNAi result despite being statistically significant does not actually indicate a relationship between \textit{vvl} and \textit{shd}. This is because we only observed a minor reduction of \textit{shd} expression (1.77-fold) in the \textit{vvl} RNAi animals which may fall within the error of transcript level detection using qPCR. In contrast, \textit{dib} showed both a statistically significant and more than two-fold difference in mRNA levels between the treatment and control group in the anterior region of the nymph. The results would be supported by further investigation of the effect of \textit{vvl} knockdown on ecdysone biosynthesis genes in another instar, such as the fourth instar.

\textbf{Vvl regulates oogenesis in \textit{Oncopeltus} adults}

In addition to the conserved role of \textit{vvl} in nymphal/larval molts across the hemimetabolous and holometabolous boundary, I also found that \textit{vvl} plays a critical role during \textit{Oncopeltus} reproduction. In \textit{vvl} dsRNA-injected females, oocyte maturation stops, and the ovary is smaller in comparison to ovaries in wildtype females. JH and ecdysone both have varying
roles in ovarian maturation in insects. In most insects except dipterans, JH regulates vitellogenin synthesis, and ecdysteroids play critical roles in ovarian growth and oocyte maturation (Parthasarathy et al., 2010; Belles et al., 2015; Sieber and Spradling, 2015). Similarly, JH has been shown in Drosophila to regulate vitellogenic oocyte development, while ecdysteroids appear to have an antagonistic effect causing apoptosis at this stage (Soller et al., 1999).

These studies implicate an endocrine role of vvl during oocyte maturation in Oncopeltus. Vvl could be regulating either JH or ecdysone biosynthesis during the adult stage similar to the way it affects biosynthesis of developmental hormones during larval/nymphal development. Vvl may also regulate both JH and ecdysone biosynthesis. In addition to our results implicating vvl in ecdysone biosynthesis, Cheng et al. (2014) have found that vvl knockdown in Tribolium also leads to reduced expression of the JH biosynthesis gene, jhamt3. Thus, vitellogenesis may be affected due to lack of JH, and/or oocyte maturation may be disrupted due to the lack of ecdysteroids. Our attempts to rescue oogenesis by simultaneous injection of JH and 20E only led to the production of a few eggs, however, so additional studies using different concentrations of 20E and different timing of JH application are necessary to resolve the precise function of Vvl during reproduction. Even though the precise targets of Vvl during oogenesis still needs to be investigated further, it is clear that vvl knockdown affects oocyte maturation. A possible role of Vvl in hormone biosynthesis during oocyte maturation in addition to its role in hormone biosynthesis during larval/nymphal development may also suggest an overall importance of Vvl in controlling the production of JH and ecdysteroids throughout various insect life history stages.
Sites of \textit{vvl} expression in \textit{Oncopeltus}

Whole mount \textit{in situ} hybridization showed that \textit{vvl} is expressed in multiple sites in the elongated germ band to the retracted germ band stages of \textit{Oncopeltus} embryos. \textit{vvl} expression was seen in the prothoracic glands, brain region, central nervous system and in putative tracheal pits and oenocytes. Dorn and Romer (1976) propose the abdominal oenocytes as sites of ecdysone biosynthesis in \textit{Oncopeltus} embryos because they found the PGs to be inactive at this stage. In \textit{Drosophila}, Vvl has already been shown to regulate neuronal patterning (Meier et al. 2006) and in tracheal differentiation during embryogenesis (Anderson et al., 1995). Expression of \textit{vvl} in the CNS and putative tracheal pits in the embryos suggest that the non-endocrine functions of Vvl are also conserved in \textit{Oncopeltus}.

\textbf{Vvl may link ancestral endocrine function to respiratory structures in insects}

Based on the observation that corpora allata and prothoracic glands originate from the same precursor cells that give rise to the trachea, it has been suggested that respiratory structures in an ancestral arthropod also played an endocrine function (Grillo et al, 2014). Conservation of the endocrine and tracheal functions of Vvl in the Pancrustacea would provide a molecular mechanism that supports this hypothesis. Vvl has been shown to play a key role in ecdysteroidogenesis in \textit{Drosophila} (Danielson et al., 2014), \textit{Tribolium} (Cheng et al., 2014), \textit{Bombyx} (Meng et al, 2015) and \textit{Oncopeltus} (Figure 6). In \textit{Tribolium}, Vvl has also been found to control JH biosynthesis gene, \textit{jhamt3} (Cheng et al., 2014). In addition to its endocrinal functions, Anderson (1995) also first discovered \textit{vvl} as a tracheal development gene in \textit{Drosophila}. These studies in addition to the data presented in this thesis positions \textit{vvl} as a conserved factor in
ecdysteroidogenesis in both the holometabolous and hemimetabolous insects although its respiratory functions still need to be investigated further.

Less obvious is the importance of *vvl* or class III POU transcription factors in endocrine and respiratory functions among the more basally branching arthropods, the crustaceans. In the crustacean species, *Artemia*, a Class III POU transcription factor called Artemia POU-Homeoprotein (APH-1) has been studied. Vvl is also a Class III POU transcription factor and APH-1 was the only Class III POU transcription factor found in *Artemia*. Given that *APH-1* POU domain is identical in sequence to *vvl* POU domain except for two nucleotide residues, it is likely to be a homolog of Vvl. *APH-1* has been detected in the salt gland of *Artemia* embryo, a structure that is involved in osmoregulation (Chavez et al., 1999). Even though the salt gland is not part of the respiratory function in *Artemia*, it has also been shown to express an important tracheal development transcription factor *tracheless* (*trh*) (Wang et al. 2012).

Another POU domain transcription factor Nubbin (Nub) belonging to class II POU factors is found in the book gills, a respiratory organ of the of horseshoe crabs (Damen et al., 2002) and in the first thoracic segment in *Artemia* (Averof and Cohen, 1997). With a varied expression pattern between major group of arthropods, Nub has also been found to be expressed in the CNS, the leg segments of *Oncopeltus fasciatus* and *Drosophila*, and has also been implicated in insect mouth morphogenesis. The mid ventral expression of *vvl* and the ring-like expression patterns in the legs of *Oncopeltus* embryos are noteworthy as *nub* is also observed in similar although not identical patterns (Figure 9G; Li and Popadic, 2004).

Based on these observations, I suggest several possible scenarios (Figure 10). First, Class II and Class III POU factors may have been used interchangeably in various pancrustacean species. In insects, Vvl was selected to play the key endocrine (and tracheal) roles (Figure 10A).
Second, Vvl may have played a minor role in an unrelated structure in the Pancrustaean ancestor. As the common ancestor of insects transitioned onto land, Vvl acquired novel endocrine and tracheal functions (Figure 10B). Third, Vvl may have played an endocrine role in the crustaceans and its role was retained in insects (Figure 10C). Regardless of the scenarios, our finding that *Oncopeltus* vvl plays a key role in hormonal biosynthesis suggests that Vvl’s endocrine role arose relatively early and has been maintained throughout more than 350 million years that separate the *Oncopeltus* and *Drosophila*.
Figure 10. Hypothetical scenarios for the evolution of Vvl’s role in tracheal and endocrine development. (A) Scenario 1: Class III (Vvl homolog) and Class II (Nub homolog) POU transcription factors were interchangeably involved in tracheal and endocrine development in the pancrustacean ancestor. Vvl assumed a tracheal and endocrine role in the insect lineage. Nubbin assumed a limb development function. Table summarizes what Class III/Vvl and Class II/Nub POU factors would transcriptionally regulate in this scenario. ✓ = transcriptionally regulates. (B) Scenario 2: POU III/Vvl played roles in unrelated structures (e.g. salt gland) in the pancrustacean ancestor and acquired novel endocrine and tracheal functions in insect lineage. (C) Scenario 3: POU III/Vvl played a transcriptional role in the tracheal and endocrine development in the pancrustacean ancestor.
REFERENCES


